NOVEL MECHANISMS FOR THE ALTERATION OF RET RECEPTOR TYROSINE KINASE SIGNAL TRANSDUCTION

by

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To my family for all their love and support.

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Receptor tyrosine kinases (RTKs) are the second largest family of transmembrane receptors, and these proteins regulate numerous cellular processes including cell survival, metabolism, proliferation and differentiation. Mutations that affect the activity, abundance, cellular distribution or regulation of these receptors often leads to diseases such as cancer, making our understanding of the basic biology of these receptors, especially the regulation of their expression and signaling, critically important. One such RTK, RET, is a receptor for glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) and has several functions in the developing embryo, including crucial roles in kidney morphogenesis, spermatogenesis and development of numerous neuronal populations. Regulation of RET through mechanisms such as intracellular trafficking, turnover and activation change the signaling capabilities of RET and have been well studied. Here I present two additional novel mechanisms of action of RET.

In the first investigation, I explored the effects of novel 5'-alternative splicing on RET protein structure and function. These isoforms, which arise from exon skipping, are full-length RET proteins with deletions in the extracellular domain and are referred to as $RET^{\Delta E3}$ and $RET^{\Delta E345}$. I found that these isoforms differ from full-length RET in both their biochemical properties as well as their signaling capabilities. In the second study, I investigated the interaction of RET with p75, a pro-apoptotic protein that is a member of the tumor necrosis factor receptor (TNFR) superfamily. While I discovered that RET and p75 interact, and that this association is increased by the presence of pro-apoptotic

stimuli *in vitro*, I identified a p75-independent role for RET in programmed cell death in the sympathetic nervous system during development *in vivo*. These data provide two novel mechanisms for the regulation of RET which, along with future studies, will further our understanding of RET biology under physiologic and pathologic conditions.

CHAPTER 1

INTRODUCTION

Receptor tyrosine kinases are a large family of cell surface receptors.

Receptor tyrosine kinases (RTKs) are a large family of cell surface proteins that act as receptors for hormones and growth factors. They are the second largest family of transmembrane receptors, the largest being G-protein coupled receptors (GPCRs), and are comprised of 58 RTKs that fall into 20 subfamilies (Lodish et al., 2008). RTKs regulate numerous cellular processes such as proliferation and differentiation, cell survival and cellular metabolism, and their importance can be appreciated by their high conservation between multiple species, from *C. elegans* to humans (Lemmon and Schlessinger, 2010).

All RTKs have a similar molecular architecture that includes an extracellular ligand-binding region, a single transmembrane α-helix, and an intracellular tyrosine kinase domain (Lodish et al., 2008). In the absence of ligand, most RTKs are monomeric, and have very low intrinsic kinase activity. Upon ligand binding, two receptor monomers are induced to dimerize, allowing the kinase domain of one monomer to phosphorylate one or several tyrosine residues in the catalytic site of the other monomer (Lodish et al., 2008). This leads to a conformational change in the dimeric structure of the proteins, allowing for binding of ATP to some receptors or protein substrates in other receptors (Lemmon and Schlessinger, 2010). The resulting conformational change enhances the kinase activity of the RTK, causing

phosphorylation of additional tyrosine residues in the cytosolic domain of the receptor (Lodish et al., 2008). These phosphorylated tyrosines serve as docking sites for additional adaptor molecules and signaling proteins, which trigger activation of multiple intracellular signaling pathways (Lemmon and Schlessinger, 2010). However, not all RTKs dimerize upon ligand binding – some RTKs, such as the insulin receptor, form pre-complexed dimers in the absence of hormone. In this case, binding of the ligand insulin alters the conformation of the receptor to allow for its activation (Lodish et al., 2008).

RTKs have been widely studied not only due to their important roles throughout development, but also because they play numerous roles in diseases due to genetic mutations that affect the activity, abundance, cellular distribution or regulation of the receptors (Mulligan, 2014). One common theme is that aberrant activation of RTKs are causally linked to cancers (Schlessinger, 2014), diabetes (Schlessinger, 2014), inflammation (Wang and Hankey, 2013), bone disorders (Segaliny et al., 2015), arteriosclerosis (Hopkins, 2013) and angiogenesis (Jeltsch et al., 2013). Understanding the basic biology of these receptors and how cells regulate their expression and signaling is important to better understand their role in disease.

The receptor tyrosine kinase, RET, is a receptor for glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs).

RET is a single-pass, type 1 transmembrane receptor tyrosine kinase that is conserved from humans to *Drosophila melanogaster* (Anders et al., 2001). The gene encoding *RET* was first identified in 1985 when DNA from a T cell lymphoma was

transfected into NIH/3T3 cells resulting in foci formation (Takahashi et al., 1985). The cause of this was a DNA rearrangement that occurred during the transfection process resulting in the recombination of two unrelated DNA sequences, and thus the authors proposed calling the previously undescribed gene, *RET*, which is an acronym for "REarranged during Transfection." Although the name *RET* originally referenced the recombined gene, the name was kept to designate the gene coding for the receptor tyrosine kinase portion of the original oncogene (Takahashi et al., 1985).

RET is activated by and acts as a common receptor for the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) (Fig. 1.1), of which there are four: GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN) (Airaksinen and Saarma, 2002). GDNF was purified in 1993 and characterized as a neurotrophic factor for the survival of embryonic dopaminergic neurons *in vitro*, and in 1996, several groups reported that GDNF was the ligand for RET (Lin et al., 1993, Durbec et al., 1996, Trupp et al., 1996). The identification of NRTN soon followed when it was discovered that an unknown factor in conditioned medium from Chinese hamster ovary (CHO) cells could support the survival of sympathetic neurons in culture. Amino acid sequencing indicated that this was a novel ligand and was related to GDNF (Kotzbauer et al., 1996). Thereafter, ARTN and PSPN were identified by database searches and homology-based PCR screening (Milbrandt et al., 1998, Baloh et al., 1998).

Unlike other ligand-RTK signaling complexes, GFLs do not bind directly to RET, but first bind to and create a binary complex with one of four cognate glycosylphosphatidylinositol (GPI)-anchored co-receptors known as the GDNF Family Receptor-αs (GFRα1-4). RET is the only known receptor tyrosine kinase that does not

directly bind its ligands. Each ligand has a preferential affinity for one of the GFRαs; GDNF binds to GFRα1, NRTN binds to GFRα2, ARTN binds to GFRα3, and PSPN binds to GFRα4 (Airaksinen and Saarma, 2002). Upon associating, the GFL-GFRα complex binds to RET, recruiting it into a cholesterol-rich membrane subdomain known as a lipid raft (Tansey et al., 2000, Paratcha et al., 2001, Ledda et al., 2002). Here, RET monomers are induced to dimerize, allowing for the autophosphorylation of multiple tyrosine residues within the RET intracellular tyrosine kinase domain, which initiates the association of adaptor proteins and enzymes that trigger multiple second messenger cascades (Airaksinen and Saarma, 2002).

Experiments conducted in knockout mouse models have helped identify the functions of the GFLs, GFRαs and RET in the developing embryo, including crucial roles for RET in kidney development, spermatogenesis and development of the nervous system. In the renal system, RET is required for early induction of growth, branching and morphogenesis of the ureteric bud in the developing metanephric kidney (Schuchardt et al., 1994, Schuchardt et al., 1996, Vega et al., 1996, Chi et al., 2009, Davis et al., 2014). In the testis, RET is responsible for the maintenance and self-renewal of spermatogonial stem cells and for the regulation of germ cell differentiation process in testes (Meng et al., 2000, Naughton et al., 2006, Jijiwa et al., 2008). In the nervous system, RET signaling is critical for the development of sympathetic (Enomoto et al., 2001), motor (Gould et al., 2008), and sensory nervous systems (Luo et al., 2009, Golden et al., 2010), for the development and maintenance of the enteric nervous system (Schuchardt et al., 1994, Schuchardt et al., 1996), and also for the postnatal maintenance of dopaminergic midbrain neurons (Kramer et al., 2007).

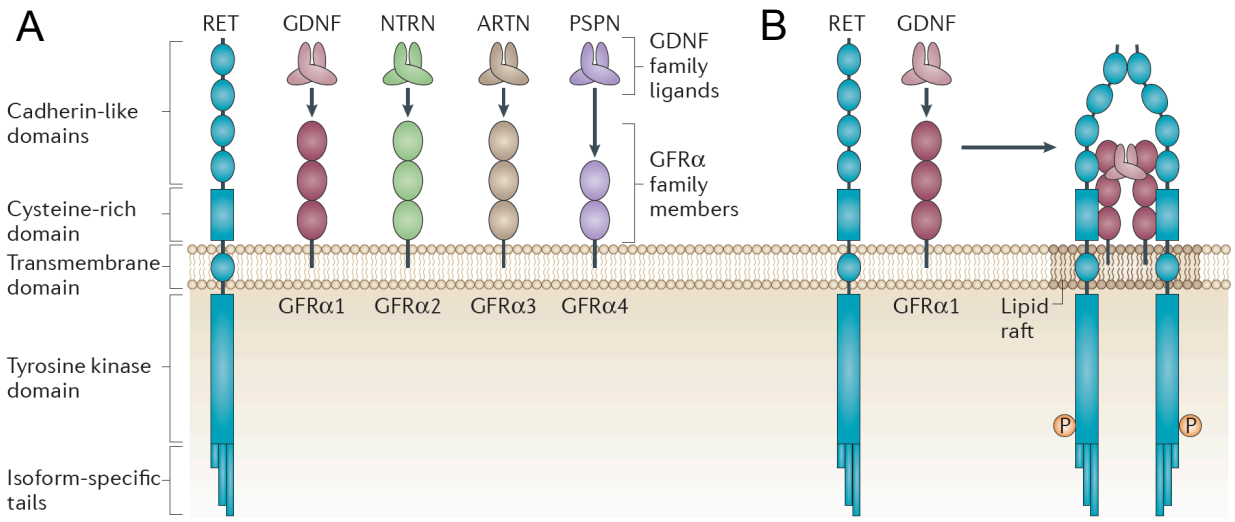

Figure 1.1: RET is a receptor for the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs). (A) The GFLs, GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN) are dimerized ligands that first bind to their respective GPI-anchored coreceptors, the GDNF Family Receptor-α (GFRα). (B) GFL-GFRα binding to the extracellular domain of RET leads to its dimerization and activation via autophosphorylation of the intracellular tyrosine kinase domain. Figure adopted from (Mulligan, 2014).

RET encodes two major protein isoforms that have biologically distinct functions

The central dogma of molecular biology, established by Francis Crick in 1956, describes the flow of genetic information, wherein DNA is transcribed to RNA, which is then processed and translated to protein. One of the most interesting and complicated steps in this progression is the regulated post-transcriptional processing of RNA in the nucleus. When DNA is first transcribed into RNA, the resulting ribonucleic acid is comprised of protein-coding exonic sequences as well as non-coding intronic sequences, and is referred to as pre-mRNA. The cell uses a complex of small nuclear RNAs and proteins, known as the spliceosome, to remove or "splice out" the intronic sequences, and then joins together the protein-coding sequences into one continuous mRNA transcript that can be translated by the ribosome (Clancy, 2008).

It was discovered that during the processing of pre-mRNA, the splicing machinery pieces together the exonic sequences in different ways such that multiple mRNA transcripts can be generated from a single gene (Lee and Rio, 2015). For example, in certain tissues an exon can be included to encode one protein product, but in another tissue, the exon can be excluded to encode a similar, yet different protein. This process has been termed "alternative splicing," and is now appreciated as one of many processes that mediate gene regulation in metazoans, allowing for the generation of numerous transcripts from a single protein-coding gene that creates proteomic diversity without expanding the genome. Interestingly, the frequency of alternative splicing increases with species complexity; Of the approximately 25,000 genes encoded by the human genome, ~95% produce transcripts that are alternatively spliced (Wang et al., 2008, Pan et al., 2008). With such an enormous increase in complexity comes an

increased susceptibility to malfunction, and defects in pre-mRNA splicing processes have emerged as a common disease-causing mechanism (Lee and Rio, 2015).

By expanding the proteome through the synthesis of various protein isoforms, alternative splicing allows for increased protein diversity, with isoforms performing selective biological functions by differing in their protein-protein interactions, cellular localization and/or catalytic ability. One example of this is fibroblast growth factor receptors (FGFRs). FGFRs are a subfamily of four RTKs that are receptors for fibroblast growth factors (FGFs), of which there are 18 ligands (Turner and Grose, 2010). Alternative splicing of the FGFRs in the region coding for the extracellular ligand-binding domain of the receptors allows for differential ligand binding, and thus, differential signaling events (Turner and Grose, 2010). Additionally, several neurotrophic factor receptors encode multiple isoforms, such as TrkB. One *trkB* transcript produces a truncated form of the receptor, TrkB.T1, that lacks catalytic activity and acts as a dominant negative receptor that can inhibit TrkB signaling (Eide et al., 1996). This truncated form sequesters and translocates its ligand, brain-derived neurotrophic factor (BDNF), and can induce filopodia and neurite outgrowth, stimulate intracellular signaling cascades and modify cytoskeletal structures (Fenner, 2012). Additionally, upregulation of this truncated TrkB isoform specifically in male mice leads to a loss of sensory innervation in mammary glands during development (Liu et al., 2012).

RET consists of 20 exons, and encodes two major protein isoforms and one minor isoform, all of which are generated from alternative 3' splicing. These isoforms are named for the number of unique amino acids found at the C-terminus of each protein; either 9, 43 or 51 (Myers et al., 1995). The two major isoforms, RET9 and

RET51, are highly conserved among vertebrates, and a third isoform, RET43, has been identified in humans, but has not been found to be conserved in other species (Lee et al., 2003).

Although RET9 and RET51 only differ in their C-termini, these proteins have distinct physiological functions and have unique adaptor protein interactions, leading to separate signaling abilities (Tsui-Pierchala et al., 2002). For example, mice lacking RET9 have renal malformations, defects in enteric nervous system innervation and die shortly after birth similar to *Ret* null mice (de Graaff et al., 2001). However, mice lacking RET51 appear normal and survive into adulthood (de Graaff et al., 2001). Additionally, there are differences in expression of the two isoforms, both within tissues and during developmental times, such as in the kidneys (Ivanchuk et al., 1998). Mice lacking RET9 have renal malformations due to the early expression of *RET9* in development of the kidney during branching of the ureteric bud (Ivanchuk et al., 1998). *RET51* expression increases later in renal development, and likely has a differentiating role rather than a mitogenic role in early kidney induction like RET9 (Ivanchuk et al., 1998).

The two RET isoforms are also regulated in different ways. For example, in the sympathetic neurons of the superior cervical ganglion (SCG) where RET51 is more highly expressed, Cbl E3 ubiquitin ligase binds to this isoform and rapidly degrades the protein via the proteasome (Tsui-Pierchala et al., 2002, Scott et al., 2005). RET9, on the other hand, is not degraded nearly as rapidly in the SCG. This is largely due to both the absence of a key tyrosine residue in the alternatively spliced C-terminal tail that acts as a docking site for the adapter molecules necessary for its degradation, as well as for RET translocation into lipid rafts upon activation, which sequesters it from the

degradation machinery (Pierchala et al., 2006). However, in sensory neurons of the DRG, the GDNF-GFRα1-RET complex is not degraded and is instead retrogradely transported to the cell body to support neuron survival and maintenance (Tsui and Pierchala, 2010). Taken together, 3'-splicing of *Ret* to produce RET9 and RET51 isoforms serves to increase the diversity of the possible signaling capacities of the GFLs.

Structure of the RET extracellular and kinase domains and associated intracellular signaling pathways

Since the discovery of RET, numerous experiments have investigated the overall structure of the RET protein and how it interacts with the GFLs and GFRαs. A major driving force for this is due to the fact that mutations which cause overexpression of RET, and therefore, increased signaling, or mutations that cause constitutive activation of the RET receptor, are found in a number of cancers (Mulligan, 2014). It has been extremely important to characterize the structure, especially of the intracellular kinase domain, to aid in the creation of small molecule inhibitors to treat these cancers. Additionally, understanding the overall structure of the protein is important for understanding why certain mutations lead to gain-of-function or loss-of-function phenotypes.

As previously stated, the gene encoding *RET* is 20 exons long. Exons 1-10 of the gene encode the extracellular region of the protein, which is the least conserved region between vertebrates, with 40% amino acid sequence identity among vertebrates, and 30% amino acid sequence identity after including *D. melanogaster* Ret (Kjaer et al.,

2010). Exon 11 encodes the transmembrane domain of the receptor, and exons 12-20 encode the intracellular domain. Conservation analysis has shown that the cytoplasmic kinase domain is the most highly conserved region of the protein, with 90% amino acid sequence identity among several vertebrate species. When adding the *D. melanogaster* Ret amino acid sequence to this comparison, the conservation of the kinase domain decreases to 65% (Kjaer et al., 2010).

The extracellular domain of RET

The extracellular domain is comprised of four cadherin-like repeats (CLD1-4) (human amino acid sequence, residues 29-516) followed by a membrane-proximal cysteine-rich domain (CRD) (residues 517-635) (Anders et al., 2001). The CLDs are structurally similar to the extracellular cadherin (EC) domains of cadherin proteins. The EC domains of cadherins are important for mediating cell-cell contact and adhesion at adherens junctions (Shapiro and Weis, 2009). RET CLDs, in contrast, do not function in this manner and instead function to stabilize the overall structure of the RET dimer. EC domains are characterized by a repeating amino acid sequence of approximately 110 residues with a calcium-binding domain positioned between each EC domain, and calcium binding is structurally important for the proper orientation and binding of cadherin ectodomains (Shapiro and Weis, 2009). The RET CLDs are also approximately 110 residues in size, but only contain one calcium-binding domain that is located between CLD2 and CLD3. While the loss of the calcium binding domains between CLD1-2 and CLD3-4 is thought to allow for increased flexibility of RET in binding to GFL-GFRα binary complexes, calcium binding between CLD2-3 is still crucial

for the proper folding, secretion and signaling functions of RET (Anders et al., 2001, Nozaki et al., 1997, Kjaer et al., 2010).

The membrane-proximal CRD is necessary for proper protein conformation as well as binding to ligand. Initial mutagenesis experiments argued that the binding of the GFL-GFRα binary complex occurs in CLDs1-3; however this interaction was not observed in cross-linking experiments (Amoresano et al., 2005, Kjaer and Ibanez, 2003). Instead the CLD4 and CRD domains of RET are required for the binding of RET to the GDNF-GFRα1 binary complex (Amoresano et al., 2005).

Understanding the three-dimensional structure of the RET extracellular domain has been lacking mainly due to the inability to obtain a full crystal structure. However, Kjaer et al. did determine the crystal structure of a CLD1 and CLD2 fragment, which has a “clam-shell” shaped structure that forms a secondary dimer between two RET monomers following ligand-dependent dimerization (Kjaer et al., 2010). The authors proposed a tentative GFL-GFRα-RET ternary complex model in which CLD4 and the CRD domains of two RET monomers directly interact with the GLF-GFRα binary structure. Upon associating, the membrane-distal CLD1-CLD2 regions of the two RET monomers form a clam-shell shaped secondary dimer that helps topologically trap and stabilize the GFL-GFRα binary complex (Kjaer et al., 2010). While this predicted model has yet to be disproven, additional experiments are still needed to confirm these results.

The RET intracellular domain and signal transduction

Across the plasma membrane lies the intracellular domain of RET which is comprised of a tyrosine kinase domain (residues 724-1016) and a C-terminal tail. As

discussed previously, upon GFL-GFRα binding, two RET monomers dimerize, allowing for autophosphorylation of multiple tyrosine residues within the kinase domain. This causes recruitment of adaptor proteins that bind to the phosphorylated residues and themselves become tyrosine phosphorylated by RET. This induces their activation, triggering cascades that lead to the activation of multiple downstream signaling pathways (Airaksinen and Saarma, 2002). While the activation of these pathways has been thoroughly studied, it is worth noting that because RET was an orphan receptor for a decade, a great deal of the information regarding RET signal transduction is a result of experiments performed with chimeric and/or oncogenic forms of RET (Arighi et al., 2005).

The three main signal transduction pathways downstream of RET activation are the RAS-MAPK (mitogen activated protein kinase) pathway, the PI3K-AKT pathway and the PKC (protein kinase C) pathway (Arighi et al., 2005, Mulligan, 2014). Activation of the RAS-MAPK pathway promotes growth and differentiation of neurons (Grimm et al., 2001). Adaptor proteins, such as GRB2, bind to phosphorylated tyrosine residues of RET, which in turn phosphorylates the adaptor protein, allowing further recruitment of signaling molecules (Airaksinen and Saarma, 2002). In the RAS-MAPK pathway, these adaptor proteins are linked to a guanine nucleotide exchange factor (GEF), such as SOS, that transduces the signal to a small GTP binding protein, such as RAS (Mulligan, 2014). This in turn activates the main core of this serine-threonine kinase cascade by phosphorylating and activating MAPKKK (Raf), which phosphorylates and activates MAPKK (MEK1/2), which then phosphorylates and activates MAPK (ERK) (Lodish et al., 2008). MAPK can then interact with targets in the cytosol, or it can translocate to the

nucleus and phosphorylate a variety of transcription factors, which ultimately regulates gene expression (Lodish et al., 2008).

The PI3K-AKT pathway regulates growth, proliferation, survival, transcription and protein synthesis within cells (Hemmings and Restuccia, 2012). Upon GFL-GFRα binding to RET, adaptor proteins again bind to phosphorylated tyrosine residues in the intracellular domain of RET. These adaptor proteins then activate phosphoinositide-3-kinase (PI3K), which then converts phosphatidylinositol (3,4)-bisphosphate (PIP_2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) (Hemmings and Restuccia, 2012). PIP_3 binds to protein kinase B (PKB), also known as AKT, thus recruiting it to the plasma membrane where it is phosphorylated and activated by PDK1/2 (Pitt and Chen, 2008). AKT is a serine/threonine kinase that has numerous cytoplasmic substrates (Lodish et al., 2008). Downstream targets of activated AKT include the TSC1/TSC2 complex, which is part of the mTORC-signaling pathway that regulates protein synthesis and cell proliferation (Lodish et al., 2008). Additionally, activated AKT can also promote cell survival by inhibiting the function of pro-apoptotic proteins (Pitt and Chen, 2008).

Lastly, the PKC pathway regulates cell proliferation, differentiation, survival and motility (Kang, 2014). Phospholipase C (PLCγ) is recruited to RET and becomes activated (Ibanez, 2013). PLCγ produces diacylglycerol (DAG), a lipid second messenger, which binds and activates PKC (Kang, 2014). The downstream signaling that follows activation of PKC is complex due to the PKCs being a large family of isozymes that are diverse in their mechanisms of activation, although all PKCs do phosphorylate serine and threonine residues on a large number of downstream signaling proteins (Kang, 2014).

Within the intracellular domain of RET, the shorter RET9 isoform contains 16 tyrosine residues, while the RET51 isoform contains 18. Of these many tyrosine residues, adaptor proteins have been identified that bind to eight of these tyrosine residues; the five most widely studied are Tyr905, Tyr981, Tyr1015, Tyr1062, which are located in the kinase domain, and Tyr1096 which is only present in the C-terminal tail of the RET51 isoform (Ibanez, 2013). Tyr905, which is a binding site for GRB7 and GRB10 and signals via the RAS-MAPK pathway, is also an autocatalytic tyrosine that is conserved in many RTKs and helps to stabilize the active conformation of the kinase (Pandey et al., 1995, Pandey et al., 1996). Mutation of Tyr905 to phenylalanine (Y905F) impairs the kinase activity of RET (Iwashita et al., 1996). Tyr981 is a site for SRC binding that signals via the PI3K-AKT pathway (Encinas et al., 2001). Translocation of RET into lipid rafts promotes the interaction of SRC with activated RET and enhances mitogenesis and neuronal survival (Encinas et al., 2001). Tyr1015 is a docking site for PLCγ, which activates the PKC signaling pathway and is crucial for kidney morphogenesis and migration of neuro-progenitors in the developing brain (Jain et al., 2006, Borrello et al., 1996, Lundgren and et al., 2012). Tyr1062 is a multifunctional binding site for SHC, Dok4/5, IRS-1, and FRS-2 when phosphorylated and is a binding site for Enigma in a phosphorylation-independent manner (Durick et al., 1996, Lorenzo et al., 1997, Asai et al., 1996, Arighi et al., 1997, Grimm et al., 2001, Hayashi et al., 2000, Kurokawa et al., 2001, Melillo et al., 2001b, Melillo et al., 2001a). Interactions between Tyr1062 and these adaptor proteins lead to activation of both the RAS-MAPK and PI3K/AKT pathways (Asai et al., 1996, Arighi et al., 1997, Besset et al., 2000, Kurokawa et al., 2001, Melillo et al., 2001b). Lastly, Tyr1096 is a binding site for GRB2,

which signals through the PI3K-AKT signaling pathway, but it can also recruit the CBL family of ubiquitin ligases that function in the downregulation of RET51 by initiating ubiquitination and degradation (Alberti et al., 1998, Scott et al., 2005, Tsui and Pierchala, 2008). Although many of the pathways through which RET transduces signals have been identified, far less is known about the target genes that are specifically modulated in response to receptor activation.

Role of RET in disease

Receptor tyrosine kinases play very important roles throughout development, and as such, loss or gain of function mutations in these receptors or other proteins in the signaling pathway can have deleterious affects on the organism. RET is no exception to this rule. Loss of function mutations in *RET* are responsible for renal agenesis as well as Hirschsprung's disease, a complex genetic disorder characterized by aganglionosis of variable length of the distal gastrointestinal tract (Fig. 1.2) (Mulligan, 2014). This is not surprising given the importance of RET signal transduction during development, especially in the kidneys and enteric nervous system. On the other hand, gain of function mutations in *RET* play roles in cancer, including the formation of medullary thyroid carcinomas and pheochromocytomas, and mutations in *RET* are also causal for Multiple Endocrine Neoplasia Types 2A and 2B (Fig. 1.2) (Mulligan, 2014).

