REGULATION OF IGF-1 RECEPTOR SIGNALING BY AUTOPHAGY IN HUMAN NEUROBLASTOMA CELL LINES

by

Mehrnoosh Soori

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

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To my Mother, Father, and to my Motherland Iran

The Lights of my Dark Nights and the Warmth of my Cold Days

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LIST OF ABBREVIATIONS

- Atg autophagy-related genes
- ALK anaplastic lymphoma kinase
- BCA bicinichoninic
- BSA bovine serum albumine
- EGF epidermal growth factor
- FBS fetal bovine serum
- FYAD Fmoc-Tyr-Ala-CHN(2)
- Grb-2 growth factor bound receptor protein 2
- IGF-1 insulin like growth factor 1
- IGF-1R insulin like growth factor 1 receptor
- IRS insulin receptor binding protein
- LAMP-1 lysosome associated membrane protein 1
- MAPK mitogen activated protein kinase
- PKB protein kinase B
- PTB phosphotyrosine binding domain
- SIM super resolution illumination microscopy
- SH2 Src homology 2 domain
- Tris hydroxymethyl aminomethane
- TrkB tropomyosin receptor-kinase B

ABSTRACT

This study was designed to examine the mechanism by which inhibition of lysosomal proteases causes cell death in neuroblastoma. The major lysosomal proteases are two cysteine proteases, cathepsins B and L, and an aspartic protease, cathepsin D. Inhibition of these three proteases was found to cause cellular accumulation of fragments of the IGF-1 receptor. The fragments were located in dense organelles that were characterized as autophagosomes. This novel discovery provides the first clear link between lysosIGF-1omal function, autophagy and IGF-1 mediated cell proliferation. It provides a mechanistic explanation for enhanced cytotoxicity of chemotherpautic agents when combined with inhibitors of lysosomal function and autophagy. A more in depth analysis of the IGF1 signaling pathway revealed that the MAPK pathway was particularly impaired in inhibitor treated cells, while the PKB cell survival pathway remained functional. It was discovered that Shc, an adapter protein that transmits IGF-1 signaling towards the MAPK pathway, was also sequestered in autophagosomes while IRS2, an adapter protein that transmits IGF-1 signaling towards the PKB pathway, was unaffected by cathepsin inhibition. Furthermore, Shc was sequestered in autophagasomes as its active form, indicating that autophagy is a key mechanism for down-regulating IGF-1 induced cell proliferation. Proliferating cells of neuronal origin are particularly sensitive to cathepsin inhibition. This enzyme inhibition had a greater effect on autophagic

sequestration of the neuronal specific adapter protein, Shc-C, than ubiquitously expressed Shc-A, providing mechanistic support for the enhanced sensitivity of neuronally derived tumor cells. The Shc adapter proteins are central to transducing proliferation signaling by a range of receptor tyrosine kinases and consequently cathepsin inhibition may become an important therapeutic approach to treating neuroblastoma and other tumors of neuronal origin.

Chapter 1

INTRODUCTION

The ultimate goal of this project is to develop novel treatment approaches for neuroblastoma that have fewer long-term side effects on children. The Mason laboratory has designed novel inhibitors of lysosomal proteases that induce selective apoptosis of neuroblastoma cell lines. In this thesis, I am taking a mechanistic approach to understand the process by which these inhibitors cause cell death. I hypothesize that inhibition of lysosomal proteases induces accumulation of autophagic vesicles that sequester activated IGF-1 receptor and the activated adaptor protein Shc, and thereby prevent activation of downstream signaling cascades of IGF-1 and its pro-survival effects on neuroblastoma cells. In this introduction I discuss the biology of neuroblastoma and importance of developing novel therapies to treat it, the role of cathepsins in development of the nervous system and neuronal cells, the biology of autophagy and its role in neuronal and cancer cells, and lastly the role of IGF-1 signaling in cancer, especially neuroblastoma.

1.1 Biology of Neuroblastoma

Neuroblastoma is the most common extra-cranial solid tumor of children, and the most common cancer of infancy. It accounts for 1 out of 8 cancer related deaths of

children. The median diagnosis age of neuroblastoma is 17 months and its incidence is 10.2 per million children under 15 years of age.

Neuroblastoma shows diverse clinical behaviors. While the mortality and morbidity rate for high risk neuroblastoma remains high, in some cases the disease displays a strikingly unique feature where it can spontaneously regress, even with the presence of multiple metastasis (stage 4S). Based on age at diagnosis, histological features, chromosomal abnormalities and amplification of the MYCN oncogene, a staging system has been developed for neuroblastoma patients, where the patients are assigned high, low and intermediate assessment risks (table1) [reviewed in Maris 2010, Volchenbum et al 2009, van Noesel et al. 2004].

1.1.1 Developmental origins of neuroblastoma

As a cancer of infancy, neuroblastoma is considered to be an embryonal tumor. The originating cells of neuroblastoma are incompletely committed, developing cells from the neural crest that normally give rise to the sympathetic nervous system, adrenal medulla and paraspinal ganglia. The fetal adrenal medulla consists of a mixture of neuroendocrine chromaffin cells and clusters of mature interconnecting ganglion cells. After birth the number of chromaffin cells strongly increases, in concert with a gradual loss of ganglion cells. The opposite trend is observed in the sympathetic nervous system, where the chromaffin cells rapidly disappear and the neuronal cells become the predominant cell type. Neuroblastoma tumors can contain both cell types, indicating that they originate either from both cell types or from the

pluripotent precursor cells that give rise to both cell types. Neuroblastoma tumors are usually located in the neck, chest, abdomen, and pelvis [reviewed in van Noesel et al. 2004, Gestblom et al. 1999, Cooper et al 1990]

Table 1.1 The International Neuroblastoma Staging System

Stage/Prognostic Group	Description
Stage 1	Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (i.e., nodes attached to and removed with the primary tumor may be positive).
Stage 2A	Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically.
Stage 2B	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically
Stage 3	Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement. The midline is defined as the vertebral column. Tumors originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column.
Stage 4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/or other organs, except as defined for stage 4S.
Stage 4S	Localized primary tumor, as defined for stage 1, 2A, or 2B, with dissemination limited to skin, liver, and/or bone marrow (by definition limited to infants younger than 12 months).[Taggart et al. 2011] Marrow involvement should be minimal (i.e., <10% of total nucleated cells identified as malignant by bone biopsy or by bone marrow aspirate). More extensive bone marrow involvement would be considered stage 4 disease. The results of the mIBG scan, if performed, should be negative for disease in the bone marrow.

mIBG = metaiodobenzylguanidine, http://www.cancer.gov/cancertopics/pdq/treatment/neuroblastoma/HealthProfessional/Table3

Neuroblastoma is almost nonexistent after the age of 10, and artificial induction of N-MYC into murine models can induce neuroblastoma only in the first month of life, suggesting that neuroblastoma arises only during development. However, except for a few specific subsets of patients, genetic alterations in developmental regulator genes have been not been shown in neuroblastoma, and common genetic alterations associated with advanced stages of the disease (resulting in loss of apoptotic potential and overexpression of oncogenes) are more consistent with mechanisms in other cancers of adults [van Noesel 2004].

Stage 4S is a unique form of neuroblastoma where the disease has an automatic regression rate of 70-90%, even when distant metastasis are present. The metastatic pattern of this stage (skin, liver, little involvement of bone marrow), and absence of genetic alteration (no N-MYC amplification, ALK mutations, or segmental chromosomal abnormalities) is consistent with normal (but delayed) developmental stages of neural crest, rather than classically invasive and metastatic tumors. Thus, stage 4S neuroblastoma may be considered a consequence of dysregulated development that spontaneously regresses [van Noesel 2012].

