2016

SRC FAMILY KINASES MEDIATE INDIVIDUAL CELL RESPONSES TO RECEPTOR TYROSINE KINASE SIGNALING IN NEUROBLASTOMA CELLS

Juan Enrique Palacios Moreno

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“SRC FAMILY KINASES MEDIATE INDIVIDUAL CELL RESPONSES TO RECEPTOR TYROSINE KINASE SIGNALING IN NEUROBLASTOMA CELLS”

By

Juan Enrique Palacios Moreno

Bachelor of Science in Chemistry and Pharmacy, Universidad de Valparaíso, Chile, 2008

Dissertation

presented in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Neuroscience

The University of Montana
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Approved by:

Scott Whittenburg, Dean of The Graduate School
Graduate School

Mark Grimes, Chair
Department of Biological Sciences

Jesse Hay
Department of Biological Sciences

Sarah Certel
Department of Biological Sciences

Scott Wetzel
Department of Biological Sciences

Philippe Diaz
Department of Biomedical and Pharmaceutical Sciences
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**FYN and LYN dynamically change intracellular location in response to RTK activation**

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Receptor Tyrosine Kinases (RTKs) are involved in proliferation, differentiation, cellular metabolism, and homeostasis. More than half of the RTKs in the human genome are expressed in neuroblastoma cell lines. RTKs activate downstream signaling pathways including the canonical pathways of ERK, AKT, PLCγ, and SRC Family Kinases (SFKs). Phosphoproteomic data showed high expression and phosphorylation levels of SFK scaffold PAG1, and SFKs FYN and LYN in neuroblastoma cell lines and endosomes. We hypothesized that FYN and LYN distinguish between proliferation- and differentiation-inducing signals from different RTKs in neuroblastoma cells.

To test this hypothesis, we measured changes in FYN and LYN's intracellular location using cell fractionation in response to RTK activation. We found that FYN and LYN increased in endosomes and lysosomes in response to activation of ALK and KIT, RTKs that stimulate proliferation, whereas the FYN and LYN decreased in endosomes in response to activation of RET, which stimulates differentiation.

PAG1kd cells were made using the CRISPR-CAS9 system in order to define the effect of PAG1 ablation on proliferation, differentiation, and activation of SFKs and downstream pathways. We used flow cytometry to simultaneously measure the level of intracellular phosphoproteins and DNA content in adherent neuroblastoma cells. We found that PAG1 ablation increased the levels of
activated SFKs, as well and proliferation, as shown by increased number of cells in the S and G2-M stages of the cell cycle, and an increased absorbances in MTT assays. PAG1kd cells displayed increased number of cells with high levels of pAKT, which were located in the G2-M stages of the cell cycle for both PAG1kd and wild type cells.

In addition, PAG1 ablation differentially affected the activation of the ERK pathway downstream from RTKs that induce proliferation. The levels of pERK increased after EGFR activation and were not affected by PAG1 ablation, whereas KIT activation slightly increased only long term activation of ERK, and this increase was suppressed by PAG1 ablation.

We then evaluated differentiation in PAG1kd cells. PAG1kd cells did not respond to treatment with Retinoic Acid and NGF: PAG1kd cells did not change their proliferation rates or their cell cycle distribution. PAG1kd cells dramatically decrease the expression of the neuronal marker of differentiation, β III-Tubulin. In addition, PAG1 ablation prevented the activation of ERK in response to activation of TRKA and RET.

Our data show that FYN and LYN increase in late endosomes and lysosomes in response to activation of RTKs that induce proliferation, such as ALK, and KIT. In addition, tumor suppressor PAG1 is a key regulator of proliferation, differentiation, and RTK mediated signaling in neuroblastoma cells. Future studies will define therapeutic potential of targeting PAG1 expression in the treatment of neuroblastoma.
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<th>Abbreviations</th>
<th>Definition</th>
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<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SFK</td>
<td>SRC Family Kinase</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular body</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysosomes</td>
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<tr>
<td>End1</td>
<td>Endosomal fraction of high mass and density</td>
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<td>Endosomal fraction of intermediate mass and density</td>
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<tr>
<td>Cyt</td>
<td>Cytosol</td>
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<tr>
<td>DRM</td>
<td>Detergent resistant membranes</td>
</tr>
<tr>
<td>P1M</td>
<td>Detergent soluble membranes</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>Gene name</td>
<td>Alternative names</td>
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<td>-----------</td>
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<td><strong>RTKs:</strong></td>
<td></td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic Lymphoma Kinase</td>
</tr>
<tr>
<td>NTRK1</td>
<td>TRKA, high affinity NGF receptor</td>
</tr>
<tr>
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<td>TRKB, BDNF receptor</td>
</tr>
<tr>
<td>NTRK3</td>
<td>TRKC</td>
</tr>
<tr>
<td>RET</td>
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</tr>
<tr>
<td>KIT</td>
<td>c-KIT, SCFR, SCF receptor</td>
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<tr>
<td>INSR</td>
<td>Insulin Receptor</td>
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<td>MET</td>
<td>HGFR, Hepatocyte Growth Factor Receptor</td>
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<td>IGF1R</td>
<td>Insulin-like Growth Factor Receptor</td>
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<td><strong>RTK Ligands:</strong></td>
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<td>PTN</td>
<td>Pleiotrophin, Pleiotropin</td>
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<td>NGF</td>
<td>Nerve Growth Factor</td>
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<tr>
<td>BAD</td>
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<td>BLK</td>
<td>B-Lymphoid Tyrosine Kinase</td>
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<td>FRK</td>
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<td>FYN</td>
<td>SLK, SYN</td>
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<td>GDNF</td>
<td>Glial cell line Derived Neurotrophic Factor</td>
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<td>NGF</td>
<td>Nerve Growth Factor</td>
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<tr>
<td>PI3KCA</td>
<td>p110 PI3K, PI3K catalytic subunit</td>
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<tr>
<td>PI3KR2</td>
<td>p85 PI3K, PI3K regulatory subunit</td>
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<td>PDK1</td>
<td>Piruvate Dehydrogenase Kinase 1</td>
</tr>
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<td>PDK2</td>
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<tr>
<td>PTK2</td>
<td>FAK, Focal adhesion kinase</td>
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<td>PTN</td>
<td>Pleiotrophin, Pleiotropin</td>
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<td>PTEN</td>
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<td>SRC</td>
<td>SRC proto-oncogene, non-receptor tyrosine kinase,</td>
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<td>TGF-β1</td>
<td>TGF-β1</td>
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<td>YES1</td>
<td>YES, c-YES</td>
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Chapter 1

Introduction
Significance

Receptor Tyrosine Kinase (RTK) signaling mediates cellular differentiation in many cell types (Fantauzzo & Soriano 2015; Scioli 2014; Chen 2012), and is implicated in developmental diseases (Amberger 2015; McDonell 2015), diabetes (Fountas 2015) and cancer. For example, activating mutations in the RTK ALK are the main cause of familial neuroblastoma (Mosse 2008; Ogawa 2010) and mutations in this gene have also been identified in a significant proportion of sporadic cases (Ogawa 2010). Similarly, mutations that lead to over activation of the RTK epithelial growth factor receptor (EGFR), can be the drivers of lung cancer and lead to chemoresistance (Kobayashi 2016, Luo 2013). Mutations that promote receptor phosphorylation, or decreased interaction with the phosphatases that reverse receptor phosphorylation induce increased phosphorylated-receptor lifetime, and thus create a state of sustained signaling that can lead to uncontrolled proliferation and tumor formation.

Tyrosine Kinases

Tyrosine kinases can be considered the writers of the phospho-tyrosine signaling code. The covalent addition of a highly negatively charged phosphate group to tyrosine residues can change protein conformation, protein-protein interactions, and enzymatic activity, and is usually an early event in RTK signaling (Zheng 2013). RTKs are a type of tyrosine kinase whose activity is controlled by ligand binding to the extracellular domain of the receptor. In humans there are 58 different RTKs. Some of them have been extensively studied, such as the EGFR and the Insulin Receptor (INSR), whereas others have been identified but their precise roles in development and disease have not been fully explored.
RTKs are a family of structurally related proteins that share a common architecture: one extracellular, ligand-binding domain on the N terminus, a single membrane spanning domain, and a cytosolic C-terminal domain. The C-terminus has intrinsic tyrosine kinase activity and interacts with signaling effector molecules (Robertson 2000; Locascio & Donoghue 2013). RTKs typically reside at the plasma membrane as monomers or dimers. If the RTK exists as a monomer, binding of their cognate ligands on the extracellular domain triggers receptor dimerization (Li 2010), and receptor transphosphorylation of tyrosine residues on the C-terminus. These phosphorylated residues allow intermolecular interactions between RTKs and proteins with phosphotyrosine binding domain-containing proteins.

**Phosphotyrosine interacting domains**

There are two well known phosphotyrosine-interacting protein domains that can interact with phosphorylated RTKs: the SRC Homology 2 (SH2) and the Phospho-Tyrosine Binding (PTB) domain. Proteins that contain one or more of these domains can act as adaptors/scaffolds to bring two or more proteins physically together to form a protein/signaling complex. Mutations in phospho-tyrosine interacting domains are implicated in disease. One example is SHC1, a member of the SHC family of proteins, which contains a single copy of each SH2 and PTB domain (Mooijaart 2004). SHC-PTB domain mutants that are unable to interact with phosphotyrosines delay the onset of tumor development in a rodent model of breast cancer, but increase tumor size and vascularization once tumors develop (Ahn 2013). In addition, breast cancer cells expressing a nonfunctional SH2 domain in SHC1 fail to survive, and to develop metastases in vivo (Ursini-Siegel 2012). These examples illustrate the importance of functional interactions between specific phosphorylated residues on receptor cytoplasmic domains, and that disruption of such interactions has functional biological consequences.
Phosphatases

RTK phosphorylation and signaling takes place in response to an extracellular signal, but these responses are not permanent: phosphorylation is reversible. Cellular phosphatases remove phosphate groups from proteins, including phosphorylated tyrosine, serine, and threonine residues, tightly controlling the lifespan of individual phosphorylations. Phosphatase activity in cells is high (Mayer 2013), so the duration of activation of any given RTK response could be considered a balance between kinase and phosphatase activities, and is therefore time constrained. Defects in phosphatase activity play a role in the development of cancer (Zhao 2015), in their absence, phosphorylated residues on receptors and signaling proteins are not removed and the pathways are constitutively active.

Tyrosine kinases in general act as writers, phospho-tyrosine binding domain containing proteins act as readers because they recognize the newly developed phospho-tyrosine moieties, and protein phosphatases act as erasers of the phospho-tyrosine code by removing these phospho-tyrosine moieties (Mayer 2013; Liu 2012a; Liu 2012b). These interactions may occur at the plasma membrane as well as through the endocytic pathway.

RTKs endocytosis and ubiquitination

Activated RTKs undergo endocytosis as a means to regulate RTK activity (Goh 2013). Endocytosis is the process in which a cell internalizes a portion of the plasma membrane, along with extracellular fluid and molecules dissolved or suspended in it, and gives rise to a newly formed endocytic vesicle. Of the many types of endocytosis, receptor activation- and clathrin-dependent endocytosis is the most common and well understood way in which RTKs are internalized. The endocytic vesicle fuses with and becomes part of the early endosome (Kornilova 2014), a key compartment that is characterized by the presence of the small GTPase
Rab5 (Woodman 2000). The early endosomes act as a sorting hub, and are a point of divergence in the endocytic pathway. From sorting endosomes cargo can travel back to the plasma membrane in recycling endosomes, or continue through the endocytic pathway to be included in multivesicular bodies (MVB) and degraded in lysosomes. Cargo can travel in two distinct ways to the plasma membrane: 1) directly back to the plasma membrane on recycling endosomes characterized by the presence of Rab4 (also known as the fast recycling pathway) (Daro 1996), or 2) back to the plasma membrane through the perinuclear area on recycling endosomes characterized by the presence of Rab11 (also known as the slow recycling pathway) (Sandilands 2007). Alternatively, cargo can continue in the endocytic pathway as the endosome matures, and be included into MVB/late endosomes. MVBs are characterized by the presence of Rab7 and contain intraluminal vesicles (Guerra 2016). Finally, as endosomes mature from endocytic vesicles to lysosomes the pH decreases, and cargo can be degraded in lysosomes by lysosomal hydrolases after late endosomes fuse with preexisting lysosomes.