Hirschsprung's Disease

In 1888, Harald Hirschsprung first described a condition in infants in which patients presented with the absence of spontaneous bowel movements, leading to

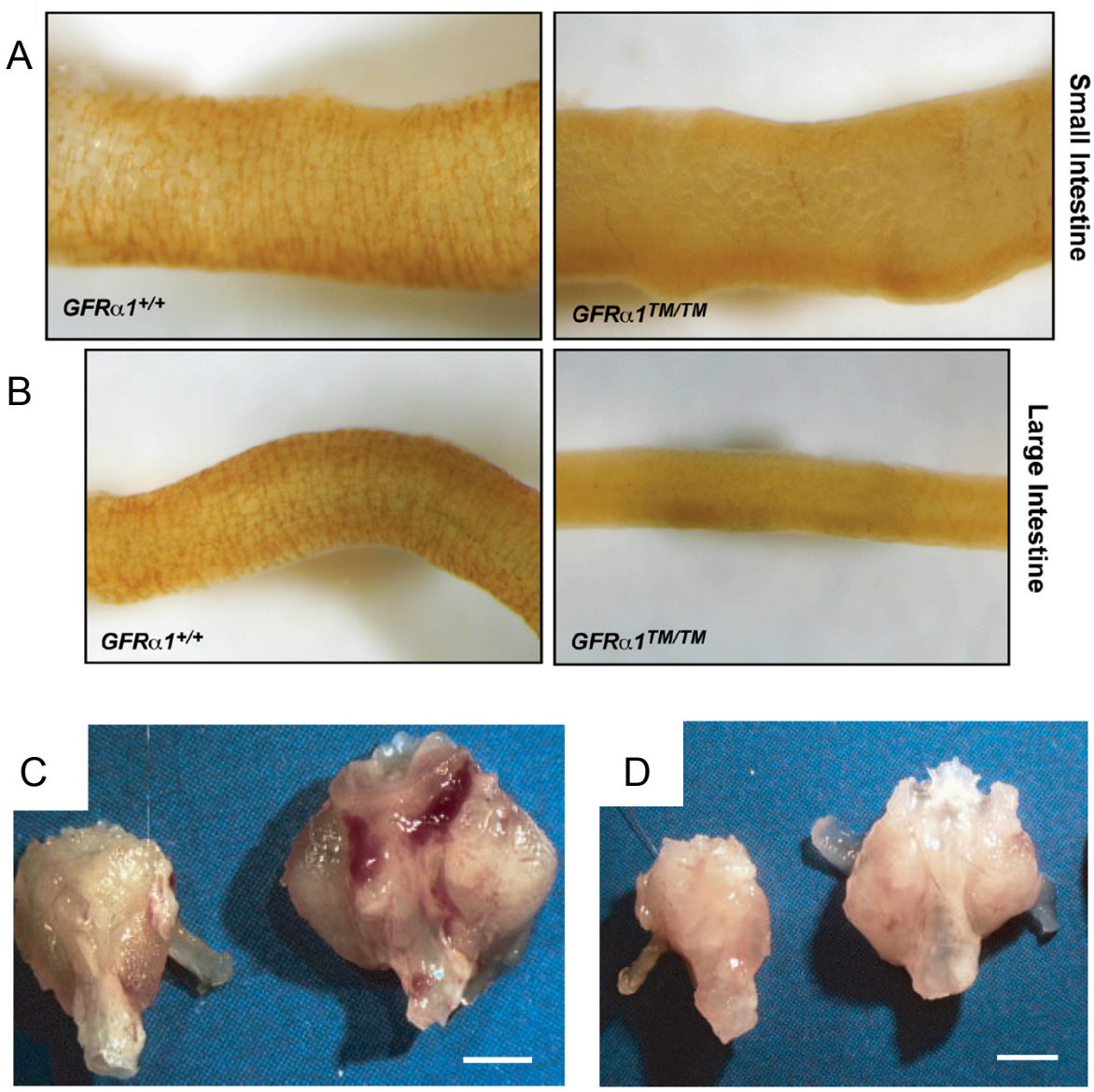

Figure 1.2: Loss-of-function or gain-of function mutations in the RET signaling pathway cause defects in mice. (A, B) Loss of enteric nervous system innervation of the small and large intestine in mice that lack the ability to translocate RET into lipid rafts for proper signaling. Visualization of the myenteric plexes was performed using acetylcholinesterase immunohistochemistry in E18.5 wildtype (A) or mutant (B) mice. Figure adopted from (Tsui et al., 2015). (C, D) Examples of medullary thyroid carcinomas (MTC) from mice harboring an activating mutation of RET, Cys634Arg, that causes ligand-independent dimerization of RET monomers. Thyroid glands from wildtype littermates are on the left of each picture, while thyroid glands exhibiting MTC from mutant mice are pictured on the right. (C) Transgenic mice backcrossed into CBA/ca genetic background. (D) Transgenic mice backcrossed into FVB/N genetic background. Figure adopted from (Cranston and Ponder, 2003)

intestinal obstruction and chronic constipation (Hirschsprung, 1888). It was later discovered that these symptoms resulted from the absence of the enteric nervous system due to the failure of neural crest cells to migrate, proliferate, differentiate and/or survive in the bowel wall (Obermayr et al., 2013). This condition has since been termed Hirschsprung's Disease (HSCR) or congenital intestinal aganglionosis, and affects 1 in every 5000 live births (Bodian and Carter, 1963).

Colonization of the gut by enteric neural crest-derived cells (ENCCs) is regulated by several local signals produced in the gut, and mutations in several of these genes have been associated with HSCR, including loss of function mutations in *RET* (Obermayr et al., 2013). Studies in animal models have shown that RET plays an essential role in the survival, proliferation, migration and differentiation of ENCCs (Heanue and Pachnis, 2007, Laranjeira and Pachnis, 2009). Interestingly, approximately half of familial cases of HSCR are associated with loss of function mutations in the *RET* gene (Angrist et al., 1995, Attie et al., 1995). Additionally, mutations in *GDNF* and *NRTN* have also been described in patients with HSCR, and mice haploinsufficient for *Gdnf* present with Hirschprung-like symptoms including intestinal obstruction and early-onset lethality (Parisi and Kapur, 2000, Shen et al., 2002).

While the pathogenic role of RET in HSCR has been widely studied, mutations in other genes and pathways have been identified as well. The endothelin type B receptor pathway has been associated with HSCR, including mutations in *EDNRB* and *EDN3* that inhibit neuronal differentiation (Obermayr et al., 2013). Interestingly, *EDNRB* was first associated with HSCR because it was linked to the same region of the

chromosome as the susceptibility to Hirschsprung Disease 2 locus (Puffenberger et al., 1994). Additional mutations in genes encoding transcription factors, such as *Hoxb5*, *Sox10* and *Phox2B,* have also been found to be associated with HSCR (Obermayr et al., 2013). Unlike *RET*, however, mutations at other individual loci are not sufficient or necessary to cause clinical disease. It is very likely that polymorphisms at several loci contribute in a combinatorial manner to cause this complex genetic disorder.

Multiple endocrine neoplasia, type 2

RET gain-of-function mutations that lead to over-activation of the receptor arise from missense changes that induce constitutive activation of the receptor, or its aberrant over-expression, allowing for ligand-independent receptor activation (Mulligan, 2014). This increase in signaling via RET is associated with several familial and sporadic cancers of neuroendocrine organs. In the autosomal dominantly inherited syndrome multiple endocrine neoplasia type 2 (MEN2), numerous causal mutations have been identified within the *RET* gene (Arighi et al., 2005). The syndrome, which affects one in every 35,000 individuals, is characterized by the presence of multiple endocrine tumors including a form of thyroid cancer known as medullary thyroid carcinoma (MTC), tumors of the adrenal gland known as pheochromocytomas, as well as benign tumors of the parathyroid glands known as parathyroid adenomas (Mulligan, 2014).

MEN2 is subdivided into three categories based upon the neoplasms of the patient and includes familial medullary thyroid carcinoma (FMTC), MEN2A and MEN2B. Patients who present primarily with MTC are classified as having FMTC. MEN2A is characterized by MTC and includes pheochromocytomas in 50% of cases and also

parathyroid adenomas. MEN2B is the most severe disease phenotype, and has the earliest onset and poorest survival of the MEN2 subtypes (Mulligan, 2014). MEN2B is characterized by MTC and pheochromocytomas, as well as other developmental anomalies that are consistent with RET function in embryonic development (Arighi et al., 2005).

The MEN2 mutations that arise in *RET* are generally single amino acid substitutions that fall in one of two regions of the receptor and ultimately lead to constitutive activation and signaling (Mulligan, 2014). Although numerous casual variants have been identified in patients with MEN2, mutations primarily affect the extracellular cysteine-rich domain (MEN2A) or the intracellular kinase domain (MEN2B). In MEN2A, more than 95% of patients have amino acid substitutions of C609, C611, C618, C620, C630 or C634, with mutations at C634 being the most common (Pasini et al., 1996). These cysteines, which lie just proximal to the transmembrane domain of RET, form disulfide bonds that are required for the proper three-dimensional structure of RET (Mulligan, 2014). However, when one of these cysteines is mutated, it leaves one of the five remaining cysteines unpaired, allowing it to form intermolecular bonds and induce ligand-independent receptor dimerization. Interestingly, amino acid substitutions at C609, C611, C618, or C620 are deemed “Janus mutations.” Individuals with substitutions at these positions within RET present with both a gain-of-function and a loss-of-function phenotype. It was found that this “mixed” phenotype is due to the lack of proper maturation of RET proteins, leading to a decrease in RET expression on the cell surface (Arighi et al., 2005). As a consequence, some patients have neuroendocrine tumors as well as kidney and enteric nervous system abnormalities (Arighi et al., 2005).

MEN2B mutations fall in the intracellular kinase domain of RET, and most commonly include amino acid substitutions of M918T and M883F (Carlson et al., 1994, Smith et al., 1997). These mutations are located near the activation loop of the kinase domain, and have been shown to change the conformation of the protein, allowing for an increase in the basal kinase activity of the receptor, especially M918T (Santoro et al., 1995). Additionally, the M918T mutation alters substrate binding by affecting the autophosphorylation sites of the receptor as well as the phosphorylation of intracellular adaptor proteins (Santoro et al., 1995).

MTCs and pheochromocytoma tumor samples have been thoroughly analyzed for other mutations in *RET* that could be causal or aid in tumor progression for MEN2. During the course of these studies, three novel *RET* transcripts were identified in pheochromocytomas that were the product of exon skipping in the 5' region of *RET*, which encodes for the extracellular domain of the protein (Lorenzo et al., 1995). Skipping of exons 3 ($RET^{\Delta E3}$) or exons 3, 4 and 5 ($RET^{\Delta E345}$) give rise to transcripts that encode for full-length RET proteins, but with deletions in the extracellular domain, specifically cadherin-like domain 1 (CLD1), or CLD1-3, respectively. We have hypothesized that these deletions change the extracellular domain structure as well as binding to GFL-GFRα complexes, and may impact overall stability of the proteins. The skipping of exons 3 and 4 ($RET^{\Delta E34}$) results in a frameshift that culminates in a premature stop. This protein encodes only a small portion of the RET extracellular domain with no transmembrane or intracellular domain (Lorenzo et al., 1995). Because this transcript is likely to be subjected to nonsense-mediated decay, it is unlikely to be involved in RET signal transduction. However, beyond analyzing the expression of

these transcripts, expression of these potential isoforms and the functional role they may play in disease formation and/or progression has not been elucidated.

The Sympathetic Nervous System as a Model to Study RET Signal Transduction and Programmed Cell Death

The sympathetic nervous system, part of the autonomic nervous system, is responsible for maintaining homeostasis within the body, and is most commonly known for controlling the body's "fight or flight" response. This subset of the nervous system is responsible for regulating many physiological processes, including but not limited to body temperature, blood pressure, respiration, cardiac output, and blood glucose levels (Janig and Habler, 2000). The importance of the sympathetic nervous system can best be observed when it becomes dysfunctional, playing roles in hypertension, congestive heart failure, dysautonomia, and various neuropathies (Glebova and Ginty, 2005).

The sympathetic nervous system consists of paired cholinergic preganglionic sympathetic neurons whose cell bodies reside in the intermediolateral horn of the spinal cord. These neurons make synaptic connections with post-ganglionic, predominantly noradrenergic sympathetic neurons that are situated as paired ganglia that run the length of the body on either side of the spinal cord. These post-ganglionic neurons project their axons to many different target tissues and glands (Figure 1.3). The anterior most pair of ganglia are the superior cervical ganglia (SCG), which innervate a number of targets within the head as well as the heart and trachea. These ganglia have been used extensively to study both RET signal transduction and programmed cell death (Glebova and Ginty, 2005).

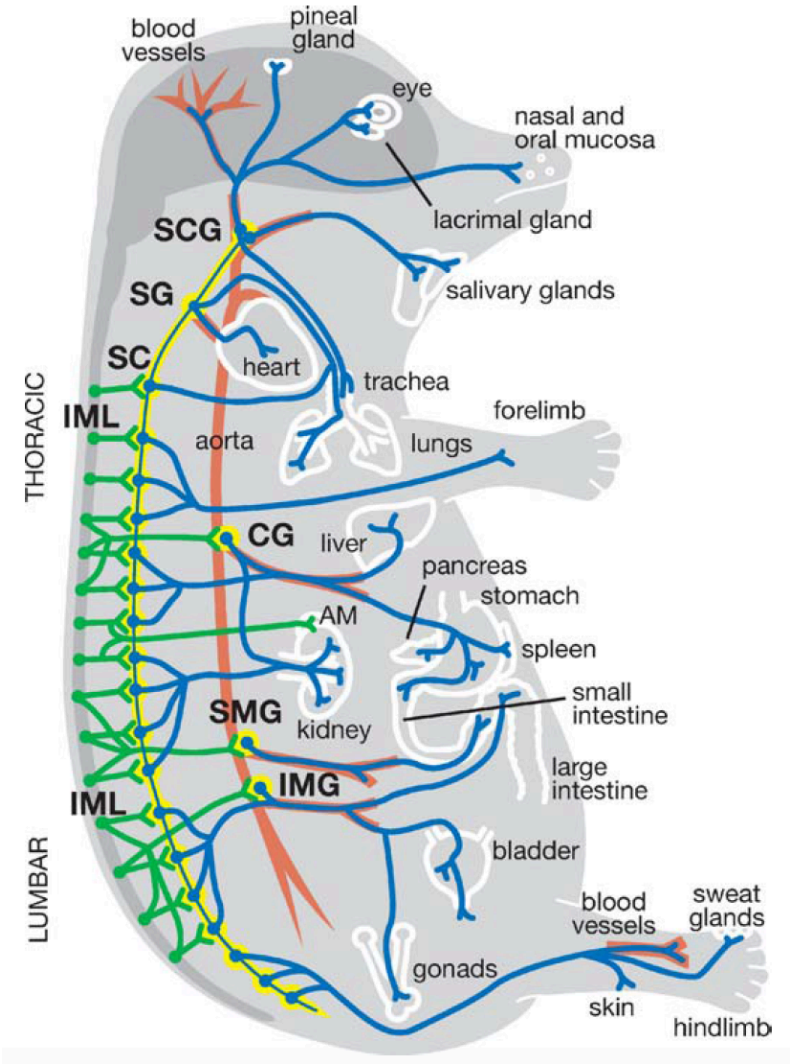

Figure 1.3: Schematic representation of the sympathetic nervous system in the mouse. Preganglionic sympathetic neurons (green) are located in the intermediolateral column (IML) at thoracic and lumbar levels of the spinal cord. These cholinergic preganglionic neurons make synaptic connections with post-ganglionic sympathetic neurons (yellow), which include the paravertebral ganglia consisting of the superior cervical ganglion (SCG), the stellate ganglion (SG) and the sympathetic chain (SC), and prevertebral ganglia, including the celiac ganglion (CG), the superior mesenteric ganglion (SMG), and the inferior mesenteric ganglion (IMG). The post-ganglionic sympathetic neurons project their axons (blue) to many different target tissues and glands. Figure adopted from (Glebova and Ginty, 2005).

RET plays several roles throughout SCG development. In *Ret*$^{-/-}$ mice, the SCG is mislocated, failing to correctly migrate to its proper rostal position (Enomoto et al., 2001). Additionally, sympathetic neuronal precursors display severe deficits in axonal projections, leading to a dramatic loss of target innervation (Enomoto et al., 2001). These developmental events are dependent on the proper signaling of ARTN/GFRα3/RET (Nishino et al., 1999, Honma et al., 2002).

In vitro models in which SCGs are isolated, dissociated and maintained as primary neuronal cultures have been used to study many aspects of basic RET biology including, but not limited to, trafficking within the cell, signal transduction and degradation (Tsui-Pierchala et al., 2002, Pierchala et al., 2006, Tsui and Pierchala, 2010, Calco et al., 2014). Additionally, GDNF and RET can support the survival of approximately 30% of neurons in primary sympathetic cultures (Yu et al., 2003).

The SCG has not only been used as a model to study RET, but it has also been used as a model to understand the molecules and underlying mechanisms important for programmed cell death. Development of the nervous system is an immensely complicated process, and requires coordination of multiple signaling events to create properly functioning circuits. To ensure the correct neural connections are formed, twice as many neurons are born than will be needed in adulthood, and those not making proper or strong enough connections will be eliminated through a process known as programmed cell death (PCD) (Oppenheim, 1991). In the mouse SCG, PCD occurs late perinatally through early postnatal stages (~E17-P20) (Bamji et al., 1998), and during this time, sympathetic neurons require nerve growth factor (NGF) for their survival (Levi-Montalcini, 1987). NGF is secreted in limited amounts from target tissues, and binds to

the receptor tyrosine kinase, TrkA, located on sympathetic axons (Glebova and Ginty, 2005). Upon binding, this NGF-TrkA complex is retrogradely trafficked to the cell body and promotes neuronal survival and inhibits apoptosis in these “winning” cells (Glebova and Ginty, 2005). However, axons that do not reach their target or arrive late to the target fail to receive enough NGF to support their survival, and these “losing” neurons will die via an intrinsic PCD mechanism (Glebova and Ginty, 2005, Conradt, 2009).

PCD in the SCG is complicated by the presence of the p75 neurotrophin receptor (p75NTR, NGFR, TNFRSF16). p75 is a promiscuous receptor, binding to TrkA and enhancing its survival signaling (Verdi et al., 1994, Barker and Shooter, 1994). However, in the absence of Trk receptors, p75 can function as a lower affinity receptor for members of the neurotrophin family, and signaling in this context can upregulate cell death-signaling pathways (Bamji et al., 1998, Aloyz et al., 1998). During PCD, “winning” neurons take advantage of this by releasing “competition factors” such as brain-derived neurotrophic factor (BDNF) that binds to p75 (Deppmann et al., 2008, Teng et al., 2010). The “winning” neurons that receive sufficient survival signals through NGF-TrkA/p75 signaling are protected from the competition factor, but the “losing” neurons are vulnerable to these cues and will die via an extrinsic BDNF-p75 mediated PCD mechanism (Bamji et al., 1998, Deppmann et al., 2008).

Interestingly, RET is expressed in a small subset of neurons in the SCG during this period of PCD *in vivo*, but its role during this time has not been elucidated. Experiments by Enomoto et al. demonstrated that RET is not required for the survival of these neurons during this time (Enomoto et al., 2001), leaving unanswered the question about the role of RET during this developmental time.

Unanswered questions in the field

Discovering how a cell manages the activity, abundance, cellular distribution and turnover of RTKs is essential to understanding the phenotypes that occur when these activities are dysregulated in pathophysiological conditions. For RET, factors have been identified that modulate transcription of the receptor, such as the DNA binding factors SPI, SP3 and EGR1, and additional factors that contribute to the tissue-specific expression of *RET* during development, such as SOX10, Phox2b and Pax3 (Andrew and et al., 2002, Leon and et al., 2009). Additionally, alternative splicing of the 3' region of *RET*, encoding the C-terminal tail, has allowed for different mechanisms of signal transduction, as well as the differential regulation of trafficking and turnover of the protein. Although much has been done to understand the biology and pathobiology of RET, there is still a need to better understand mechanisms of modulation of its signal transduction pathways.

Beyond the three isoforms that have been reported, additional transcripts have been observed in human pheochromocytomas that arise from alternative splicing in the 5' region of *RET*, as previously mentioned (Lorenzo et al., 1995). Interestingly, $RET^{\Delta E3}$ and $RET^{\Delta E345}$ transcripts are also present in normal kidney and brain tissues, but at much lower expression levels (Lorenzo et al., 1995). It is unknown whether these transcripts are translated to protein, if they interact with GLF-GFRα complexes in a ligand dependent manner, and what their potential biological and pathobiological functions may be. I explore these questions in Chapter 2.

Interestingly, p75 interacts with a number of receptors and thereby modulates

their activity and function. For example, p75 interacts with the receptor tyrosine kinase, TrkA, and increases the affinity of the NGF/TrkA interaction, thus enhancing pro-survival and pro-growth signaling (Davies et al., 1993). Preliminary data suggest that p75 interacts with RET and functions to enhance the differentiation and maintenance of non-peptidergic nociceptors in the dorsal root ganglion during development (Chris Donnelly, Kuo Fen Lee, unpublished data). However, we have found that pro-apoptotic signals can induce the association of p75 and RET in the sympathetic nervous system. Surprisingly, RET forgoes its pro-growth and pro-survival signaling to enhance the function of p75-mediated cell death, which is the first time that an RTK has been shown to enhance the functions of TNF-receptor cell death signaling pathways. In Chapter 3, I explore the interactions of p75 and RET both *in vitro* and *in vivo,* and investigate the role these proteins play during programmed cell death in the sympathetic nervous system. The implications of the results in Chapters 2 and 3 for neural development and pathophysiology of neuroendocrine tissues will be discussed in Chapter 4.

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CHAPTER 2

EXON SKIPPING IN *RET* ENCODES NOVEL ISOFORMS THAT DIFFERENTIALLY REGULATE RET SIGNAL TRANSDUCTION

Summary

RET, a receptor tyrosine kinase that is activated by the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), plays a crucial role in the development and function of the nervous system, and additionally is required for kidney development and spermatogenesis. *RET* encodes a transmembrane receptor that is 20 exons long and produces two known protein isoforms differing in C-terminal amino acid composition, referred to as RET9 and RET51. Studies of human pheochromocytomas identified two additional novel transcripts involving the skipping of exon 3 or exons 3, 4, and 5 and are referred to as $RET^{\Delta E3}$ and $RET^{\Delta E345}$, respectively. Here we report the presence of $Ret^{\Delta E3}$ and $Ret^{\Delta E345}$ in zebrafish, mice, and rats, and show that these transcripts are dynamically expressed throughout development of the CNS, PNS and kidneys. We further explore the biochemical properties of these isoforms, demonstrating that, like full-length RET, $RET^{\Delta E3}$ and $RET^{\Delta E345}$ are trafficked to the cell surface, interact with all four GFRα co-receptors, and have the ability to heterodimerize with full-length RET. Signaling experiments indicate that $RET^{\Delta E3}$ is phosphorylated in a similar manner to full-length RET. $RET^{\Delta E345}$, in contrast, displays higher baseline autophosphorylation, specifically on the catalytic tyrosine, Tyr905, and also on one of the most important

signaling residues, Tyr1062. These data provide the first evidence for a physiologic role of these isoforms in RET pathway function.

Introduction

RET is a receptor tyrosine kinase that is critical for kidney morphogenesis, spermatogenesis, and development of the nervous system (Airaksinen and Saarma, 2002, Baloh et al., 2000, Meng et al., 2000, Naughton et al., 2006). RET is activated by a family of four growth factors known as the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), which includes GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN) (Airaksinen and Saarma, 2002). Each GFL binds to one of four cognate glycosylphosphatidylinositol (GPI) anchored co-receptors known as the GDNF Family Receptor-αs (GFRαs) (Airaksinen et al., 1999, Baloh et al., 2000). The GFL-GFRα complex binds to RET, inducing RET dimerization and subsequent autophosphorylation on multiple tyrosine residues within the intracellular tyrosine kinase domain. This enhances tyrosine kinase activity and initiates the association of adaptor proteins and enzymes that trigger multiple second messenger cascades (Wells, 2009).

The presence of two major Ret isoforms, RET9 and RET51, has been extensively described in the literature, and a third isoform, RET43 has also been observed in humans (de Graaff et al., 2001, Carter et al., 2001, Tahira et al., 1990). *Ret* is 20 exons long, and *Ret9* and *Ret51* transcripts differ in alternative splicing of intron 19. Intron 19 in the *Ret51* transcript is excised properly, whereas in the *Ret9* transcript, the intron is retained, changing the reading frame and inserting a premature stop codon into the amino acid sequence. This creates a unique 9 amino acid C-terminal sequence

for RET9 and a unique 51 amino acid C-terminal sequence for RET51. Interestingly, these two different isoforms display marked differences in their degradation and function (de Graaff et al., 2001, Tsui-Pierchala et al., 2002b, Tsui and Pierchala, 2010).

Three additional *Ret* transcripts have been reported in various tumor sources as well as adult human tissues (Lorenzo et al., 1995). These novel transcripts are a product of exon skipping in the 5' region of *RET*, which encodes for the extracellular domain of the protein. Skipping of exons 3 (*RET*$^{\Delta E3}$) or exons 3, 4 and 5 (*RET*$^{\Delta E345}$) gives rise to transcripts that encode for full-length Ret proteins, but with deletions in the extracellular domain, specifically cadherin-like domain 1 (CLD1), or CLD1-3, respectively. These deletions are hypothesized to change the extracellular domain structure as well as binding to GFL-GFRα complexes, and may impact overall stability of the proteins. The skipping of exons 3 and 4 results in a frameshift that culminates in a premature stop. This protein encodes only a small portion of the RET extracellular domain with no transmembrane or intracellular domain. Because this transcript is likely to be subjected to nonsense-mediated decay, it is unlikely to be involved in RET signal transduction.

Here we show that *Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ transcripts are conserved in vertebrates, and that the mRNA and proteins of these splice variants are expressed throughout the nervous system in mice. We also show that these isoforms are trafficked to the cell surface and that both isoforms interact with all four GFRαs. Additionally, we find that RET$^{\Delta E3}$ is phosphorylated to a similar level as full-length RET. However, RET$^{\Delta E345}$ displays higher baseline autophosphorylation, specifically on the catalytic tyrosine, Tyr905, and also on an additional signaling tyrosine, Tyr1062. Taken together, these

isoforms may have unique and important unidentified roles in the development and maintenance of the nervous system and kidneys, as well as in the pathophysiology of neuroendocrine gland diseases.

Results

Exons 3, 4, and 5 of Ret are not highly conserved, but $RET^{\Delta E3}$ and $RET^{\Delta E345}$ transcripts are observed in several organisms.

It has been previously described that the intracellular domain of RET, which contains a tyrosine kinase domain, is more highly conserved than the extracellular domain (Anders et al., 2001). To better understand the level of conservation of amino acids encoded by each exon, we compared sequences from zebrafish (*Danio rerio*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), and humans (*Homo sapiens*). We found that the entire intracellular domain was more than 75% conserved at the amino acid level. This was not surprising since mutations in many of these highly conserved regions give rise to loss of function (e.g. Hirschsprung's disease) or gain of function (e.g. multiple endocrine neoplasia type 2A and 2B) phenotypes (Fig. 2.1A). Interestingly, exons 3, 4 and 5 are less than 50% conserved between these species. While this lack of conservation is suggestive that these regions of RET are not as functionally important, it could also be interpreted as allowing for amino acid flexibility between the different RET isoforms.

Our initial sequence analysis suggested that $RET^{\Delta E3}$ and $RET^{\Delta E345}$ transcripts could be expressed in additional vertebrates other than *homo sapiens*. These transcripts were originally identified in human kidney and substantia nigra fetal tissues

(Lorenzo et al., 1995). Sequence analysis demonstrated that exon skipping also encodes potential full-length transcripts in zebrafish, mice and rats (Fig. 2.1A). To determine whether these novel transcripts were expressed in these organisms, species specific primers were created to identify each of the *Ret* transcripts: full-length *Ret,* $Ret^{\Delta E3}$ and $Ret^{\Delta E345}$. RT-PCR analysis identified the presence of $Ret^{\Delta E3}$ and $Ret^{\Delta E345}$ from 48 hpf zebrafish embryos, E19.5 rat dorsal root ganglia, and E18.5 mouse spinal cord (Fig. 2.1B). Sequence analysis confirmed these amplicons to be $Ret^{\Delta E3}$ and $Ret^{\Delta E345}$ (data not shown). This is the first time these transcripts have been identified in vertebrates other than *Homo sapiens*. Taken together, these data indicate that 5' exon skipping is conserved and results in the expression of $Ret^{\Delta E3}$ and $Ret^{\Delta E345}$ transcripts.