1.1.2 Current therapies for high risk neuroblastoma and significance of developing novel therapies

Approximately 40% of neuroblastoma patients fall into the high risk group at diagnosis. In spite of progress in treating other childhood cancers such as lymphoma and leukemia, the survival rate for high risk neuroblastoma patients remains less than

40% [Volchenbum et al. 2009, Maris 2010, van. Noesel et al. 2004]. These patients are treated with a combination of high dose chemotherapy, radiation and surgery. Currently used chemotherapeutic agents such as mitosis inhibitors, alkylating agents, topoisomerase inhibitors, DNA cross linkers and intercalating agents that target all proliferating cells, have high toxicity to normal proliferating cells in a child's body [Wagner et al. 2009]. Patients who respond to cytotoxic chemotherapy and achieve long-term event-free survival suffer from severe side effects of this cytotoxic chemotherapy, such as delay in growth, learning problems, renal and cardiac dysfunction, hearing loss, infertility and second malignancies [Volchenbum et al. 2009, Wagner et al. 2009]. These severe side effects, which can last through adulthood of surviving patients, make it necessary to develop more specific therapies that selectively target proliferating, malignant neuroblastoma cells with fewer side effects for patients.

1.2 Biology of Cathepsins

Due to the origin of neuroblastoma from the developing nervous system, proteins and pathways that have specific roles in development of the nervous system may provide a better insight into mechanisms of development of the cancer and help identify therapies that can be targeted specifically against malignant neuroblastic tissue.

Studies on mice knocked out for lysosomal enzymes cathepsins B and L have provided valuable information on this matter. These two enzymes are members of the

cathepsin family; a group cysteine proteases that belong to the papain super family. They are predominantly endopeptidases localized intracellularly within endolysosomal vesicles. There are 11 different human cathepsins; cathepsins B and L are most studied. Generally, the enzymes are considered to be ubiquitously expressed and are responsible for constitutive protein turnover. However, more recent studies have identified specific roles for some of these enzymes. Notably cathepsin K in bone and cathepsin S in leucocytes, have more specific expression and function, but all have been proposed to play pathological roles, especially in cancer cell invasion and metastasis [Mohamed et al. 2006].

1.2.1 Role of cathepsins in normal development of murine nervous system

Mice deficient in cathepsin L show phenotypes in skin and cardiac muscles [Roth et al. 2000, Stypmann et al. 2002], while mice deficient in cathepsin B expression show reduced TNF (tumor necrosis factor) induced apoptosis [Reinhekel et al. 2001]. However, mice deficient in both enzymes suffer from severe brain atrophy induced by massive apoptosis of cerebral and cerebellar neurons, and die 2-4 weeks after birth. The deleterious effects start shortly after birth but are not observed prior to birth [Felbor et al. 2002]. These observations indicate that together, cathepsins B and L play a critical role in development of the nervous system.

1.2.2 Effect of inhibition of cathepsins on cell lines and murine models

An inhibitor of cathepsins B and L disrupts placenta function and causes apoptosis of embryonic neuroepithelial and neural crest cells, but not other embryonic cells and tissues [Ambroso et al. 1994]. The rodent placenta expresses a unique family of cathepsins that are structurally related to cathepsin L [Mason et al. 2004]. These may compensate for loss of cathepsins B and L in the knockout mice, delaying neurotoxicity until after birth. The placental cathepsins may also be sensitive to small molecule inhibitors, leading to embryonic neurotoxicity. Inhibitors of cathepsins B and L do not have any effect on mature rodents [Desmarais et al. 2008], and actually protect mature brain from ischemia [Anagli et al. 2007], indicating that mature neuronal cells are not sensitive to cathepsin inhibition. This led to a hypothesis that inhibition of cathepsins B and L could selectively target neuroblastoma while sparing other proliferating cells and the developed nervous system. The neonatal stage of development of neuronal tissue in mice affected by cathepsin inhibition corresponds to the third trimester fetal stage in humans, therefore cathepsin inhibition is less likely to affect normal neuronal cells in children and adults, but abnormally proliferating neuroblastoma cells may be eradicated in response to cathepsin inhibition. Previous studies with less specific inhibitors of cathepsins B and L and other enzymes or lysosomal inhibitors such as chloroquine induce apoptosis or inhibit growth of a range of cancer cell types including neuroblastoma [Geng et al. 2010, Sasaki et al. 2010, Zhu et al. 2000]. These reagents have less specific targets and broader effects on cells and therefore are less suitable for determining the mechanism that leads to selective death

of neuroblastoma cells. We overcome this problem by using novel specific inhibitors of cathepsins B and L.

1.2.3 Effect of inhibition of cathepsins B and L by FYAD on neuroblastoma cells

Fmoc-Tyr-Ala-CHN2 (FYAD) is a membrane permeable inhibitor for cathepsins B and L [Crawford et al. 1988, Wilcox et al. 1992] with similar affinity and specificity for both enzymes [Xing et al. 1998]. FYAD treatment causes selective death of neuroblastoma cell lines [Colella et al. 2010]. It causes enzyme inactivation by irreversibly binding to the active site of the enzymes and preventing their reactivation upon inhibitor withdrawal. In cell systems, complete inhibition of the enzyme can be achieved within 2-3 hours of incubation in the presence of 10µM inhibitor; however, an apoptotic response is observed only after 48-72 hours, suggesting that apoptosis is an indirect outcome of enzyme inhibition. By reducing inhibitor concentration and measuring activity of residual cathepsin activity it was shown at least 90% enzyme inhibition is required to induce cell death. Electron microscopic examination of treated cells showed time dependent accumulation of multilayered vesicles, indicative of accumulation of autophagic vesicles within treated cells. These vesicles appeared prior to observation of an apoptotic response, suggesting autophagy may be part of the mechanism that induces death of neuroblastoma cells. [Colella et al.2010].

In summary, cathepsins have an important role in development of the nervous system. Gene knockout studies and specific enzyme inhibition show that only rapidly dividing neuronal cells are affected by inhibition of cathepsins B and L, indicating that inhibition of these enzymes should not have any side effects on other normally growing tissues or the developed nervous system after birth but may result in selective death of neuroblastoma cells. Understanding the molecular mechanism that leads to apoptotic effects of cathepsin inhibition may provide a better insight to improve development of protease inhibition as a novel therapy for neuroblastoma.

1.3 Autophagy

Autophagy is a process of self digestion which allows cells to recycle their obsolete and damaged proteins and subcellular organelles. It initiates with nucleation followed by expansion and closure of a two layered membrane around a fraction of cytoplasm, resulting in formation of a two layered autophagosome. The process of sequestration can happen through selective or bulk non-selective mechanisms. The autophagosome then fuses with a late endosome or lysosome, resulting in digestion of the inner membrane and then the sequestered content (Figure 1.1). While the large scale capacity of autophagy is critically important for its biological functions, excessive degradation of cytoplasmic proteins and organelles is likely to be lethal to the cell, thus induction of autophagy beyond basal levels needs to be tightly regulated [reviewed in Mathew et al 2011].

The molecular mechanism underlying autophagy has been mainly elucidated by analysis of mutant autophagy related genes (Atg) in yeast [reviewed in Simonsen et al. 2009]. Further study of homologs of these genes in deficient mammalian cell lines and mouse models have helped identify similar mechanisms of autophagy in higher organisms, although additional studies are needed.