Much of the details of the endocytic pathway have been revealed by studying the trafficking of RTKs.

Extensive work examining RTK endocytosis has focused on EGFR, and lessons learned from EGFR biology were, for many years, the rule for all RTKs. For example, it is accepted that EGFR activation and signaling are primarily regulated, and decreased, by endocytosis (Tomas 2014, Goh 2013, Sorkin 2009). After endocytosis, EGFR remains associated with the endosome as it matures, and is finally degraded by lysosomal proteases once the mature/late endosomes fuses with the lysosome. Later work has shown that this is not the case for all RTKs. Activation of TRKA/NTRK1 by the Nerve Growth Factor (NGF) triggers the endocytosis of TRKA either via clathrin mediated endocytosis (Freund-Michel 2008; Grimes 1996), or by macropinocytosis in membrane ruffles (Shao 2002). In neurons, the changes in cellular signals and gene expression
triggered by TRKA takes place in the soma, but activation of TRKA takes place near axon terminals. In order to achieve these changes, signaling MVBs formed at axon terminals, travel to the soma on microtubules (Howe 2004). These TRKA-containing MVBs carry phosphorylated, active TRKA, and mediate the long term differentiation-inducing effects of TRKA activation (Grimes 1997; Grimes 1996). This example shows that some processes, like endocytosis, are common to all RTKs. However, for some RTKs, such as TRKA and unlike EGFR, endocytosis is not a means to decrease RTK activation, but instead mediates sustained RTK signaling.

RTKs undergo several post translational modifications, including phosphorylation and ubiquitination. The covalent addition of ubiquitin to lysine residues of RTKs by the ubiquitin ligase CBL family is critical for inclusion of activated RTKs in endocytic vesicles, and trafficking within the endosomal pathway (Mohapatra 2014; Goh 2013; Wegner 2011). Two major categories of ubiquitination have been described: mono-ubiquitination and poly-ubiquitination. Poly-ubiquitination involves the formation of ubiquitin chains linked to each other in different arrangements (K48, K63, or combinations), depending on the residue of the addition of the following ubiquitin monomer. While K48 poly-ubiquitination is a signal for proteasomal degradation, K63 poly-ubiquitination does not lead to proteasomal degradation. K63 poly-ubiquitination and mono-ubiquitination (Wegner 2011) function to generate protein-protein interactions that lead to changes in cargo’s trafficking and recognition by the ESCRT complexes in endosomes that results in RTK de-ubiquitinization by de-ubiquitinizing (DUB) enzymes, and subsequent inclusion of RTKs into intraluminal vesicles of MVBs (Mohapatra 2014; Goh 2013; Wegner 2011). An example of how different types of ubiquitination have different signaling effects is exemplified by MYC regulation, where K63 poly-ubiquitination leads to MYC activation, K48 poly-ubiquitination leads to proteasomal degradation, and mixed ubiquitin chains leads to MYC stabilization and cell cycle progression (Hammond-Martel 2012). Therefore, post-
translational modifications, such as phosphorylation and ubiquitination, can regulate the activity and intracellular trafficking of signaling proteins, including tyrosine kinases.

The intracellular location of a protein dictates what other proteins they can interact with. Proteins (including tyrosine kinases) at the plasma membrane, endosomes, or MVBs, have differential access to interacting proteins based on each protein’s cellular distribution. For example, MVB’s intraluminal vesicles may sequester specific proteins, such as Glycogen Synthase Kinase 3β (GSK3B) in the WNT signaling pathway, preventing the sequestered proteins from interacting with their cytosolic signaling partners (Taelman 2010). Similarly, cytosolic tyrosine kinase CSK, which was engineered to locate to lipid rafts in the plasma membrane, increases the level of phosphorylation of target SFKs (Sirvent 2010). In addition, signaling events have a finite duration; for example, (SH2 and PTB domain containing) SHC-1 interacts with a variety of phosphorylated proteins. In general, during early events (seconds to minutes after RTK activation) SHC-1 binds phosphorylated receptors and tyrosine phosphorylated signaling partners. During later events (minutes to hours after RTK activation) SHC1 binding partners include serine and threonine phosphorylated proteins (Zheng 2013).

**RTKs activate 4 canonical pathways**

Interactions between the phosphorylated RTK, phospho-tyrosine interacting proteins, and protein complexes assembled at the plasma membrane and in endosomes lead to the activation of four canonical pathways downstream of RTK activation: the SRC family kinase (SFK), PI3K-AKT, RAS-ERK (MAPK3), and PLCγ pathways (Figure 1). First, I will describe these four canonical pathways individually, with emphasis on the SFK pathway because it is the center of our hypothesis and research focus. Then I will discuss the complexity and interconnectivity of all four pathways. These concepts are central to my research questions.
Taken together the data shows that FYN and LYN activity and intracellular differentiation in a PAG1 dependent fashion. Whereas treatment with SCF ablation also increased the activation levels of SFKs in non-stimulated cells. We only RTK ligand that induced transient activation of the ERK1/2 pathway is EGF. Differentiation-inducing retinoic acid. Flow cytometry shows a somewhat induced cell growth to a similar level to EGF, whereas stimulation with NGF did endosomes and lysosomes, and decreased their concentration in lipid rafts, differentiation and cellular signaling by flow cytometry. We also show the effect that induce proliferation from those that induce differentiation.

hypothesize that SFKs FYN and LYN can distinguish signals from RTKs SFKs candidates for integrating signals from different receptors. Domain, along with their pattern of expression in neuroblastoma cells make the migration, and metastasis. The presence of an SH2, an SH3, and a kinase domain, along with their pattern of expression in neuroblastoma cells.

RTKs activate the same canonical pathways ERK1/2, AKT, SRC and PLCγ markers of a more differentiated state and correlate with good prognosis. All are markers of early developmental stages, whereas NTRK1/TRKA and RET are.

Expression of NTRK1/TRKA and RET are markers of early developmental stages, whereas NTRK1/TRKA and RET are.

Figure 1. RTKs activate four canonical pathways: SFKs, PI3K-AKT, RAS-ERK, and PLCγ pathways.
SFKs

SFKs are a family of structurally related, non-receptor tyrosine kinases whose activity is controlled by phosphorylation of hydroxyl groups on amino acids tyrosine, serine, and threonine residues (Roskoski 2005). There are ten SFKs identified to date in the human genome: SRC, FYN, LYN, YES1, HCK, LCK, BLK, FGR, FRK, and PTK6. The tissue expression of the SFKs vary: some are ubiquitously expressed, like SRC, YES, and FYN, whereas others are restricted to specific tissues, such as HCK and FGR, which are restricted to myeloid cells (Thomas 1997; Lowell 1996; Peng 2014). Early studies showed that LYN expression was restricted to B cells and the brain (Corey 1999, Lowell 1996), but it is now known to be widely distributed (proteinatlas.org).

The SFKs contain four domains and a linker sequence: from N-terminus to C-terminus, they contain a SH4 unique domain, a SH3 proline-rich binding domain, a SH2 phosphotyrosine binding domain, a proline rich linker, and a SH1 tyrosine kinase domain. The presence of acylation site(s) in the SH4 domain leads to the preferential location of the SFKs in biological membranes, especially lipid rafts at the plasma membrane. The presence of SH2 and SH3 domains in all SFKs allows them to interact with a wide range of proteins. In addition, the kinase domain allows the SFKs to control the activity of a wide range of downstream proteins through phosphorylation (Senis 2012) (Figure 2).

In some cases, since SFKs have similar structure and contain similar domains, one SFK can compensate for the absence of another. Because of this, understanding the specific roles of each SFK is complicated, as overexpression of SFKs induces growth and migration, and single SFK knockout mice may not have a phenotype. For example, HCK and FGR expression is
restricted to myeloid cells. Knockout mice for either of these SFKs display no phenotype, but knocking out both HCK and FGR compromises host immune responses (Corey 1999).

While some SKFs can partially compensate for the absence of a single SFK, there are SFK functions that are specific for each SFK and cannot be compensated by another SFK. For instance, different phenotypes are observed for knockout mice for ubiquitously expressed SRC and YES1. SRC knockout mice display osteopetrosis due to malfunction of osteoclasts in bone resorption, while YES1 knockout mice display no phenotype (Corey 1999). However, knocking down both SRC and YES1 is lethal (Corey 1999). Another study focused on the developing mouse brain, where SRC is expressed during brain development throughout the cortical plate, and mediates neuronal branching (Wang 2015). In this model, SRC phosphorylates Focal Adhesion Kinase (FAK, PTK2) in Y925, which mediates FAK mediated activation of ERK and may mediate the dissociation of FAK from focal adhesions (McLean 2005). SRC phosphorylation of FAK results in decreases in the phosphorylation of actin depolymerizing factor Cofilin. FAK and SRC cooperate to stabilize neuronal processes and the migration of cortical neurons throughout development (Wang 2015). Similarly, in the chicken lens, different SFKs play different roles. SRC, expressed in undifferentiated epithelial cells, is associated with newly formed N-Cadherin junctions, and prevents cells from differentiating, whereas FYN signaling after mature N-Cadherin have been established induces differentiation (Leonard 2013).

The unique domain is key to the differential activity of each SFK. SFKs undergo differential post translational modifications (acylation and phosphorylation) in the SH4 unique domain. With the exception of PTK6 and FRK that lack the N terminal myristoylation site, all SFKs undergo single myristoylation in the unique domain (Anneren 2003). Further acylations are also possible for
different SFKs; for example, LYN is mono palmitoylated, and FYN is dually palmitoylated (Sato 2008, Okada 2012). These acylations affect SFK localization and allow the SFKs to be tightly associated with endosomes, the plasma membranes, and lipid rafts (Sato 2008, Okada 2012, Palacios-Moreno 2015). In addition, the unique domain is also differentially phosphorylated, and these phosphorylations allow the SFKs to selectively interact with different patches of the plasma membrane (Amata 2014). The unique role of each SFK in human health may be due to a combination of the differential pattern of expression, acylations, and phosphorylation of different sites that regulates their tyrosine kinase activity.

**SFK activity regulation**

Unlike RTKs that are activated by a ligand, SFK activity is controlled by phosphorylation on specific residues that trigger inter- and intra-molecular interactions. In unstimulated cells, 90% of SFKs are in the inactive conformation, phosphorylated on the C-terminal inhibitory tyrosine (Zheng 2000; Shenoy 1992; Cooper 1986). In this conformation (Figure 2), the phosphorylated C-terminal inhibitory tyrosine interacts with the SH2 domain of the SFK, while the SH3 domain interacts with the proline-rich linker sequence, leaving the kinase domain unreachable to signaling partners.
Figure 2. SFK structure and intramolecular interactions. A. General structure of SFKs. B. Structure of SFKs in their catalytically active state. C. Structure and intramolecular interactions (indicated by curved lines) of the SFKs in their inhibited conformation.