***Exon skipping creates large deletions in the extracellular domain of* RET.**

To understand the impact deletions in the extracellular domain would have on RET, we analyzed the deletions in terms of a CLD1-4 model derived from electron microscopy and SAXS analyses (Goodman et al., 2014). The main consequence of deletion of exon 3 is the removal of half of CLD1 (residues 113 to 150) and half of CLD2 (residues 154 to 208). Each domain adopts a β-sandwich cadherin fold comprised of two β-sheets, separated by numerous buried hydrophobic residues. Elimination of exon 3 removes one sheet from CLD1 and a several β-strands from both sheets of CLD2 (Fig. 2.1C). Therefore, it is likely that both CLD domains are substantially perturbed leaving β-strands and exposed hydrophobic residues. Exon 3 removal also eliminates a highly constrained Cis-Pro loop (cysteine137-cysteine142) unique to higher vertebrate RET sequences. The portion of CLD1-2 removed coincides with regions stabilizing the

clam-shell arrangement of CLD1-2 (Kjaer et al., 2010). This would result in a more open and extended arrangement for the remaining parts of CLD1 and CLD2 with much greater flexibility between the domains. An odd number of cysteine residues (3 in total) are deleted by removing exon 3 including the Cys157-Cys197 disulfide. Cys166 is also removed leaving its partner Cys243 unpaired but buried. The two unpaired cysteines, Cys87 and Cys216, that constitute a known folding bottleneck in wild type RET are left untouched. We expect $RET^{\Delta E3}$ to be a less stable, transmembrane protein with a short half-life that retains at least some ligand-binding properties.

For $RET^{\Delta E345}$, removal of exons 3, 4 and 5 effectively eliminates much of CLD1-3 but leaves CLD4-CRD intact (Fig. 2.1C). This region by itself was shown to be insufficient to bind ligand but retains at least one important binding epitope (i.e. necessary for ligand binding but not sufficient) (Goodman et al., 2014). Many receptor tyrosine kinases have extracellular domains that have auto-inhibitory functions in the absence of ligand. Deletion of such regions can lead to auto-activation in a ligand-independent manner. By analogy with these other systems, removal of CLD1-3 could leave a truncated form of RET that would be constitutively activated in the absence of ligand and likely would be unresponsive to the GFLs.

$Ret^{\Delta E3}$ and $Ret^{\Delta E345}$ are co-expressed in several tissues with Ret throughout development.

To determine where and when *$Ret^{\Delta E3}$* and *$Ret^{\Delta E345}$* transcripts are expressed, and whether their expression levels change over time, we analyzed tissues in which full length *Ret* is expressed at several developmental time points. Within the central

nervous system, we analyzed the brain, as signaling via Ret is important for the survival of central noradrenergic neurons and dopaminergic neurons in the ventral midbrain, as well as the spinal cord where GDNF/GFRα1/Ret signaling is critical for axon guidance into the hindlimbs and survival of γ-motor neurons (Arenas et al., 1995, Horger et al., 1998, Bonanomi et al., 2012, Shneider et al., 2009, Gould et al., 2008, Kramer et al., 2006). In the peripheral nervous system, we analyzed sensory neurons of the dorsal root ganglia (DRG) where NRTN/GFRα2/Ret signaling is important for the survival and maintenance of mechanoreceptors during development (Luo et al., 2009, Bourane et al., 2009). Additionally, shortly after birth, a subclass of nociceptors transition from being TrkA positive to expressing GFRα1/Ret to support the development and survival of nonpeptidergic nociceptors (Molliver et al., 1997, Luo et al., 2007, Franck MC, 2011). Sympathetic neurons of the superior cervical ganglia (SCG) were also analyzed as it has been well established that ARTN/GFRα3/Ret signaling is necessary for proper sympathetic chain migration and axon guidance during development (Nishino et al., 1999, Enomoto et al., 2001, Honma et al., 2002). Lastly, the kidney was analyzed because GDNF/GFRα1/Ret signaling is crucial for ureteric bud branching and morphogenesis of the kidney (Moore et al., 1996, Pichel et al., 1996, Sanchez et al., 1996, Cacalano et al., 1998, Enomoto et al., 1998). Using qPCR, we examined the relative expression of *Ret, Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ in each of these tissues in mice and found they were detected in all five tissues. The relative expression of *Ret, Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ was lower in brain, spinal cord, and kidney (Fig. 2.2A) compared to the DRG and SCG (Fig. 2.2B). Interestingly, in the spinal cord, DRG and SCG there was a significant increase of *Ret*$^{\Delta E3}$ expression at E19.5 that preceded a significant increase of

Ret at P3. The expression of all three transcripts was highest in the DRG, particularly at E19.5 and P3, when the IB4+ subpopulation of nociceptors is emerging.

5' and 3' alternative splicing of Ret transcripts are not mutually exclusive.

Alternative splicing of intron 19 in *Ret* allows for the translation of two isoforms of Ret, RET9 and RET51. We sought to determine whether alternative splicing could occur simultaneously, both 5' and 3' in *Ret* transcripts, allowing for increased diversity of encoded RET proteins. cDNA was isolated from E19.5 mouse DRGs, as we found this tissue to have the highest relative expression of *Ret, Ret$^{\Delta E3}$,* and *Ret$^{\Delta E345}$* (Fig. 2.2B). Forward primers specific for each 5' splicing event were paired with reverse primers that would detect either *Ret9* or *Ret51* alternative splicing. Full-length *Ret9* and *Ret51* transcripts were detected, as expected, at 3.33 kb and 4.38 kb, respectively. We observed *Ret$^{\Delta E3}$* and *Ret$^{\Delta E345}$* with intron 19 retention, thus encoding *Ret9$^{\Delta E3}$* and *Ret9$^{\Delta E345}$* transcripts with amplicons of 3.05 kb and 2.61 kb. However, we were only able to detect *Ret$^{\Delta E345}$* with a properly excised intron 19 at an amplicon size of 3.66 kb, encoding a *Ret51$^{\Delta E345}$* transcript. While we were unable to detect a *Ret51$^{\Delta E3}$* transcript (4.10 kb), this is likely due to the generally low expression levels of *Ret$^{\Delta E3}$.* Taken together, these data indicate that the *Ret* locus encodes for at least three previously unidentified transcripts, and at least five total Ret isoforms - *Ret9, Ret9$^{\Delta E3}$, Ret9$^{\Delta E345}$, Ret51* and *Ret51$^{\Delta E345}$*.

RET$^{\Delta E345}$ proteins are detected in vivo.

Although we can detect *Ret$^{\Delta E3}$* and *Ret$^{\Delta E345}$* mRNA transcripts, which are likely

being processed for translation, we sought to detect the presence of these isoforms at the protein level. Plasmids encoding human *RET51*, *RET51*$^{\Delta E3}$ and *RET51*$^{\Delta E345}$ were transfected into NIH/3T3 cells, and lysates were run as size standards to aid in detecting Ret isoforms of the appropriate molecular weights from E18.5 mouse brain. The molecular weight of native RET51 protein is 120 kDa, but due to post-translational modifications, specifically glycosylation of the extracellular domain, RET51 has a molecular weight of ~180 kDa. The native molecular weights of RET$^{\Delta E3}$ and RET$^{\Delta E345}$ were predicted to be 109 kDa and 93 kDa, respectively. In transfected cells we consistently observe the mature, processed proteins to have molecular weights of ~150 kDa (RET$^{\Delta E3}$) and ~125 kDa (RET$^{\Delta E345}$) (Fig. 2.2D).

Whole brains from *Ret*$^{+/+}$ or *Ret*$^{-/-}$ E18.5 littermate mice were lysed, divided in two, and immunoprecipitations were performed to isolate either RET9 or RET51 proteins. Although the qPCR data suggested that *Ret* transcripts have an overall lower relative expression in the brain compared to PNS tissues, the brain was chosen for analysis because of the abundant amount of protein that could be isolated. *Ret*$^{-/-}$ mice were used as a control to demonstrate specificity of the Ret immunoblotting, and to show the presence of RET proteins at the same molecular weight as RET$^{\Delta E3}$ and RET$^{\Delta E345}$. Since we previously observed more highly expressed *Ret9*$^{\Delta E345}$ and *Ret51*$^{\Delta E345}$ transcripts compared to those of *Ret*$^{\Delta E3}$, we hypothesized that we would be most able to detect both RET9$^{\Delta E345}$ and RET51$^{\Delta E345}$ proteins. By immunoblotting for RET, we were able to detect full-length RET9 and RET51 proteins, as expected (Fig. 2.2D). Additionally, we observed RET9 and RET51 bands with similar molecular weights to RET$^{\Delta E345}$, suggesting the presence of RET9$^{\Delta E345}$ and RET51$^{\Delta E345}$ proteins in

mouse brain (Fig. 2.2D). Because the antibody used for immunoprecipitations of RET9 and RET51 are to the C-terminus, and the RET antibody for immunoblotting is to the common N-terminal region, this RET protein is unlikely to be a degradation product. However, we cannot exclude the possibility that this is an immature, non-glycosylated full-length RET protein.

RET$^{\Delta E3}$ and RET$^{\Delta E345}$ are trafficked to the cell surface

To examine the biochemical properties of RET$^{\Delta E3}$ and RET$^{\Delta E345}$, we transitioned to an *in vitro* system for these experiments. Although RET$^{\Delta E3}$ and RET$^{\Delta E345}$ encode proteins that may be capable of signaling, this does not guarantee that they will be correctly trafficked to the cell surface for activation. To determine if RET$^{\Delta E3}$ and RET$^{\Delta E345}$ are trafficked to the plasma membrane, NIH/3T3 cells were transfected with C-terminally epitope tagged *Ret* constructs that also contain an IRES-GFP to serve as an indicator for transfected cells. Taking advantage of a RET antibody specific for the extracellular domain and using a HA antibody to label the C-terminus of RET, we were able to selectively visualize RET located on the plasma membrane. Full-length RET was detected on the cell surface of transfected NIH/3T3 cells, as expected (Fig. 2.3K). Permeabilization revealed the C-terminal HA tag, confirming the integrity of the plasma membrane (Fig. 2.3P). Similar to full-length RET, both RET$^{\Delta E3}$ and RET$^{\Delta E345}$ were trafficked to the cell surface as determined with the extracellular RET antibody in non-permeabilized cells (Fig. 2.3S, Fig. 2.3A'). Transfection of GFP alone showed no labeling with the RET and HA antibodies, regardless of permeabilization, confirming the specificity of our immunostaining (Fig. 2.3A-H). Thus, RET$^{\Delta E3}$ and RET$^{\Delta E345}$ are

trafficked properly to the cell surface where they can participate in GFL-mediated activation.

RET$^{\Delta E3}$ and RET$^{\Delta E345}$ bind to GFRα1, GFRα2, GFRα3 and GFRα4

Previous structural analysis (Fig. 2.1C) suggested that interactions between RET$^{\Delta E3}$ and the GFRαs may be unstable and that RET$^{\Delta E345}$ would be unlikely to bind to the co-receptors due to deletions of important binding residues in CLD1 and CLD3. To confirm these results, we co-transfected HEK293T cells with *RET$^{\Delta E3}$* or *RET$^{\Delta E345}$* and HA epitope tagged *GFRα* constructs. Immunoprecipitations were performed using a stringent immunoprecipitation buffer (modified RIPA buffer) to eliminate any weak, non-specific interactions. Surprisingly, we found that both RET$^{\Delta E3}$ and RET$^{\Delta E345}$ bind to each of the four GFRαs (Fig. 2.4). Co-transfection of the GFRαs with TrkA, another receptor tyrosine kinase, was performed as a negative control to show selectivity of binding between the GFRαs and RET (Fig. 2.4). As expected, TrkA did not associate with any of the GFRαs. There were no apparent differences between the four different GFRαs in regard to the extent of their association with RET$^{\Delta E3}$ and RET$^{\Delta E345}$, suggesting that these splice variants could potentially be activated by all four GFLs (Fig. 2.4).

RET$^{\Delta E3}$ and RET$^{\Delta E345}$ heterodimerize with full-length RET

In addition to being potential signaling molecules on their own, we observed that these transcripts are normally expressed in tissues where full-length RET is also expressed (Fig. 2.2). Although these isoforms may be present in different cells within these tissues, these observations raise the question of whether RET$^{\Delta E3}$ and RET$^{\Delta E345}$

could heterodimerize with full-length RET and affect its function. To test this possibility, HEK293T cells were co-transfected with HA epitope tagged *RET51* and untagged *RET$^{\Delta E3}$* or *RET$^{\Delta E345}$*. Immunoprecipitations were performed using a HA antibody to select for full-length RET51 and determine if the different isoforms could interact with one another (Fig. 2.5A). In an additional experiment, FLAG tagged *RET51$^{\Delta E345}$* (FLAG:: RET51$^{\Delta E345}$) was co-transfected with *RET51* and *RET51$^{\Delta E3}$*, and immunoprecipitations were performed using a FLAG antibody to select for RET51$^{\Delta E345}$ (Fig. 2.5B). We found that both RET51$^{\Delta E3}$ and RET51$^{\Delta E345}$ associated with full-length RET51, and that RET51$^{\Delta E3}$ and RET51$^{\Delta E345}$ could also bind to one another (Fig. 2.5). This is consistent with former experiments suggesting that overexpression of RET proteins allows for ligand-independent dimerization via the transmembrane domain (Kjaer et al., 2006).

RET$^{\Delta E345}$ has increased ligand-independent activation compared to RET and RET$^{\Delta E3}$.

In order to determine levels of activation of RET$^{\Delta E3}$ and RET$^{\Delta E345}$, the basal phosphorylation of tyrosine residues of the splice variants were compared to full-length RET phosphorylation. Overexpression of the RET isoforms *in vitro,* either in the presence or absence of a GFRα, allows for ligand-independent dimerization and activation of RET. The level of activation can be assessed by determining the summated level of all of the phosphorylated tyrosines using a pan phosphotyrosine antibody, or individual tyrosine residues can be analyzed using residue-specific RET phosphotyrosine antibodies. We evaluated the level of total phosphotyrosine (P-Tyr) between the RET isoforms, as well as individually evaluating phosphorylation at two

tyrosine residues of RET, Tyr905 and Tyr1062. Tyrosine 905 in the RET kinase domain is an autocatalytic tyrosine that is conserved in many receptor tyrosine kinases (RTKs) and is a binding site for GRB7 and GRB10 (Durick et al., 1996, Pandey et al., 1995, Pandey et al., 1996). Additionally, mutation of tyrosine 905 to phenylalanine (Y905F) impairs the kinase activity of RET (Iwashita et al., 1996). Tyrosine 1062 is a binding site for SHC, Dok4/5, IRS-1, and FRS-2 when phosphorylated and is a binding site for Enigma in a phosphorylation-independent manner (Durick et al., 1996, Lorenzo et al., 1997, Asai et al., 1996, Arighi et al., 1997, Grimm et al., 2001, Hayashi et al., 2000, Kurokawa et al., 2001, Melillo et al., 2001b, Melillo et al., 2001a). Interaction between Tyr1062 and these adaptor proteins leads to activation of Ras/ERK and PI3K/AKT pathways (Asai et al., 1996, Arighi et al., 1997, Besset et al., 2000, Kurokawa et al., 2001, Melillo et al., 2001b).

To evaluate RET phosphorylation, NIH/3T3 cells were transfected with *RET51*, *RET51*$^{\Delta E3}$ or *RET51*$^{\Delta E345}$ constructs in the presence or absence of *GFRα1*, and the total levels of phosphorylated RET, or the level of phosphorylation at Tyr905 and Tyr1062, were assessed. To analyze total P-Tyr levels of RET, immunoprecipitations of RET51 were performed followed by immunoblotting for P-Tyr (Fig. 2.6A). An increase in the level of RET$^{\Delta E345}$ phosphorylation compared to full-length RET was observed, but this was not statistically significant (p=0.0837). We did detect, however, a significant increase in the P-Tyr level of RET$^{\Delta E345}$ co-expressed with GFRα1 compared to full-length RET co-expressed with GFRα1 (p=0.0366). The phosphorylation of RET$^{\Delta E3}$ co-expressed with GFRα1, or expressed alone, was not significantly different than full-length Ret (p=0.6231).

To determine levels of phospho-Tyr905 and phospho-Tyr1062, pre-IP lysates were collected from samples in the previous experiments, and immunoblotting was performed using antibodies specific for these two phosphorylated tyrosine residues (Fig. 2.6B). Levels of phospho-Tyr905 and phospho-Tyr1062 were significantly elevated for $RET^{\Delta E345}$ compared to RET (p=0.0439, p=0.0411 respectively), and also for $RET^{\Delta E345}$ co-expressed with GFRα1 compared to full-length RET co-expressed with GFRα1 (p=0.0106, p=0.0096, respectively). However, levels of phospho-Tyr905 and phospho-Tyr1062 were unchanged for $RET^{\Delta E3}$ compared to RET, and also for $RET^{\Delta E3}$ co-expressed with GFRα1 compared to full-length RET co-expressed with GFRα1. Taken together, these data suggest that $RET^{\Delta E3}$ has a similar activation level to full-length RET, whereas $RET^{\Delta E345}$ has an elevated activation level compared to RET in a ligand-independent manner. Additionally, the lower total phosphotyrosine level for $RET^{\Delta E345}$ compared to phospho-Tyr^{905} and phospho-Tyr^{1062} suggests that one or more of the other tyrosine residues in RET is not phosphorylated as highly as Tyr^{905} and Tyr^{1062} compared to full-length RET.

Discussion

Alternative splicing of precursor mRNA is one of many processes that mediates gene regulation in metazoans, and the frequency of alternative splicing increases with species complexity (Pan et al., 2008, Wang et al., 2008). For example, of the approximately 25,000 genes encoded by the human genome, ~95% produce transcripts that are alternatively spliced (Wang et al., 2008, Pan et al., 2008). By expanding the

proteome through the synthesis of various protein isoforms, alternative splicing allows for increased protein diversity with isoforms performing different biological functions.

Here we provide evidence for two alternative splicing events in *Ret* that, in combination with *Ret9* or *Ret51* alternative splicing, give rise to at least five *Ret* transcripts (Fig. 2.2C). The splicing events for $RET^{\Delta E3}$ and $RET^{\Delta E345}$ have only been previously described in human tissues (Lorenzo et al., 1995). Because an additional *Ret* transcript, *RET43*, is only expressed in humans, we examined the expression of $RET^{\Delta E3}$ and $RET^{\Delta E345}$ in additional vertebrate tissues to determine whether these might also be human specific transcripts (Myers et al., 1995, Carter et al., 2001). To this end, we discovered that $RET^{\Delta E3}$ and $RET^{\Delta E345}$ are not human-specific *RET* transcripts, but are also expressed in normal developing tissues of zebrafish, mice and rats (Fig. 2.1C). Functionally important transcripts are transcriptionally conserved between species, arguing that $RET^{\Delta E3}$ and $RET^{\Delta E345}$ have physiologically important functions.

Analysis of an experimentally validated structural model for the RET cadherin-like domains 1-4 was made to evaluate the consequence of exon deletions of *RET* alternative splicing on the ligand binding and activation characteristics of $RET^{\Delta E3}$ and $RET^{\Delta E345}$ (Fig. 2.1B). Previous experiments indicate that the GDNF-GFRα1 complex binds directly to CLD4 and the CRD (Amoresano et al., 2005).This suggested that both $RET^{\Delta E3}$ and $RET^{\Delta E345}$ should be able to bind to the GDNF-GFRα1 complex, and indeed this interaction was observed not only for GFRα1, but also for GFRα2, GFRα3 and GFRα4 (Fig. 2.4). While it was originally thought that CLD1-3 was involved in direct binding from results obtained through mutagenesis studies, a direct interaction between CLD1-3 and the GDNF-GFRα1 complex has not been observed (Amoresano et al.,

2005, Kjaer and Ibanez, 2003). Instead, it has been proposed that CLD1-3 contributes to the stability of the tertiary structure of the GDNF-GFRα1-RET complex by forming a secondary dimerization site to trap the GDNF-GFRα1 binary complex (Goodman et al., 2014). These studies predict that $RET^{\Delta E3}$, which forms a fused CLD1-CLD2 region, likely gives rise to a less stable tertiary GDNF-GFRα1-RET complex compared to full-length RET. This may result in a receptor variant with differential ligand binding specificities. Fibroblast growth factor receptors (FGFRs) are a subfamily of four RTKs that are receptors for fibroblast growth factors (FGFs), of which there are 18 ligands (Turner and Grose, 2010). Alternative splicing of the FGFRs in the region coding for the extracellular ligand-binding domain of the receptors allows for differential ligand binding. It is possible that the alternative splicing responsible for $RET^{\Delta E3}$ similarly modifies GFL-GFRa affinity. Alternatively, the truncated extracellular domain of $RET^{\Delta E3}$ may cause alterations in the conformational changes that occur upon GFL-GFRa binding that induces receptor autophosphorylation. This would cause different autophosphorylation kinetics in $RET^{\Delta E3}$ compared to full-length *RET* and, therefore, differential activation of downstream second messenger cascades. Biochemical experiments using an *in vitro* system that allows for the direct ligand activation of $RET^{\Delta E3}$ or full-length *RET* in isolation will be necessary to distinguish between these possibilities.

The large deletion in the extracellular domain of $RET^{\Delta E345}$, including the Ca^{2+} binding domain which is important for RET folding and ligand-dependent signaling, is predicted to have dramatic effects on $RET^{\Delta E345}$ activation. Indeed, it is likely that $RET^{\Delta E345}$ functions as a constitutively active form of RET, consistent with the mechanistic data shown in Figure 2.6. Autophosphorylation of $RET^{\Delta E345}$ is increased

compared to full-length RET in the presence and absence of GFRα1 *in vitro* (Fig. 2.6B). Constitutively active RTKs have not been found to exist in normal tissues, and usually arise from germline and somatic mutations where they are most commonly found to cause neoplasias. Activating mutations in RET have been shown to be responsible for multiple endocrine neoplasia, type 2, including neoplasias such as medullary thyroid carcinoma and pheochromocytomas (Mulligan, 2014). Unlike other constitutively active forms of RET, $RET^{\Delta E345}$ may be post-transcriptionally or translationally regulated *in vivo* in a manner that is not recapitulated in the *in vitro* system used here. In this way, the signaling of $RET^{\Delta E345}$ may be under tight regulation in order for this isoform to perform specific functions *in vivo*. Importantly, this is the first constitutively active RTK identified in normal tissues (Fig. 2.2).

To interrogate the functions of $RET^{\Delta E3}$ and $RET^{\Delta E345}$ in primary neurons and tissues, we attempted to generate isoform specific antibodies. Likewise, we also tried to create siRNAs to selectively knockdown $Ret^{\Delta E3}$ and $Ret^{\Delta E345}$ transcripts. Neither of these experimental approaches were successful at targeting $RET^{\Delta E3}$ and $RET^{\Delta E345}$ without also affecting full-length RET. Therefore, in order to determine the physiologic functions of $RET^{\Delta E3}$ and $RET^{\Delta E345}$, a transgenic approach in a vertebrate will be required to selectively remove these individual transcripts. This could be accomplished by deleting specific introns and fusing the flanking exons in frame to inhibit exon skipping (e.g. remove intron 3 and fuse exons 2 and 3 together to inhibit alternative splicing for $Ret^{\Delta E3}$). How this approach would affect RET expression, and other splicing events, however, is unknown. In addition, due to the invariably coincident expression of $Ret^{\Delta E3}$ and $Ret^{\Delta E345}$ in the same tissues as *Ret*, it is possible that full-length RET may

compensate for the functions of $RET^{\Delta E3}$ and $RET^{\Delta E345}$. As an alternative approach, production of knock-in animals in which only $Ret^{\Delta E3}$ or $Ret^{\Delta E345}$ is expressed would determine whether these isoforms are capable of supporting the normal development of the nervous system and kidneys.

Lastly, to gain a mechanistic understanding of the factors responsible for the alternative splicing of *Ret,* it would be interesting to explore RNA motifs encoding exon splicing enhancers within the locus that impact the formation of $Ret^{\Delta E3}$ and $Ret^{\Delta E345}$. Understanding the spliceosome machinery involved in this process may help us understand why the expression of these transcripts are elevated in tumors, such as in pheochromocytomas (Lorenzo et al., 1995). Overall, it may be necessary to return to a disease model, such as neuroendocrine gland tumors where these *RET* transcripts were originally identified, and use the pathobiology to understand the signaling capacities of $RET^{\Delta E3}$ and $RET^{\Delta E345}$, in order to identify their functions in normal development.

EXPERIMENTAL PROCEDURES

Molecular modeling - This analysis was performed as previously described (Goodman et al., 2014). Models of cadherin-like domains 1 to 4 were taken from PDB code 4UX8 defined by a combination of EM and SAXS analyses. The figure was made using PyMol software (Schrodinger, USA).

RNA isolation, RT-PCR, qPCR - Total RNA was isolated from zebrafish, mouse or rat tissues using TRIzol reagent (Thermo Fisher Scientific) according to manufacturer's

instructions. cDNA synthesis was performed from 1 µg of total RNA to generate polyA first-strand cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Life Technologies). For RT-PCR reactions with amplicons less than 200 bp, GoTaq Green master mix (Promega) and species-specific oligonucleotide primers for *Ret*, $Ret^{\Delta 3}$, and $Ret^{\Delta 345}$ were used. RT-PCR reactions with amplicons greater than 200 bp were performed using the Phusion High-Fidelity DNA Polymerase kit (NEB) following the manufacturer's instructions. qPCR was performed using FastStart Universal SYBR Green Master Mix (Roche) and run on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). Quantitative analyses were performed by calculating the ΔCt (Ct of transcript divided by Ct of the housekeeping gene, *actin*) for each gene analyzed and transforming that value to $\log_2$. These values are reported as the relative expression.