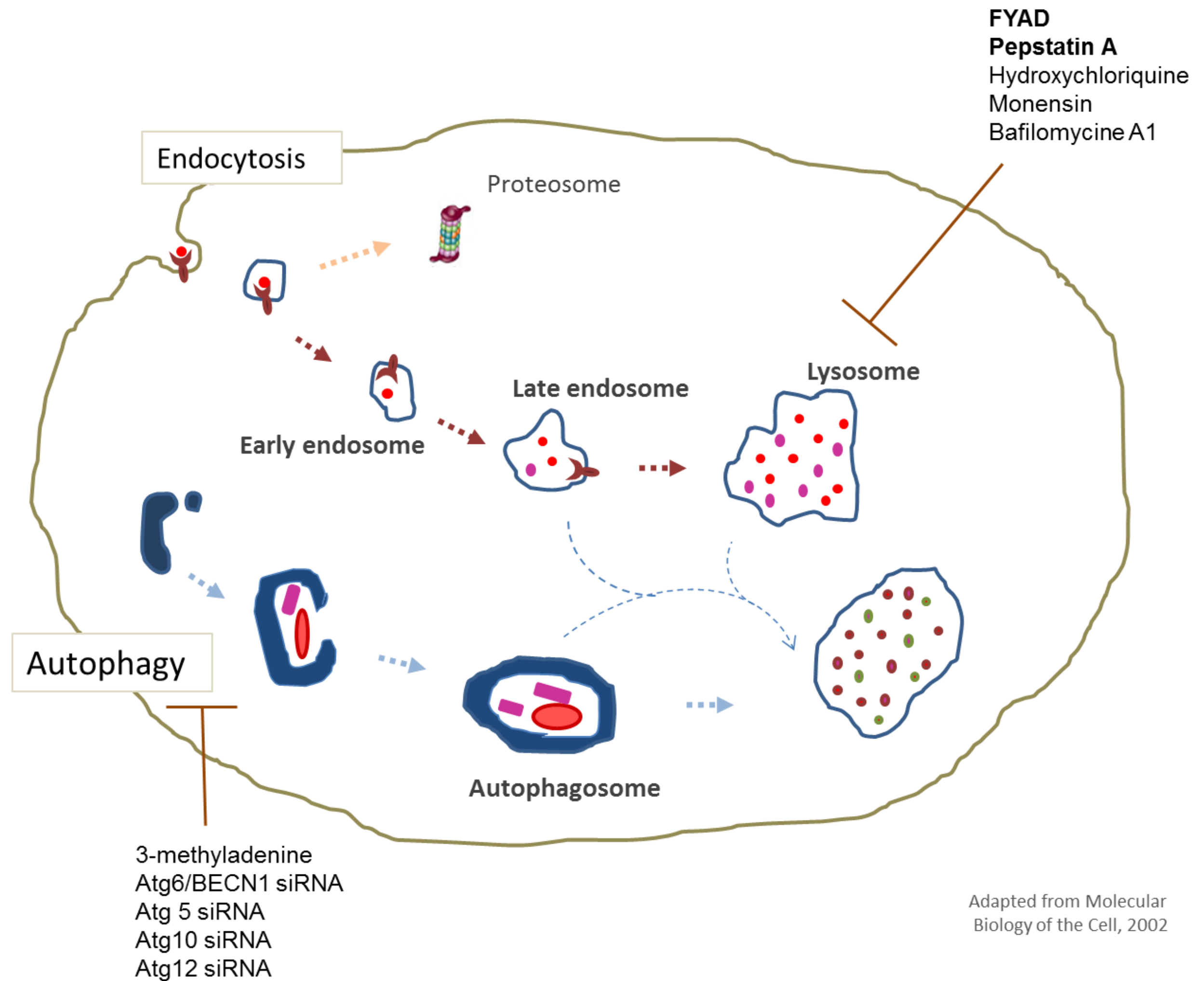


Figure 1.1 Stepwise stages of autophagy.
The first step of macro-autophagy consists of the step-wise envelopment of cytoplasmic material (cytosol and/or organelles) in the isolating membrane, which finally sequesters cytoplasmic material in autophagosomes (also called autophagic vacuoles) lined by two membranes. This two-membrane structure is a definitive characteristic of autophagic vacuoles. Autophagosomes then undergo maturation by fusion with endosomes (or multi-lamellar bodies) and/or lysosomes. The latter step creates autolysosomes (also called autophagolysosomes) in which the inner membrane and the luminal content of the autophagic vacuoles are degraded by lysosomal enzymes.

1.3.1 Physiological roles of autophagy

Autophagy is generally known as a pro-survival pathway, especially under starvation and stress inducing circumstances. Degradation of damaged proteins and organelles of the cell regulates the size of the cell, and inhibits its inappropriate growth and proliferation in the absence of appropriate trophic signals. Recycled organelles and proteins provide biosynthetic and bioenergetic resources for cell survival, while their sequestration and degradation prevent cytotoxic effects caused by their accumulation and malfunction in the cytosol [reviewed in Mathew and White, 2011; Degenhardt et al. 2006, Lum et al. 2005].

1.3.2 Role of autophagy in cancer

The role of autophagy in cancer is context dependent. While continuous autophagy helps prevent formation of cancer cells and prevent initial stages of carcinogenesis, it helps survival of cancer cells in the hostile microenvironment of established tumor, thereby facilitating cancer progression [reviewed in Su et al. 2013, Shen et al.2012, White 2012].

1.3.2.1 Role of autophagy in cancer initiation

There is a significant amount of data to indicate that autophagy is important for preventing formation of malignant cells and cancer initiation. A role of autophagy in cancer was first identified by monoallelic deletion of the Atg gene beclin 1 in human tumor specimens, [Eccles et al. 1992]. Several Atg genes, including LC-3 (a

mammalian homolog of Atg8) and beclin 1 (mammalian homolog of Atg6), are down-regulated or have inactivating point mutations in many human cancers, establishing a direct role of reduced autophagy in cancer initiation [reviewed in Wu et al, 2012].

Consistent with reports from clinical tumor specimens, studies in mice with heterozygous deletion of beclin 1 show a decreased level of autophagy, and a higher rate of spontaneous lymphoma and lung tumors [Yue et al. 2003, Qu et al. 2003, Liang et al. 1999]. Mosaic deletion of Atg5 and liver specific deletion of Atg7 in mice results in a high incidence of liver adenoma, indicating that autophagy suppresses initial stages of tumor development [Takamura et al. 2011].

Continuous autophagy prevents cancer cell formation by maintaining genomic stability [reviewed in Vessoni et al 2013]. Cells deficient in Atg genes show a higher rate of mitochondrial DNA mutation, possibly due to decreased turnover of damaged mitochondria and increased oxidative and genotoxic stress. The chromosomal abnormalities that are also higher in Atg5 and beclin 1 deficient cells could be caused by this elevated oxidative stress [Kongara et al. 2012, Mathew et al. 2007]. In the absence of proper trophic signals, autophagy prevents inappropriate nuclear division, and growth and proliferation of cells, thereby preventing expansion of cells with aneuploidy or genetic abnormalities. If autophagy is reduced, this could lead to tumor cell formation and proliferation [Matsui et al. 2013, Rosenfeldt et al. 2013].

1.3.2.2 Role of autophagy in cancer progression

Contrary to its suppressive role in cancer initiation, autophagy helps survival of tumor cells in the microenvironment of established tumors. In established tumors, cancer cells have to survive in a microenvironment with poor vascularization, nutrient deprivation, high inflammation and hypoxia. In such microenvironments, autophagy provides a mechanism to recycle damaged cellular compartments and protein aggregates and reuse them as bioenergetic resources. In addition, autophagy prevents excessive cell growth and proliferation and regulates cell size, thereby helping the cancer cells to adjust their demand for bioenegertic resources in hostile tumor microenvironment [Reviewed in Rabinowitz et al. 2010, Simonsen et al.2009].

Increased autophagy and shrinkage of cancer cell size is also suggested to be part of the mechanism that leads to cancer cell dormancy. The dormant cells are smaller, non-proliferating, and have a slower metabolic rate. These cells are able to survive hostile microenvironments for long periods of time and when conditions improve cells can re-enter the cell cycle and continue to proliferate, causing cancer relapse [Chaterjee et al. 2011, Lu et al, 2008, Amaravadi 2008, Degenhardt et al. 2006].