CSK phosphorylates and inhibits SFKs

The main kinase responsible for the phosphorylation of the SFKs in the C-terminal inhibitory tyrosine is the cytosolic SH2 and SH3 domain-containing C-terminal SRC Kinase, CSK (Okada 2012). CSK localization is critical for CSK activity: CSK engineered to localize to lipid rafts,
where SFK concentration is highest, decreases SFK activity and SFK-dependent invasiveness of colorectal cancer cells (Sirvent 2010). In order to phosphorylate its SFK targets, CSK needs to interact with a membrane-bound scaffold to be in the vicinity of SFKs at membrane surfaces. The main scaffold that accomplishes this function is the lipid raft transmembrane protein PAG1 (PAG, Phosphoprotein Associated with Glycosphingolipid-enriched microdomains; CBP; CSK Binding Protein), but others can also act as scaffolds, like Caveolin-1 (Cao 2002) and Paxillin (Rathore 2007), as well as LAT and LIME in lymphoid cells (Tedoldi 2006). The SH2 and SH3 domains of SRC are required for CSK dependent phosphorylation of the C-terminal inhibitory tyrosine (Superti-Furga 1993; Okada 2012).

The most active conformation of the SFKs requires the removal of the phosphorylation on the C-terminal inhibitory tyrosine and the phosphorylation of the tyrosine at the activating loop in the kinase domain. Dephosphorylation of the C-terminal inhibitory tyrosine is catalyzed by cytoplasmic phosphatases PTP1B (Bjorge 2000), PTPN6/SHP-1 and PTPN11/SHP-2 , as well as transmembrane CD45, PTPRA, PTPRE, and PTPRL (Roskoski 2005). Removal of the phosphate on the C-terminal inhibitory tyrosine triggers the disruption of the intermolecular interaction between the C terminal tail and the SH2 domain, leaving the tyrosine at the activating loop in the kinase domain available for auto/trans phosphorylation; binding of the SH2 domain to phosphorylated RTKs facilitates autophosphorylation of the tyrosine at the activating loop in the kinase domain (Roskoski 2005, Roskoski 2004, Brown 1996). These modifications leave the kinase domain exposed and available to interact with, and phosphorylate, their substrates (Figure 2). There are many other tyrosine, serine, and threonine residues phosphorylated on different SFKs, although the biological implications of these phosphorylations are not fully understood (Phosphosite.org).
**Tumor Supressor PAG1 SFK signaling**

The scaffolds, including PAG1, that bring CSK in proximity to SFKs at the plasma membrane are an important regulatory component of SFK signaling. PAG1 is composed of a small extracellular domain, a transmembrane domain, and a long intracellular domain. The intracellular domain is highly phosphorylated on tyrosine, serine, and threonine residues (phosphosite.org). PAG1 interacts with the SFKs not only by bringing CSK to the plasma membrane, but also by directly interacting with SFKs through the SFK’s SH3 domain and PAG1’s proline rich domain, and through protein-protein interactions with the SFK’s SH2 domain and phosphotyrosine residues of PAG1 (Ingley 2006). All of these interactions decrease the activation of SFKs, and since over activation of SFKs is a common feature of cancer, PAG1 is classified as a tumor suppressor (Agarwal 2016, Oneyama 2008). For example, in neuroblastoma, high levels of expression of PAG1 is correlated with higher rates of survival *in vivo*, and decreased proliferation *in vitro*, whereas the opposite was observed under low levels of PAG1 expression (Agarwal, 2016). In esophageal cancer cell cultures, overexpression of PAG1 resulted in decreased SFK expression, migration, invasion, and proliferation (Zhou, 2015). These and other (Oneyama 2008) clinical examples demonstrate the tumor suppressor role of PAG1.

**Endosomal trafficking is involved in SFK activity**

In addition to interactions among scaffolds and CSK, endosomal trafficking is involved in the regulation of the non palmitoylated SFKs, such as SRC. It has been shown that functional recycling endosomes are required for SRC activation (Reinecke 2014, Sandilands 2007). Although most of the SFKs reside at the plasma membrane, a proportion of SRC resides in organelles in the perinuclear area. In response to RTK activation, perinuclear non-activated SRC travels to the plasma membrane in Rab11+ recycling endosomes (Reinecke 2014,
This trafficking event also takes place for non-palmitoylated FYN but does not occur for palmitoylated FYN or palmitoylated mutant SRC (Sandilands 2007). These results highlight the importance of acylations in the unique domain for SFK trafficking and activation.

Once activated, SFKs phosphorylate a myriad of cellular targets, including membrane proteins, RTKs, other kinases, phosphatases, Guanine Exchange Factors (GEFs), GTPase Activating Proteins (GAPs), and proteins involved in cytoskeleton rearrangement and cell adhesion. SFK targets are involved in several signaling pathways, including those downstream of FAK, PDGF, HGF, and IGF1R. In turn, these signaling pathways impact many biological processes such as cell movement, the cell cycle, growth, proliferation, platelet activation, metabolism of amino acids, nucleic acids, and carbohydrates (Senis 2012, Ferrando 2012). Interestingly, SRC also phosphorylates and promotes degradation of CBL proteins (Bao 2003). The CBL family is a group of ubiquitin ligase that mediates ubiquitination of tyrosine kinases at the plasma membrane, thus by increasing the degradation of CBL, SRC indirectly regulates RTK signaling.

This detailed description of the SFK pathway provides the necessary elements to understand the focus of our research on SFKs. However, the SFK pathway is highly interconnected with the other three RTK activated canonical pathways (Figure 3). Therefore, the members of the other canonical pathways will be briefly described to provide context for some experiments that analyze different RTK signaling outputs. For each pathway, I will describe the overall architecture of the pathways, key members of the pathway, and important signaling targets.
Figure 3. The canonical pathways activated by RTKs are highly interconnected. The ERK, AKT, and SFK nodes are key signaling nodes in the canonical pathways network (Green letters indicate key signaling hubs. Black arrows indicate activation within each pathway, blue arrows indicate stimulatory crosstalk between pathways, red arrows indicate inhibitory interaction).
**PI3K-AKT pathway**

The PI3K/AKT pathway is key to many cellular processes including cell proliferation, angiogenesis, metabolism, differentiation, and cell survival (Porta 2014, Hemmings 2012). After RTK activation, the 2 subunit enzyme Phosphatidylinositol 3 Kinase (PI3K) is recruited to the RTK through a protein adaptor (i.e. GRB2, IRS2, SHC1). PI3K phosphorylates Phosphatidylinositol (4,5)-bisphosphate (PIP2), creating Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on the inner leaflet of the plasma membrane. This phosphorylation is antagonized by the activity of PTEN phosphatase, which converts PIP3 back to PIP2. The serine/threonine kinase AKT (PKB) binds PIP3. AKT migrates to the newly formed patches of the plasma membrane rich in PIP3, and there it is phosphorylated and activated by PDK1 and other kinases. After activation, AKT is released to the cytoplasm where it phosphorylates a wide range of proteins (Hemmings 2012).

**PI3K structure/activation**

Lipid rafts are patches of the plasma membrane that are rich in cholesterol and PIP2. PI3K activation in lipid rafts has been shown to play an important role in cell survival (Resi-Sobreiro 2013). PI3K is a heterodimer: PI3K p85 is the regulatory subunit and PI3K p110 is the catalytic subunit. The p85 regulatory subunit has different isoforms, all of which contain two SH2 domains with an inter-SH2 domain that mediates interaction with the catalytic p110 subunit. p85 can bind phosphorylated RTKs directly or through the formation of a protein complex using protein adaptors, such as IRS1, IRS2, GRB2, or SHC1. p85 localizes PI3K to the plasma membrane through these adaptors upon RTK activation (Liu 2009; Vivanco 2002). The catalytic p110 subunit has five domains: a p85-binding domain, a RAS-binding domain, a putative membrane binding C2 domain, a helical domain, and the catalytic domain in the C terminal.
Once activated, PI3K increases the levels of plasma membrane PIP3, which attracts AKT to the membrane, where AKT is activated.

AKT contains 3 domains: a PH domain, followed by a kinase domain, and a regulatory C terminus domain. Two phosphorylation sites are critical for AKT activity: T308 in the kinase domain and S473 in the regulatory C terminal domain. Phosphorylation of these two residues is required for maximal AKT activation. T308 is phosphorylated at the plasma membrane by PH domain containing PDK1/PDPK1 (PDPK1, 3-Phosphoinositide Dependent Protein Kinase 1), or by Integrin-Linked Kinase (ILK). S473 is phosphorylated by different kinases in different cellular locations: at the plasma membrane by PDK2 (PDPK2, 3-Phosphoinositide Dependent Protein Kinase 2); or in the cytoplasm by the mammalian Target Of Rapamycin Complex 2 (mTORC2), or by DNA-dependent Protein Kinase (DNA-PK) (Liu 2009). In growing cells, AKT is selectively activated during the G2/M phase of the cell cycle (Liu 2014).

Serine-threonine kinase AKT phosphorylates a wide range of substrates, including cytosolic proteins and transcription factors. Targets of AKT include MDM2, FOXO1, NFκB, BAD, GSK3β, S6K, and mTOR. By phosphorylating these targets, AKT mediates anti-apoptotic, pro-survival, and proliferative signaling (Liu 2009; Vivanco 2002).

In vitro studies using inhibitors of the AKT pathway have shown the involvement and importance of the AKT pathway in neuroblastoma (Pierce 2011) and they display the importance of this pathway in NB. Currently in vitro and in vivo research is taking place using inhibitors of the PI3K pathway in neuroblastoma to define their efficacy in this disease (Kushner 2016; King 2015; Spunt 2011).
**RAS-ERK pathway**

The RAS-ERK pathway regulates the transcription of target genes, cell cycle progression, prevents apoptosis, and is involved in a wide variety of cancers (McCubrey 2007). RTK activation leads to the formation of a protein complex at the plasma membrane that triggers the activation of RAS GTPase. In turn, GTP-bound RAS mediates the phosphorylation and activation of a kinase cascade where each kinase phosphorylates and activates the following kinase. These kinases are RAF, MEK and ERK. Activated ERK phosphorylates cytosolic proteins and transcription factors, thus influencing gene expression.

At the plasma membrane, the RAS-ERK cascade can be initiated after RTK activation from both lipid rafts and non-raft membranes (Hancock 2003). Phosphorylation of RTK cytoplasmic tails triggers the assembly of a protein complex that includes GRB2, and the RAS guanine exchange factor for SOS1 (Neuzillet 2013), which triggers the activation of membrane-associated RAS. Activated RAS then mediates the serial phosphorylation of RAF, MEK, and ERK. Activating mutations in any of the nodes of the pathway, especially in the RAF isoform BRAF (Wellbrock 2004), lead to over activation of the pathway, cell survival, and contributes to cancer.

There are two main isoforms of ERK: ERK1 and ERK2. These isoforms share high amino acid sequence homology and substrate specificity. MEK phosphorylates ERK at T202 and Y204 in ERK’s activation loop, triggering the activation of ERK’s kinase activity. ERK has a wide range of protein targets, including proteins of its own pathway, other signaling proteins, as well as transcription factors. Activated ERK can phosphorylate and inhibit SOS1, as well as RAF, and MEK (Caunt 2015), destabilizing the RAS protein complex at the plasma membrane and the
cytosolic kinase cascade, preventing its own over-activation, forming a negative feedback loop. Other targets of ERK include ETS-1, c-JUN and c-MYC (McCubrey 2007), retinoblastoma (RB) (Rodriguez 2010), RSK and MSK (Kyriakis 2012).

Studies have related the persistency of RTK mediated ERK activation with proliferation and differentiation processes. It has been shown that long lasting activation of ERK, for example by activation of TRKA, correlates with differentiation in neurons and neuron-like cells (Chen 2012, Marshall 1995). On the other hand, transient activation of ERK, for example, by activation of EGFR, correlates with proliferation (Chen 2012; Marshall 1995).