Primer sequences: The primers used for this study are as follows: ZF.RetFL.F: 5'-TCG TAG TTT ACG CAG CGG CTC A-3'; ZF.RetFL.R: 5'-TCG CGA TTT TCA GTG ATG TG-3'; ZF.Ret3.F: 5'-CAG TAG TTT ACG ATC TTC TGT ACC G-3'; ZF.Ret3.R: 5'-TTC AGT GTC AGT CCC GTT GA-3'; ZF.Ret345.F: 5'-CAG TAG TTT ACA GCT GAA ACT CAG TC-3'; ZF.Ret345.R: 5'-TGA CAT TGG AGA AGC GAA TG-3'; Mouse.RetFL.F: 5'-AGC ATC CGC AAT GGT GGT TT-3'; Mouse.RetFL.R: 5'-TGT TCT CCC TGA CTC GGA AG-3'; Mouse.Ret3.F: 5'-AGC ATC CGC AGG GAT AGT CT-3'; Mouse.Ret3.R: 5'-ACA CTG TCA CTG GGA AGG AC-3'; Mouse.Ret345.F: 5'-AGC ATC CGC AAG CTG ATT CT-3'; Mouse.Ret345.R: 5'-TTC ACT GGG AAG GAG TAG GC-3'; Rat.RetFL.F: 5'-AGC ATC CGC AAT GGC GGC TT-3'; Rat.RetFL.R: 5'-TAG CAT GCG

GAA CTG GTA GA-3'; Rat.Ret3.F: 5'-AGC ATC CGC AGG GAC GGT CT-3'; Rat.Ret3.R: 5'-CCG CTT AAA CTC CAC CAC AG-3'; Rat.Ret345.F: 5'-AGC ATC CGC AAG CTG GTT CT-3'; Rat.Ret345.R: 5'-TAG GCC ATG GGT AGG TTC AG-3'; Mouse.Ret9.R: 5'-ATT TAC TGT CCA TTG CAA GGC-3'; Mouse.Ret51.R: 5'-CCT ATC AGT GCT TTA AGT CTG-3'.

Mice – Wild-type C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Ret*$^{-/-}$ mice have been previously described and were maintained in a mixed genetic background (Schuchardt et al., 1994). For timed matings, noon of the day on which a vaginal plug was detected was considered as E0.5. All housing and procedures performed on mice were approved by the University of Michigan Animal Care and Use Committee (UCUCA).

Detergent extraction and preclearing of whole tissues – Tissues were harvested, placed in a 2.0 mL tube with 250 µL immunoprecipitation (IP) buffer lacking NP-40 (Tris-buffered saline [TBS], pH 7.4, 10% glycerol, 500 µM sodium vanadate and protease inhibitors) along with a steel grinding ball (5mm, 69989, Qiagen, Valencia, CA) and mechanically homogenized using a TissueLyzer II (Qiagen) for 2 minutes at a frequency of 20 Hz. Following this, 250 µL of 2% NP-40-containing IP buffer was added to homogenates and incubated for one hour at 4°C under gentle agitation. Homogenates were then centrifuged for 10 minutes at 16,000 x g and subjected to an initial preclearing step with protein A and protein G alone at 4°C for two hours under gentle

agitation. A second preclearing step was performed with protein A, protein G and a species-matched nonspecific control IgG for two hours under gentle agitation.

Immunoprecipitations and immunoblotting - Plates were placed on ice, gently washed twice with 1X PBS, pH 7.4, and lysed with modified RIPA buffer (TBS, pH 7.4, 10% glycerol, 1% Triton X-100, 0.1% SDS, 500 μM sodium vanadate and protease inhibitors). Proteins were immunoprecipitated using anti-RET9 (goat, Santa Cruz), anti-RET51 (goat, Santa Cruz), anti-HA (mouse, Millipore), or anti-FLAG (rabbit, Cell Signaling) selective antibodies. Immunoprecipitates were subjected to SDS-PAGE followed by electroblotting onto polyvinylidene difluoride membranes (PVDF, Immobilon P; Millipore Corporation). Immunoblotting was performed on blots using antibodies selective for RET (1:500, goat, R&D), HA (1:5000, rabbit, Cell Signaling), Trk (1:1000, rabbit, Santa Cruz), phospho-tyrosine (1:1000, mouse, Millipore), phospho-RET (Tyr^{905}) (1:500, rabbit), or phospho-RET (Tyr^{1062}) (1:500, rabbit). Phospho-RET (Tyr^{905}) and phospho-RET (Tyr^{1062}) have been previously described (Tsui-Pierchala et al., 2002a). Lysates collected prior to immunoprecipitations served as loading controls to assess protein expression levels, and were also subjected to immunoblotting for actin (1:1000, goat, Santa Cruz). Each biochemical experiment was performed three to four times with similar results.

Mammalian cell culture and transfections – NIH/3T3 and HEK293T cells (ATCC) were maintained at 37°C with 5% CO_2 in DMEM medium supplemented with FBS (10% by volume) and a penicillin/streptomycin/glutamine (PSQ) mixture. Cells were plated into 6-

well tissue culture plates and allowed to proliferate until cells were 70% confluent. Transfections of NIH/3T3 and HEK293T cells were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). For experiments testing the interaction of proteins, a total of 4 µg of plasmid DNA was added per well, using a GFP plasmid as a control for transfecting equal amounts of DNA. For *in vitro* activation experiments, 2 µg of HA::GFRα1 and 1 µg of one of the RET isoforms were transfected for a total of 3 µg of DNA per condition, with GFP plasmid used as a control for transfecting equal amounts of DNA. *RET51*$^{\Delta E3}$ and *RET51*$^{\Delta E345}$ constructs were subcloned from *RET51* constructs into pcDNA3.1 (Thermo Fisher Scientific). A *Ret51* construct encoding mouse RET51 fused at the C-terminus with an HA epitope tag (RET51::HA) was acquired from Dr. Ben Allen. This plasmid was subcloned to create similarly tagged *Ret51*$^{\Delta E3}$ and *Ret51*$^{\Delta E345}$ constructs, which were sub-cloned into pLentilox-IRES-GFP (University of Michigan Vector Core). The HA::GFRα1 (mouse) plasmid was generously donated by Dr. Ben Allen. HA::GFRα2 (rat), HA::GFRα3 (mouse), and HA::GFRα4 (chicken) plasmids were generously donated by Dr. Carlos Ibáñez. The TrkA (rat) plasmid was generously donated by Dr. Christin Carter-Su.

Quantification of Immunoblots - Scanned images of X-ray films were imported into ImageJ (National Institutes of Health) and processed using the gel analysis tool. Integrated density values obtained from immunoblotting were reported as mean values ± SEM, with arbitrary units on the vertical axis. Values were normalized to the level of RET51 phosphorylation in each experiment. Statistical analyses were performed using

a Student's *t*-test for each experiment, and all biochemical experiments were performed at least three times with similar results.

Immunocytochemistry – Transfected cells that were plated on glass coverslips were fixed for 8 minutes in 4% PFA. Cells were washed twice in 1X PBS, and briefly blocked in immunofluorescence blocking solution (3% BSA, 1% normal donkey serum in 1X PBS, pH 7.4) with or without 0.1% Triton X-100. Primary antibodies were diluted in blocking buffer [RET (1:50, goat, R&D), HA (1:1000, mouse, Millipore)], and slides were incubated at 4°C overnight. Cells were washed twice with 1X PBS and then were incubated at room temperature for one hour with secondary antibodies [donkey anti-mouse Alexa Fluor 633 (Thermo Fisher Scientific), donkey anti-goat 543 (Biotium)] diluted in blocking solution. Lastly, cells were washed three times in 1X PBS and mounted with DAPI Fluoromount-G (Southern Biotech). Cells were imaged using a Zeiss Axiovert 200 M epifluorescence microscope with a 40X objective.

Acknowledgments

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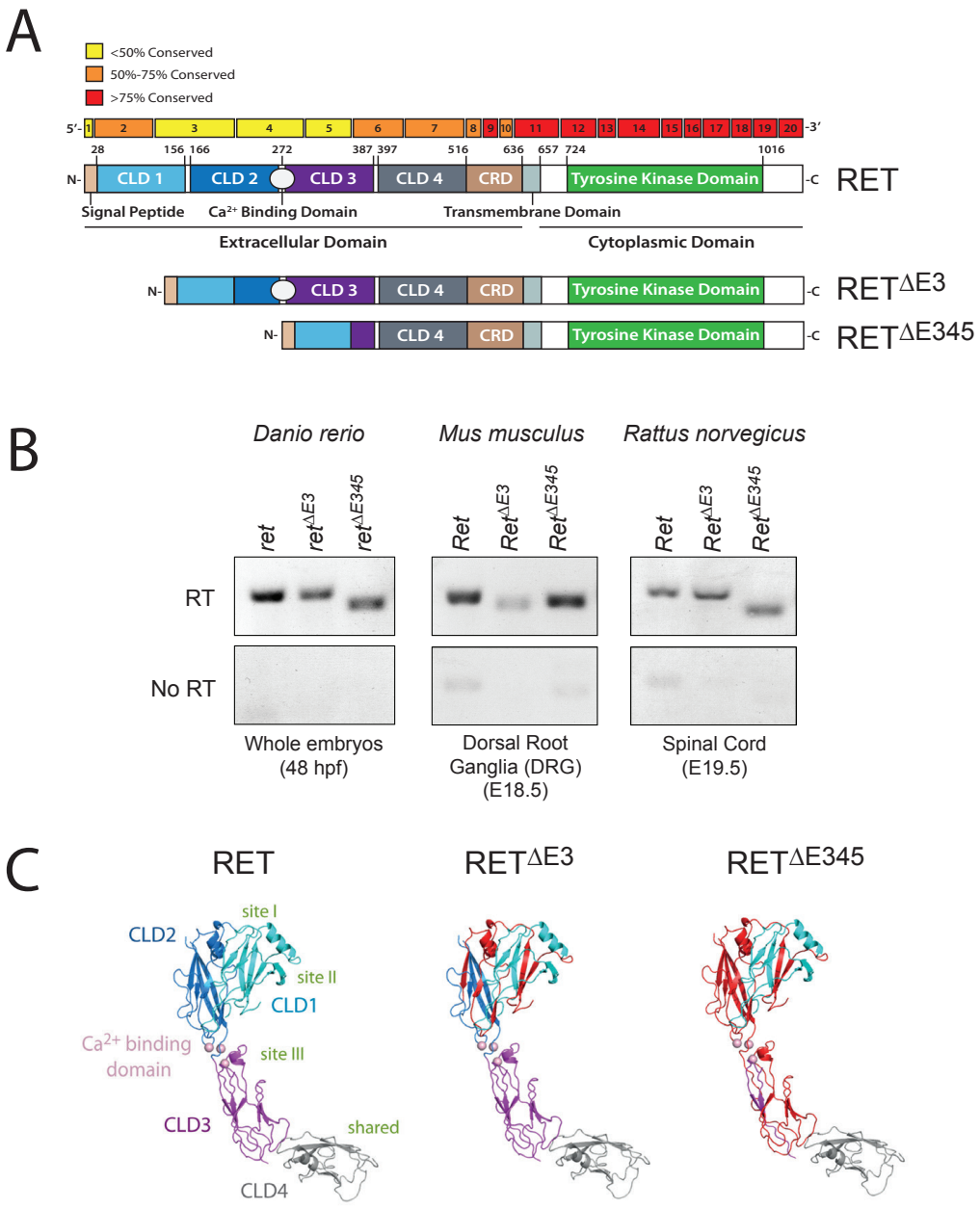

Figure 2.1: Removal of exons 3 or 3, 4 and 5 of *RET* creates proteins with large deletions in the extracellular domain, and transcripts encoding these receptors are expressed in multiple organisms.

(A) Comparison of the amino acid sequences of RET between zebrafish, mice, rats, and humans was performed, and the conservation within each exon is indicated by color. Interestingly, the amino acids encoded in exons 3, 4, and 5 are the least conserved. Additionally, the impact of exon skipping on the extracellular domain within the protein is shown. (B) *Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ transcripts are detectable in zebrafish (*Danio rerio*), mice (*Mus musculus*), and rats (*Rattus norvegicus*) by qPCR. Like human *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$, alternative splicing of exon 3 or exons 3, 4 and 5 in these vertebrates are predicted to encode full-length, transmembrane RET proteins with deletions in the extracellular domain. (C) Deleted regions within the crystal structure of the extracellular domain of RET are colored in red.

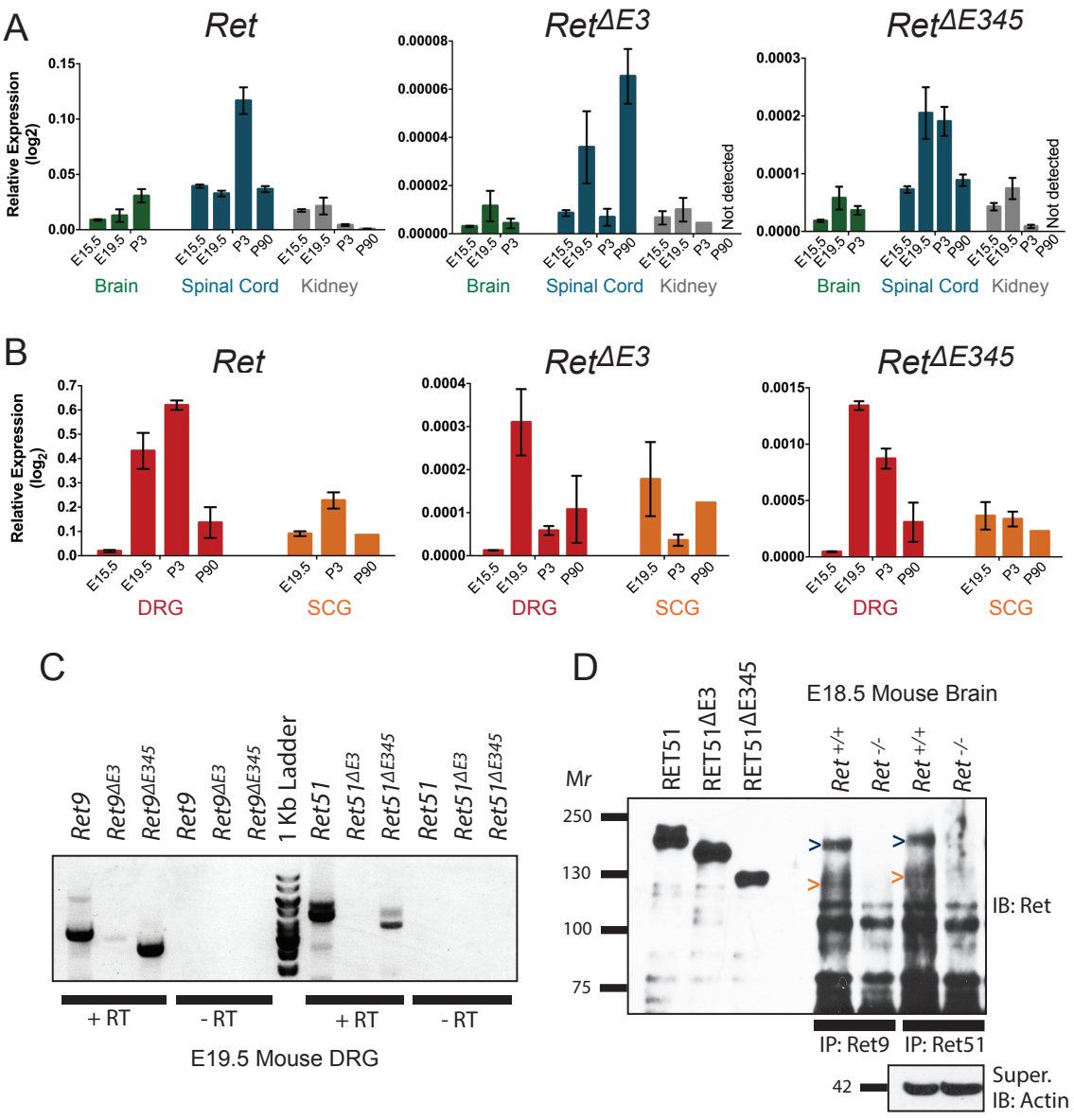

Figure 2.2: ***Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ are dynamically expressed throughout development, and RET proteins with similar molecular weights to RET$^{\Delta E345}$ are detect *in vivo*.**
(A) Expression of *Ret*, *Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ in mouse brain (E15.5, E19.5 P3), spinal cord (E15.5, E19.5, P3, adult), and kidney (E15.5, E19.5 P3, adult) by qPCR is shown as relative expression compared to *actin*. (B) Expression of *Ret*, *Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ in mouse sympathetic (E19.5, P3, adult) and DRG sensory neurons (E15.5, E19.5, P3, adult) by qPCR is graphed as in (A). (C) cDNA from E19.5 DRGs was synthesized from polyA RNA, and RT-PCR was performed to determine the presence of *Ret* transcripts with both 5' and 3' alternative splicing. *Ret9*, *Ret9*$^{\Delta E3}$, *Ret9*$^{\Delta E345}$, *Ret51*, and Ret51$^{\Delta E345}$ transcripts were identified. (D) E18.5 mouse brains were lysed and immunoprecipitations for RET9 or RET51 were performed. Immunoblotting for RET detected full-length RET9 and RET51, as expected. The presence of RET9 and RET51 proteins at the same molecular weight as RET$^{\Delta E345}$ suggested the existence of RET9$^{\Delta E345}$ and RET51$^{\Delta E345}$ isoforms.

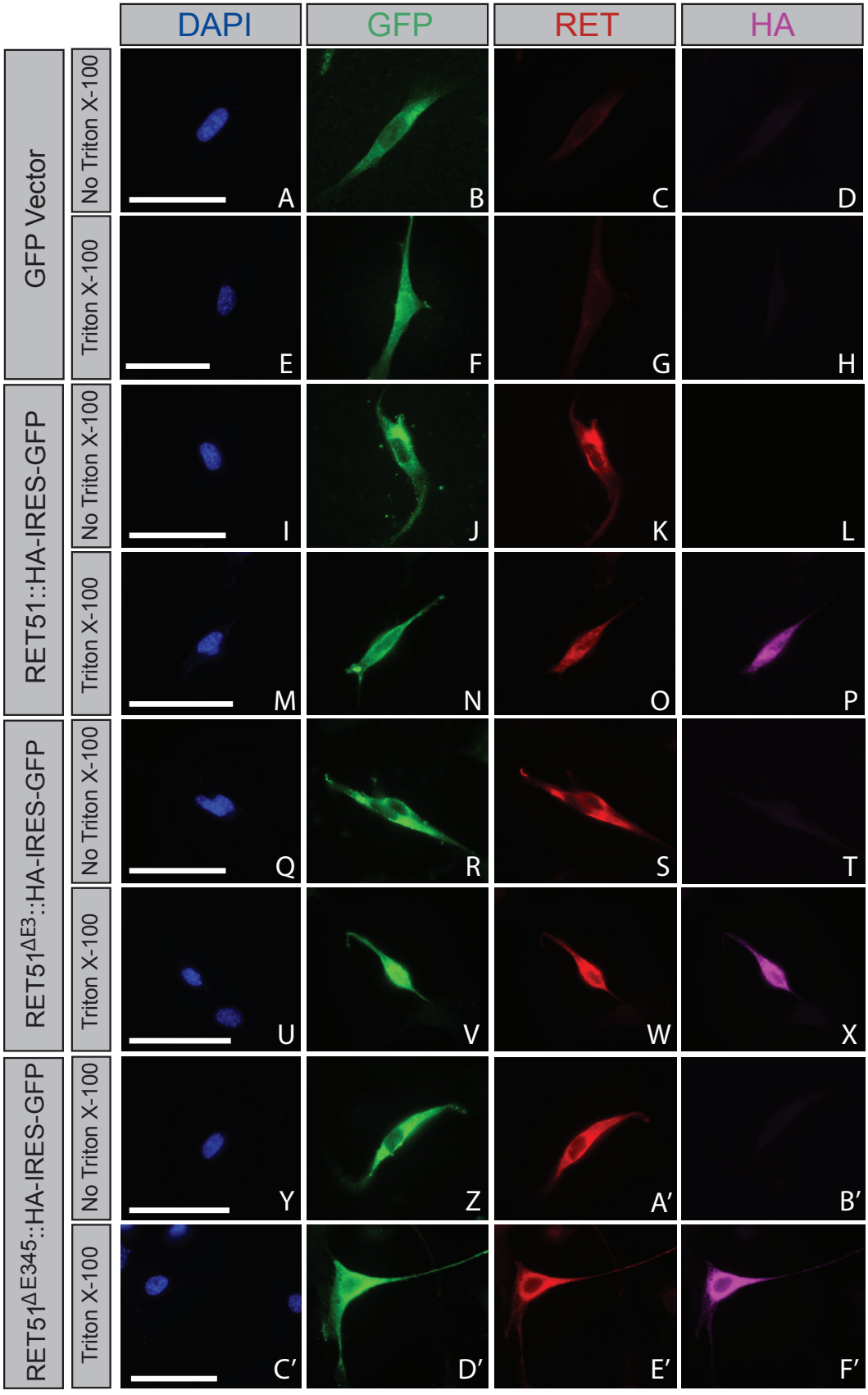
DAPI
GFP
RET
HA
GFP Vector
RET51::HA-IRES-GFP
RET51ΔE3::HA-IRES-GFP
RET51ΔE345::HA-IRES-GFP
No Triton X-100
Triton X-100
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
A′
B′
C′
D′
E′
F′

Figure 2.3: **RET$^{\Delta E3}$ and RET$^{\Delta E345}$ are trafficked to the cell surface.**
NIH/3T3 cells were transfected with *GFP* (A-H), *Ret51::HA* (I-P), *Ret51$^{\Delta E3}$::HA* (Q-X) or *Ret51$^{\Delta E345}$::HA* (Y-F'). Constructs for the RET51 isoforms were bicistronic, allowing for expression of GFP as an indicator for positive transfection, which was encoded by an IRES-GFP following RET cDNA sequences. Cells were washed in 1X PBS and fixed briefly in 4% PFA 24 hours post-transfection. Cells were then blocked in immunofluorescence blocking solution with or without Triton X-100 to allow for cell membrane permeabilization. In the absence of Triton X-100, antibodies specific for the RET extracellular domain were able to bind to RET51::HA (K), RET51$^{\Delta E3}$::HA (S), and RET51$^{\Delta E345}$::HA (A'). However, using an antibody against the HA epitope which would detect the C-terminus located intracellularly for RET51::HA (L), RET51$^{\Delta E3}$::HA (T), and RET51$^{\Delta E345}$::HA (B') showed no binding/fluorescence in the absence of Triton X-100. When immunofluorescence blocking solution with Triton X-100 was used, fluorescence was detected using the anti-HA antibody for RET51::HA (P), RET51$^{\Delta E3}$::HA (X), and RET51$^{\Delta E345}$::HA (F'). These data show that RET51$^{\Delta E3}$ and RET51$^{\Delta E345}$ are trafficked to the cell surface similarly to full-length RET51.

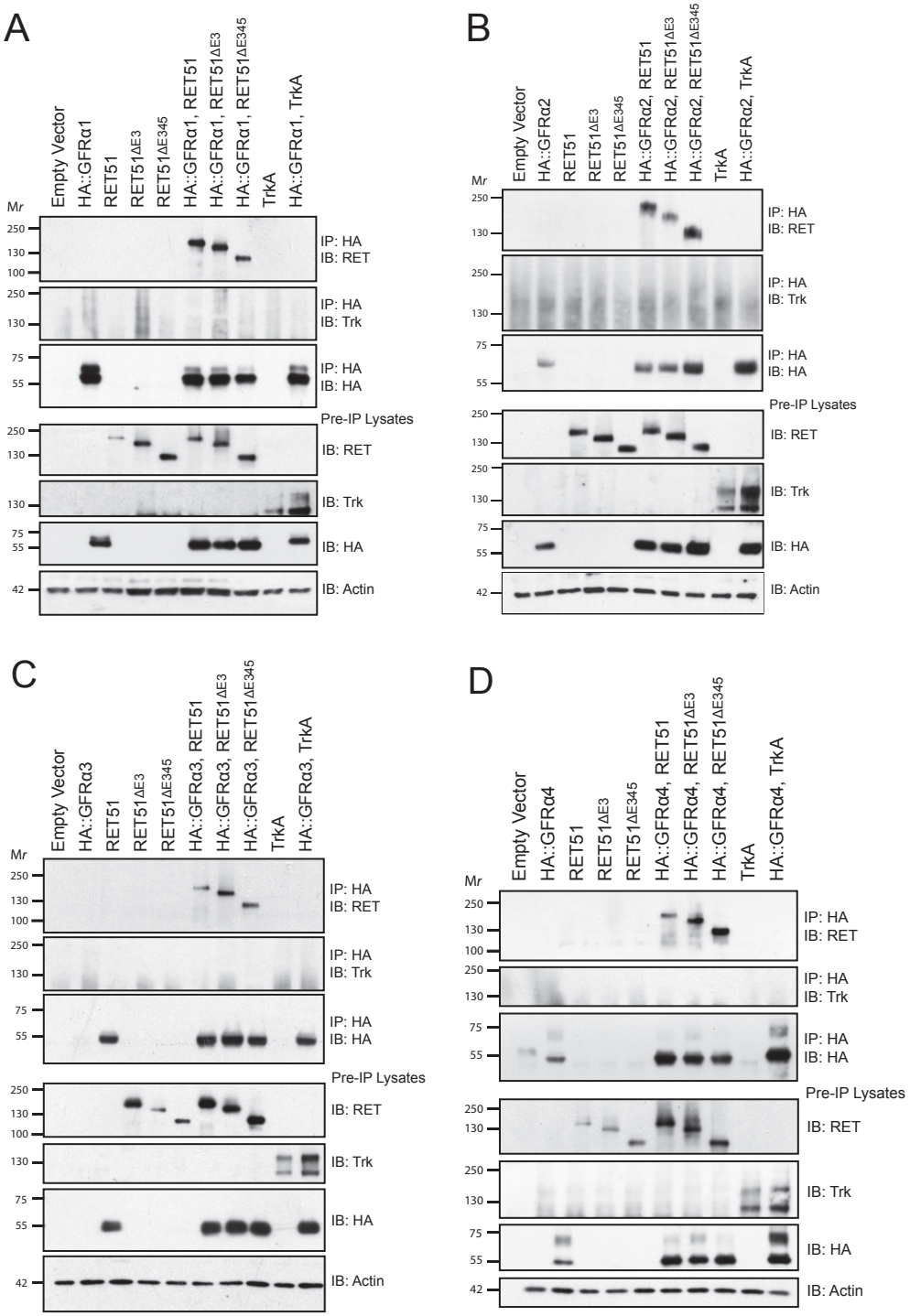

Figure 2.4: RET$^{\Delta E3}$ and RET$^{\Delta E345}$ bind to GFRα1, GFRα2, GFRα3 and GFRα4. *RET51*, *RET51$^{\Delta E3}$* and *RET51$^{\Delta E345}$* were co-transfected with HA epitope tagged *GFRα1* (A), *GFRα2* (B), *GFRα3* (C), or *GFRα4* (D). Immunoprecipitations were performed using an HA antibody to select for the GFRαs. Immunoblotting for RET showed the interaction of RET51$^{\Delta E3}$ and RET51$^{\Delta E345}$ with all four GFRα proteins. Co-transfection of HA::GFRα1, HA::GFRα2, HA::GFRα3 or HA::GFRα4 with TrkA was performed as a negative control, and as expected, TrkA did not associate with any of the GFRαs.