Cytotoxicity caused by chemotherapeutic agents and radiation also results in cellular stress and accumulation of damaged proteins. Consequently, if autophagy can be reduced in cancer cells it may accelerate death of these damaged cells, and prevent their dormancy [reviewed in Sosa et al. 2013].

Immunohistochemical analysis of several tumor specimens consistently show increased levels of the autophagy markers LC-3 and GABARAP (another mammalian homolog of Atg8) in the center of tumors, compared to normal tissue. The expression of these proteins correlated with tumor progression and poor patient prognosis, supporting a positive role of autophagy for survival of established tumors [Miao et al. 2010, Fujii et al. 2008]

While inhibition of autophagy could result in initiation of tumor formation, reduced autophagy may make tumors more sensitive to chemotherapy or reduced nutrition. Preclinical and clinical studies are currently in progress to further clarify *in vivo* effects of autophagy inhibition on tumor growth and eradication. These studies will need to consider the effect of reduced autophagy in increasing genetic instability that may lead to deleterious mutations that result in chemo-resistance and abnormal growth.

1.3.2.3 Regulation of autophagy

Nutrient and growth factor availability are important regulators of autophagy. Mammalian target of rapamycin (mTOR), is one of the important negative regulators of autophagy [Hosokawa et al. 2009]. It gets phosphorylated and activated in response to nutrient availability. Upon activation, mTOR binds to and inhibits activation of protein complexes that nucleate phagophores, thus preventing autophagosome formation. In the absence of nutrients, mTOR is inactive and autophagy induced. Autophagy may also be regulated by mTOR independent pathways, some of which are

regulated by amino acid catabolism and the level of nitrogen resources and byproducts in the cell. [reviewd in Jung et al. 2010, and Rabinowitz et al. 2010, Cheong et al. 2011, Eng et al. 2010].

Autophagy and apoptosis crosstalk at several points, and are closely regulated [reviewed in Su et al. 2013]. Important regulators of autophagy are the anti-apoptotic Bcl-2 family proteins. Bcl-2 proteins bind to beclin 1 and maintain formation of autophagic vesicles at the level of basal autophagy that is necessary for normal cellular homeostasis [Pattigre et al. 2005 and 2006].

1.3.2.4 Targeting autophagy to treat cancer

Most cancer therapies target rapidly proliferating cancer cells. Cancer cells that reside within established solid tumors that exist in a hostile microenvironment with poor nutrient supply will proliferate more slowly and can use autophagy as a survival mechanism. Cellular stress in response to cancer therapeutic agents can also induce autophagy. It was originally proposed that autophagic cell death was part of the mechanism of action of these drugs, but more recent evidence suggests that autophagy is part of the response of the cells to overcome the cytotoxic stress caused by drug treatment, rather than a cause of the cell death [reviewed in, Shen et al. 2012]. This happens both in the case of chemotherapeutic agents that have cytotoxic effects, and targeted therapies such as inhibitors of mTOR, PI3K-PKB, Bcl-2 and pathways that crosstalk with autophagy [reviewed in Cheong et al. 2012]. Combination therapies of chemotherapeutic agents with inhibitors of autophagy may therefore exhibit

synergistic effects to enhance efficiency of drugs, and also preventing tumor cell dormancy and subsequent relapse. Among inhibitors of autophagy, chloroquine and hydroxychloroquine are being evaluated in clinical trials. These drugs are used as antimalarial agents that function by increasing pH of lysosomes and autophagolysosomes, thereby inhibiting digestion of their content. Pre-clinical studies using a combination of hydroxychloroquine and chemotherapy showed an increase in cell death and decrease in the rate of relapse after chemotherapy [Rosenfeld et al. 2014, Rangawal et al. 2014, Mahalingam et al. 2014, Ji et al. 2014, Liu et al. 2014, Bokobza 2014, Selvakumaran et al. 2013, Donohue et al. 2013, Shen et al. 2013, Wu et al. 2013, Chang et al. 2013, Seitz, et al. 2013, McAfee et al. 2012, Carew et al. 2011 and 2012, Amaravadi et al. 2007 and 2009, Maclean et al. 2008]. Chloroquine and hydroxychloroquine are in clinical trials to treat a range of adult cancers (see clinicaltrials.gov).

1.3.3 Role of autophagy in neuronal cells

While blocking of autophagy is showing promise in cancer treatment, there may be concerns of the effect of blocking this process in neuronal function. Demand for turnover and recycling of damaged proteins and organelles by autophagy may be more critical in cells such as mature neuronal cells that do not divide after differentiation, and do not have the capacity for self renewal. Although the level of basal autophagy and number of detectable autophagosomes are low in neuronal cells [Nixon et al. 2005], neuronal specific knock out of the autophagy related genes

beclin1 and Atg5 causes progressive motor neuron deficit and abnormal reflexes in mice, accompanied by accumulation of ubiquitin-positive inclusion bodies in their neurons [Hara et al. 2006, Komatsu et al. 2006].

The importance of autophagy to neuronal cells is even more evident in neuronal disease conditions. Accumulation of diffuse [Hara et al. 2006] or aggregated [Bjorkoy et al. 2005, Iwata et al. 2005] cytosolic forms of mutant, misfolded proteins are detected in many neurodegenerative diseases such as Huntington, spinalcerebellar ataxia and familial Parkinson, and it is proposed that some of this accumulation could be due to reduced autophagy [Rubinsztein et al. 2005 and 2006]. In Alzhiemers disease, autophagosome-like structures accumulate in dystrophic neurites of affected cells, possibly due to impairment of autophagosome to autolysosome maturation [Yu et al. 2005]. The immunosuppressant Rapamycin blocks the function of mTOR and results in enhanced autophagy. This drug has shown promising results in mouse and *Drosophila* models of neurodegenerative diseases [Sarkar et al. 2007, Ravikumar et al. 2004].

Overall, it appears that enhanced autophagy may improve neuronal cell survival whereas blocking of autophagy may lead to neuronal cell death. Clearly potential neurotoxicity of any cancer treatment that targets autophagy should be carefully considered. Despite these concerns, many clinical trials that target autophagy to treat a variety of cancers are underway. It may be that neuronal cells are primarily sensitive to inhibition of autophagy during pre-natal development and later in life, and that if tumors of the nervous system are especially sensitive to inhibition of

autophagy that treatment benefits may outweigh potential toxic side effects on neuronal function.

1.4 Role of IGF-1 Receptor Signaling in Cancer

The major discoveries in this study relate to the role of the lysosomal proteases in regulation of the IGF-1 signaling system. This section introduces the key elements of this system and its known relationship to cancer progression.

1.4.1 IGF-1 receptor structure

The IGF-1 receptor is a type 2 tyrosine kinase receptor. The human receptor consists of a 4101 nucleotide ORF, which encodes a 1367 amino acid protein. An N-terminal 30 amino acid signal peptide is cleaved co-translationally as it enters the endoplasmic reticulum. The protein is then glycosylated, folded and dimerized by disulfide bond formation in the endoplasmic reticulum before transport to the Golgi apparatus where it is cleaved to produce the mature tetrameric protein (Figure 1.2).