ERK inhibitors have proven effective in decreasing proliferation of neuroblastoma cells in vitro (Tanaka 2016; Woodfield 2016; Singh 2015; Sartelet 2012). However, studies testing the efficacy of inhibition of the ERK pathway in neuroblastoma patients has not reach clinical stages.
**PLCγ pathway**

Phospholipase Cγ1 (PLCγ1) is the ubiquitously expressed PLC isoform that is phosphorylated and activated in response to RTK activation. PLC-γ1 expression in tissues is tightly regulated, and it decreases as differentiation takes place (Di Giacomo 2005, Lee 1995). PLCγ1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 diffuses into the cytoplasm where it mediates the release of calcium from intracellular stores, increasing the concentration of calcium ions in the cytosol, and therefore the activity of calcium-binding proteins. DAG is retained at the plasma membrane and activates Protein Kinase C (PKC), which can phosphorylate a range of targets to induce proliferation or differentiation.

PLCγ1 contains a PH domain near the N terminus, an EF hand domain, an X domain, a split PH domain with two consecutive SH2 and one SH3 domain between the two PH halves, followed by a Y domain and a C2 domain. The PH domains allow PLCγ1 to bind membrane patches rich in PIP3; the SH2 domains allow PLCγ1 to interact with proteins phosphorylated on tyrosine residues; and the C2 domain allows PLCγ1 to interact with phospholipids and calcium (Choi 2007). Upon RTK activation, PLCγ1 is phosphorylated on Y783 and other residues which stimulate its phospholipase activity. Phosphatase PTP1B and PTPN11 remove these phosphorylations and return PLCγ1 to its inactive state. Active PLCγ1 catalyzes the hydrolysis of PIP2 into DAG and IP3. DAG remains embedded in the plasma membrane and mediates the activation of PKC, whereas soluble IP3 diffuses throughout the cytoplasm, where it binds to its receptor, a ligand gated calcium channel, on the endoplasmic reticulum which leads to the release of calcium ions, and increase in the activity of calcium regulated proteins.
Activated PKCs phosphorylate and modulate the activity of several signaling proteins involved in gene transcription, cell survival, and proliferation. These targets include GSK3, PKD, Rho GTPases, and IkK. On the other hand, transient increases in calcium concentration mediate cellular processes such as muscle contraction, synaptic communication, neuronal plasticity, and apoptosis.

PLCγ1 is involved in different cancer processes. PLCγ1 activity is required for differentiation of neuronal-like PC12 cells (Choi 2001), and other cell types such as keratinocytes (Xie 1999). PLCγ1 activity also increases cell motility in response to a wide range of RTKs, which is an important aspect of metastasis (Wells 2003), and PLCγ1 inhibition decrease invasiveness in breast cancer cells (Kassis 1999). However, given the multiple isoforms of PLC, there are currently no targeted therapies for this pathway (Mochly-Rosen 2012).
The 4 canonical pathways activated by RTKs are involved in cancer and are highly interconnected

The knowledge amassed during the last several decades has evolved from the linear concept of RTK signaling transduction to the idea of a highly interconnected signaling network that takes place in specific intracellular compartments, dynamically changes over time, and controls complex cellular processes such as cell survival, cell death, migration, differentiation, and proliferation. Overactivation of each of the canonical pathways has been implicated in cancer: the SFK (Liu Pham, 2014, Vlaeminck-Guillem 2014, Irby 2000), the AKT (Mundi 2016), the ERK (Yu 2015), and the PLC-γ (Lattanzio 2013) pathways. These 4 canonical signaling pathways are highly interconnected at different levels: by directly modifying the activity of members of another pathway, by modifying the same signaling substrates/targets, by inducing or inhibiting gene expression of the same genes, or by causing the expression of proteins that modify enzymatic activity. Due to the many possible interactions, signaling networks are extremely complex.

Each of the 4 canonical pathways directly interacts with at least one other pathway (Figure 3). SFKs phosphorylate and increase the kinase activity of both ARAF (residues Y299/Y300) and RAF1 (residues Y340/Y341) (Marais 1997). The SFKs also mediate the phosphorylation and activation of PI3K (Summy 2005). AKT directly phosphorylates RAF in S259 (Gurumurthy 2010), located in RAF’s RAS-binding domain, and results in reduced RAF kinase activation (Mendoza 2012, Kubicek 2002). AKT also phosphorylates PLCγ1 at S1248 (Wang 2006), which reduces PLCγ1 activity (Park 1992). RAS directly binds p110-PI3K, and changes its conformation allosterically to activate PI3K (Mendoza 2011, Rodriguez-Viviana 1994). This interaction is required for transformation induced in vitro and in vivo by KRAS and HRAS (Castellano 2011, Sun 2010). ERK phosphorylates GAB2 (scaffold that mediates activation for PI3K), which
results in decreased activation of AKT (Mendoza 2012). PLC-γ1 can directly bind and activate SOS1, leading to the activation of RAS (Kim 2000). Activated under PLC-γ1, PKC can activate ERK (Chang 2012), and CaMKII phosphorylates AKT in Ser 473 (Li 2011), and MAP2K1 in Ser 221 (Li 2009). These examples illustrate the high level of cross talk between these pathways.
The Hallmarks of Cancer

Aberrations in the canonical pathways may lead to the development of cancer. All cancer cells within a tumor have acquired at least the following capabilities: they can evade apoptosis, are self sufficient in growth signals, are insensitive to anti-growth signals, have the capability of invading other tissues and metastasize, have limitless replicative potential, and display sustained angiogenesis (Hanahan 2011, Hanahan 2000). Additionally, cancer cells change their metabolic pathways towards a mainly glycolytic metabolism, and are able to survive the immune system (Hanahan 2011, Liu 2015). One cellular process that is involved in these hallmarks is RNA splicing, as alternative RNA splicing is implicated in the RNA processing of proteins that participate in all of these 8 hallmarks (Liu 2015).

Neuroblastoma

Neuroblastoma cells are a good model to study RTK signaling. They express a wide range of the RTKs, more than half of the RTKs in the human genome (Palacios-Moreno 2015). They retain their capability to proliferate (Teppola 2016), to migrate (Becker 2012), and to differentiate (Yang 2016, Teppola 2016), and they respond to RTK activating ligands and RTK inhibitors (Ding 2014, Schonherr 2011).

Neuroblastoma is the most common solid extracranial pediatric tumor, and a developmental malignancy that is characterized by its high clinical and biological heterogeneity. Compared to other solid tumors, clinical outcomes for neuroblastoma have improved the least during the last few decades. Neuroblastoma can be considered the result of a failure in the differentiation program of a cell of the neural crest. (Kobayashi 2013, Mohlin 2011, Westermark 2011, Dupin 2007).
The neural crest is a transient group of highly migratory multipotent cells formed after gastrulation that migrate in waves (Sadaghiani 1990) and give rise to a variety of different cell types including osteocytes, chondrocytes, glial cells, melanocytes, smooth muscle cells, and neurons (Sauka-Spengler 2008, Knetch 2002). During their differentiation process the cells of the neural crest respond to different cues: soluble growth factors, hormones, extracellular matrix proteins, and other cells. These signals are integrated during migration in order to appropriately differentiate in the physiological context. A sub group of the neural crest is destined to become the peripheral sympathetic nervous system, including the adrenal medulla. Neuroblastoma arises from the sympathoadrenal lineage of neural crest when one or more of these cells proliferate uncontrollably. Neuroblastoma presents a clinical challenge because the biology of every individual case of neuroblastoma is dependent on the particular stage at which the first cell became malignant (Dupin 2007, Joseph 2004). As a consequence of the migratory nature of the neural crest cells, at the moment of diagnosis neuroblastoma has often undergone metastasis.

Neuroblastoma is a devastating disease. It is the most commonly diagnosed pediatric solid tumor and the average age of diagnosis is under 1 year of age. It affects 1 in 8,000 live births, and has an incidence of 1 in 100,000 children below the age of 15 years, representing about half of infant and roughly 10% of childhood cancers, and around 15% of all pediatric oncological deaths (Heck 2009, Maris 2007).

Neuroblastoma is often asymptomatic. Its discovery frequently relies on identification of symptoms related to the growing tumor mass and/or metastasis, or it is inadvertently discovered during the course of another medical procedure (Schwab 2003). While some patients experience a very aggressive form of the disease, others experience spontaneous regression of
the tumor, or remission after minimal chemotherapy. The most common symptoms include pain and weight loss. Depending on the site of the primary tumor and/or metastasis, weakness of voluntary movement of the lower limbs may occur when a tumor puts pressure on the spinal chord. In addition to symptoms directly caused by the tumor mass, symptoms in tissues distant from the tumor itself can develop, mediated by cytokines and/or hormones released by the tumor. This group of symptoms are collectively called paraneoplastic syndrome, and can arise in neuroblastoma cases. Some examples include: high blood pressure due to increased secretion of catecholamines, and diarrhea that responds poorly to regular treatment due to increased secretion of Vasoactive Intestinal Peptide. The majority (over 30%) of the cases present the primary tumor in the adrenal medulla (Schwab 2003).

Neuroblastoma patients are clinically categorized as low-risk or high-risk according to their age, stage of progression, histology, MYCN status, and tumor cell ploidy (Bhatnagar 2012, Maris 2007, Schwab 2003). Good prognosis indicators include young age at diagnosis, low stage of progression, no metastasis, little to no neovascularization, no MYCN amplification, and the presence of diploid cells. On the other hand, older age at diagnosis, metastasis, neovascularization, MYCN amplification, and aneuploidy are indicators of poor prognosis. All these factors are considered in order to classify the patient as low- or high- risk. Patients with low-risk disease are submitted to a light chemo- and radiotherapy treatment, and often have a successful outcome; patients with high-risk disease are submitted to a more rigorous and extensive chemo- and radiotherapy regimen, often with a negative outcome, experiencing further metastasis and growth, as well as developing resistance to chemotherapy. For both low- and high- risk cases, surgical resection of the tumor is recommended when possible. Commonly used non-targeted chemotherapeutic drugs include carboplatin, cyclophosphamide, doxorubicin, ifosfamide, cisplatin, vincristine, melphalan, etoposide, teniposide, and topotecan. For relapsing
cases, targeted treatments are used and include monoclonal antibodies, retinoids, RTK small molecule inhibitors, and histone deacetylase inhibitors (Bhatnagar 2012). The overall survival ranges from 10% for high-risk cases to 95% for low risk cases. Despite new drugs and treatments that have been developed for different types of cancers, including pediatric cancers, the clinical outcomes for neuroblastoma have not improved accordingly, and a new approach is required.

Correlations have been established between neuroblastoma prognosis and the expression of specific molecular markers. Expression of the RTKs NTRK1 (TRKA) and NTRK3 (TRKC) correlate with good prognosis and patient survival (Brodeur 1997, Nakawagara 1993). In addition, RET collaborates with TRKA to induce differentiation in neuroblastoma (Oppenheimer 2007, Peterson 2004). Expression of these two RTKs represent a more differentiated state, more likely to respond chemotherapy. On the contrary, expression of NTRK2 (TRKB) correlates with poor prognosis (Brodeur 1997, Nakagawara 1994), and reflects a less differentiated state, and often display poorly response to chemotherapy.

Other RTKs may also have clinical implications. Activating mutations in the Anaplastic Lymphoma Kinase (ALK) gene are the main cause of familial neuroblastoma (Mosse 2008) and are present in roughly 10% of sporadic cases (Caren 2008). Expression of KIT is increased in cases with MYCN amplification, which makes these cases most aggressive and proliferative in vitro (Vitaly 2003). In addition, the coexpression of soluble SCF and KIT in neuroblastoma cells suggests an autocrine loop that promotes proliferation (Vitaly 2003).