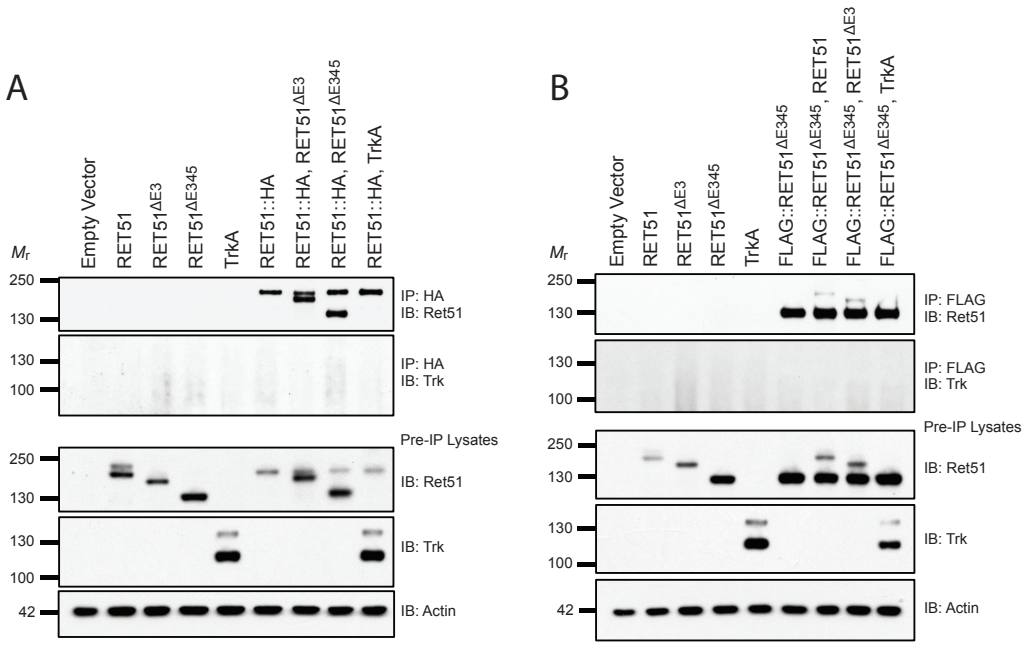

Figure 2.5: RET$^{\Delta E3}$ and RET$^{\Delta E345}$ can heterodimerize with full-length RET.
(A) HA epitope tagged *RET51* (RET51::HA) was co-transfected with untagged *RET51$^{\Delta E3}$* or *RET51$^{\Delta E345}$* in HEK293T cells. Immunoprecipitations were performed with an HA antibody to select for RET51::HA. Immunoblotting for RET51 showed co-immunoprecipitation of RET51$^{\Delta E3}$ and RET51$^{\Delta E345}$ with full-length RET51. (B) A similar experiment was performed using FLAG epitope tagged *RET51$^{\Delta E345}$* (*FLAG:: RET51$^{\Delta E345}$*). Immunoprecipitations with an anti-FLAG antibody selected for FLAG:: RET51$^{\Delta E345}$. Immunoblotting for RET51 demonstrated the association of RET51 and RET51$^{\Delta E3}$ with FLAG:: RET51$^{\Delta E345}$. Co-transfections with TrkA were performed for both experiments as a negative control, and immunoblotting for TrkA showed no binding with either RET51::HA or FLAG::RET51$^{\Delta E345}$, as expected.

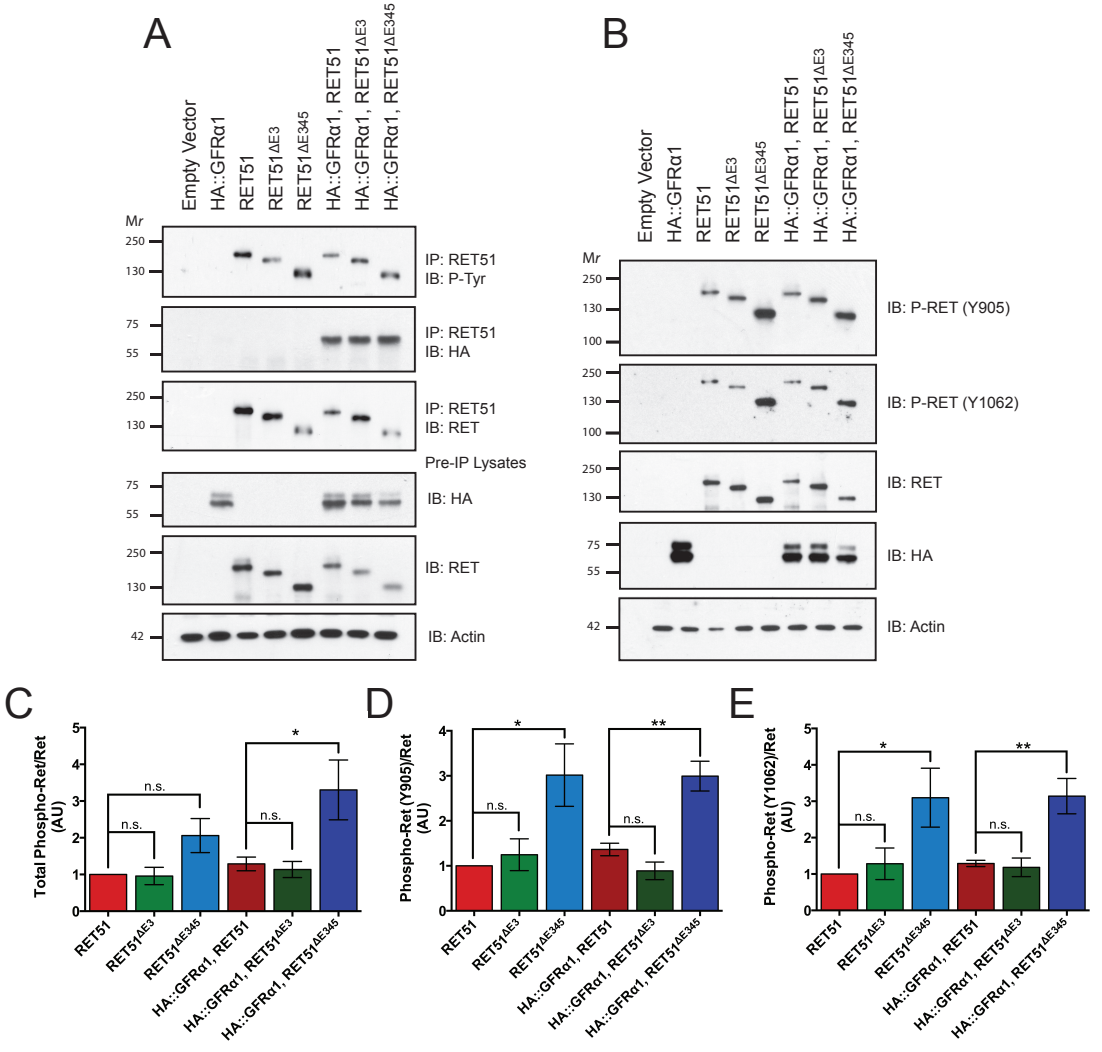

Figure 2.6: RET$^{\Delta E345}$ displays increased ligand-independent activation compared to full-length RET.
(A) *RET51* was co-transfected with HA epitope tagged *GFRα1* in NIH/3T3 cells. Immunoprecipitations were performed with a RET51 antibody to select for the RET isoforms. Immunoblotting for phospho-tyrosine and RET was performed, and integrated density values were calculated for each. (B) Pre-IP lysates from the previous experiment were subjected to SDS-PAGE, and Western blotting was performed to determine the levels of residue-specific tyrosine phosphorylation of RET. Immunoblotting was performed for phospho-RET (Tyr^{905}), phospho-RET (Tyr^{1062}), and RET, and integrated density values were calculated for each. (C) Ratios of phospho-RET/RET, (D) phospho-RET (Tyr^{905})/RET, and (E) phospho-RET (Tyr^{1062})/RET are reported, with values normalized to RET51. A single asterisk (*) denotes a p-value < 0.05 and a double asterisk (**) denotes a p-value < 0.01.

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CHAPTER 3

LOSS OF RET RESULTS IN A DECREASE IN APOPTOSIS DURING PROGRAMMED CELL DEATH

Summary

Target-derived neurotrophic factors, such as the neurotrophins and the glial cell-line derived neurotrophic factor (GDNF) family of ligands (GFLs) are crucial components aiding in the establishment of proper connections between neurons and their targets. In the peripheral nervous system (PNS), GFLs signal through their common receptor tyrosine kinase, RET, to produce signals critical for neuronal survival, migration, and axonal growth. Recently, we discovered that RET associates with p75, a member of the tumor necrosis factor family of death receptors, *in vitro* and in *vivo*. This interaction is surprising because p75 is a critical mediator of apoptosis upon activation by proneurotrophins, while RET is canonically involved in pro-survival signaling as a receptor for the GFLs. Interestingly, the interaction between p75 and RET is increased upon stimulation with apoptotic signals that induce p75-dependent apoptosis in sympathetic neurons. These findings suggest a functional role of RET in p75-mediated pro-apoptotic signaling. Furthermore, we find that genetic deletion of *Ret* results in a decrease in programmed cell death (PCD) *in vivo*. Unexpectedly, genetic deletion of *p75* resulted in no difference in programmed cell death *in vivo*. Collectively, these data implicate RET as a novel co-receptor for p75-mediated apoptosis *in vitro,* but the *in vivo*

mechanisms behind p75-mediated cell death during PCD may need further evaluation.

Introduction

During the development of the peripheral nervous system there is a widespread overproduction of neurons, with most populations producing twice the number of neurons that are present in adulthood (Oppenheim, 1991). Neurons that do not make appropriate or sufficiently extensive connections and, therefore, do not receive a sufficient amount of target-derived survival factors are eliminated through a process known as programmed cell death (PCD) (Oppenheim, 1991). One population of neurons that has been used extensively as a model to study this process is post-ganglionic sympathetic neurons of the superior cervical ganglia (SCG) (Glebova and Ginty, 2005). In the mouse SCG, PCD occurs late perinatally through early postnatal stages (~E17-P20) (Bamji et al., 1998), and during this time, sympathetic neurons require nerve growth factor (NGF) for their survival (Levi-Montalcini, 1987). NGF is secreted in a limited amount from target tissues, and binds to the receptor tyrosine kinase, TrkA, located on sympathetic axons (Glebova and Ginty, 2005). Upon binding, this NGF-TrkA complex is retrogradely trafficked to the cell body and promotes neuronal survival by inhibiting apoptosis in these “winning” cells (Ehlers et al., 1995, Riccio et al., 1997).

In addition to the competition for survival factors, evidence also points to the involvement of pro-apoptotic factors in developmental cell death via TNF family receptors such as the p75 neurotrophin receptor (p75NTR, NGFR, TNFRSF16) (Bamji

et al., 1998, Deppmann et al., 2008). p75 is a promiscuous receptor that interacts with TrkA, thereby increasing its affinity for NGF, as well as enhancing TrkA-mediated survival signaling (Verdi et al., 1994, Barker and Shooter, 1994). In the absence of Trk receptors, however, p75 can function as a receptor for members of the neurotrophin family, as well as proneurotrophins, and signaling in this context can trigger cell death pathways (Teng et al., 2010). It has been proposed that "winning" neurons release pro-apoptotic p75 ligands, such as brain-derived neurotrophic factor (BDNF), that enhance apoptosis in the vulnerable "losing" neurons that are not protected by the survival pathways activated by NGF-TrkA/p75 signaling (Teng et al., 2010). Genetic evidence in support of this competition model includes the significant lengthening of the period of PCD in the SCG of *p75* null mice (Bamji et al., 1998, Deppmann et al., 2008).

An additional neurotrophic factor receptor, RET, is also expressed in the SCG during the period of PCD (Enomoto et al., 2001), but its function during this time has not been elucidated. Like TrkA, RET is a receptor tyrosine kinase, but it is activated by a family of four growth factors known as the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), which includes GDNF, neurturin, artemin and persephin (Airaksinen and Saarma, 2002). These ligands do not bind directly to RET, but bind to one of four cognate glycosylphosphatidylinositol (GPI) anchored co-receptors known as the GDNF Family Receptor-αs (GFRαs) (Airaksinen and Saarma, 2002). Once this GFL-GFRα complex forms, it can then bind to RET, allowing for activation of the receptor. RET signaling has been shown to be critical for survival in a number of neuronal populations including subpopulations of sensory neurons of the dorsal root ganglia (DRG) (Luo et al., 2009, Golden et al., 2010), enteric neurons (Schuchardt et

al., 1994, Schuchardt et al., 1996) and γ-motor neurons of the motor nervous system (Gould et al., 2008). Moreover, specifically in the SCG, RET signaling has been shown to be important for sympathetic chain ganglia migration, coalescence of the ganglia and early axon pathfinding (Enomoto et al., 2001). These deficits lead to the early apoptotic death of SCG neurons prior to the period of PCD, thereby limiting investigation of the role of RET signaling in PCD of the SCG.

We report here that *Ret* is expressed in a subset of sympathetic neurons of the SCG during PCD that express both p75 and tyrosine hydroxylase. We also observe that these *Ret* positive neurons are lost during this developmental time window. Furthermore, we find that RET can interact with p75 both *in vitro* and *in vivo*, and that this interaction is enhanced in the presence of apoptotic stimuli, suggesting that RET may be able to enhance p75-mediated cell death. Genetic deletion of *Ret* in the developing embryo using a tamoxifen inducible Cre recombinase at a late perinatal time showed that loss of RET leads to a reduction in cell death during PCD. Surprisingly, genetic deletion of *p75* utilizing both a tamoxifen inducible Cre recombinase approach as well as a germline deletion approach showed no difference in the amount of cell death occurring between mutant or control animals. This potential dichotomy will be considered in detail in the discussion of this chapter as well as in Chapter 4.

RESULTS

Ret is expressed in a subset of p75-positive noradrenergic neurons during programmed cell death

RET is required for the proper differentiation of sympathetic precursors, for migration of the sympathetic chain ganglia, and also for sympathetic axon guidance during development (Enomoto et al., 2001). Within the superior cervical ganglia (SCG), *Ret* expression is high until E15.5 (Enomoto et al., 2001). After this time, *Ret* expression becomes restricted to a subset of neurons within the SCG, but its role during this developmental period has not been elucidated (Enomoto et al., 2001). Interestingly, late prenatal expression of *Ret* overlaps with the period of PCD, and we postulated that RET may be involved in this process. Using a reporter line in which GFP was knocked into the *Ret* locus, Enomoto et al. showed that at any time throughout PCD, approximately 5-6% of neurons in the SCG are *Ret* positive (Enomoto et al., 2001). However, this approach did not allow for the authors to distinguish if a subset of neurons were *Ret* positive and remained *Ret* positive during this time, or if cells that upregulated *Ret* were then actively being turned over. To examine this possibility, we performed a tamoxifen pulse experiment using *Rosa26*$^{LSL\text{-}tdTomato}$ mice crossed to *Ret*-Cre/ER^{T2}, in which a tamoxifen inducible Cre recombinase is knocked into the endogenous *Ret* locus (Luo et al., 2009). By administering tamoxifen to these animals, cells that are endogenously expressing *Ret* at that time are labeled with fluorescent tdTomato protein. We administered tamoxifen (100 µg) to pregnant dams at embryonic day 16.5 (E16.5) or 18.5 (E18.5), and then isolated SCGs from embryos at E19.5 (Fig. 3.1A). Because a consistent percentage of cells are *Ret* positive during this time period (Enomoto et al., 2001), we hypothesized that if *Ret* positive cells were turning over, the mice receiving tamoxifen at E16.5 would have fewer *Ret* positive cells than the animals receiving tamoxifen at E18.5. However, if a stable population of cells were expressing

Ret, then we would expect to observe a similar number of *Ret* positive cells at E19.5 from animals injected at either timepoint. Interestingly, we observed a decrease in the number of *Ret* positive cells from pups receiving tamoxifen at E16.5 compared to those receiving tamoxifen at E18.5, suggesting that these *Ret*-expressing cells are lost throughout this period of developmental cell death (Fig. 3.1B). Consistent with this hypothesis, many of the *Ret*-expressing neurons labeled at E16.5 appeared atrophic at E19.5 with the presence of early pyknotic nuclei forming, indicating these neurons were starting to undergo apoptosis (Fig. 3.1C). However, when staining for the late apoptotic marker, cleaved caspase-3, we did not observe co-localization of cleaved caspase-3 in *Ret*-expressing cells (data not shown). This lack of co-localization is likely due to degradation of tdTomato protein in the *Ret*-expressing cells at this late apoptotic time point.

p75 is critically involved in programmed cell death in the SCG (Bamji et al., 1998, Deppmann et al., 2008); to determine whether *Ret* was co-expressed with p75 in the same cells within the SCG, *Rosa26*$^{LSL\text{-}tdTomato}$*; Ret*-Cre/ER^{T2} mice were again used and pregnant dams were given tamoxifen at E18.5. The embryos were collected one day later and SCGs were isolated and then processed for cryosectioning. Immunofluorescence staining for p75 was performed, and the co-expression of *Ret* labeled by tdTomato and p75 was assessed. As a control, the p75 antibody was validated by immunostaining *p75* null SCGs (Fig. 3.2C). Of the small subset of neurons that were *Ret*-positive, 100% of these *Ret*-positive cells were also p75-positive (Fig. 3.2A), indicating that p75 is co-expressed in *Ret*-positive SCG neurons.

In the stellate ganglia (STG), the majority of cells are noradrenergic, but a subpopulation of *Ret*-positive cholinergic neurons has been described that emerge during late prenatal development (Huang et al., 2013). To determine if this subset of *Ret*-positive neurons in the SCG was not noradrenergic, and therefore most likely cholinergic, immunofluorescence staining for tyrosine hydroxylase (TH), a marker for noradrenergic neurons, was performed. We observed that *Ret*-positive neurons were also co-expressing TH, indicating that, unlike in the STG, *Ret*-positive neurons in the SCG are not a subpopulation of cholinergic neurons (Fig. 3.2B). Taken together, these data indicate that *Ret*-expressing cells in the SCG are noradrenergic, co-express with p75, and actively turn over during PCD.

p75 and RET interact in vitro and in vivo

p75 is a promiscuous receptor that directly interacts with other cell surface proteins including TrkA, Ephrins, and Nogo receptor (Meeker and Williams, 2014). To determine if p75 interacts with RET, we used an *in vitro* transfection system to co-express p75 with either the RET9 or RET51 isoforms. Cells were transfected with RET9, RET51 or p75 alone, or were co-transfected with one of the RET isoforms along with p75. Immunoprecipitations were performed using a RET antibody specific for the extracellular domain of both RET isoforms. Via immunoblotting, we observe co-immunoprecipitation of p75 with both RET9 and RET51 (Fig. 3.3A) Thus, using this *in vitro* system, p75 interacts with RET.

To determine if p75 and RET interact under basal conditions *in vivo*, spinal cords were isolated from $Ret^{fl/fl}$ mice that were crossed to *UBC*-Cre/ER^{T2} mice in which the

expression of Cre recombinase fused to an estrogen receptor is driven off of the ubiquitin C promoter, allowing for the chimeric protein to be ubiquitiously expressed. Tamoxifen was administered to females that had recently given birth (P0) to allow the transfer of tamoxifen via lactation to the pups. The pups were sacrificed at P5, and spinal cords were isolated. The tissues were detergent-extracted and immunoprecipitations for both RET9 and RET51 were performed to isolate both RET isoforms. To determine whether RET and p75 interact *in vivo*, immunoblotting of these immunoprecipitates was performed for p75; we did observe a robust interaction (Fig. 3.3B). As a control to confirm the specificity of our antibodies, immunoprecipitations were performed for RET9 and RET51 from spinal cord in which RET had been conditionally deleted. Immunoblotting for p75 from these spinal cords showed no co-immunoprecipitation (Fig. 3.3B), indicating that our antibodies are specific. Taken together, RET interacts with p75 both *in vivo* and *in vitro.*

Apoptotic stimuli induce the interaction of RET and p75.

During the process of PCD, apoptosis can occur through several mechanisms including lack of NGF trophic support as well as binding of competition factors, either BDNF or Pro-BDNF, to p75 (Glebova and Ginty, 2005, Conradt, 2009). We postulated that the interaction of RET and p75 may be induced by the presence of these apoptotic signals. To test this hypothesis, primary sympathetic cultures were treated with one of three conditions: (1) α-NGF to create a lack of trophic support (Fig. 3.4A), (2) BDNF (Fig. 3.4B) or (3) Pro-BDNF (Fig. 3.4C); the latter two create a pro-apoptotic, competition ligand system. Upon treatment with α-NGF, BDNF and Pro-BDNF, we

observed an increase in association over time between RET and p75. These data suggest that RET may be involved in p75-mediated apoptotic signaling.

Apoptosis is reduced during PCD upon deletion of RET

To determine if *Ret* is important for enhancing cell death in the SCG during the period of programmed cell death, *Ret* was conditionally deleted from mice at a later postnatal stage. *Ret* null mice were not used as it has been shown that neuronal precursors of the sympathetic nervous system fail to migrate and project axons properly (Enomoto et al., 2001). As a consequence, sympathetic nerve trunks are misrouted and there is increased cell death of sympathetic neurons due to neuronal precursors failing to properly develop (Enomoto et al. 2001). Instead, $Ret^{fl/fl}$ females were mated to $Ret^{fl/fl}$;*UBC*-Cre/ER^{T2} males. Pregnant females were given tamoxifen (100 µg) and progesterone (50 µg) once a day for four consecutive days beginning at E14.5 (E14.5-E17.5). Pregnant females were euthanized at E19.5, the embryos were isolated, and SCGs were dissected and processed for cryosectioning. Additionally, spinal cords were isolated and detergent extracted, and these extract were subjected to Western blotting to determine the level of knockdown of RET protein. We found that greater than 95% of RET was eliminated in $Ret^{fl/fl}$;*UBC*-Cre/ER^{T2} as compared to control $Ret^{fl/fl}$ littermates (Fig. 3.5C).

To assess the amount of cell death occurring in the SCG, immunofluorescence staining for cleaved caspase-3 was performed on serially sectioned SCGs. Quantification of the number of cleaved caspase-3 positive cells showed a significant

decrease in the number of apoptotic cells in *Ret*$^{fl/fl}$*;UBC*-Cre/ER^{T2} mice as compared to *Ret*$^{fl/fl}$ littermates (Figure 3.5B).

Deletion of *p75* is reported to result in a reduction of cell death as measured by total neuronal cell counts. We employed cleaved caspase-3 staining so that we could examine levels of apoptosis as it was actively occurring. To determine how *p75* deletion affected actively progressing apoptosis, we used the same genetic deletion strategy as was used to delete *Ret*. Germline heterozygous *p75 (p75*$^{+/-}$) females were mated to *p75*$^{fl/fl}$;*UBC*-Cre/ER^{T2} males, and as previously performed, pregnant females were administered tamoxifen from E14.5-E17.5 to delete *p75* in the SCG during PCD. *p75*$^{fl/+}$ pups were used as positive controls while *p75*$^{fl/-}$*;UBC*-Cre/ER^{T2} pups were analyzed as the experimental group. *p75*$^{fl/fl}$;*UBC*-Cre/ER^{T2} animals were not used as we were unable to achieve consistent knockdown of p75 in these animals (data not shown). Surprisingly, there was no significant difference in the number of cleaved caspase-3 positive cells in the SCG between *p75*$^{fl/+}$ and *p75*$^{fl/-}$*;UBC*-Cre/ER^{T2} mice (Figure 3.6A,B). We confirmed, however, that our deletions were effective by immunoblotting for p75 from spinal cord extracts (Fig. 3.6C).

Because these results seemed at odds with the previous germline knockout data (Bamji et al., 1998), we performed a similar experiment utilizing *p75* germline knockout mice (*p75*$^{-/-}$) to see if we observed a difference in cell death during PCD in the SCG of these animals (Bogenmann et al., 2011). Pregnant females were euthanized at the same age as the others, E19.5, embryos were isolated, and SCGs were dissected and processed for cryosectioning. Surprisingly, we again observed no significant difference in the number of cleaved caspase-3 positive cells in the SCG between *p75* null and *p75*

wild-type mice (Figure 3.7A,B), and we confirmed that p75 protein was absent in *p75* null mice (Fig. 3.7C). These data suggest that RET plays a role in programmed cell death at E19.5, but that p75 may not.

Discussion

During late embryonic development, the function of RET in the developing SCG has remained elusive. Experiments in primary neuron cultures suggested that RET could interact with p75 (Chris Donnelly, unpublished data), a known pro-apoptotic protein that plays a role in programmed cell death during late embryonic and early postnatal development. Because RET does not support the survival of neurons in the SCG after E15.5 (Enomoto et al., 2001), we hypothesized that, instead, RET may play a role in programmed cell death.

Through a tamoxifen pulse experiment utilizing *Rosa26*$^{LSL\text{-}tdTomato}$*; Ret*-Cre/ER^{T2} mice, we were able to specifically label cells expressing *Ret* at late embryonic time points. The SCGs from these embryos were isolated at E19.5, and the number of cells expressing tdTomato was quantified. This experimental design allowed us to determine if *Ret* positive cells were turning over during this time. Although we need to increase our sample size, we did observe a reduction in the number of *Ret*-positive cells at E19.5 from an earlier tamoxifen pulse at E16.5 versus a tamoxifen pulse at E18.5, suggesting that early *Ret* positive cells are turning over, unlike what had previously been reported (Enomoto et al., 2001).

Additionally, these *Ret* positive cells were co-expressed with p75 and tyrosine hydroxylase, demonstrating that these cells are noradrenergic. It was previously

hypothesized that this subset of *Ret* positive cells was a cholinergic subpopulation, similar to the RET and vesicular acetylcholine transporter (VAChT) positive subpopulation that arises in the stellate ganglia during late postnatal development (Huang et al., 2013). However, it appears that this is not the case. More interestingly, we see ubiquitous expression of p75 throughout the SCG at E19.5, potentially due to its role in enhancing the survival signaling of TrkA during PCD (Barker and Shooter, 1994, Verdi et al., 1994), although this has not been definitively shown to occur.

Furthermore, we found that RET can interact with p75 both *in vitro* and *in vivo*, and that this interaction is enhanced in the presence of apoptotic stimuli, suggesting that RET may be able to enhance p75-mediated cell death. To assess our hypothesis *in vivo*, *Ret* was conditionally deleted using a ubiquitously expressed, tamoxifen inducible Cre recombinase during late prenatal development. We observed that a loss of RET results in a reduction in the number of cells actively undergoing apoptosis during PCD.

As a control experiment, we deleted *p75* utilizing both a tamoxifen inducible Cre recombinase approach as well as a germline deletion approach, and surprisingly, observed no difference in either genetic model in the number of cells actively undergoing apoptosis in p75-deficient SCGs versus wild-type littermate controls at E19.5.

There are a few potential reasons for the dichotomy between our results and previously reported findings that p75 functions in PCD in the SCG. The first difference is that we normalized our data to quantify the number of cleaved caspase-3 positive cells per area (mm^2) to correct for any loss of sections during cryosectioning and/or immunostaining, whereas previous studies did not. The second difference is in the

assessment of cell death. In our experiment, we chose to analyze cells actively undergoing apoptosis by evaluating the number of cleaved caspase-3 positive cells. Previous studies quantified cell death by performing total cell counts, and established that PCD occurs between P0 and P20 (Bamji et al., 1998, Deppmann et al., 2008). The most appropriate readout for cell death during PCD has yet to be determined.