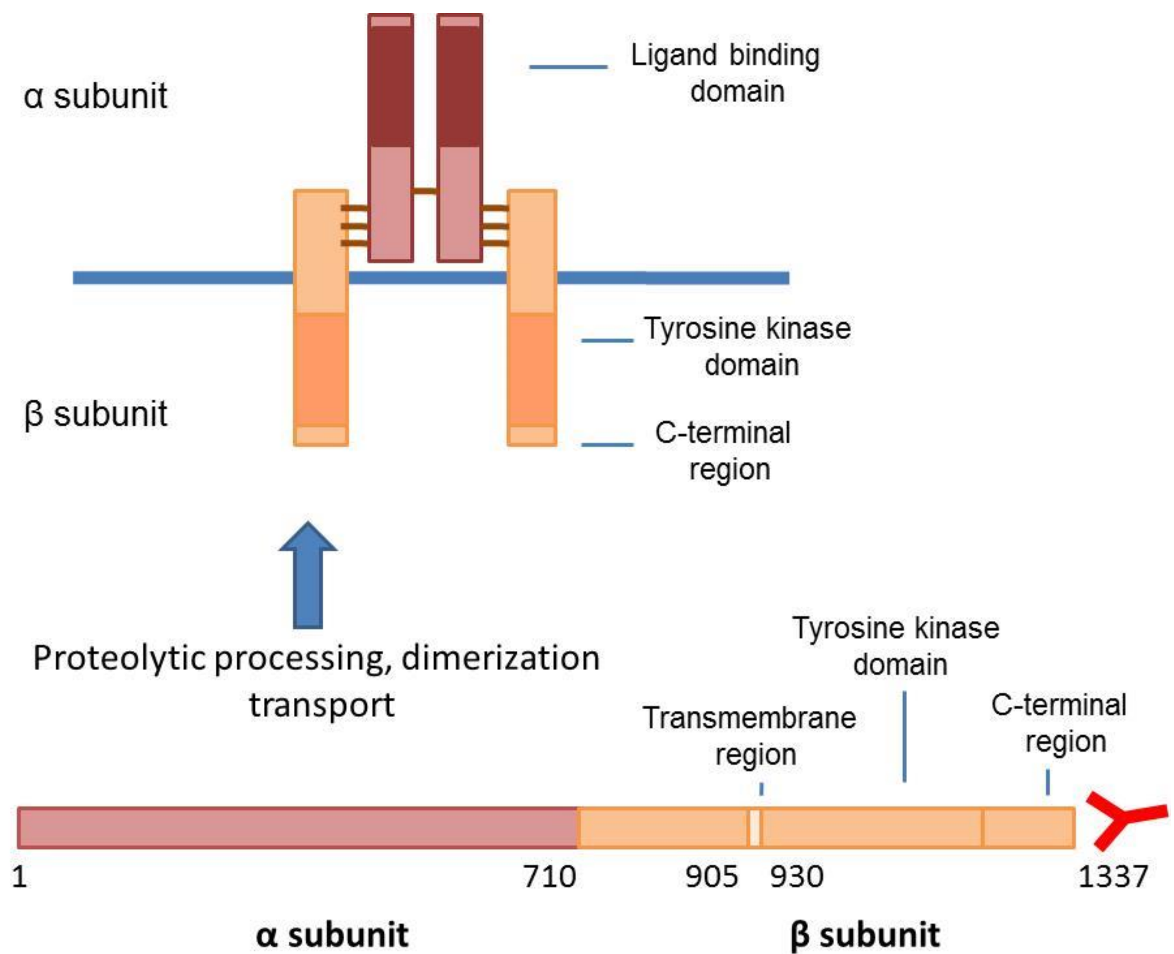


Figure 1.2 Structure of IGF-1 receptor.
IGF-1 receptor is synthesized from a precursor which is glycosylated, cleaved, and dimerized to produce heterodimeric receptor. The fully processed receptor consists of two extracellular, ligand binding subunits, as well two transmembrane beta subunits with tyrosine kinase activity KDa. An antibody against the C-terminal end of the beta subunit is used in this study for immunoblotting and immunefluorescent staining.

The mature IGF-1R consists of two 130-135 KDa extracellular alpha subunits and two 90-95 KDa transmembrane beta subunit. The variation in size is due to differential glycosylation. The subunits are covalently bound together by extracellular alpha-alpha and alpha-beta disulfide bonds. The alpha subunit is extracellular and creates a multi-contact binding site for IGF-1 ligand. The transmembrane beta subunits are responsible for transducing signals into the cell. The intracellular portion of the beta subunit consists of a central tyrosine kinase domain, flanked by juxta-membrane and C-terminal regions. The juxtamembrane region is involved in receptor internalization and docking of the adapter proteins IRS (1-4) and Shc proteins at tyrosine 950, which is in a NPEY motif [Dey et al. 1996, Jiang et al. 1996, Craparo et al. 1995, Hernandez-sanchez et al. 1995]. These adapter proteins also bind to other regions of the IGF-1R, but binding is primarily regulated by phosphorylation of tyrosine 950 [Brodt. et al. 2001, Leahy et al. 2004, Tartare-Deckert et al. 1995]. The kinase domain contains an ATP binding site at lysine 1003 and an activation loop that includes three critical tyrosine residues at 1131, 1135, 1136. In the basal, inactive state of the receptor, the activation loop blocks the active site, keeping it in an inaccessible closed position. Ligand binding to the extracellular domain of the receptor induces a conformational change in the loop that makes the active site accessible to ATP. Sequential phosphorylation of the critical tyrosine residues stabilizes the activation loop and fixes it in an open position to allow access to other protein substrates. Adapter protein substrates are recruited to the autophosphorylated NPEY motif in the juxtamembrane region and subsequently phosphorylated at multiple tyrosine residues by the activated

receptor. The C-terminal region of IGF-1R also includes two tyrosine residues at 1250-1 that can bind to a range of protein substrates. The role of these proteins in cell survival/proliferation is not yet clear and their biological relevance may be context specific [Pollak et al. 2008, , De Meyts et al. 2002, Favelyuki et al. 2001, Pautsch et al. 2001, Jansson et al. 1997].

1.4.2 IGF-1 receptor signaling pathways

Two main signaling cascades are activated by IGF-1 binding to its receptor (Figure 1.3.). In one cascade Shc docking proteins are recruited to the activated receptor where they are phosphorylated and subsequently recruit and activate Grb-2, and Ras, leading to activation of the MAPK cascade and downstream nuclear factors that ultimately induce cell proliferation [Pollak et al. 2008, Grey et al. 2003, Hermanto et al. 2000, Myers et al. 1994, Giorgetti et al. 1993]. The second signaling cascade is activated by recruitment of IRS to the receptor that recruits PI3K and leads to phosphorylation of the p85 subunit to activate the downstream PKB pathway, which in turn triggers an anti-apoptotic effect, which is especially important in non-proliferative differentiated cells.

1.4.3 Role of IGF-1 signaling in the nervous system

IGF-1 signaling has a well established role in normal growth and development, and in function of the nervous system. Several *in vitro* and *in vivo* studies have shown expression of IGF system components in neuronal stem cells, neuronal progenitors,

and neurons, as well as various regions of the nervous system, starting at early stages of development and continuing throughout adulthood. IGF-1 and its receptor are expressed in a tempo-spatial manner, and their biological effects depend on the

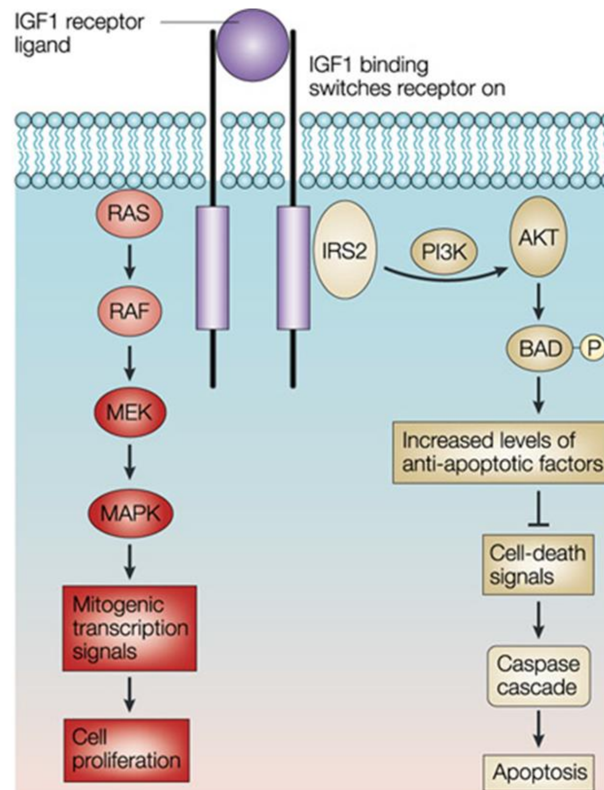


Figure 1.3 IGF-1 receptor signaling pathways.
Two main signaling cascades are activated by IGF-1 binding to its receptor. In one cascade Shc docking proteins are recruited to the activated receptor (not shown) that through Grb2 (not shown) then activates RAS and the MAPK cascade and downstream nuclear factors that ultimately induce cell proliferation. The second signaling cascade is activated by recruitment of IRS to the receptor that leads activation of the downstream PKB pathway, which triggers an anti-apoptotic effect (Bok et al. 2002, used with permission).

specific cell types, local microenvironment, and stage of development.