Since neuroblastoma is a good model to study RTK signal transduction mechanisms, and the study of these mechanisms has clinical relevance to neuroblastoma, and perhaps other types of
cancer, we used neuroblastoma cells to identify RTK signaling pathways. Preliminary phosphoproteomic data identified more than half of the RTKs in the human genome expressed and phosphorylated in neuroblastoma cell samples (Palacios-Moreno 2015). In addition, whole cell and endosomal fractions of neuroblastoma cell lines contained high levels of phosphorylated PAG1, and phosphorylated SFKs FYN and LYN (Palacios-Moreno 2015). These data lead us to formulate the following hypothesis.
Hypothesis

The SFKs FYN and LYN distinguish between proliferation- and differentiation-inducing signals from different RTKs in neuroblastoma cells.
Chapter II

Preliminary Data
Introduction

Work by Marshall et al. in the 1990s (Marshall 1995) established that the duration of activation of signaling pathways, especially ERK, relates to cell fate decisions, such as differentiation and proliferation. They showed in PC12 cells that sustained ERK activation takes place in response to treatment with NGF (ligand for TRKA), and that this sustained ERK activation induces differentiation. On the other hand, transient activation of ERK takes place in response to treatment with EGF (ligand for EGFR), and that this transient ERK activation induces proliferation (Marshall 1995). More recent work (Chen 2012) has shown that the AKT pathway is also an important piece of the puzzle for cell fate decision making. PC12 cells with a high pERK/pAKT ratio, such as that induced by treatment with NGF, differentiate into neurons and cease proliferation, whereas cells with a lower pERK/pAK ratio, such as that induced by treatment with EGF, proliferate. In addition, in different cell models, the SFKs can act upstream from the ERK and AKT pathways (Agarwal 2016, Summy 2015, Marais 2007). Because of these observations, we hypothesized that the pERK/pAKT ratio would be different in cells treated with different RTK ligands, and that the inhibition of the SFKs would alter the pERK/pAKT ratio.

In order to interrogate our hypothesis we measured the levels of activated ERK, and activated AKT in response to RTK ligands, and then the effect of inhibition of the SFKs by SFK inhibitors PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine), and SU6656 (2,3-Dihydro-N,N-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl)methylene]-1H-indole-5-sulfonamide).
Figure 4. Preliminary experiments indicate that activation of different RTKs changes the pERK/pAKT ratio in LAN6 cells. A. LAN6 cells were exposed to 5nM NGF, 5nM GDNF, or 50nM PTN for 60 minutes in suspension. Whole cell extracts were prepared, and western blot analysis was performed for phospho-ERK1/2 and phospho-AKT. Error bars represent SD. n=2. B. Western blot of one of the two experiments is displayed. Differences did not reach statistical significance.
Figure 5. Preliminary experiments indicate that inhibition of SFKs increases the pERK/pAKT ratio in RTK stimulated LAN6 cells. A. LAN6 cells were preincubated with 500nM PP2 or 500nM SU6656 prior to exposure to 5nM NGF, 5nM GDNF, or 50nM PTN for 60 minutes in suspension. Whole cell extracts were prepared, and western blot analysis was performed for pERK and pAKT. Error bars represent SD. n=2. B. Western blot of one of the two experiments is displayed. Differences did not reach statistical significance.
Results

In order to test our hypothesis, we performed 60 minutes long stimulations with different RTK ligands. In addition, since the SFKs have been shown to be upstream of the ERK and AKT pathways, we used SFK inhibitors PP2 and SU6656 to measure the impact of SFK inhibition on the activation of these pathways. In order to compare our results with similar experiments performed by Chen et al. (2012), we analyzed the pERK/pAKT ratio.

Does RTK activation change the pERK/pAKT ratio? The preliminary data presented indicates that the activation of TRKA by NGF, and RET by GDNF slightly increased the pERK/pAKT ratio, whereas the activation of ALK by PTN decreased it (Figure 4).

Does inhibition of the SFKs by PP2 and SU6656 change the pERK/pAKT ratio? The preliminary data presented indicates that inhibition of the SFKs by PP2 and SU6656 increased the ratio pERK/pAKT (Figure 5).

Discussion

Activation of TRKA and RET, RTKs known to induce differentiation in neuroblastoma, slightly increased the pERK/pAKT ratio (Figure 5), similar to the results obtained by Chen et al. (2012), whereas the activation of ALK, known to induce proliferation in neuroblastoma, decreased the pERK/pAKT ratio. The fact that inhibition of the SFKs increased the the pERK/pAKT ratios in all conditions (Figure 5) shows that these pathways are intimately interconnected in neuroblastoma cells.

These results were in the direction indicated by Chen (2012), but not statistically robust. The indication that inhibition of the SFKs increased the pERK/pAKT ratio motivated the examination of the intracellular location of SFKs FYN and LYN and their response to RTK activation.
Chapter III

FYN and LYN dynamically change intracellular location in response to RTK activation
Introduction

Our preliminary data identified that inhibition of the SFKs increased the pERK/pAKT ratio, which would imply decreased proliferation following Chen’s (2012) results, and it is known that intracellular trafficking is important for SFK regulation, therefore, it is important to examine SFK trafficking in neuroblastoma cells in response to RTK activation.

Sandilands et al. (2007, 2008) have performed research on the involvement of endosomal trafficking in the activation of SRC. They showed that Rab11+ recycling endosomes are required for redistribution from perinuclear organelles to the plasma membrane and activation of SRC. However much less is known of the RTK mediated changes in the intracellular distribution of FYN and LYN in neuroblastoma cells. We hypothesized that activation of RTKs that induce proliferation would induce cellular redistribution of FYN and LYN different than that of RTKs that induce differentiation. To test this hypothesis we set out to define the intracellular location of FYN and LYN and possible changes in their intracellular distribution in response to activation of different RTKs. In addition, we measured proliferation in response to activation of RTKs known to induce proliferation or differentiation in neuroblastoma cells.
Results

Does RTK stimulation change the distribution and activation levels of SFKs in
neuroblastoma cells? In order to identify changes in distribution of SFKs FYN and LYN in
response to RTK activation, we performed organelle fractionation experiments in LAN6 cells
stimulated with RTK ligands. Cells were mechanically disrupted using a cell cracker. The
cracked cells were separated by centrifugation in two fractions: the plasma membrane and the
organelles. The plasma membrane fraction was further separated by treatment with IGEPAL into
detergent resistant (DRM) and soluble membranes (P1M). The organelle fraction containing the
lysosomal, endosomal, and cytosolic fractions was submitted to a velocity iodixanol gradient,
yielding the lysosomes (Lys), endosomes of high mass and density (E1), endosomes of
intermediate mass and density (E2), endosomes of low mass and density (E3), and cytosol. Lys,
and E1-E3 fractions were further submitted to an equilibrium iodixanol gradient (at 100,000xg),
yielding a membrane containing, floating fraction (F) and a non floating fraction (NF).

We identified the distribution of FYN and LYN in untreated cells (Figure 6). FYN and LYN reside
mostly in lipid raft containing detergent resistant membranes. A much smaller fraction of these
SFKs resides in the detergent soluble fraction of the plasma membrane (P1M), and a small
fraction of FYN and LYN is detected in endosomes and lysosomes. The association of SFKs
with the endosomal and lysosomal membranes is very strong since FYN and LYN are only
detected on the floating (F) fractions of endosomes and lysosomes. We also detected FYN and
LYN in the cytosol (Figure 6 A-C).
Figure 6. SFKs FYN and LYN reside primarily at the plasma membrane, especially in lipid rafts, and are also present and strongly bound to endosomal and lysosomal membranes.

A. Western blot displaying FYN and LYN in different intracellular compartments; because of their sample size, only 5% of the DRM, 2% of the P1M and 10% of the cytosolic fractions were loaded. 100% of the sample for all other fractions was loaded. B and C. Bar graphs representing the percentage of total cellular B, FYN, and C, LYN in each fraction. To simplify, the endosomes (E1, E2, and E3 endosomal fractions) were summed into the total END fraction. Error bars represent SD. Lys = Lysosomes, E1 = Endosomes of high mass and density, E2 = Endosomes of intermediate mass and density, E3 Endosomes of low mass and density, DRM = detergent resistant membranes, P1M = detergent soluble membranes, Cyt = cytosol, F = Floating fraction, NF = Non floating fraction. Error bars represent SEM.
Activation of ALK by PTN and KIT by SCF increased FYN and LYN in endosomes, especially in
the organelles of highest mass and density (lysosomal and E1 fractions, Figure 7, Figure 9). In
contrast, activation of RET by GDNF decreased the overall amount of FYN and LYN in
dendosomes, and changed the distribution of FYN and LYN in the organelle fractions (Lys, E1-
E3, Figure 7). Activation of ALK and KIT also changed the amounts of FYN and LYN in the
fractions where FYN and LYN primarily reside, the plasma membrane’s P1M and DRM (Figure
8), and induced an increase in FYN and LYN in lysosomes and endosomes (Figure 9, arrows).
(Figures 8 and 9 were created by pooling data obtained by myself and Lauren Foltz).
Figure 7. FYN and LYN dynamically change intracellular location in response to RTK activation. Western blot displaying intracellular location of FYN and LYN in response to the activation of ALK by 50nM PTN and of RET by 5nM GDNF for 60 minutes.
Figure 8. Plasma membrane distribution of FYN and LYN in response to the activation of ALK by PTN and KIT by SCF. Box plots represent the percentage of cellular FYN (blue) and LYN (red) in plasma membrane fractions. DRM = detergent resistant membranes, P1M = detergent soluble membranes after stimulation of ALK by 50nM PTN and of KIT by 5nM SCF for 60 minutes.
Figure 9. ALK and KIT activation dynamically change the intracellular distribution of FYN and LYN, and increase the presence of FYN and LYN in endosomes, especially in endosomal fractions of high mass and density. Fold change over control was calculated in each intracellular compartment for FYN and LYN in response to activation of ALK by 50nM PTN and of KIT by 5nM SCF for 60 minutes. Arrows highlight the increase in FYN and LYN in the high mass and density E1 fraction. Error bars represent the standard error of the mean (SEM).
Since we detected an increase in the presence of FYN and LYN in the late endosomes (fraction E1), we hypothesized that the activation of FYN and LYN in this fraction would be affected by signaling from different RTK. The activation status of FYN and LYN was detected by immunoprecipitating with antibodies for the phosphorylated active site of the SFK, followed by a second immunoprecipitation of the remaining sample with an antibody for the non-phosphorylated inactive SFK, and western blotting for FYN and LYN. The E1 fraction contained both active and inactive FYN and LYN (Figure 10), though the strongest immunoreactivity in all conditions was displayed by the active form of FYN and LYN.

**Figure 10. Populations of active and not active FYN and LYN coexist in endosomes.** High mass and density endosomal fractions (E1) of LAN6 cells exposed to 5nM SCF or 50nM PTN for 60 minutes were submitted to immunoprecipitations using phosphorylated SFK (pSRC), followed by a second immunoprecipitation of the remaining sample with non-phosphorylated SFK (non-pSRC) antibodies. The samples were then submitted to western blot analysis for A. FYN, and B. LYN.
We also found that stimulation of SH-SY5Y neuroblastoma cells with EGF and SCF increased proliferation, whereas the stimulation with NGF or RA did not (Figure 11). The small increase over control in cells in the latter case likely represents increased cell survival in low serum conditions.

Figure 11. Activation of EGFR and KIT induces proliferation in SH-SY5Y cells. MTT assay was employed to determine growth rates (absorbance at 96h/absorbance at time 0) in SH-SY5Y cells exposed to 5nM NGF, 5nM SCF, 5nM EGF, or 10µM Retinoic Acid (RA); in low serum (2% FBS) medium. * represents p-value < 0.05 on Student’s t-test.
Discussion

Our results show increased FYN and LYN in endosomes and lysosomes with a corresponding decrease at the plasma membrane in response to activation of RTKs that induce proliferation (ALK, KIT) (Figure 12). In contrast, activation of RTKs that induce differentiation (TRKA and RET) decreased the amount of FYN and LYN in endosomes and lysosomes with a corresponding increase at the plasma membrane (Figure 12).