Additionally, the previous studies have used a different *p75* null mouse model to understand the role of p75 in cell death in the SCG. In this mouse, exon 3 of *p75* is removed ($p75^{\Delta exon3/\Delta exon3}$), and no p75 protein product was detect from this targeted deletion, which created a *p75* null mouse (Lee et al., 1992). Interestingly, an alternatively spliced transcript of p75 was identified in $p75^{\Delta exon3/\Delta exon3}$ mice in which exon 3 was skipped, and a p75 protein product, s-p75, was identified in primary Schwann cells at the appropriate 62 kDa molecular weight (von Schack et al., 2001). While the presence of s-p75 is still debated, it is interesting to consider how the protein may function; s-p75 is able to bind to Trk receptors, but the protein cannot bind neurotrophins (von Schack et al., 2001), thus it would eliminate the "competition factor" model that is widely accepted to occur during PCD (Deppmann et al., 2008).

Due to the controversy surrounding the true deletion of *p75* in the $p75^{\Delta exon3/\Delta exon3}$ mouse, we used the *p75* null mouse generated by Bogenmann et al. (Bogenmann et al., 2011), which is described in greater detail in Chapter 4. Bogenmann et al. showed that this was a true *p75* knockout, however our analysis in the SCGs of these mice is the first evaluation of PCD in this model. Additional experiments are needed to ascertain the period of PCD in this line for $p75^{+/+}$ and $p75^{-/-}$ mice, analyzing both total cell counts and

cleaved caspase-3 immunofluorescence staining to determine the most appropriate readout for cell death during this time.

Lastly, it would be interesting to determine if deletion of p75 specifically in *Ret* positive cells results in a loss of cell death. In our genetic approach, other members of the TNFR superfamily, such as TNFR1 and DR6, may somehow compensate for the ubiquitous deletion of p75. Specifically deleting *p75* in this small subset of *Ret* positive cells may avoid this potential compensation. However, the bigger question still remains: What is the function of RET in the SCG during late embryonic development? This topic is explored further in Chapter 4.

Experimental Procedures

Culture and Transfection of Immortalized Cell Lines **-** NIH/3T3 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were plated on 6-well tissue culture plates (Falcon) and allowed to proliferate until an approximate density of 50% confluence was obtained prior to transfection. Transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). A total of 5 µg plasmid DNA was added per well, using a plasmid encoding GFP to keep the total amount of DNA constant between treatments. The plasmid encoding *p75* was generously provided by Phil Barker.

Production of Primary Superior Cervical Ganglion Neurons - Superior cervical ganglia (SCGs) were surgically dissected from E19-P1 Sprague-Dawley rats (Charles

River, Portage, MI), and enzymatically dissociated via incubation in type I collagenase (Worthington) and a 1:1 ratio of HBSS: TrypLE (Invitrogen). Neurons were plated on gas-plasma treated 35 mm^2 dishes (Harrick Plasma; Ithaca, NY) coated with type I collagen (BD Biosciences, San Jose, CA). For all biochemical experiments using rat neurons, cells were plated as mass cultures at a density of 3 ganglia per plate. Neurons were maintained in minimum essential medium (MEM) containing 50 ng/ml NGF (Harlan), 10% (FBS), anti-mitotic agents aphidicolin (3.3 ug/ml) and 5-fluoro-2-deoxyuridine (20 uM; Sigma, St. Louis, MO), 2 mM glutamine, and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Neurons were maintained at 37°C with 8% CO_2 with medium changes every 3-4 days until treatment. Growth factor treatments were performed as described in the figure legends.

Immunoprecipitations and Quantitative Immunoblotting **-** Cells were stimulated as indicated in the figure legends. Following treatment, dishes were placed on ice, gently washed with PBS and lysed with immunoprecipitation (IP) buffer (Tris-buffered saline, pH 7.4, 1% Nonidet P-40, 10% glycerol, 500 μM sodium vanadate, and protease inhibitors) as described previously (Tsui and Pierchala, 2008). Antibodies for α-p75 (5 ul; 07-476, Millipore, Billerica, MA), α-RET51 and/or α-RET9 (8 μL; C-20 and C-19-G, respectively; Santa Cruz Biotechnology) were added along with protein A and protein G (Invitrogen, Carlsbad, CA) and incubated overnight at 4°C under gentle agitation. Immunoprecipitates were then washed three times with IP buffer and prepared for sodium dodecyl sulfate-polyacrylate gel electrophoresis (SDS-PAGE) by adding 2X

sample buffer (tris-buffered saline, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 0.1% bromophenol blue and 4% SDS) and boiling the samples for 10 minutes.

Samples for western blotting were subjected to SDS-PAGE followed by electroblotting onto polyvinylidene difluoride membranes (Immobilin P; Millipore). Western blot analysis was performed using the following antibodies at the indicated concentrations: α-Ret51 (C-20, Santa Cruz, 1:500-1:1000), α-Ret9 (C19R, Santa Cruz, 1:1000), α-Ret (AF482, R&D, 1:1000), α-p75 (Advanced Targeting Systems, 1:1000), α-actin (JLA-20, Iowa Hybridoma, 1:2000), α-Sortilin (ab16640, Abcam, 1:1000). Blots were developed using a chemiluminescent substrate (Supersignal, ThermoFisher). For quantifications, scanned images of X-ray films were imported into ImageJ (National Institutes of Health) and processed using the gel analysis tool. Integrated density values obtained from immunoblotting were reported as mean values ± SEM, with arbitrary units on the vertical axis. Values were normalized to the appropriate control: for co-immunoprecipitation studies, values were normalized to the precipitated protein, and values were normalized to actin (used as a loading control) for all other samples. All biochemical experiments were performed at least three times with similar results.

Detergent Extraction and Immunoprecipitation from Whole Tissues - Spinal cords were harvested separately from E19.5/P0 $Ret^{fl/fl}$; *UBC*-Cre/ER^{T2} mice, $p75^{fl/-}$; *UBC*-Cre/ER^{T2} mice or $p75^{-/-}$ mice, placed in a 2.0 mL tube with 250 µL IP Buffer lacking NP-40 along with a steel grinding ball (5mm, 69989, Qiagen, Valencia, CA) and mechanically homogenized using the TissueLyzer II (Qiagen). The homogenates were then mixed with 250 µL of 2% NP-40-containing IP buffer and incubated for 1 hour at 4°C under

gentle agitation. Homogenates were centrifuged for 10 minutes at 16,100xg and subjected to an initial pre-clearing step with protein A and protein G alone at 4°C for 2 hours under gentle agitation, followed by pre-clearing with protein A, protein G, and a species-matched nonspecific control IgG for 2 hours under gentle agitation. Following pre-clearing, immunoprecipitations were performed as described above.

Mouse Lines and Production of Embryos - *UBC*-Cre/ER^{T2} (Ruzankina et al., 2007), *Ret*$^{fl/fl}$ (Luo et al., 2007), *p75*$^{fl/fl}$ (Bogenmann et al., 2011), *p75*$^{-/-}$ (Bogenmann et al., 2011), *Ret*-Cre/ER^{T2} (Luo et al., 2009), and *Rosa26*$^{LSL\text{-}tdTomato}$ mice (Madisen et al., 2010) have all been previously described, and all mice were maintained in mixed genetic backgrounds except for *Rosa26*$^{LSL\text{-}tdTomato}$ which was maintained in a C57BL/6J background. For timed matings, noon of the day on which a vaginal plug was detected was considered as E0.5. *Rosa26*$^{LSL\text{-}tdTomato}$ mice were crossed to *Ret*-Cre/ER^{T2} mice and given one intraperitoneal injection of tamoxifen (100 µL, 10 mg/mL) at E18.5. Additionally, *Ret*$^{fl/fl}$ and *p75*$^{+/-}$ female mice crossed to either *Ret*$^{fl/fl}$*; UBC*-Cre/ER^{T2} or *p75*$^{fx/fx}$*; UBC*-Cre/ER^{T2} males, respectively, and were given intraperitoneal injections of tamoxifen (100 µL, 10 mg/mL) and progesterone (100 µL, 5 mg/mL) consecutively for four days beginning at E14.5. All pregnant females were euthanized at E19.5 and embryos were recovered for analysis. The housing and all procedures performed on these mice were approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC).

Fixation, Sectioning and Immunostaining of Superior Cervical Ganglia - Superior cervical ganglia (SCG) were fixed with 4% paraformaldehyde at 4ºC for 2-3 hours, washed in PBS three times for 10 minutes, and cryoprotected at 4ºC in 1X PBS containing 30% sucrose overnight. Tissues were embedded in OCT (Tissue Tek), frozen and stored at -80ºC until use. SCGs were serially sectioned at 7 µM on a cryostat (CM1950, Leica Biosystems, Germany), with every third section being mounted onto the same slide for collective analysis. Tissue sections were washed with PBS and blocked with 5% normal goat serum in PBS-T (0.1% Triton X-100 in 1X PBS) for 1 hour, followed by incubation with primary antibody (diluted in blocking solution) in a humidified chamber overnight at 4ºC. Sections were washed with PBS-T, and incubated with secondary antibody (1:500) diluted in blocking solution for 2 hours. Sections were washed again with PBS-T and mounted in fluoromount-G with DAPI (Southern Biotech). Images were taken using an inverted fluorescence microscope (Axiovert 200M, Zeiss Microsystems, Germany). Antibodies used include α-cleaved caspase-3 (1:300, Cell Signaling), α-tyrosine hydroxylase (1:1000, Millipore), and α-p75 [NGFr] (1:200, Advanced Targeting Systems). The number of cleaved caspase-3 positive neurons were counted on every third section by an observer naïve to the genotypes of the mice. Area measurements of SCGs were performed in the AxioVision software (Zeiss) using the “Outline” function.

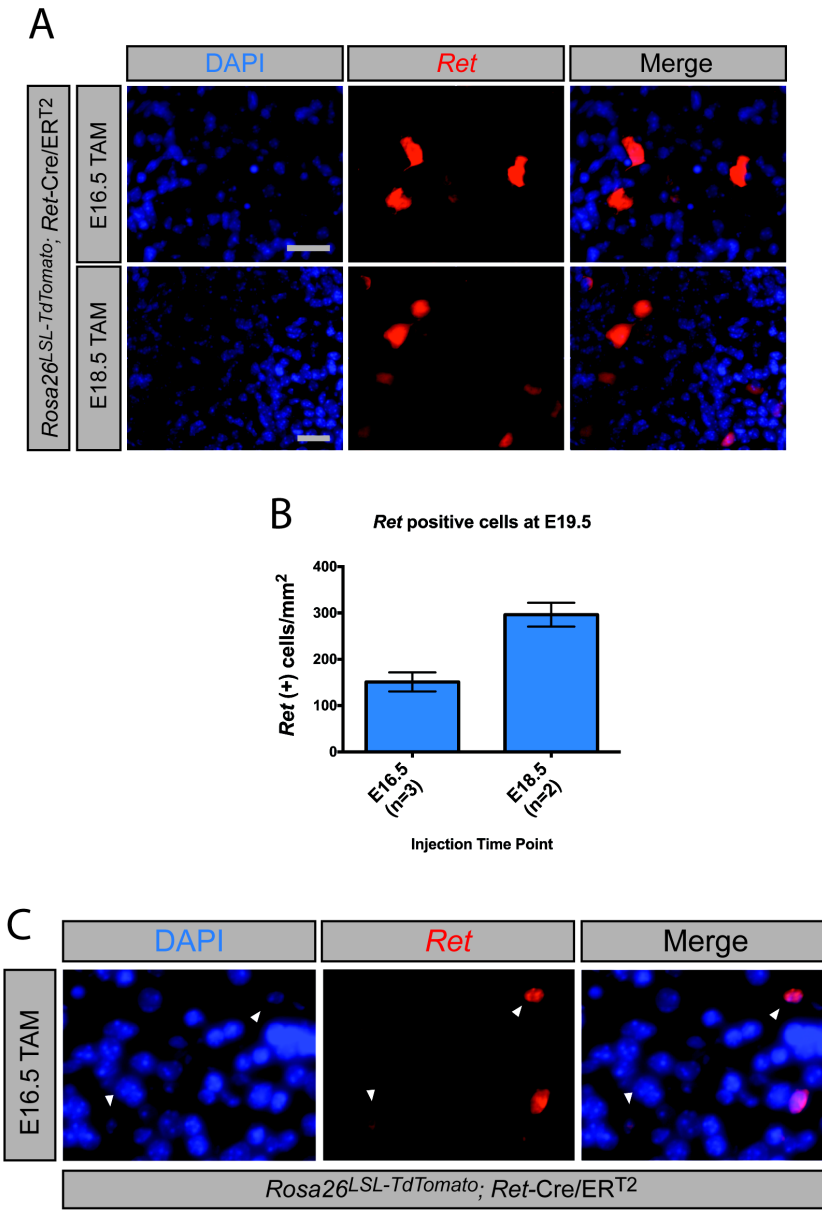

Figure 3.1: *Ret* is expressed in a subset of neurons during late prenatal development. (A) *Rosa26*$^{LSL\text{-}tdTomato}$ females were crossed to *Ret*-Cre/ER^{T2} males, and a pulse experiment was performed in which pregnant dams were injected with tamoxifen at E16.5 or E18.5. SCGs were then isolated from embryos at E19.5. Representative images from each set of SCGs are shown. The scale bars denote 50 μm. (B) The number of *Ret* positive cells are quantified from mice injected with tamoxifen at E16.5 (n=3) or E18.5 (n=2). (C) *Ret* positive cells from embryos receiving tamoxifen at E16.5 appear apoptotic. Arrows designate *Ret* positive cells that also have karyolytic or pyknotic nuclei.

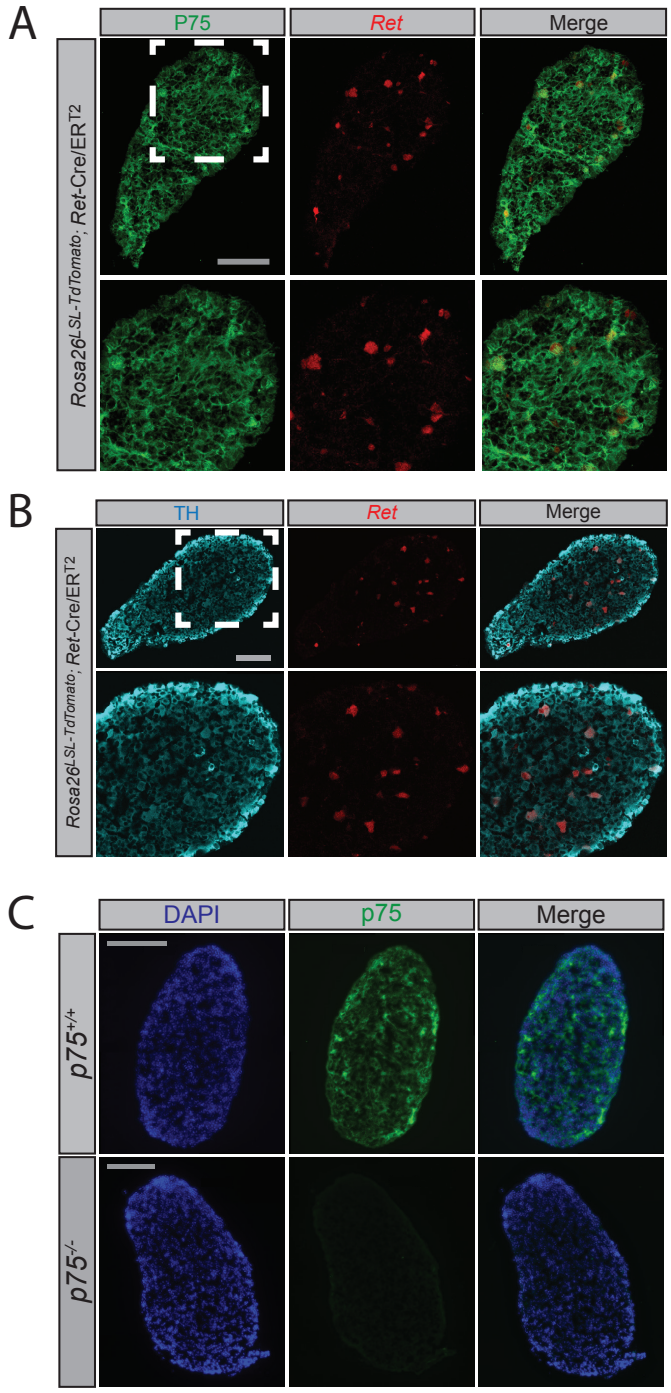

Figure 3.2: *Ret* is expressed in a subset of p75-positive noradrenergic neurons during late embryonic development. (A) *Ret* is expressed in p75 positive cells of the SCG at E19.5. Scale bars denote 200 µm. (B) *Ret* is expressed in cells also expressing tyrosine hydroxylase, a marker of noradrenergic neurons. Scale bars denote 200 µm. (C) The specificity of the p75 antibody was determined by performing immunofluorescence staining on SCGs from E18.5 wild-type or germline *p75* null littermates. We observed a loss of all p75 staining in SCGs of $p75^{-/-}$ mice. Scale bars denote 200 µm.

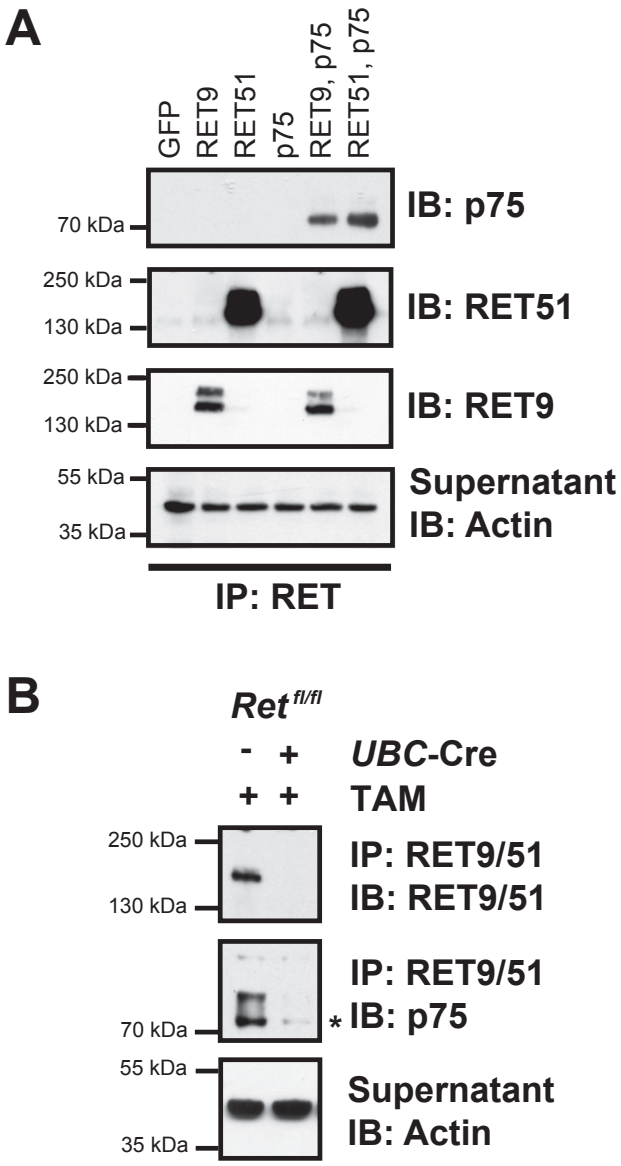

Figure 3.3: RET and p75 interact *in vitro* and *in vivo*. (A) Cells were transfected with RET9, RET51 or p75 alone, or were co-transfected with one of the RET isoforms along with p75. Immunoprecipitations for RET were performed, and immunoblotting for p75 revealed co-immunoprecipitation of p75 with both RET9 and RET51. (B) Spinal cords were isolated from P5 $Ret^{fl/fl}$ or $Ret^{fl/fl}$;*UBC*-Cre/ER^{T2} that had been treated with tamoxifen since birth. Immunoprecipitations for RET9 and RET51 were performed to isolate both RET isoforms. Immunoblotting for p75 showed co-immunoprecipitation of p75 with RET under basal conditions. In spinal cords where *Ret* was conditionally deleted, immunoblotting for p75 showed no co-immunoprecipitation, indicating that our antibodies are specific. Experiments performed and data provided by Olivia Stephens and Chris Donnelly.

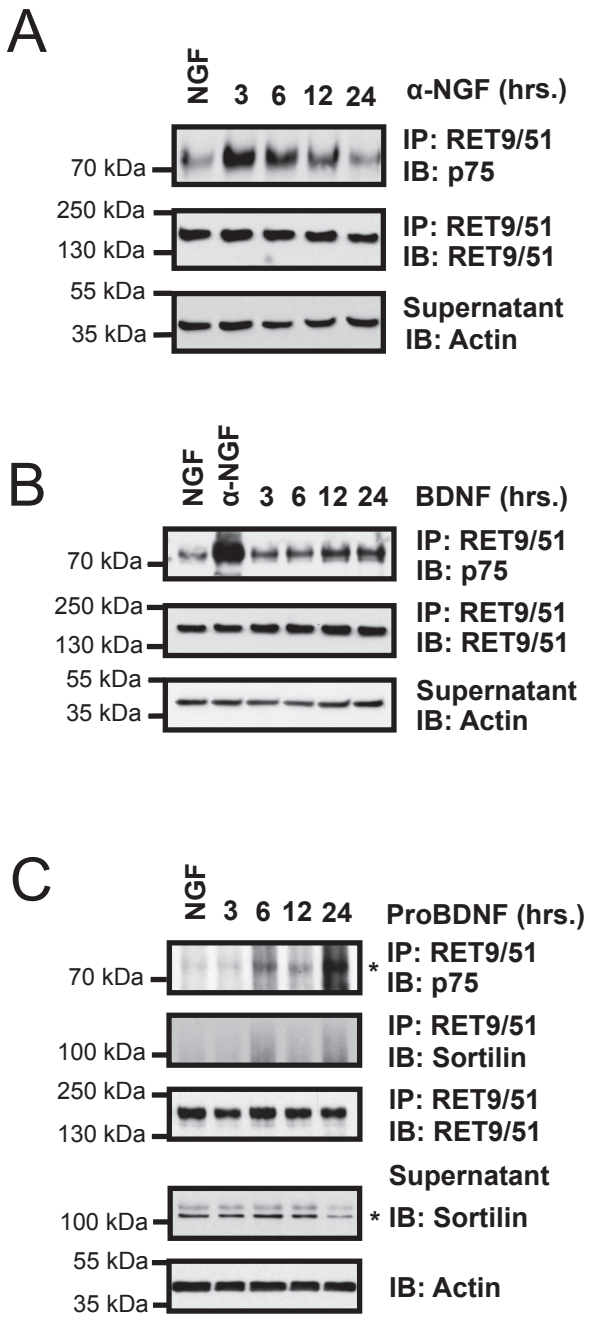

Figure 3.4: Apoptotic stimuli induce the association of RET and p75 *in vitro*. Primary sympathetic neurons were treated with (A) α-NGF, (B) BDNF or (C) proBDNF for 3, 6, 12 or 24 hours. At each time point, immunoprecipitations for RET were performed and immunoblotting for p75 was done to assess stimuli dependent interactions between RET and p75. Neurons treated with α-NGF (A) had a faster association that dissipated over time, whereas neurons treated with BDNF (B) or proBDNF (C) showed an increase in association of the two proteins over time. Immunoblotting for sortilin (C) a protein that has been shown to associate with p75 for proBDNF pro-apoptotic signaling was not observed to associated with the RET-p75 complex. Experiments performed and data provided by Chris Donnelly.

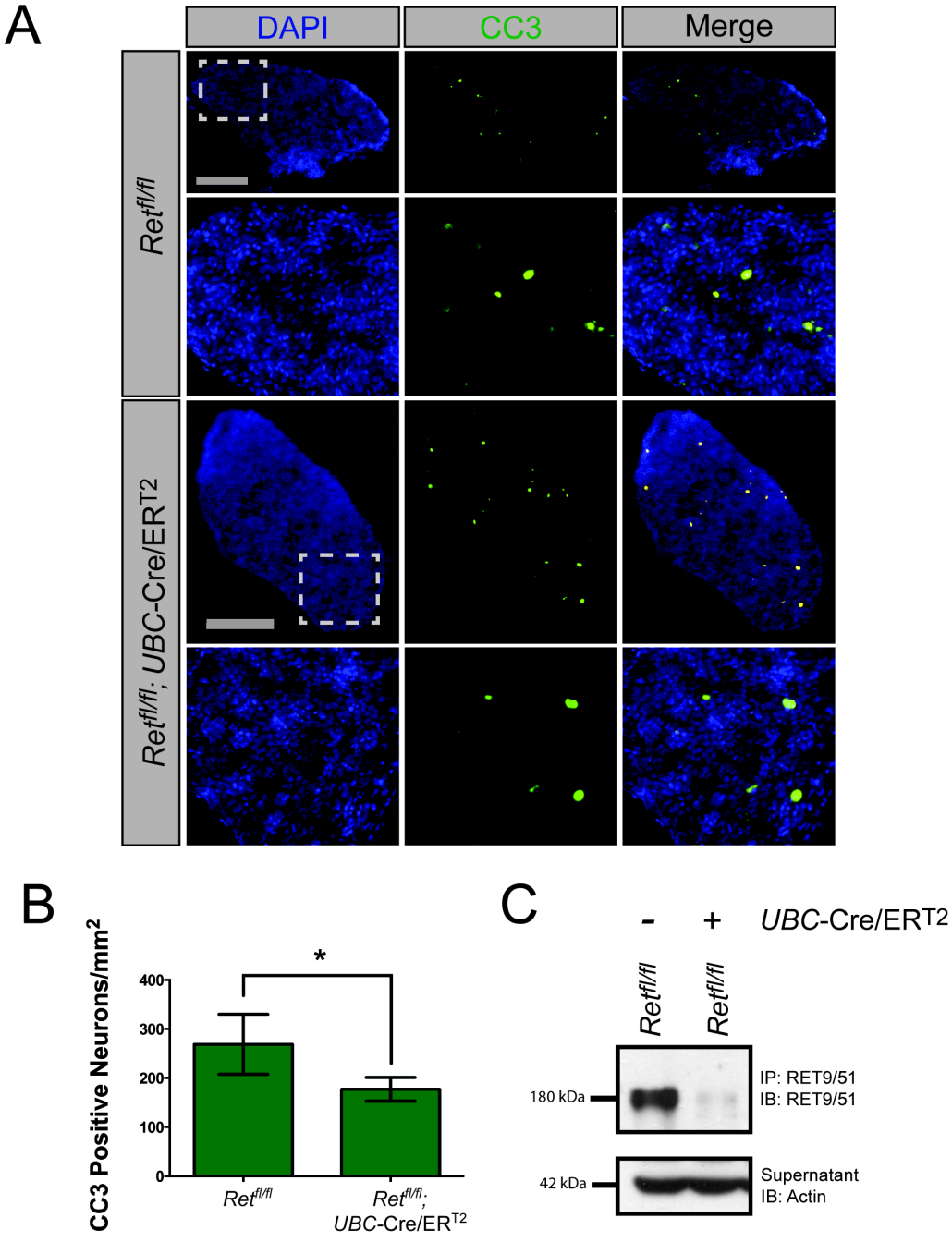

Figure 3.5: Conditional deletion of *Ret* results in a reduction in the number of cleaved caspase-3 positive cells at E19.5. (A) Representative images of *Ret*$^{fl/fl}$ and *Ret*$^{fl/fl}$;*UBC*-Cre/ER^{T2} E19.5 SCGs with DAPI to label nuceli and cleaved caspase-3 staining to label actively dying cells. Embryos received tamoxifen from E14.5 to E17.5. Scale bars denote 200 µm. (B) Quantifications of the number of cleaved caspase-3 positive cells per area (5 SCGs per genotype, one-tailed, paired t-test; p=0.0420). (C) Spinal cords were isolated from *Ret*$^{fl/fl}$ and *Ret*$^{fl/fl}$;*UBC*-Cre/ER^{T2} embryos, and knockdown of RET was analyzed. We found that greater than 95% of RET was eliminated in *Ret*$^{fl/fl}$;*UBC*-Cre/ER^{T2} spinal cords compared to *Ret*$^{fl/fl}$ littermate controls.