In rodents IGF-1 expression is detectable in all brain regions as early as embryonic day 11. It peaks at the second week after birth, and then gradually declines throughout adulthood. Expression of IGF-1 and its receptor correlates with local growth and proliferation rates of neural precursors and progenitors and high levels of it protect cells from apoptosis. The positive regulatory role of IGF-1 signaling on proliferation of neuronal stem cells and progenitors is confirmed by several *in vitro* studies, *in vivo* studies with transgenic mice, and clinical data from patients with mutations in IGF-1 or its receptor. *In vitro* studies on isolated neural progenitors show IGF-1 signaling induces proliferation and protection against apoptosis. Transgenic mice defective for IGF-1 or its receptor show dramatic decrease in brain size and neuron number, whereas over expression of either of these proteins has the opposite effect. Patients with mutations in IGF-1 or its receptor suffer from severe growth failure, mental retardation and hydrocephaly [reviewed in O'Kusky et al. 2012, D'Ercole et al. 2008].

In the adult nervous system, IGF-1 and its receptor are expressed at lower levels. The majority of neurogenesis and neural development occurs prior to birth, although lower levels of active neurogenesis continues in two areas of brain, the hippocompal dentate gyrus and the subventricular zone throughout adulthood. Expression of IGF-1 and its receptor has been detected in these areas of active cell proliferation, and peripheral infusion of IGF-1 to adult rats induces growth and proliferation of progenitors in these areas of the brain. Production of IGF-1 is

markedly increased in response to brain injury, and IGF-1 mediates exercise induced neurogenesis in adult brain [reviewed in Ma et al. 2010, Gould et al. 2007].

In addition to promoting survival and proliferation of neural progenitors in both the developing and adult brain, IGF-1 signaling also has other context specific roles in differentiated neurons. Several *in vivo* and *in vitro* studies have shown that IGF-1 signaling regulates neuronal lineage differentiation, mylination, synaptogenesis, axonal remodeling and neuronal excitation [reviewed in O'Kusky et al. 2012].

1.4.4 Role of IGF-1 signaling in cancer

IGF-1R expression is elevated in several human cancers [Sekyi-otu et al. 1995, Freund et al. 1994, Chen 1991]. Mechanistic studies show that IGF-1 activates pro-survival pathways in cancer cells, increasing their resistance to chemotherapy and apoptosis [Reviewed in Kurmasheva et al. 2006, Kai et al. 2009, Dallas et al. 2009, Grothey et al. 1999, Dunn et al. 1997], acts as a mitogen for several cancers [Ankrapp et al. 1993, Myal et al. 1984] and increases transforming activity of oncogenes [Sell et al. 1993].

Components of IGF signaling pathways are elevated in tissues from a variety of cancers, including lung [Minuto et al. 1986], prostate [Liao et al. 2005], breast [Yee et al. 1989, Paik et al. 1992], ovary [Yee et al. 1991], liver [D'Errico et al. 1994, Lamas et al. 1991, Su et al. 1989, Cariani et al. 1988], and carcinomas of gastrointestinal tract such as colorectal [Freier et al. 1999, Tricoli et al. 1986], gastric [Furukawa et al. 2005, Chung et al. 1992] and pancreatic cancers [Bergmann et al.

1995]. Activation of the IGF system has also been observed in cancers of the nervous system such as glioma [Sandberg-Nordqvist et al. 1993, Sandberg et al. 1988], and meningioma [Wrobel et al, 2005, Hultberg et al. 1993, Antoniades et al. 1992].

Epidemiologic evidence from prospective studies support a role of IGF signaling in cancer development and progression. High circulating levels of IGF-1 and 2, or low levels of IGF binding proteins are associated with higher risk of developing breast, prostate, lung, colorectal, endometrial and bladder cancer [Allen et al. 2005, Cui et al. 2003, Wu et al. 2003, Petridou et al. 2003, Palmqvist et al. 2002, Toniolo et al. 2000, Giovannucci et al. 2000, Hankinson et al. 1998, Stattin et al. 2000, Yu et al. 1999, Ma et al. 1999, Chan et al. 1998]. Some discrepancies exist in the reports that can be due to differences in analysis methods and experimental procedures, but these positive correlations suggest an endocrine, paracrine and/or autocrine role of IGF system in various stages of cancer development and progression.

1.4.5 Role of IGF-1 signaling in neuroblastoma

IGF-1 and its receptor are expressed at high levels by human neuroblastoma cell lines [Martin et al. 1992]. IGF-1 stimulates growth and proliferation, and prevents apoptosis of neuroblastoma cells in an autocrine and paracrine manner [Meghani et al. 1993, Martin et al. 1993, Singleton et al. 1996, van Golen et al. 2000, Van Golen et al. 2000]; activating MAPK and PI 3-kinase pathways [Kurihara et al. 2000, Kim et al. 1997,]. Overexpression of the N-myc oncogene, a marker of poor prognosis for neuroblastoma, induces upregulation of IGF-1 receptor expression [Chambery et al.

1999]. The growth stimulatory and pro-survival effects of IGF-1 signaling in neuroblastoma make IGF-1 receptor signaling an attractive target for developing therapies against neuroblastoma. Although a number of clinical trials have been initiated to target IGF1 signaling to treat a variety of cancers, success has been limited. There are some concerns about tumors using alternative growth factor signaling pathways to survive and grow in the absence of IGF1 signaling, and consequently alternative approaches are needed to target multiple signaling pathways.

1.5 Significance of This Study to Neuroblastoma Treatment

As described in section 1.1, neuroblastoma is the most common extra cranial tumor of children and the survival rate for high risk neuroblastoma remains less than 40%. Currently used chemotherapeutic agents were originally designed to treat adult cancers and do not discriminate between cancer cells and cells that rapidly divide in growing children. Clearly, there is a high demand for developing novel therapies with fewer side effects for neuroblastoma.

Neuroblastoma arises from pluripotent neuroblastic cells that can potentially differentiate normally to neuroendocrine chromaffin or mature ganglion cells. In spite of the neuroblastic nature of neuroblastoma, mechanisms that are involved in normal development of neuronal cells have been not targeted to treat neuroblastoma.

Inhibitors of cathepsins B and L are attractive alternatives for treating neuroblastoma for a number of reasons. Inhibitors are not toxic to mature animals, indicating that the enzymes are not essential for normal cellular functions. By contrast,

knocking out both cathepsins B and L causes severe defects in development of the mouse nervous system, resulting in death in the first few weeks after birth. This indicates that the enzymes are uniquely required for survival of developing neuronal cells. Neuronal development in the sensitive neonatal period in mice corresponds to the third trimester in humans and consequently severely affected neuroblastoma patients who are usually not diagnosed before the age of two may have less neurological side effects if these enzymes are inhibited. The neuroblastoma cells retain characteristics of the rapidly dividing neural crest precursor cells and would therefore remain sensitive to enzyme inhibition. Thus, targeting these enzymes in neuroblastoma patients may help selectively eradicate proliferating neuroblastoma cells without affecting other normally growing cells within a child's body.