**Figure 12. Model for FYN and LYN redistribution in response to RTK stimulation.**

Activation of RTKs that are associated with proliferation increase migration of FYN and LYN from the plasma membrane into endosomes, whereas the activation of RTKs that are associated with differentiation increases the presence of FYN and LYN at the plasma membrane and decreases the presence in endosomes. EE = Early Endosome, LE Late Endosome.
We found that, in control conditions, FYN and LYN reside mostly at the plasma membrane, especially in lipid raft containing detergent resistant membranes (DRM), as previously shown by many research groups (Palacios-Moreno 2015, Minami 2011, Tamzin 2008, Kannan 2008, Pereira 2007) (Figure 6 A-C). Other researchers have shown that SFKs arrive to the plasma membrane in different ways depending on the acylation state of their unique domain: myristoylated-palmitoylated LYN travels to the plasma membrane using the Golgi apparatus/the secretory pathway, whereas myristoylated-dually palmitoylated FYN is directly targeted to the plasma membrane after translation and subsequent dual palmitoylation (Sato 2009). In addition, a population of SFKs has been shown to reside in the perinuclear organelles (Sandilands 2007). These perinuclear SFKs translocate to the plasma membrane in response to RTK activation, for example after PDGFR stimulation, on Rab11+ recycling endosomes (Sandilands 2007). We found that activation of ALK and KIT differentially changed the levels of FYN and LYN in detergent soluble and detergent resistant regions of the plasma membrane (Figure 8), which may have direct implications for cellular signaling since different signaling nodes reside in different cellular compartments (Palacios-Moreno 2015, Gao 2011, Otahal 2010, Blouin 2010).

The removal of receptors from the plasma membrane via endocytosis was understood for many years as a means to decrease receptor signaling, by mechanically removing them from exposure to the extracellular environment, and by inducing receptor degradation in lysosomes. The signaling endosome hypothesis has been developed to explain the long lasting signaling activated by TRKA in neurons (Howe 2004). This way of signaling is part of the mechanism by which receptors such as TRKA normally communicate their signal (Marlin 2015, Cui 2007, Grimes 1997, Grimes 1996). This hypothesis has been widely accepted. In neurons, after meeting NGF at the axonal terminal, TRKA undergoes endocytosis and is transported to the neuron soma on microtubules, where it can mediate long term changes in signaling molecules.
(such as ERK5) that lead to the changes in gene expression that induce differentiation and survival (Marlin 2015, Geetha 2013, Cui 2007, Grimes 1997, Grimes 1996).

Our data adds to the signaling endosome hypothesis and suggests that endocytosis of specific RTKs induces different patterns of SFK trafficking. We detected the presence of FYN and LYN in endosomes and lysosomes. In response to ALK and KIT activation, FYN and LYN increased in organelles that had a sedimentation velocity that coincided with that of Rab7, a marker of late endosomes (fraction E1), (McCaffrey 2009), and acid phosphatase, marker of lysosomes (fraction Lys) (Figure 9) (McCaffrey 2009). We also detected FYN and LYN in the cytosol (Figure 6 A-C). There are at least three potential explanations for FYN and LYN in the cytosol: that they are newly translated proteins before acylation; they are the product of lipases that have removed the palmatoyl groups, and thus weakening their association with membranes; or they are part of cytosolic signaling particles (MacCormick 2005).

We detected active and inactive populations of FYN and LYN in late endosomes (Figure 10). Lack of phosphorylation by CSK in the inhibitory site of the SFKs results in increased SFK degradation, and decreased SFK stability (Harris 1999). Activated SFK may undergo degradation via the ubiquitination and lysosomal degradation pathway (Hakak 1999). Similarly, v-SRC (that lacks the inhibitory site and is constitutively active), is highly ubiquitinated and degraded in lysosomes (Kim 2004). One interpretation of our data could be that active FYN and LYN may be on their way to lysosomal degradation, whereas inactive FYN and LYN may be recycled to the plasma membrane in Rab11+ recycling endosomes (Reincke 2014, Sandilands 2007). The presence of these SFKs in endosomes supports the notion that endosomes are potential active signaling compartments for SFKs, or play a role in regulating SFK activity by sequestration in intraluminal vesicles of MVBs.
Since we find activated FYN and LYN in late endosomes, it is important to note that the SFKs can influence their own trafficking. Once active, the SFKs can phosphorylate and thus control the activity of a range of substrates, including components of the endocytic pathway such as clathrin (Wilde 1999), or AP2 subunit AP2B1, which lead to increased receptor endocytosis (Zimmerman 2009), components of the ESCRT-0 complex such as ubiquitin binding HRS (Bache 2002), which enhances the association of HRS to endocytic membranes (Gasparrini 2012) and incorporation of cargo into multivesicular bodies.

Whether the active form of each of these SFKs is located to the outer membrane of late endosomes or to the intraluminal vesicles, resembling GSK-3 in WNT signaling (Taelman 2010), remains to be defined. Further research is required to define if the increase of FYN and LYN in late endosomes we have shown in proliferating cells takes place in vivo. If that’s the case, drugs targeted to keep SFKs at the plasma membrane could be employed in the treatment of neuroblastoma, and hopefully improve its clinical outcomes. This principle could also be applied to tissue engineering for proliferation control of cellular populations.

Our results shed light on how individual RTKs differentially communicate signals to downstream effector pathways such as the SFKs. Specifically, our findings indicate RTKs that promote proliferation in neuroblastoma, such as ALK and KIT induce an increase of FYN and LYN in endosomes, whereas RTKs that promote differentiation in neuroblastoma, such as TRKA and RET, decrease the presence of FYN and LYN in endosomes (Figure 12).
Chapter IV

PAG1 ablation increases proliferation, prevents differentiation, and modifies signaling responses to RTKs
**Introduction**

Our previous work (Palacios-Moreno 2015) established that the intracellular distribution of FYN and LYN dynamically changes in response to RTK activation. However, collecting organelles and fractions of the plasma membrane requires pooling a huge number of cells into one sample, which obscures the detection of subpopulations of cells within the culture, as the results display the combination of millions of cells. In order to identify single cell signals, and potential subpopulations in our cultured cells, we used flow cytometry.

PAG1 plays a critical role in regulating the levels of activation of the SFKs by bringing CSK to the plasma membrane, where CSK can phosphorylate the SFKs in the C-terminus inhibitory site, and thus decrease SFK activity. In order to measure the impact of PAG1 ablation on RTK signaling, proliferation, and differentiation at the single cell level, we generated PAG1 knockdown (PAG1kd) SH-SY5Y cells with the help of Lauren Foltz, utilizing the CRISPR-CAS9 system, and measured proliferation and differentiation, and activation of signaling molecules upon RTK activation.
Results

We performed flow cytometry in order to assess the levels of activated SFKs in PAG1kd cells in normal cell culture conditions (10% FBS in RPMI1640). We hypothesized that because of the role of PAG1 in SFK inhibition, PAG1kd cells would have higher levels of activated SFKs. Indeed, we did detect increased levels of activated SFKs in PAG1kd cells (blue peak, Figure 13).

![Flow Cytometry Graph]

**Figure 13.** PAG1kd SH-SY5Y cells express higher levels of activated SFKs than wild type cells. The levels of activated SFKs in wild type and PAG1kd cells were measured by flow cytometry. Data displayed are from one experiment and are representative of at least three independent experiments.
Because of the elevated levels of activated SFKs in PAG1kd cells, we hypothesized that PAG1kd cells would exhibit higher levels of proliferation than wild type cells. We detected an increased proportion of cells in the S and G2/M stages of the cell cycle in cells growing in culture (10% FBS in RPMI1640) (Figure 14 A1 and A2). We then asked the question: do the levels of activated SFKs change with long exposure (96h) to low serum (2% FBS) conditions in wild type cells, in wild type cells exposed to PP2, and in PAG1kd cells? Expectedly, we detected a very small proportion of cells in the S and G2/M stages of the cell cycle, as neuroblastoma cells do not actively proliferate in low serum conditions (Figure14 B). Similar results were seen in wild type cells exposed to 500nM PP2 (Figure 14 B). In contrast, PAG1kd cells displayed an increased proportion of cells in both the S and G2/M stages of the cell cycle, despite the low serum conditions (Figure 14 B).

In addition, because we had already identified these changes in the cell cycle, we decided to confirm the increase in proliferation using MTT assays. WT and PAG1 cells were exposed to low serum conditions for 96 hours in the presence or absence of the SFK inhibitor PP2. Confirming our findings, wild type cells, both in the presence and absence of PP2, did not proliferate in low serum conditions. Again, in contrast, PAG1kd cells had vastly higher proliferation rates (Figure 14 C, Blue boxes).
### Percentage of cells in each stage of the cell cycle

<table>
<thead>
<tr>
<th>Stage</th>
<th>WT</th>
<th>PAG1kd</th>
</tr>
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<tbody>
<tr>
<td>G0-G1</td>
<td>80</td>
<td>54</td>
</tr>
<tr>
<td>S</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>G2-M</td>
<td>12</td>
<td>25</td>
</tr>
</tbody>
</table>

### Percentage of cells in each stage of the cell cycle

<table>
<thead>
<tr>
<th>Subset</th>
<th>C</th>
<th>C+PP2</th>
<th>PAG1kd</th>
</tr>
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<tbody>
<tr>
<td>G0-G1</td>
<td>85</td>
<td>91</td>
<td>58</td>
</tr>
<tr>
<td>S</td>
<td>13</td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td>G2-M</td>
<td>2</td>
<td>2</td>
<td>14</td>
</tr>
</tbody>
</table>
Figure 14. PAG1 ablation increases proliferation in SH-SY5Y cells. Wild type cells (A1) and PAG1kd cells (A2) were stained with Hoescht 33342 to identify the proportion of cells in each stage of the cell cycle by flow cytometry. The table indicates the percentage of cells in each stage of the cell cycle. B. Wild type and PAG1kd cells were seeded on collagen coated plates, and were exposed to low serum (2% FBS) conditions for 96h. The cells were then stained with Hoescht 33342 and measured by flow cytometry. Percentage of cells in each stage of the cell cycle, G0-G1, S, and G2-M, is displayed in the table for each condition. Data displayed from 1 experiment representative of at least 3 independent experiments. C. WT and PAG1kd cells were exposed for 48h to low serum conditions in the presence (purple boxes) or absence (blue boxes) of differentiation inducing ligands NGF and Retinoic Acid (RA+N), and proliferation was evaluated using the MTT assay. The graph represents data of 3 independent experiments, and * represents p-values <0.05 by t-test.
Since the SFKs have been shown to be upstream of the AKT pathway (Figure 3), and PAG1kd cells have increased levels of activated SFKs (Figure 13) we hypothesized that the levels of activated AKT are higher in PAG1kd cells. Also, because it has been shown that the activation of AKT is cell cycle stage specific (Liu 2014), we hypothesized that the cells with the highest pAKT levels reside in the G2/M stages of the cell cycle. To test these hypotheses it was necessary to develop simultaneous DNA content and levels of activated AKT measurements by flow cytometry.

With this approach, we identified a distinct population of larger sized cells with very high levels of pAKT in both wild type and PAG1kd cells (Figure 15 A1 and A2), and found that these cells with high levels of pAKT reside mostly in the G2/M stages of the cell cycle in both wild type and PAG1kd cells (Figure 15 A1 and A2). We also found that the proportion of cells with high pAKT levels was higher in PAG1kd cells (Figure 15 B, arrow).
A1) Wild type

A2) PAG1kd

B) 

Figure 15. **pAKT expression is highly cell cycle stage specific.** The cell population with high levels of pAKT is shown for wild type cells (A1) and PAG1kd cells (A2). High expressing pAKT cells resided mostly in the G2-M stages of the cell cycle. B. PAG1kd cells had a proportionally larger population of high pAKT cells (arrow). Data displayed from one experiment are representative of at least three independent experiments.
Next, we decided to investigate downstream signaling responses to RTK ligands that induce proliferation in wild type and PAG1kd cells. We hypothesized that PAG1kd cells would have higher levels of pERK and pSRC in response to EGFR (Figure 16 A and B) and KIT activation (Figure 16 C-D). We measured the early (5 minutes) and late (60 minutes) responses of pERK and pSRC levels after RTK stimulation.