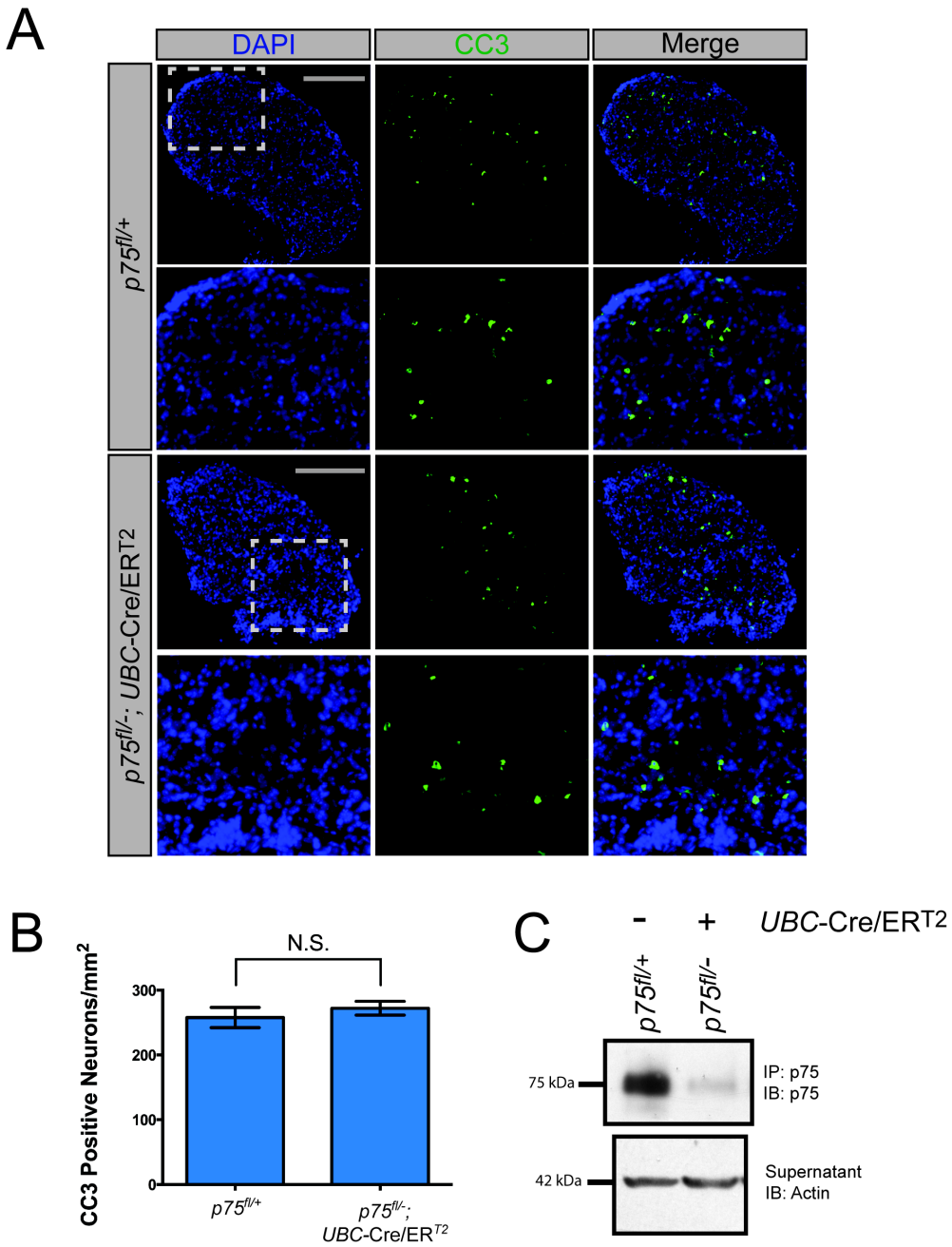

Figure 3.6: Conditional deletion of *p75* does not result in a reduction of the number of cleaved caspase-3 positive cells at E19.5. (A) Representative images of $p75^{fl/+}$ and $p75^{fl/-}$;*UBC*-Cre/ER^{T2} E19.5 SCGs with DAPI to label nuceli and cleaved caspase-3 staining to label actively dying cells. Embryos received tamoxifen from E14.5 to E17.5. Scale bars denote 200 µm. (B) Quantifications of the number of cleaved caspase-3 positive cells per area (3 SCGs per genotype, one-tailed, paired t-test; p=0.2515). (C) Spinal cords were isolated from $p75^{fl/+}$ and $p75^{fl/-}$;*UBC*-Cre/ER^{T2} embryos, and knockdown of p75 was analyzed. We found that greater than 90% of p75 was eliminated in $p75^{fl/-}$;*UBC*-Cre/ER^{T2} spinal cords compared to $p75^{fl/+}$ littermate controls.

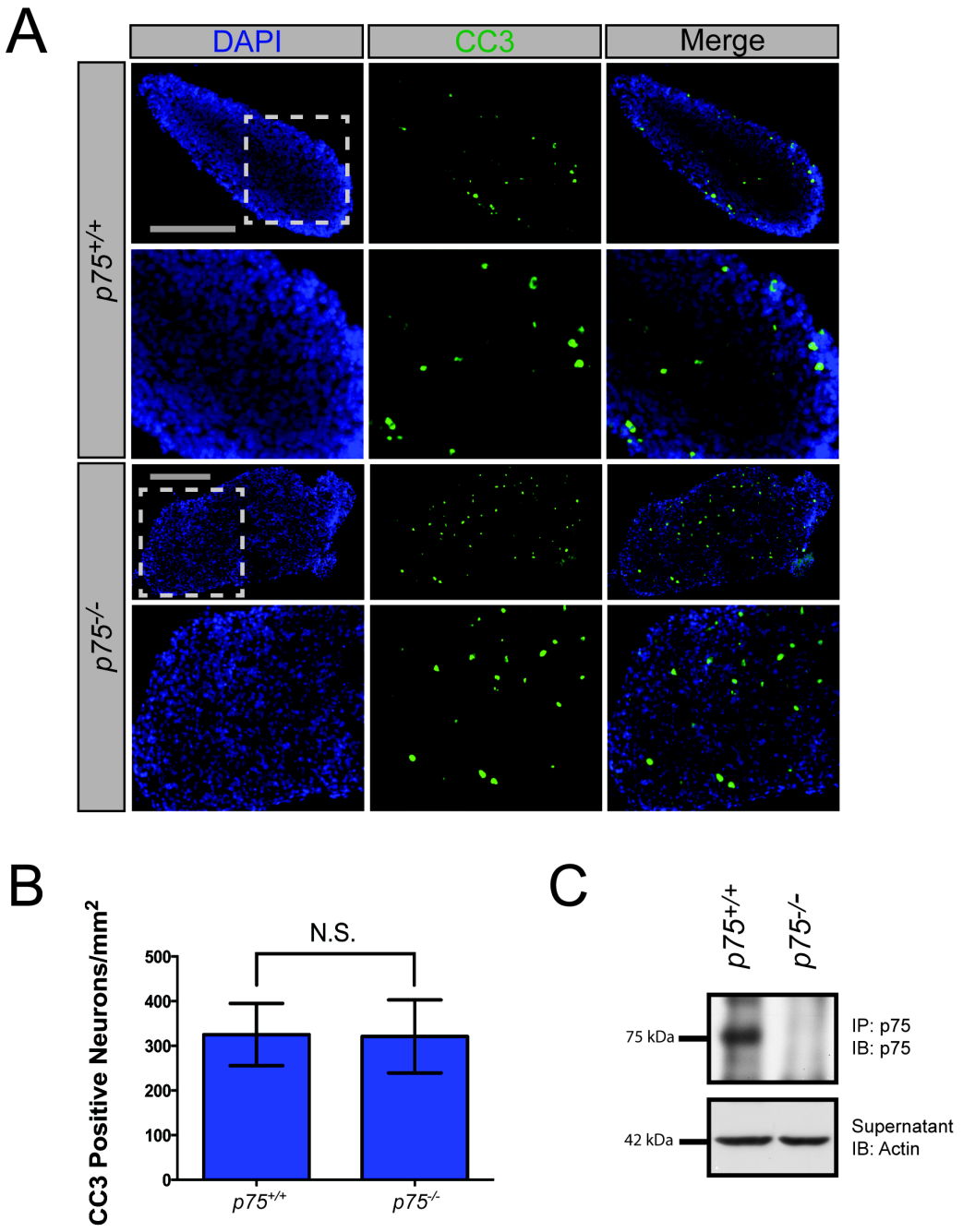

Figure 3.7: Germline deletion of *p75* does not result in a reduction of cleaved caspase-3 positive cells at E19.5. (A) Representative images of $p75^{+/+}$ and $p75^{-/-}$ E19.5 SCGs with DAPI to label nuceli and cleaved caspase-3 staining to label actively dying cells. Scale bars denote 200 μm. (B) Quantifications of the number of cleaved caspase-3 positive cells per area (3 SCGs per genotype, one-tailed, paired t-test; p=0.3935). (C) Spinal cords were isolated from $p75^{+/+}$ and $p75^{-/-}$ embryos, and knockdown of p75 was analyzed. We were unable to detect any p75 protein band in $p75^{-/-}$ spinal cords whereas a p75 band was present in $p75^{+/+}$ littermate controls.

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CHAPTER 4

DISCUSSION

Exon Skipping in RET Encodes Novel Isoforms that Differentially Regulate RET Signal Transduction: Discussion and Future Directions

In this thesis I have shown that *Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ transcripts, which were originally identified in humans, are conserved in zebrafish, mice and rats. We additionally observed that these mRNAs are expressed dynamically throughout nervous system development in mice. Interestingly, I found that the *Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ transcripts encoded full-length RET proteins in all species analyzed, but with deletions in the extracellular domain. I assessed species conservation because transcripts that are highly conserved between multiple species usually play an important role for the organism. Furthermore, *RET43*, an additional *RET* transcript that arises from 3' alternative splicing, has only been observed in humans, and I wanted to confirm that *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$ were not solely human *RET* transcripts (Myers et al., 1995, Lee et al., 2003).

For the *Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$ expression experiments, I evaluated tissues where full-length *RET* was expressed because the original expression studies performed in humans also analyzed tissues in which *RET* is known to be expressed, such as the kidney and substantia nigra. *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$ are co-expressed in these tissues (Lorenzo et al., 1995, Ivanchuk et al., 1998). Analyzing additional tissues where *RET* is

not known to be expressed to see if *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$ are expressed independently of full length *RET* would be intriguing. Additionally, Lorenzo et al. found an increase in expression of *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$ in human pheochromocytomas compared to normal tissues (Lorenzo et al., 1995). From these observations, it would be interesting to determine if the 5' alternative splicing that occurs in *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$ is enhanced in a disease setting. The RNA splicing machinery may become unregulated in disease contexts, allowing for the formation of these *RET* transcripts that, when translated, give rise to proteins that enhance tumor progression. This idea will be discussed further in this chapter.

In accordance with the low transcript expression of *Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$, I struggled to observe RET$^{\Delta E3}$ and RET$^{\Delta E345}$ protein isoforms in mouse tissues. I scaled up our protocols to isolate RET from larger volumes and higher concentrations of total protein, including tissues of the brain and spinal cord, in the hope that I would isolate enough protein of these lower expressed isoforms. While I have observed molecular weight RET proteins that run at the appropriate molecular weight for RET$^{\Delta E345}$, I have not been able to isolate enough protein to successfully confirm by protein sequencing and/or mass spectrometry the identity of these protein bands. Additionally, technical complications related to the glycosylation of RET arose in our collaborations with the University of Michigan Proteomics Core and the Harvard University Proteomics Core, and so far I have been unable to detect RET protein via liquid chromatography mass spectrometry (LC-MS) from tissues in which RET has been immunoprecipitated. Follow up experiments are being performed to determine if RET$^{\Delta E3}$ and RET$^{\Delta E345}$ are expressed in normal tissues.

As for additional experimental methods to detect the $RET^{\Delta E3}$ and $RET^{\Delta E345}$ isoforms, we created isoform specific antibodies against the amino acids at the exon-exon junctions of these proteins. I performed control experiments in which NIH/3T3 cells were transfected with expression plasmids encoding *RET51*$^{\Delta E3}$ and *RET51*$^{\Delta E345}$. RET proteins were isolated 48 hours post-transfection via immunoprecipitations using a RET51 specific antibody. Western blot analyses using RET isoform-specific antibodies failed to detect the recombinant $RET51^{\Delta E3}$ and $RET51^{\Delta E345}$ proteins.

Because these deletions in the extracellular domain are highly conserved, we were interested in understanding the signaling capabilities and biological functions these proteins may possess. We assessed this by first determining if these RET isoforms were trafficked to the cell surface, given that mutations in RET, such as M918T, have been shown to create unstable proteins that are not trafficked as efficiently (Runeberg-Roos and Saarma, 2007). Utilizing two different approaches, a cell surface biotinylation assay (data not shown) and an immunocytochemistry approach (Fig. 2.3), I successfully detected $RET51^{\Delta E3}$ and $RET51^{\Delta E345}$ at the cell surface.

Once I determined that the RET isoforms were trafficked to the plasma membrane, I wanted to determine whether they could interact with GPI-anchored GFRα co-receptors. Interactions with the GFRαs would be required in order for $RET^{\Delta E3}$ and $RET^{\Delta E345}$ to function as receptors for the GFLs. Although the affinity for the GFRαs is increased in the presence of ligand, overexpression of the GFRαs and RET in an *in vitro*, cell transfection setting allows for the interaction of these proteins that are otherwise thought to be kept separate under physiologic conditions by lipid rafts (Tansey et al., 2000). I hypothesized that both $RET^{\Delta E3}$ and $RET^{\Delta E345}$ would be able to

interact with all four GFRαs since the necessary domains, specifically CLD4 and the CRD, are present in both isoforms. Indeed, we did observe interactions between GFRα1, GFRα2, GFRα3 and GFRα4 with both $RET^{\Delta E3}$ and $RET^{\Delta E345}$. However, questions still remain unanswered about how the deletions in the extracellular domains of these RET isoforms affects the binding affinity of the GFRαs. I am currently investigating this question using an enzyme-linked immunosorbent assay (ELISA) in which GFRα1 fused to human IgG1 Fc is added with or without GDNF at differing concentrations to cells expressing RET, or to RET protein lysates. An antibody against human IgG conjugated to horseradish peroxidase (HRP) is then added, and binding is assessed using a colorimetric readout. Our preliminary experiments show that $RET^{\Delta E3}$ and $RET^{\Delta E345}$ bind to GFRα1-Fc and GFRα1-Fc with GDNF, but with lower affinity than full-length RET. Because $RET^{\Delta E3}$ retains much of the extracellular domain and can potentially function as a receptor for the GFLs, our hope is to use different GFRα-Fc recombinant proteins and their cognate ligands to determine if $RET^{\Delta E3}$ has a higher affinity for selective GFL-GFRα binary complexes.

We showed that $RET^{\Delta E3}$ and $RET^{\Delta E345}$ can bind to all four GFRαs, but are they able to signal? As a first step, we asked this question using a ligand independent approach. Overexpression of RET in transfected cell lines allows for ligand-independent association of RET monomers, which induces dimerization and autophosphorylation of the proteins. We assessed the amount of activation by quantifying the level of phosphorylated RET versus the total amount of RET. In our transfected cell line approach, we found that $RET^{\Delta E3}$ is phosphorylated to a similar level as full-length RET. However, $RET^{\Delta E345}$ displays higher baseline autophosphorylation at

both the catalytic tyrosine, Tyr^{905}, and also on an additional signaling tyrosine, Tyr^{1062}. This was not surprising given we had hypothesized that, due to the large structural deletions in the extracellular domain including the calcium binding domain, $RET^{\Delta E345}$ likely acts as a constitutively active form of RET. While this information was informative, we were still interested in addressing if $RET^{\Delta E3}$ and $RET^{\Delta E345}$ could be activated in a ligand-dependent manner.

Technical difficulties arose while trying to develop a ligand-dependent RET activation system *in vitro*. Preliminary experiments in NIH/3T3 cells suggested that the $RET^{\Delta E3}$ isoform could be activated in a ligand-dependent manner. However, the $RET^{\Delta E345}$ was not activated in a ligand-dependent manner, as expected, and also exhibited increased phosphorylation overall in either the absence or presence of GDNF. Replication of this result in transfected NIH3T3 cells was unsuccessful, and countless troubleshooting attempts were made to re-establish this technique. We recently established a modified ligand-induced activation assay in Neuro2A cells, but the basal phosphorylation levels remain too high for us to observe ligand-dependent phosphorylation of RET. As an alternative, we have assessed two additional factors as a readout of RET activation: (1) ligand dependent association of GFRα1 with RET, and (2) ligand-dependent poly-ubiquitination of RET. RET is poly-ubiquitinated following activation to signal internalization and degradation of the protein by the proteasome to limit the signaling life of the receptor (Tsui-Pierchala et al., 2002). Our preliminary results using this assay suggest our earlier findings were correct – $RET^{\Delta E3}$ can be activated in a ligand-dependent manner while $RET^{\Delta E345}$ is not. In our new assay, we observe a ligand-dependent association of GDNF-GFRα1 with both $RET^{\Delta E3}$ and

$RET^{\Delta E345}$, which was surprising. However, $RET^{\Delta E3}$ appears to become poly-ubiquitinated within 30-60 minutes after GDNF treatment, similar to full-length RET, but $RET^{\Delta E345}$ does not become poly-ubiquitinated in a ligand-dependent manner (data not shown).

Taken together, these isoforms may have unique and important unidentified roles in the development and maintenance of the nervous system and renal system, and may also be involved in the pathophysiology of neuroendocrine gland diseases, and as such, a number of interesting questions still remain.

Future Directions:

Further exploration of $Ret^{\Delta E3}$ and $Ret^{\Delta E345}$ in neuroendocrine gland tumors

The alternative splicing that gives rise to *$RET^{\Delta E3}$* and *$RET^{\Delta E345}$* was first observed in adrenal gland tumors, but only expression of the transcripts was determined (Lorenzo et al., 1995). No experiments were performed to determine if these transcripts, which seemed to have a higher relative expression in pheochromocytomas compared to normal tissues, could be detected at the protein level in these human tumor samples (Lorenzo et al., 1995). It would be intriguing to determine whether $RET^{\Delta E3}$ and $RET^{\Delta E345}$ could be isolated from human pheochromocytoma tissues, which may be pursued in the future, potentially via the University of Michigan Adrenal Biorepository Effort, although our initial attempts to procure samples were unsuccessful. It would also be interesting to take advantage of pheochromocytoma cell lines to assess the signaling and the function of $RET^{\Delta E3}$ and $RET^{\Delta E345}$ in a disease context. I would recommend that two different experimental designs be used:

In the first set of experiments, a pheochromocytoma cell line that does not overexpress RET could be used, as not all pheochromocytomas do, such as PC-12 cells (Powers et al., 2003, Powers et al., 2009). These cells would be co-transfected with one of the RET isoforms and GFRα1, and then stimulated with GDNF. Driving RET activation in pheochromocytoma cell lines causes differentiation of the cells to a more neuronal-like morphology (Powers et al., 2009). Quantification of the number of differentiated cells could then be analyzed as a physiological readout of RET isoform signaling. Additionally, the transfections and GDNF stimulations in this cell line could be assessed as an additional *in vitro* ligand-dependent activation system, and biochemical analysis of phospho-RET levels could be determined via immunoblotting, if there is not the problem of excessive levels of basal Ret autophosphorylation.

In the second set of experiments, a pheochromocytoma cell line that does overexpress RET could be used. Since $RET^{\Delta E3}$ and $RET^{\Delta E345}$ transcripts were detected in adrenal gland tumors that overexpress RET, this cell line could be used to determine if the $RET^{\Delta E3}$ and $RET^{\Delta E345}$ isoforms are present. Initial experiments would need to be performed to determine if $RET^{\Delta E3}$ and $RET^{\Delta E345}$ transcripts are present, initially by performing Western blotting to detect full-length RET and the presence of smaller molecular weight RET proteins. If RET bands of the appropriate molecular weight are present, siRNAs which target the exon-exon junctions of $RET^{\Delta E3}$ and $RET^{\Delta E345}$ could be used to determine if these lower molecular weight RET proteins are truly the $RET^{\Delta E3}$ and $RET^{\Delta E345}$ isoforms. Additionally, if these isoforms are detected, a number of experiments could be performed to determine the pathobiological functions of these proteins in an *in vitro* context, including analysis of phospho-RET levels before and after

GDNF stimulation as well as investigating the adaptors proteins that interact with each RET isoform.

Identifying the mechanism by which exon skipping in RET is regulated

As mentioned previously, the *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$ transcripts are upregulated in adrenal gland tumors, but the reason for this has not been elucidated (Lorenzo et al., 1995). My preliminary bioinformatics analysis predicts several exonic splicing enhancers in exons 3, 4 and 5 (data not shown). In cells that endogenously express *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$ transcripts, it would be exciting to mutate these 6-bp sites with a CRISPR/Cas9 system to create synonymous mutations that do not affect the amino acid sequence. The expression levels of *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$ transcripts could then be analyzed by qPCR; by mutating these sites, we would expect to lose expression of these alternatively spliced transcripts. Additionally, if a pheochromocytoma cell line were observed to have increased expression of *RET*$^{\Delta E3}$ and/or *RET*$^{\Delta E345}$*,* it would be an attractive system to investigate essential pre-mRNA splicing factors, such as SR proteins, involved in exon skipping of these *RET* transcripts.

Interrogating the biological function of the RET$^{\Delta E3}$ and *RET*$^{\Delta E345}$ *isoforms*

We have so far been unable to successfully establish an experimental system that is capable of generating a ligand-dependent physiological readout of RET activation, a problem that is not new to the field of RET biology. Important questions, however, still remain regarding the function of RET$^{\Delta E3}$ and RET$^{\Delta E345}$. Can they support

cell survival? Are they needed for differentiation of specific cell types? Do they function in axon guidance?

To address their role in cell survival, primary sympathetic neuron cultures may be able to be utilized. SCGs from *UBC*-Cre/ER^{T2}; *Ret$^{fl/fl}$* mice could be isolated and cultured, and then treated with 4-hydroxy-tamoxifen to conditionally delete *Ret.* This would bypass the developmental period when RET is necessary for proper development of the SCG. Endogenous RET could then be replaced by transducing the cells with a virus encoding *RET$^{\Delta E3}$* or *RET$^{\Delta E345}$*. These primary sympathetic neurons could then be treated with anti-NGF to block survival signaling and stimulated with GDNF to assess the amount of cell survival via RET activation. Full-length RET can support the survival of approximately 30-50% of mouse neurons *in vitro* (Yu et al., 2003). We performed preliminary experiments in which primary sympathetic neurons were treated with lentiviruses to deliver HA epitope tagged full-length *RET*, *RET$^{\Delta E3}$* or *RET$^{\Delta E345}$* to SCG neurons. We observed, however, little to no expression of our exogenous RET isoforms in these cells. Surprisingly, it appears that sympathetic neurons do not express proteins well when driven by a *CMV* promoter, which was the promoter we used given that it is the most common promoter in lentiviral vectors. Using a GFP-encoding lentiviral vector, SCG neurons seem to express proteins consistently using the neuron-specific promoter *Synapsin* (Fig. 4.1). Further troubleshooting of this system remains to be completed, but this may be the most ideal *in vitro* system to interrogate the signaling and function of RET$^{\Delta E3}$ or RET$^{\Delta E345}$ in primary neurons. It should be noted that we also attempted to express these *RET* splice variants in SCG neurons using HSV expression vectors, which use the chicken β-actin promoter, but the expression was so robust and rapid

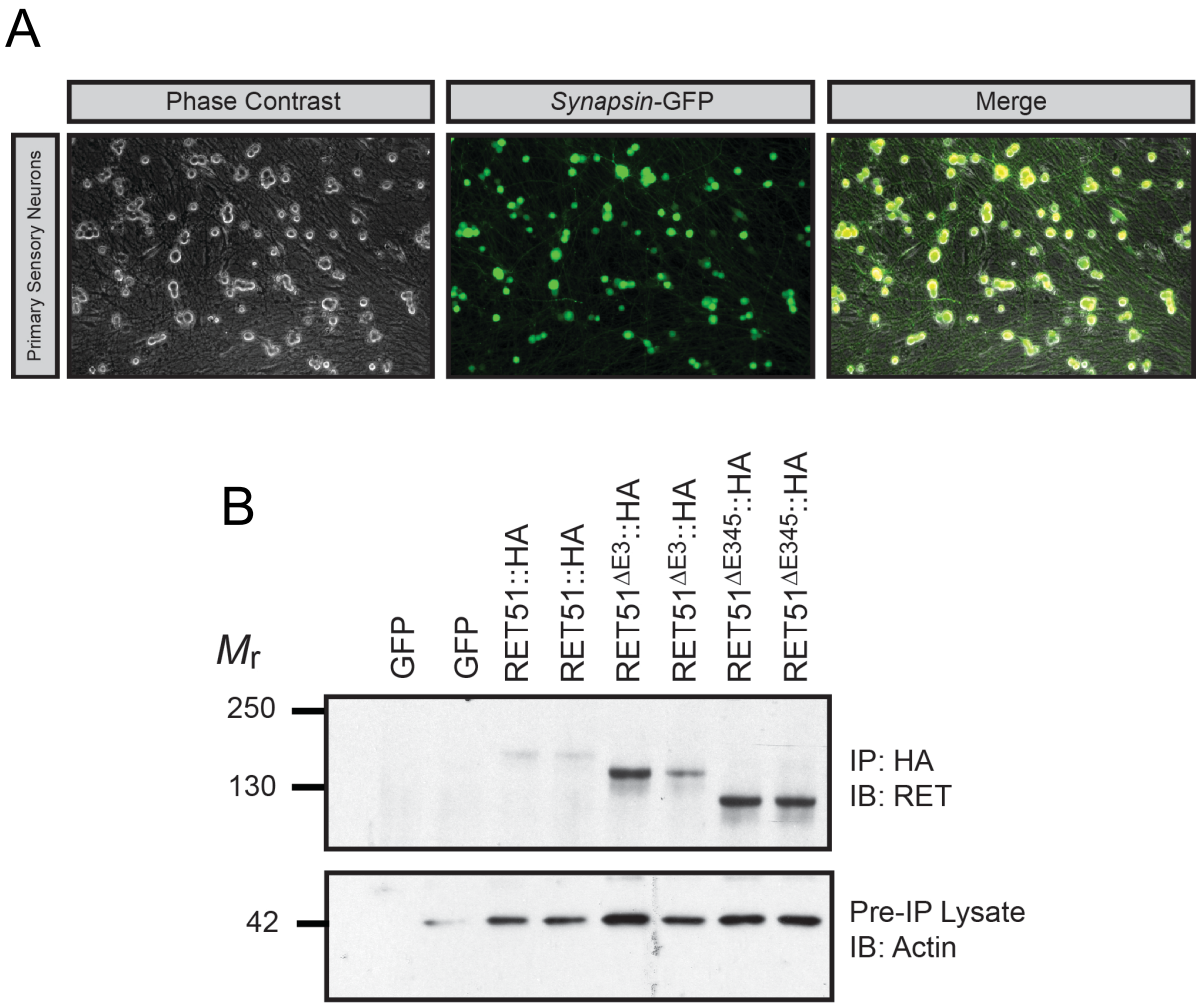

Figure 4.1: Infections of primary neuronal cells with lentivirus. (A) Primary sensory neurons isolated from the dorsal root ganglia were infected with a 1:1000 dilution of lentivirus delivering expression of GFP driven from the *Synapsin* promoter. We observed robust GFP expression in both sensory and sympathetic neurons treated with this lentivirus after 72 hours. (B) Primary sympathetic neurons isolated from the superior cervical ganglia were infected with a 1:50 dilution of lentivirus delivering expression of HA epitope tagged RET isoforms driven from the *CMV* promoter. Expression of the RET isoforms was observed after 7 days, however the treatment with this concentration of lentivirus negatively affected the viability of the neurons. This is likely due to the high titer of lentivirus needed to successfully express the RET isoforms.