The mechanism by which inhibition or genetic deletion of cathepsins B and L causes neuronal cell death is not known. Accumulation of autophagic vacuoles is apparent, and similar results have been seen in cells treated with chloroquine [reviewed in Kumaru et al. 2013]. This latter compound raises the pH in intracellular organelles, which both inhibits function of lysosomes and blocks organelle fusion processes [Poole et al. 1981]. In this study, I investigate the potential role of down-regulation of cell surface growth factor receptor function as a mechanism that leads to death of neuronal cells after inhibition of cathepsins B and L. The IGF-1 receptor is highly expressed in neuroblastoma cell lines and tumor specimens, indicating that it may have an important role in regulating survival, growth and proliferation of

neuroblastoma. My studies are focused on this receptor system because of its well established role in cell survival and proliferation.

1.6 Significance of This Study to Cancer Treatment in General

While inhibition of autophagy may promote early stages of carcinogenesis, early clinical trials with chloroquine indicate that blocking of autophagy may cause regression of established tumors. Blocking autophagy may also enhance cytoxicity of chemotherapeutic agents, and prevent formation of dormant cells that can cause relapse [Cheong et al. 2012]. Whereas chloroquine might be expected to affect all hydrolytic functions of lysosomes and consequently affect growth of a broad range of cancers, inhibition of only cathepsins B and L may only affect tumors of neuronal origin. Some of these cancers, that include glioblastoma and melanoma, are amongst the most aggressive cancer types and more effective treatments are needed. Our primary focus at A.I. duPont Hospital for Children is on cancers of childhood, but the potential to treat more cancers will make development of protease inhibitor therapies more valuable.

FYAD is a novel, specific, irreversible inhibitor of cathepsins B and L, that causes selective apoptosis and death of neuroblastoma cell lines without affecting other normal or cancer cells. This apoptotic effect is preceded by cell cycle arrest and accumulation of autophagic vesicles. I also observed accumulation of fragments of IGF-1 receptor in FYAD treated cells suggestive of alterations in IGF-1 signaling pathway and inhibition of its growth stimulatory effects.

This study will shed light on the mechanisms that lead to apoptosis of neuroblastoma cell lines. It will further clarify the crosstalk between autophagy and IGF-1 receptor signaling. The observations from IGF-1 receptor signaling may be expandable to other receptor tyrosine kinases that have more specific, critical roles in neuroblastoma.

Based on these observations I hypothesize that IGF-1 receptor signaling is disrupted by cathepsin inhibition, and this leads to death of neuroblastoma cells lines. In this project I explore IGF-1 receptor signaling as a model to determine how inhibition of lysosomal function can lead to death of tumor cells.

The ultimate goal of this project is to develop novel therapeutics that specifically target neuroblastoma with fewer side effects, potentially by use of specific lysosomal enzyme inhibitors. These inhibitors may also be used in combination with currently used chemotherapeutics and increase efficiency of the therapy.

1.7 Objectives

In this thesis I have used specific inhibition of lysosomal proteases, cathepsins B, L, and D, to induce autophagy and apoptosis of neuroblastoma cell lines. Inhibition of these lysosomal proteases results in accumulation of C-terminal fragments of IGF-1 receptor in neuroblastoma cell lines, accompanied by apoptosis and accumulation of autophagic vesicles. I postulate that cathepsin inhibition disrupts IGF-1 receptor signaling, by blocking autophagic turnover of proteins in the IGF-1 signaling pathway. To address this hypothesis I first examined alterations in levels of components of IGF-

1 signaling cascade, lysosomes and the autophagic machinery. Secondly, I examined the subcellular localization of components of IGF-1 signaling cascade in relation to components of the autophagic pathway.

Specific Aim 1. To determine effects of cathepsin inhibition on IGF-1 receptor signaling. In this aim I will examine levels of components of IGF-1 signaling in inhibitor treated cells compared to control cells by immunoblotting. I will also examine the effect of cathepsin inhibition on the function of the IGF-1 signaling pathway.

Specific Aim 2. To determine the relative localization of components of the IGF-1 signaling pathway autophagic vesicles. In this aim I will separate fractions of control and inhibitor treated cells based on solubility in Triton X-100 or density. I will further address co-localization using super resolution illumination microscopy (SIM).

Chapter 2

MATERIALS AND METHODS

2.1 Cell lines and Culture

SK-N-SH, IMR-32 and NB-1691 cells were from ATCC (Manassas, VA). NB-1691 cells were a gift from Peter Houghton (St Jude's Children's Hospital, Memphis, TN). GM-11027 cells were from Coriell (Camden, NJ). IMR-32 and SK-N-SH cell lines are more established cell lines. IMR-32 cells represent neuronal type, more aggressive, N-MYC amplified tumors. SK-N-SH cell are a mixed population of stromal and neuronal cells without N-Myc amplification. They represent stromal type, less aggressive tumors. NB-1691 cells like IMR-32 cells, are N-Myc amplified aggressive tumor cells. GM11027 is a less well established cell line derived from primary tumor tissue passaged in a nude mouse. These cell lines were chosen to give a wide variety of samples of neuroblastoma cells with different phenotypes

All cell lines were maintained in MEM (Mediatech Inc., Manassas, VA, USA, #10-010-CV) supplemented with 10% FBS (# 35-015-CV, Mediatech Inc. Manassas, VA), 1% sodium pyruvate (Mediatech Inc., Manassas, VA, #25-30-CI) and 1% none essential amino acids (Mediatech Inc., Manassas, VA, #25-30-CI). IMR-32 cells were cultured in 75 cm^2 flasks and passaged upon reaching 80-90% confluence every 3-4 days by mechanical disruption. Other cell lines were cultured in 100mm tissue culture plates, and passaged by treating with 0.25% trypsin (Mediatech Inc. Manassas, VA, #

25-050-CI) upon reaching 80-90% confluence every 2-4 days. Cells were kept at 37˚C in a humidity saturated chamber containing 95:5; v/v air: CO_2 atmosphere.

2.2 Immunoblotting

At the termination of experiments, cells were scraped in the original plates and transferred to conical tubes, the supernatant was spun down at 350 g for 5 minutes and washed once with 5 ml phosphate saline buffer (PBS) (Mediatech Inc. Manassas, VA, 21-040-CV), then transferred to 1.5 ml vials and washed twice with 0.5 ml PBS followed by lysis with 2D lysis buffer (table 2.1) or 1% Triton-X-100 in PBS, containing 2X protease inhibitor cocktail (#p8465, Sigma Alrich. St Louis, MO) and 2X phosphatase inhibitor cocktail (#p5726, Sigma Alrich. St Louis, MO). Protein concentrations were subsequently determined by Biorad protein assay dye reagent (Biorad, Hercules, CA, USA, #500-006), or BCA protein assay kit (Thermo Scientific, Waltham, MA, USA #23228, #1859078). Samples with equal amounts of protein were added to 8X sample buffer (table 2.1) and heated at 95°C for 3 min. Proteins were separated by SDS-PAGE system using pre-cast 10% (w/v), or 4-15% (w/v) polyacrylamide gel in tris-glycine buffer (Biorad, #465-1033, #465-1086.) and running buffer (table 2.1). The gels were then transferred onto a PVDF membrane (Whatman International Ltd, Maidstone, England.) using a wet transfer system in transfer buffer (table 2.1) for 1.5-2 h at 110-120 volts. The membranes were then blocked for 1 h at room temperature with blocking buffer (table 2.1.) and incubated with primary antibody (table 2.2) in incubation buffer (table 2.1.) overnight at 4°C. The

blots were then rinsed twice and washed 3 times with TBS-T (table 2.1), and then probed with appropriate secondary horse radish peroxidase (HRP) conjugated antibody (Table 2.2) for 1 h at room temperature. The blots were rinsed twice and washed 3 times 5 min each in wash buffer (table 2) and developed using enhanced chemiluminescent (ECL) reagent (NEL101001 EA, PerkinElmer, Waltham, MA, USA) and X-ray films (#7940067, PerkinElmer, Waltham, MA, USA) to visualize signal. Relative intensities were measured using ImageJ software (NIH, Bethesda, MD, USA).