A) EGF

![Histograms showing pERK levels in WT and PAG1kd cells at 5m and 60m](image)

B) NGF

![Histograms showing pSRC levels in WT and PAG1kd cells at 5m and 60m](image)
B) EGF

WT 5m pSRC

![Histogram](image1)

WT 60m pSRC

![Histogram](image2)

PAG1kd 5m pSRC

![Histogram](image3)

PAG1 kd 60m pSRC

![Histogram](image4)
C) SCF

- Unstimulated cells
- FMO control
- SCF-stimulated cells

WT 5m pERK

PAG1kd 5m pERK

WT 60m pERK

PAG1 kd 60m pERK
D) SCF

WT 5m pSRC  

![Graph showing pSFK levels in WT 5m pSRC](image)

PAG1kd 5m pSRC

![Graph showing pSFK levels in PAG1kd 5m pSRC](image)

WT 60m pSRC

![Graph showing pSFK levels in WT 60m pSRC](image)

PAG1kd 60m pSRC

![Graph showing pSFK levels in PAG1kd 60m pSRC](image)

**Figure 16.** Consequences of PAG1 ablation after short term (5 minutes) and long term (60 minutes) activation of the SFK and ERK pathway in response to activation of RTKs that induce proliferation. Effect of EGFR activation by EGF (5nM) on the levels of pERK (A) and pSFK (B). Effect of KIT activation by SCF (5nM) on the levels of pERK (C) and pSFK (D). Ligand stimulated RTK activation was performed on adherent cells for 5 and 60 minutes prior to fixation and cells were stained with fluorescent antibodies for pERK, and pSFKs. Data displayed from 1 experiment representative of at least 3 independent experiments.
Activation of EGFR induced a large increase in the levels of pERK in wild type cells and elicited an even stronger response in PAG1kd cells (Figure 16 A). Changes in pERK activation were not seen in response to the activation of KIT in both cell types (Figure 16 C). The levels of activated SFKs slightly increased in the wild type cells after stimulation of both EGFR (Figure 16 B) and KIT (Figure 16 D) at 60 minutes, but not in the PAG1kd cells. These results indicate that PAG1 ablation affects the activation of the SFKs and downstream ERK differently for different RTKs.

Collectively, these proliferation and signaling data highlight the role of PAG1 in controlling proliferation and the levels of activation of the SFKs, as well as other downstream signaling pathways, such as the ERK pathway.
Since final differentiation in neurons involves turning off proliferation, it is likely that actively proliferating neuroblastoma cells are not differentiating, and we hypothesized that differentiation is impaired in PAG1kd cells. In Figure 14 C, we saw that treatment with RA and NGF (purple), known inducers of differentiation in neuroblastoma cells, increased the survival of wild type cells in low serum conditions, and that this did not happen in the presence of PP2. PAG1kd cells did not increase nor decrease proliferation in response to treatment with RA and NGF in low serum conditions. Figure 17 A shows the response to treatment with RA and NGF in wild type cells, wild type cells exposed to PP2, and PAG1kd cells (control cells not exposed to RA and NGF are displayed in Figure 14 B). Wild type cells in response to RA and NGF, both in the absence and presence of PP2, display a lower percentage of cells in S, and G2/M. In contrast, PAG1kd cells display an almost identical distribution as untreated PAG1kd cells (14 B and 17 A), indicating no response to differentiation signals. Treatment with RA and NGF in low serum (2% FBS) for 96 hours, slightly increased the levels of activated SFK in wild type cells regardless of the presence of PP2 (Figure17 B and C). The levels of activated SFKs in PAG1KD cells did not change in RA and NGF stimulated cells (Figure17 D), although the levels of activated SFKs remained higher than in wild type cells, even after 96h of low serum conditions (Figure 17 E). In addition, we measured the levels of expression of β III-Tubulin, a marker of differentiation in the neuronal lineage. We found that PAG1kd cells express much lower levels of β III-Tubulin, which indicates a de-differentiation response to PAG1 ablation (Figure 17 F).
A) 

![Graphs showing cell cycle distribution for different conditions](image)

<table>
<thead>
<tr>
<th>Percentage of cells in each stage of the cell cycle</th>
<th>C RA+N</th>
<th>C+PP2 RA+N</th>
<th>PAG1kd RA+N</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0-G1</td>
<td>98</td>
<td>94</td>
<td>59</td>
</tr>
<tr>
<td>S</td>
<td>2</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>G2-M</td>
<td>0</td>
<td>1</td>
<td>15</td>
</tr>
</tbody>
</table>

B) 

![Histogram showing pSFK expression for WT, RA+N, and No treatment](image)

C) 

![Histogram showing pSFK expression for WT+PP2 condition with FMO control, RA+N, and No treatment](image)

D) 

![Histogram showing pSFK expression for WT+PP2 condition with FMO control, RA+N, and No treatment](image)
Figure 17. PAG1kd cells fail to respond to differentiation signals. A. Wild type and PAG1kd cells were seeded on collagen coated plates, and were exposed to 10uM RA in combination with 5nM NGF in low serum (2% FBS) conditions for 96h. The cells were then stained with Hoescht 33342 and measured by flow cytometry. The percentage of cells in each stage of the cell cycle, G0-G1, S, and G2-M, is shown for each condition. (B-D) Levels of activated SFKs in B. wild type cells, C. wild type cells exposed to 500nM PP2, and D. PAG1kd cells, exposed to RA and NGF. E and F show the levels of E. activated SFKs and F. β III-Tubulin in wild type and PAG1kd cells. The figures display data from one experiment representative of three independent experiments.

Because PAG1kd cells failed to respond to differentiation signals, we hypothesized that PAG1kd cells would not activate the downstream pathways of ERK and SFK pathways to the same extent as wild type cells in response to RTK ligands that induce differentiation. To test this hypothesis we measured the levels of pERK and pSRC in response to activation of TRKA (Figure 18 A and B) and RET (Figure 18 C and D). We measured the early (5 minutes) and late (60 minutes) levels of pERK and pSRC after RTK stimulation.

The levels of pERK increased after activation of TRKA (Figure 18 A) or RET (Figure 18 C), but were similar to control in PAG1kd cells. In contrast, the transient increases in the levels of
activated SFKs, were not dramatically affected by PAG1 ablation (Figure 18 B and D). These data show that PAG1 ablation prevents the induction of differentiation by the combined treatment of RA and NGF.

E) NGF

![Graphs showing pERK levels in WT and PAG1kd cells under different conditions.](image)
F) NGF

WT 5m pSRC

PAG1kd 5m pSRC

WT 60m pSRC

PAG1 kd 60m pSRC
G) GDNF

WT 5m pERK

WT 60m pERK

PAG1kd 5m pERK

PAG1 kd 60m pERK

Unstimulated cells
FMO control
GDNF-stimulated cells
Figure 18. **Consequences of PAG1 ablation after short term (5 minutes) and long term (60 minutes) activation of the SFK and ERK pathways in response to activation of RTKs that induce differentiation.** Effect of TRKA activation by NGF on the levels of pERK (A) and pSFK (B). Effect of RET activation by GDNF on the levels of pERK (C) and pSFK (D). Ligand stimulated RTK activation was performed on adherent cells for 5 and 60 minutes prior to fixation and cells were stained with fluorescent antibodies for pERK, and pSFKs. Data displayed from one experiment representative of at least three independent experiments.
Discussion

Flow cytometry was developed in the late 1960s, and has been refined and widely used in the study of immunology. Blood cells present a great advantage as samples for flow cytometry because they are non-adherent cells. Flow cytometry for adherent cells presents several challenges, as the cells naturally form cell-cell junctions that makes the cells prone to clump. In addition, detection of phosphorylated intracellular proteins presents another set of challenges, including the fixation and permeabilization methods, and the ability of antibodies to reach their epitopes in the intracellular space. Moreover, measuring DNA content simultaneously presents added technical difficulties, and therefore, these kind of studies are scarce. In particular, all these challenges have prevented the application of these experimental approaches to neuroblastoma research. We were able to successfully measure DNA content simultaneously with intracellular phosphoproteins (pERK, pAKT, pSFKs) in adherent neuroblastoma cells.

Using this approach, for the first time we have demonstrated consequences for PAG1 ablation at the single cell level in SH-SY5Y neuroblastoma cells. Loss of PAG1 leads to increased levels of activated SFKs (Figure 13), and differences in cellular responses to activation of specific RTKs, including proliferation, differentiation, and activation of signaling pathways.

We found that in control conditions, PAG1kd SH-SY5Y cells, generated in our laboratory using CRISPR/CAS9, have an increased proportion of actively proliferating cells (Figure 14). We also found that there is a distinct population with high levels of phosphorylated, active AKT, in both control and PAG1kd cells, and that the cells in this population are primarily in the G2/M stages of the cell cycle (Figure 15). When we examined the PAG1kd cells, we found a relative increase
in the population expressing high levels of pAKT compared to control (Figure 15 B arrow); and a concomitant increase in the percentage of cells in the G2/M stages of the cell cycle (Figure 13). Our results on the levels of activated AKT are in agreement with similar experiments by Liu et al. (Liu 2014), where they show that AKT activation is highly tied to the progression of the cell cycle. They showed cell cycle stage specific increases in the levels of pAKT (at T308) that mirror the levels of cyclin A; levels start to increase in S and are highest in G2. They also showed that Cdk2/CyclinA and the mTORC2 complexes phosphorylate and activate AKT in HeLa cells. This explains, in part, why conditions that induce proliferation, like treatment of neuronal stem cells with EGF and BDNF (Zhang 2011), display increased levels of activated AKT, and that populations of cells and tumors with elevated levels of activated AKT are often associated with increased proliferation, and decreased apoptosis. In addition, Chen et al. (Chen 2012), have shown that PC12 cells with higher pAKT expression choose to proliferate over differentiating, and that inhibition of the PI3K-AKT pathway, by inhibitors like LY294002, prevents proliferation.

Our data showed that PAG1 ablation lead to increased levels of activated SFKs in normal serum (10% FBS) or low (2% FBS) serum conditions (Figure 13, Figure 17 E). In addition, we found that the population with high levels of activated AKT was bigger in PAG1kd cells (Figure 15 B, arrow).

Other researchers have found that in dendritic cells, SFKs act upstream of the AKT pathway (Singhal 2011). Activation of MET by HGF induces the activation of SRC, which is necessary for the formation of a protein complex that directs the activation of PI3K and downstream AKT (Singhal 2011). The SFKs can activate PI3K in response to activation of other kinds of receptors, such as the IL2R, where LCK is activated by IL2R and in turn phosphorylates PI3K to
increase its activity (Cuevas 2001). In our data however, we did not detect increases in the levels of activated AKT in response to short (5 minutes) or long (60 minutes) term RTK activation (Data not shown). It is possible that the activation and return to the inactivated state of AKT had taken place before the earliest (5 minutes) measurement we made.

Our results suggest that the increase in activated SFKs as a consequence of PAG1 ablation leads to increases in the S and G2-M stages of the cell cycle, and that because activated AKT is almost exclusively activated in the G2-M stages of the cell cycle, the increase in the high phospho-AKT population is most likely a consequence of the increase in the G2/M stages of the cell cycle.