(high levels within 4-6 hours) that the ectopically expressed RET proteins were highly autophosphorylated, as in cell lines (Fig. 4.2).

Additionally, the ability of $RET^{\Delta E3}$ and $RET^{\Delta E345}$ to support the differentiation of cells could be investigated. The differentiation of the neuroblastoma cell line, Neuro2A (N2A), has been used as a physiological readout of proper RET signaling (Tansey et al., 2000). N2As express low levels of RET and do not express GFRαs. These cells, however, are highly transfectable, and transfection with an expression plasmid encoding *GFRα1* allows for GDNF-induced activation of RET (Tansey et al., 2000). This activation induces N2As to differentiate into a more neuronal-like phenotype in which cells extend neurite-like projections within 24-48 hours (Tansey et al., 2000).

Initial experiments should be performed in which cells are transfected with GFRα1, and then stimulated with GDNF, to obtain a baseline level of differentiation. Cells could then be transfected with either full-length *RET, $RET^{\Delta E3}$* or *$RET^{\Delta E345}$* and the level of differentiation examined without any additional experimental treatments. An interesting question to ask first is whether $RET^{\Delta E345}$ can induce differentiation in the absence of ligand and/or a GFRα. From our experiments thus far, it seems that $RET^{\Delta E345}$ may function as a constitutively active receptor, and we predict that cells expressing $RET^{\Delta E345}$ to differentiate in the absence of ligand and co-receptor. In the next experiments, cells would be co-transfected with full-length *RET, $RET^{\Delta E3}$* or *$RET^{\Delta E345}$* and *GFRα1*, and then ligand-dependent differentiation would be assessed.

Ultimately we are interested in determining whether these splice variants have physiologic functions *in vivo*. To this end, I recommend the use of one of two possible transgenic strategies. The first would take advantage of the mutations to the 6 bp

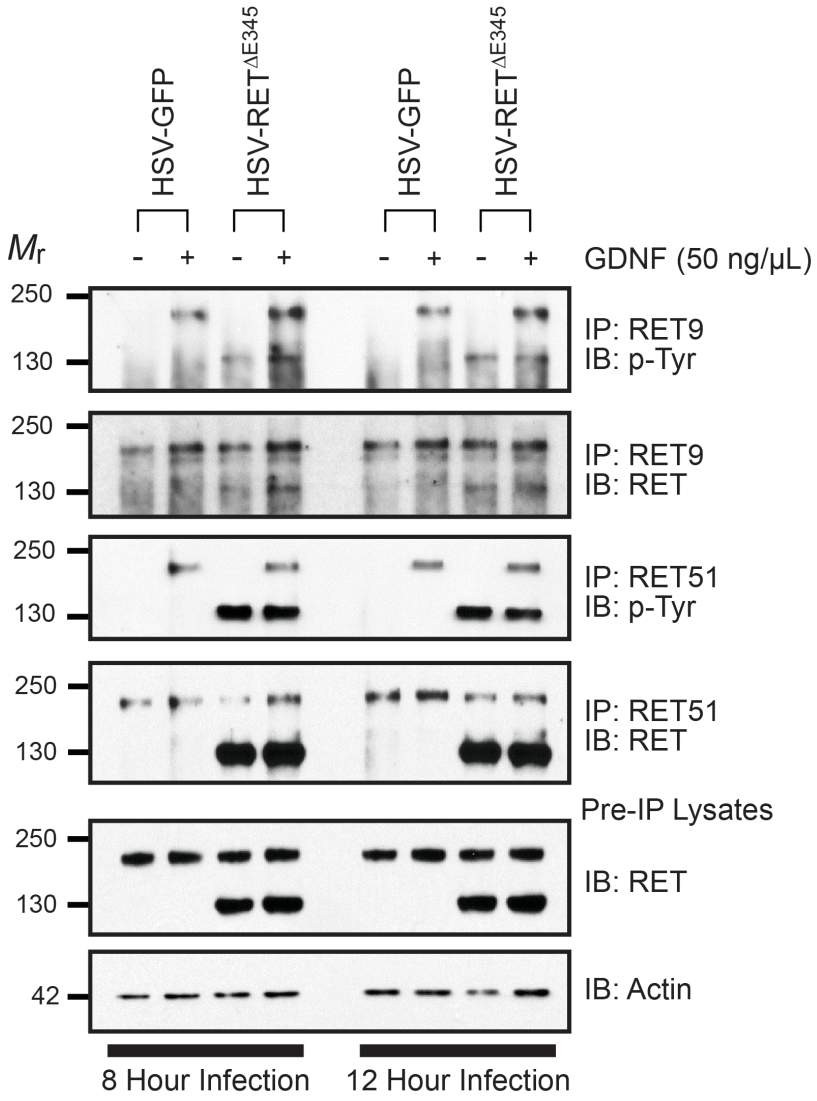

Figure 4.2: Ectopic expression of RET51$^{\Delta E345}$ does not alter ligand-dependent activation of endogenous RET. Sympathetic neurons were infected with HSV to deliver *RET51$^{\Delta E345}$* to cells. We observed very robust expression of RET51$^{\Delta E345}$ within 8 hours after infection and additionally RET51$^{\Delta E345}$ was also highly autophosphorylated. Interestingly, immunoprecipitations for RET9 and immunoblotting for RET revealed an interaction between endogenous RET9 and exogenous RET51$^{\Delta E345}$. Expression of RET51$^{\Delta E345}$ with endogenous RET9 and RET51 did not appear to have any effect on the activation of either protein in the presence or absence of GDNF.

exonic splicing enhancers described previously. Knock-in mutations of these enhancers would presumably eliminate, or at least significantly reduce, the production of a specific splice variant without altering the production of full length RET. A second approach would be to produce knock-in mice in which the intron between exons three and four, or between exons five and six, would be removed in frame such that the splicing could not occur to produce *RET*$^{\Delta E3}$ and *RET*$^{\Delta E345}$, respectively. The CRISPR/Cas9 system would be an ideal method for the production of such mice. Once these mice were made and they were confirmed to be deficient in the targeted splice variant, we would initially examine kidney morphogenesis, enteric nervous system development and motor axon guidance into the hindlimbs.

Further investigation of the alternative splicing creating a truncated RET isoform

Lorenzo et al. also detected an additional exon-skipping event in which exons 3 and 4 were excised (Lorenzo et al., 1995). This deletion causes a shift in the reading frame of the transcript, resulting in the insertion of a premature stop codon. This potential protein product would be very small, mainly encoding a portion of CLD1 and a small portion of CLD3. While this transcript would likely undergo nonsense-mediated decay, our preliminary experiments suggested that this isoform, RET$^{\Delta E34}$, may function to enhance the signaling of full-length RET. Additionally, like *Ret*$^{\Delta E3}$ and *Ret*$^{\Delta E345}$, *Ret*$^{\Delta E34}$ was also present in zebrafish, mice and rats, and its expression was also dynamically expressed throughout development in mice (Fig. 4.3). A number of experiments could be performed to interrogate the function of this isoform, and the potential role that it plays during development of the nervous and renal systems.

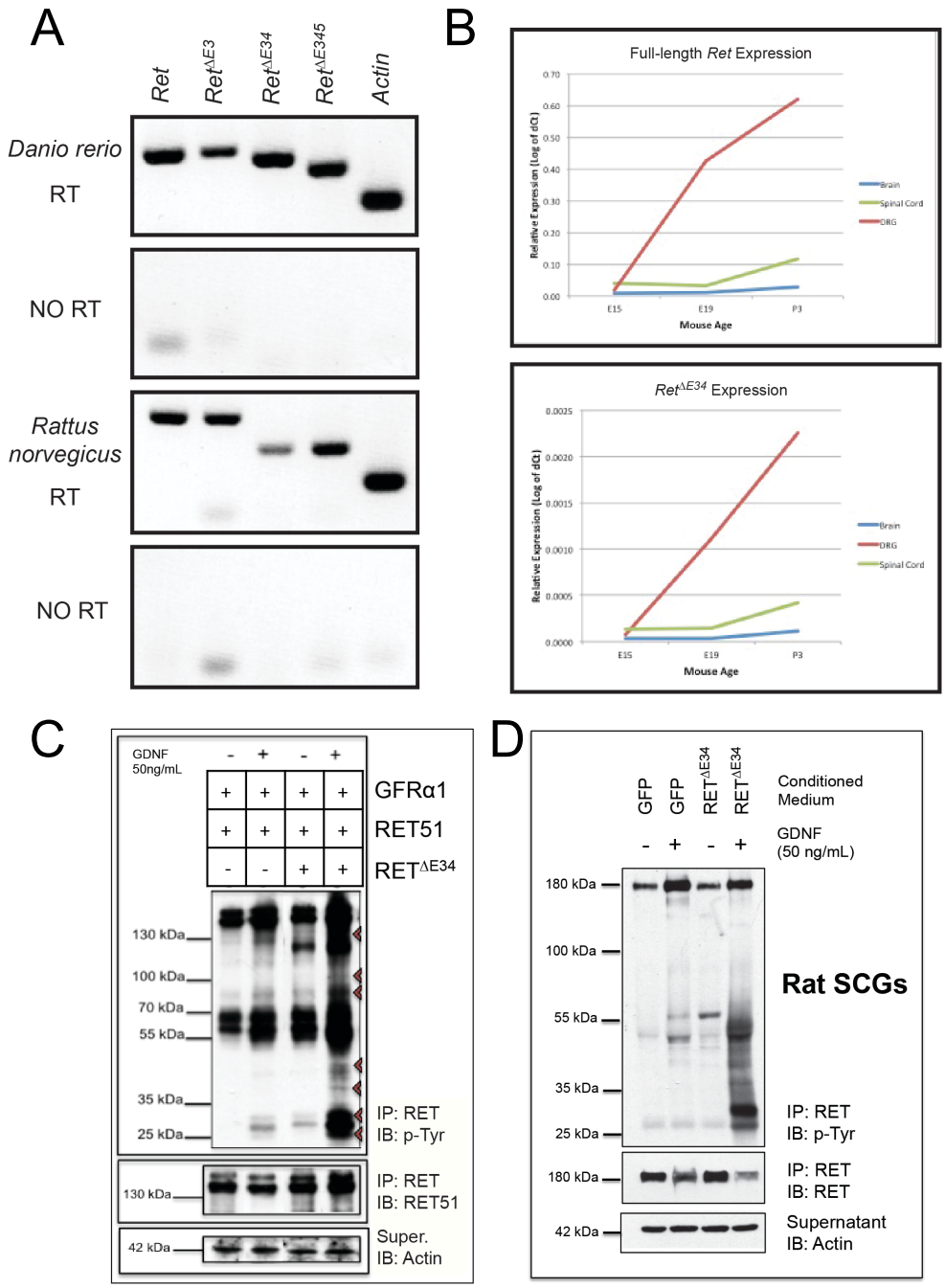

Figure 4.3: *RET*$^{\Delta E34}$ is expressed during development in multiple species and the protein augments full-length RET activation *in vitro.* (A) RT-PCR analysis of *Ret* transcripts in *Danio rerio* (48 hpf) and *Rattus norvegicus* (E19.5 spinal cord). (B) qPCR analysis of *Ret* and *Ret*$^{\Delta E34}$ in developing brain, spinal cord and DRG. (C) NIH/3T3 cells co-transfected with *RET*$^{\Delta E34}$ exhibit increased phosphorylation of full-length RET as well as interacting adaptor proteins. (D) Primary sympathetic neurons treated with or without RET$^{\Delta E34}$ conditioned medium in the presence or absence of GDNF. Neurons receiving both RET$^{\Delta E34}$ conditioned medium and GDNF had higher levels of phosphorylation of smaller molecular weight proteins, which are likely interacting adaptor proteins.

Loss of RET Results in a Decrease in Apoptosis during Programmed Cell Death: Discussion and Future Directions

We discovered that *Ret* is expressed in a subset of sympathetic neurons of the SCG during PCD that also express both p75 and tyrosine hydroxylase, and additionally we observed that these *Ret* positive neurons are lost during this developmental time window. Enomoto et al. showed previously that a subset of cells in the developing SCG express *Ret* during PCD using the Ret^{TGM} mouse in which GFP was knocked into exon 1 of *RET* and expression was driven by the endogenous promoter (Enomoto et al., 2001). The authors observed in $Ret^{TGM/TGM}$ mice that this subset of cells in the SCG still upregulated *Ret* in this null background, and that the percentage of cells upregulating *Ret* in either $Ret^{TGM/TGM}$ or $Ret^{TGM/+}$ mice did not differ (Enomoto et al., 2001). Upon examination of whether these neurons died upon the deletion of Ret in the $Ret^{TGM/TGM}$ mice, the authors did not see co-immunostaining between GFP and the apoptotic marker cleaved caspase-3. The authors interpreted these results that these *Ret* positive cells were not dying, thus RET was not supporting their survival. Our approach, however, let us distinguish if these *Ret* positive cells were being lost over time, and we did observe a large reduction in the number of *Ret* positive cells during our pulse experiments. Thus we postulated that *Ret* was a "marker" for a subset of dying cells in the SCG or that RET could potentially be participating in programmed cell death during PCD.

Since RET has not been implicated to function as a death receptor, we sought to determine whether it was functioning in conjunction with p75, a member of the TNF receptor family, which functions to eliminate "losing" neurons during PCD in the SCG

(Bamji et al., 1998, Teng et al., 2010). Since p75 has been found to interact with other receptors, including other RTKs such as TrkA, we determined if p75 and RET could interact. Using a transfected NIH/3T3 system, we observed interactions between p75 and both RET isoforms. Furthermore, we found in cultured sympathetic neurons that the interaction between RET and p75 was enhanced by apoptotic stimuli such as α-NGF, BDNF and proBDNF. This suggested that RET may be able to enhance the function of p75-mediated cell death *in vivo*.

Importantly, we found through the genetic deletion of *Ret* in developing embryos that loss of RET leads to a reduction in cell death during PCD. Surprisingly however, genetic deletion of *p75,* utilizing both a tamoxifen inducible Cre recombinase approach as well as a germline deletion approach, showed no difference in the amount of cell death occurring, as assessed by the number of activated caspase-3 positive cells, between mutant or control animals.

It is worth noting that a majority of the research on the role of p75 in cell death during PCD has utilized a germline mouse model in which exon 3 of *p75* is removed, referred to as $p75^{\Delta Exon3/\Delta Exon3}$. However, von Schack et al. detected a transcript in $p75^{\Delta Exon3/\Delta Exon3}$ mice corresponding to the skipping of exon three, which still created a full-length *p75* transcript, but with deletions in the extracellular domain of p75, termed s-p75 (von Schack et al., 2001). These deletions were predicted to inhibit the binding of p75 to the neurotrophins, and the authors observed no specific neurotrophin binding of NGF, BDNF, NT-3 and NT4/5 in 293 cells expressing recombinant s-p75 (von Schack et al., 2001). Brain and spinal cord samples were used to detect the $p75^{\Delta Exon3/\Delta Exon3}$ transcript, but these tissues were not analyzed for the translated protein product, and

the authors do not address this. Instead, total protein extracts from primary Schwann cells were prepared from either wild-type mice or *p75*$^{\Delta Exon3/\Delta Exon3}$ mice, and by Western blot it was shown that a smaller protein product corresponding to the correct molecular weight of s-p75 was detected from *p75*$^{\Delta Exon3/\Delta Exon3}$ mice (von Schack et al., 2001). The authors commented that the wild-type cells express high levels of FL-p75, which masks detection of s-p75 due to differences in the glycosylation state of FL-p75 (von Schack et al., 2001). However, this argument could have been strengthened if the authors had performed deglycosylation of p75 to demonstrate that this was in fact the case. Surprisingly, the Western blot that the authors provide in the figure clearly does not show a smaller molecular weight band in the wild-type sample, and the authors do not analyze any other tissues other than primary Schwann cells for the detection of the s-p75 protein product (von Schack et al., 2001). The authors went on to create a new *p75* null mouse in which exon 4 was targeted for deletion, and these mice presented with a considerably worsened phenotype compared to the *p75*$^{\Delta Exon3/\Delta Exon3}$ mice (von Schack et al., 2001).

Further investigation by Paul et al. argued that there was no 62 kDa molecular weight s-p75 band detected from *p75*$^{\Delta Exon3/\Delta Exon3}$ mice (Paul et al., 2004). These data, however, are complicated by the fact that the authors used high percentage SDS page gels when running the protein samples, so there is not a good separation of protein bands at the 60-80 kDa range, thereby making it quite difficult to decipher between bands that are 62 kDa and 75 kDa (Paul et al., 2004). While only light exposures of the p75 Western blots were provided, Paul et al. did investigate the expression of the s-p75 isoform in a number of tissues, and they reported not detecting a corresponding lower

molecular weight band (Paul et al., 2004). In the *p75*$^{\Delta Exon4/\Delta Exon4}$ mouse, the authors did detect a lower molecular weight band of 26 kDa, containing the extracellular stalk region and the entire intracellular domain of p75 (Paul et al., 2004). It was shown that this truncated p75 protein was able to activate apoptotic p75 signaling cascades, leading to increased cell death signaling and a more severe phenotype in these mice (Paul et al., 2004). Although these two different mutant mouse strains illuminated new functions of p75, the controversy still remains unresolved about the presence of multiple p75 isoforms and their potential functions.

To circumvent these issues, Bogenmann et al. created a *p75* null mouse model in which exons 4-6 were targeted for deletion and germline mice (*p75*$^{-/-}$*),* or mice with a conditional *p75* allele (*p75*$^{fl/fl}$), were generated (Bogenmann et al., 2011). The germline *p75*$^{-/-}$ mice exhibited a similar phenotype to the *p75*$^{\Delta Exon3/\Delta Exon3}$ mice. Importantly, *p75*$^{fl/fl}$ mice crossed to a *Wnt1*-Cre driver, where *Wnt1*-Cre can induce recombination in early migratory neural crest cells (NCCs), showed loss of p75 in the dorsal root ganglion (DRG), while its expression in the lateral motor column was unaffected (Bogenmann et al., 2011). Due to the controversy that has surrounded the use of the other *p75* knockout mouse models, we used the mice generated by Bogenmann et al., both *p75*$^{-/-}$ and *p75*$^{fl/fl}$ mice, for our experiments. These mice, however, have not been analyzed to determine if PCD is delayed in the SCG, as has been previously found in *p75*$^{\Delta Exon3/\Delta Exon3}$ mice (Bamji et al., 1998, Deppmann et al., 2008).

One reason we may not detect a significant difference in cell death in our *p75* null animals is because we examined the wrong time point for cell death in regards to p75 function. Although we chose E19.5 because it is the reported height of

programmed cell death in the SCG, previous experiments by Bamji et al. reported that in *p75*$^{\Delta Exon3/\Delta Exon3}$ mice, sympathetic neurons of the SCG do not decrease in number from postnatal day P1 to P21, which they deemed the normal period of naturally occurring cell death (Bamji et al., 1998). In support of this, Majdan et al. showed a significant decrease in TUNEL staining of *p75*$^{\Delta Exon3/\Delta Exon3}$ SCGs in postnatal day 2 (P2) mice compared to wild-type P2 mice (Majdan et al., 2001). Additionally, Deppmann et al. reported that at E18 there is no significant difference in neuron number between wild-type and *p75*$^{\Delta Exon3/\Delta Exon3}$ mice (Deppmann et al., 2008). However, by P0, there was a significant increase in the total number of cells in the SCG of *p75*$^{\Delta Exon3/\Delta Exon3}$ mice as compared to wild type mice. From these examples, it may be that we were too early in development to observe differences in naturally occurring cell death that are related to p75 function. Importantly, we reasoned when designing these experiments that since our readout for apoptosis marks cells actively undergoing cell death (cleaved caspase-3), that this would serve as an earlier marker of apoptosis rather than waiting until the cells had fully been eliminated (and would then be observed by total cell counts, as the p75 knockout studies have generally reported). An important future direction will be to repeat these experiments with our *p75*$^{-/-}$ and *p75*$^{fl/fl}$ mice at a later, postnatal time point and assess if we observe a delay in PCD that has been previously reported (Bamji et al., 1998, Deppmann et al., 2008).

Our *in vivo* data suggest that RET is not enhancing p75-mediated cell death *in vivo*, at least during the time period from E14.5-E19.5, because the loss of *p75* does not affect cell death during this time. The important question remains – what is the role of RET during this period from E15.5-E19.5, when Ret expression becomes restricted to a

subset of cells in the SCG (Enomoto et al., 2001)? One hypothesis is that during this period just after the massive innervation of targets by sympathetic axons, a population of these neurons is not receiving enough NGF from their targets to support survival. These cells may upregulate *Ret* in an attempt to continue axonal pathfinding in order to innervate another target that will provide the appropriate amount of NGF they need to survive. In this scenario, the deletion of *Ret* starting right at the beginning of this period may cause neurons that upregulate *Ret* and revert back to this axon pathfinding phenotype to undergo cell death earlier than expected. In this case, deletion of *Ret* could cause a bolus of cell death to occur earlier in development, which is missed in our experiments because we analyze the SCGs later at E19.5. Although it is more challenging, these experiments could be initiated again at E14.5 and the SCGs could be analyzed at E16.5. Because the SCG has not fully migrated to its final proper position at this time point, we would need to section whole embryos and perform immunofluorescence staining for tyrosine hydroxylase (to distinguish the SCG from surrounding structures), PECAM-1 as a marker for the vasculature, and cleaved caspase-3 to label apoptotic cells. The SCG uses cues from blood vessels (ARTN) to promote its proper migration, thereby making PECAM-1 an ideal marker to locate the migrating SCG. $Ret^{fl/fl}$ and *UBC*-Cre; $Ret^{fl/fl}$ mice would again be used, and cleaved caspase-3 would be analyzed as a readout for cell death. If this hypothesis is correct, we would expect to see an increase in cell death in SCGs from *UBC*-Cre; $Ret^{fl/fl}$ mice.

Interestingly, our results *in vitro* suggest that RET enhances p75-mediated cell death in primary sympathetic neurons, although almost all cells are *Ret* positive in our culture system, unlike what we observe *in vivo*. Our data further indicate that deleting

Ret decreases the amount of p75-mediated apoptosis *in vitro,* but the loss of *Ret* does not completely block cell death (Chris Donnelly, unpublished data). In fact, p75-mediated cell death still occurs in the absence of *Ret in vitro*, but it is delayed, and not as effective, as when RET is expressed. While primary sympathetic neurons have been a useful system to study RET signal transduction, they may not be the most accurate system to understand the function of RET during PCD. It is also possible that RET has more than one function during the period of PCD, an earlier function in survival that we uncovered in these experiments, and a later p75-dependent cell death function that will be revealed by looking at postnatal cell death time periods. In addition, a number of interesting questions still remain to be answered.

Future Directions

Determining if there are differences in programmed cell death between $p75^{\Delta Exon3/\Delta Exon3}$, $p75^{-/-}$ and UBC-Cre/ERT2; $p75^{fl/fl}$ mice

The *p75* germline and conditional mice generated by Bogenmann et al. were not analyzed to determine if PCD was occurring in the SCG in a manner that was previously described (Bamji et al., 1998, Deppmann et al., 2008). To fully examine this, SCGs from $p75^{-/-}$ and wild-type littermates should be isolated at previously described intervals, from E18.5 to P21, and total cell counts, as well as cleaved caspase-3 counts, should performed to assess PCD in these mice. Additionally, it would be intriguing to look at a few earlier time points in the SCG between $p75^{-/-}$ and wild-type littermates, such as E16.5 and E17.5, to assess the amount of cell death occurring before birth. This experiment, while time consuming, would help establish the entire period of PCD, and

determine what the appropriate readout of cell death should be – total cell counts or cleaved caspase-3 immunostaining.

Determining the protein domains necessary for the interactions of p75 and RET

As stated previously, p75 is a very promiscuous receptor. It has been found to interact with a number of other proteins including TrkA, Nogo receptor, Ephrins and Sortilin (Meeker and Williams, 2014). It would be interesting to determine whether the binding region that has been identified for TrkA and p75, especially since TrkA is an RTK, is similar in amino acid composition or structure to the binding region for RET and p75. This information would also be useful to better understand how p75 has the ability to interact with so many receptors.

Interrogating p75-RET interactions in other tissues derived from neural crest cells

In addition to our findings reported here, the interaction between p75 and RET appears to enhance the differentiation and maintenance of non-peptidergic nociceptors in the dorsal root ganglion during development (Chris Donnelly, Kuo Fen Lee; unpublished data). Because DRG sensory neurons are also derived from the neural crest similar to sympathetic neurons, it would be interesting to investigate other tissues derived from NCCs to determine whether p75 and RET function together as a complex, either p75 enhancing the pro-survival function of RET or RET enhancing the pro-apoptotic function of p75. One interesting tissue to begin with would be the enteric nervous system. It would be exciting to determine if p75 enhances RET dependent enteric neural crest-derived cell (ENCC) migration during development, or if RET

enhances p75-mediated cell death such as in Hirschsprung's disease (Huang et al., 2015).

Larger implications of this research

RTKs regulate numerous cellular processes including cell survival, metabolism, proliferation and differentiation throughout development and adulthood of many organisms. Mutations that affect the activity, abundance, cellular distribution or regulation of these receptors often leads to diseases such as cancer, making our understanding of the basic biology of these receptors, especially the regulation of their expression and signaling, critically important. In this context, understanding the regulation of RET via novel mechanisms, such as those discussed in this dissertation, is essential. Along with future studies, I hope these data will further our understanding of RET biology under both physiologic and pathologic conditions.

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