In the case of neurons, final differentiation involves turning off proliferation. Thus, it is expected that actively proliferating neuroblastoma cells are not differentiating. We found that in addition to increased proliferation, PAG1 ablation also compromises differentiation. Retinoic Acid is known to induce differentiation in neurons, and neuroblastoma cells, both in vitro (Xun 2012, Teppola 2016), and in vivo (Park 2016), and is used clinically in certain cases of neuroblastoma. NGF is also known to induce differentiation in neurons (Yuan 2013, Liu 2014). As expected, wild type SH-SY5Y cells were able to respond to differentiation inducing ligands RA and NGF, as shown by the reduction in the number of cells in the S and G2-M stages of the cell cycle, even in the presence of the SFK inhibitor PP2 (Figure 14 B and 17 A). In contrast to their wild type counterparts, PAG1kd cells did not respond to treatment with RA and NGF, as shown by similar proportion of cells in all stages of the cell cycle (Figure 14 B and 17 A), and continued to actively proliferate (Figure 14 C). It is important to note that wild type cells proliferated more than control conditions when exposed to RA and NGF (Figure 14 C). These ligands may have provided survival signals (Ma 2009) in the harsh, low serum conditions of this experiment, which
translated into increased numbers of cells. Other researchers have shown that changes in PAG1 expression have profound consequences in a variety of cellular processes. Low levels of PAG1 expression in neuroblastoma correlate with low rates of patient survival (Agarwal 2016). shRNA knockdowns of PAG1 induced with higher levels of proliferation, tumorigenesis, and active SFKs, pAKT and pERK induced by shRNA against PAG1 in vitro (Agarwal 2016). PAG1 overexpression studies in esophageal cancer cells decreased proliferation, migration and invasiveness, and the levels of expression of SFKs (Zhou 2015). Our results highlight the importance of PAG1 expression in the regulation of proliferation and differentiation of neuroblastoma cells.

RTK signaling is differentially affected by PAG1 ablation. Activation of TRKA triggered increased levels of pERK that were not seen when PAG1 was ablated, but the increase in the levels of pSRC after short term stimulation (5 minutes) remained intact (Figure 18 E, F). In contrast, activation of ERK in response to the activation of EGFR remained intact in PAG1kd cells, and increases in the levels of activated SFKs seen in wild type cells were suppressed, probably because the levels of activated SFKs are already high as a consequence of PAG1 ablation. Activation of RET and KIT displayed a behavior similar to that of TRKA. TRKA mediated activation of ERK was dependent on PAG1 status, whereas EGFR mediated activation of ERK was PAG1 independent. Our results suggest that PAG1 plays a more active role in TRKA, RET, and KIT signaling than in EGFR signaling at these time points (5 and 60 minutes). RTK initiated signaling pathways are highly interconnected (Figure 2), and in different cellular contexts one pathway can be upstream from another. While we focused on RTK mediated changes in the levels of activation of these canonical pathways, other researchers have looked at these pathways with different approaches, ligands, and receptors. The SFKs can act upstream of ERK and AKT in different cell types. For example, in osteoblast-like ROS cells, inhibition of SFKs by
PP2 decreases the activation of ERK after stimulation with TGF-β1 (Zhang 2010). Similarly, the SFKs act upstream of ERK in keratinocytes, where treatment with high concentrations of PP2 prevented the activation of ERK and AKT in response to treatment with 20GPPD (Lim 2015). Our experiments demonstrate that PAG1 controls not only the levels of activation of the SFKs, but also the levels of activation of the ERK pathway in response to RTK activation in SH-SY5Y cells. Additionally, our results indicate that PAG1 is required for normal activation of signaling proteins downstream from RTKs, and that signaling of particular RTKs, such as TRKA, are more influenced by PAG1 ablation than others, such as EGFR.

While our findings improve our understanding of how the SFKs and PAG1 influence RTK signaling, further work to completely understand the mechanisms by which these protein exert their activity is required. It is unclear if the changes we have detected are due to the changes in the levels of total SFKs, the level of activation of each specific SFK, and/or the ratio of one SFK to another. The antibody we used to measure the levels of phosphorylated SFKs reacts with a conserved site present in all SFKs, therefore reflecting the level of activation of the SFKs as a whole. It can be expected that the level of expression of specific SFK, or the ratio of one SFK to another might differ. This point is important because specific SFKs play specific roles in differentiation in neuroblastoma and in neurons; for example, it is known that expression of FYN and LYN is involved in differentiation and decreases with the increase of the tumor stage in neuroblastoma (Berwanger 2002; Bielke 1992). On the other hand, SRC is highly expressed in several types of cancer, including prostate (Vlaeminck-Guillem 2014) and colon cancers (Chen 2014; Varkaris 2014), and contributes to cancer development, maintenance, and metastasis. The differences in the levels of specific SFKs in PAG1kd cells remains unclear.
Our research indicates that PAG1 is required for differentiation in SH-SY5Y cells, and supports the role of PAG1 as a tumor suppressor and key regulator of RTK signaling (Agarwal 2016; Oneyama 2008).
Conclusions

We started this project with the hypothesis that the SFKs FYN and LYN distinguish between proliferation and differentiation inducing signals from different RTKs in neuroblastoma cells. Preliminary data suggested an important role of SFKs influencing the activation of the ERK and AKT pathways. We then determined that FYN and LYN change intracellular distribution differentially in response to activation of specific RTKs (Figure 9). Activation of ALK and KIT, which are involved in proliferation in neuroblastoma, increased the presence of FYN and LYN in late endosomes and lysosomes, whereas the activation of RET, which is involved in differentiation in neuroblastoma, decreased the presence of FYN and LYN in late endosomes and lysosomes. In addition, RTK activation changed the proportion of FYN and LYN in the detergent soluble fraction, and the lipid raft containing detergent resistant fraction of the plasma membrane. We also determined that PAG1 is a key signaling node in the SFK pathway in neuroblastoma cells. PAG1 ablation not only increased the levels of activated SFKs, as expected, but also differentially affected the activation of the ERK pathway in response to the activation of different RTKs. In addition, PAG1 ablation increased proliferation and decreased differentiation in neuroblastoma cells. These novel findings support the notion that PAG1 acts as a tumor suppressor, and provide us with new information about SFK regulation in neuroblastoma cells. In addition, we found that PAG1 tumor suppressor activity is strong in SH-SY5Y cells. PAG1 ablation increased proliferation, and decreased the ability of these cells to differentiate in response to RA and NGF, and decreased the expression of the neuronal marker of differentiation β III-Tubulin. In addition, PAG1 ablation differentially affected the activation of SFKs and ERK in response to the activation of RTK that induce proliferation, or differentiation. Future experiments will define the therapeutic potential of PAG1 in neuroblastoma patients, and perhaps other malignancies where the SFKs play a central role in the disease.
Methods
Cell culture conditions

LAN6 and SH-SY5Y cells were grown on polystyrene tissue culture plates until ~90% confluency in 2.2g/L NaHCO3 RPMI 1640 medium, supplemented with 10% Fetal bovine serum.

RTK ligand and drug concentrations

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Ligand</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP2</td>
<td>500nM</td>
<td>PTN</td>
<td>50nM</td>
</tr>
<tr>
<td>SU6656</td>
<td>500nM</td>
<td>EGF</td>
<td>5nM</td>
</tr>
<tr>
<td>9-cis-RA</td>
<td>10uM</td>
<td>SCF</td>
<td>5nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NGF</td>
<td>5nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GDNF</td>
<td>5nM</td>
</tr>
</tbody>
</table>

Whole cell extracts

In our preliminary data, whole cell extracts were prepared after RTK stimulation in suspension by centrifugation and resuspension in urea buffer. The samples were sonicated and heated for 10 minutes at 55°C prior to loading in polyacrylamide gels.

Westernblot

6-12% gradient or 8% polyacrylamide gels were prepared fresh prior to electrophoresis. Samples were run at constant voltage overnight at 16 mamps per gel, and transferred onto a nitrocellulose membrane in transfer buffer at constant voltage at 400 mamps for 4 hours.

Organelle fractionations

LAN6 cells were grown to ~90% confluency, and serum starved for 2h prior to cell harvesting. The cells were then consecutively washed with cold PEE and PGB buffers, and resuspended in cold PGB buffer containing the RTK ligand. The cells were then rotated for at 4°C for 1 hour, washed with PGB, resuspended in cold PGB and incubated for 60 minutes at 37°C. The cells
were then quenched in ice water, washed consecutively with PEE, and bud buffer, and resuspended in bud buffer. The cells were then mechanically permeabilized using a Balch homogenizer (cell cracker). The cracked cells were centrifuged, to separate the cell ghosts and plasma membranes from the organelles. The plasma membrane and cell ghosts were separated into detergent resistant membranes (DRM) and detergent soluble membranes (P1M) using a mild detergent (IGEPAL), and the organelles were layered on top of an iodixanol gradient (2.5-25%) and separated into the lysosomes (Lys), endosomes of high mass and density (E1), endosomes of intermediate mass and density (E2), endosomes of low mass and density (E3), and the cytosol (Cyt) using high speed centrifugation (100,000 x g, Figure 19). In addition, lysosomal and endosomal fractions were submitted to an equilibrium iodixanol gradient (2-30%), and centrifuged to equilibrium to separate membranes in the floating fraction (F), from loosely bound proteins in the non floating fraction (NF). Each organelle and cytosolic sample was purified using the methanol/chloroform method prior to resuspension in urea buffer, and the membrane samples were directly resuspended in urea buffer.

![Figure 19. Organelle fractionation procedure.](image)
Flow cytometry

SH-SY5Y cells were seeded at different cell densities so that the cells would not be crowded by
the time they were stimulated with RTK ligands. The cells were seeded and let attach to the
plates for 24-48h prior to treatment. The cells were then serum starved for 2h and washed with
PBS. The attached cells were exposed to their treatments for in serum free medium for 5 or 60
minutes, or in 2% FBS medium for the 4 day long exposures. The cells were then quickly
harvested with cold PEE buffer and fixed with PFA to a final concentration of 4% PFA in PBS.
The cells were treated with Benzonase in FACS buffer for 10 minutes at room temperature (in
order to reduce cell clumping) prior to permeabilization with ice cold methanol. The cells were
then incubated with Hoescht 33342 and or fluorescent antibodies for pERK, pAKT, pSFK
(pSRC), or β III-Tubulin. FMO controls were prepared from the control conditions within each
experiment. The data from our samples were obtained in a NxT Acoustic Focusing Cytometer,
Life technologies, Carlsbad, CA, using the Attune NxT Software, v2.4, and data were analyzed
Buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, pH 7.4</td>
</tr>
<tr>
<td>Transfer buffer</td>
<td>20mM TRIS, 150mM glycine, 20% methanol.</td>
</tr>
<tr>
<td>PEE</td>
<td>1mM EDTA, 1mM EGTA in PBS.</td>
</tr>
<tr>
<td>PGE</td>
<td>0.1% glucose, 0.1% bovine serum albumin in PBS.</td>
</tr>
<tr>
<td>FACS buffer</td>
<td>2% bovine serum albumin, 0.02% NaN₃ in PBS.</td>
</tr>
<tr>
<td>Bud buffer</td>
<td>38 mM each of the K⁺ salts of aspartic acid, glutamic acid and gluconic acid, 20 mM MOPS pH 7.1 at 37°C, 10mM potassium bicarbonate, 0.5mM magnesium carbonate, 1 mM EDTA, 1mM EGTA, 5mM GSH, 1mM Na₃VO₄, protease inhibitors 0.1 μg/mL aprotinin, chymostatin, leupeptin, and pepstatin A.</td>
</tr>
</tbody>
</table>

Antibodies

Westernblot:

pS473 AKT, CST 4058. pT202/Y204 ERK1/2, CST 9101. FYN, CST 4023. LYN, CST 2796.

Immunoprecipitation:


Flow cytometry.

pAKT-PE, BD 558275. pERK-AF488, CST 4344. pSRC-PerCP-eFluor710, Ebioscience 46-9034-42. β III-Tubulin-AF647, Biolegend 657406.
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