2.3 Primary Antibody Conjugation

A primary antibody against LC-3B (Table 2.2.) was conjugate to Alexa flour 488 using Apex antibody labeling kit (# A10468, Invitrogen, Life Technologies, Carlsbad, CA, USA) according to manufacturer's guidelines. In order to remove small interfering buffer components, the antibody carrier buffer was replaced by PBS using centrifugal filter units (#UFC503096, Millipore, Carrigtwohill, Ireland) prior to labeling. Antibody concentration and fluorescent labeling was measured by nano-drop 2000C spectrophotometer (#UX-83061-00, Thermo Scientific, Waltham, MA, USA) before and after labeling.

2.4 Sequential immunofluorescence (IF) Staining

SK-N-SH cells were trypsinized, washed and then plated on glass coverslips in 6 well plates at 50-80% confluence. The next day media was removed from the cells

and replaced with treatment media for 18 h. Then the media was removed and cells were washed thoroughly with ice cold PBS followed by fixation in 4% paraformaldehyde (Electron microscopy sciences, Hatfield, CA, USA, #15710) on ice for 30 min. The cells were then washed with ice cold PBS and permeabilized with methanol (Fisher scientific, USA, #A454-4) at -20°C for 15 min. Cell were then washed with ice cold PBS to remove residual methanol. Non-specific binding of IgG was blocked in blocking buffer (table 2.1) for 1 h at room temperature. Primary antibodies (table 2.2) were incubated overnight at 4°C. Cells were then rinsed twice and washed 3 times 5 min each, then incubated with appropriate secondary antibodies (table 2.2). Cells were then rinsed twice and washed 3 times with PBS and fixed for 30 min in 4% paraformaldehyde, followed by a wash and 1 h incubation with 10 mM glycine. The cell were then washed and incubated overnight at 4°C with the fluorescent conjugated labeled primary antibody followed by 3 washes 5 min each and then mounted in slow fade dapi mounting media (S36939, Molecular probes, Eugene, OR,USA) visualized by Structured Illumination Microscopy (SIM) and data was processed using Zen software (Applied Precision, Issaquah, WA, USA). Further 3 dimensional image processing was performed using Volocity software (Perkin Elmer Inc. Waltham, MA, USA).

2.5 Inhibitor and Cytokine Treatment

FYAD and Pepstatin A stock solutions were made in ethanol at 2 mM and 1 mM concentrations, respectively. To treat cells with inhibitors, media was removed

and cells were washed once with PBS. Treatment media was made with 5 μM FYAD and/or 10 μM Pepstatin A (# 26305-03-3, Sigma Aldrich, St. Louis, Mo, USA), or the vehicle control (ethanol), and added to the cells.

For cytokine treatment, media was removed from cells and they were washed 3 times with PBS, followed by 3 h of incubation with serum free medium. The cells were then treated with 100ng/ml IGF-1 (#3039, Cell signaling technologies, Danvers, MA, USA) or EGF (#8916, Cell signaling technologies, Danvers, MA, USA) for 5 min. The media was removed and cells were put on ice in ice-cold PBS for 5 min. The PBS was then removed and cells were homogenized in 1% Triton-X-100 in PBS containing 2X protease and phosphatase inhibitor cocktails.

2.6 Immunoprecipitation (IP) and Triton Fractionation

Cells were treated according to the experimental procedure in 100 mm plates, the media was removed and 5ml ice cold PBS was added to plates. The plated were kept on ice for 5 min, then the PBS was removed, and cells were lysed on the bottom of the plates by 250μl ice cold IP lysis buffer (table 2.1.) containing 2X phosphatase and protease inhibitor cocktail, then scraped off the plates. The volume of cell suspension was then adjusted to 500 μl. Samples were then centrifuged at 10,000 g for 15 min at 4°C. The resulting supernatant was transferred to a fresh vial (labeled Triton soluble fraction) and the pellet (Triton insoluble) was rinsed twice with ice cold PBS And then dissolved in 2D lysis buffer (table 2.1). Primary antibody was added to the

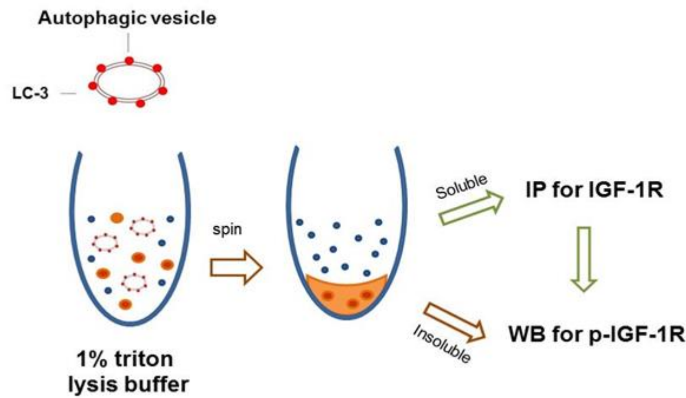


Figure 2.1 Schematic diagram of triton fractionation and Immunoprecipitation

Triton soluble fraction (table 2.2) and incubated overnight at 4°C. Protein G beads (#20398, Thermo scientific, Waltham, MA, USA) were added to vials at 1:50 dilution, followed by 1 h incubation at 4°C. The beads were then separated by centrifugation at 300 g for 2 min, rinsed twice with ice cold PBS, and then proteins solubilized by SDS sample buffer, separated on gels and western blotted

2.7 Percoll Gradient Fractionation

At the termination of experiments, cells were scraped and resuspended in media in the original culture dishes. The cell suspension was then centrifuged at 350 g for 5 min and pelleted cells washed with 5 ml PBS. Cells were then transferred to 1.5 ml vials and washed twice with 0.5 ml PBS. The cell pellet was then resuspended in 3-5 time its volume ice cold homogenization buffer (Table 2.1) containing phosphatase and protease inhibitors, and cells were broken using a Dounce homogenizer with 30-50 strokes. Cells were checked visually under the microscope to ensure that the

majority of the cells were broken. The homogenate was then centrifuged twice at 1000 g for 5 min to separate the nuclear pellet. The post-nuclear supernatant was used for Percoll gradient fractionation. The gradient was made by layering 1.5 ml sucrose cushion solution (Table 2.1) in the bottom of centrifugation tubes and adding 7ml 35% Percoll (#17081-01, GE healthcare, Uppsala, Sweden) solution. The tubes (#46910 Thermo electron corporation, Asheville, NC, USA.) were then centrifuged at 35000 g for 1 h. 14 fractions, each 0.5ml, were then collected from each tube. 100 µl of each sample was mixed with SDS/PAGE sample buffer and heated to 95°C for 3 min. Samples were then centrifuged at 16000g for 5 min, and the resulting supernatant was used for determination of protein concentrations with the BCA protein assay kit (Thermo Scientific, Waltham, MA, USA #23228, #1859078). Samples diluted with additional sample buffer to adjust protein concentrations and the separated on 4-15% tris-glycine gradient gels prior to western blot analysis.

Table 2.1 Solutions

Solution	Components
2D lysis buffer	7M urea, 2M Thiourea, 1% CHAPS 30 mM Tris, pH 8.5
Blocking buffer (immunoblotting)	5% non-fat dry milk in TBS
Blocking buffer (immunoflouresence)	10% goat serum in PBS
Homogenization buffer	0.25M Sucrose, 20mM Tris, 10mM EDTA, 2X protease and phosphatase inhibitors
Incubation buffer (Immunoblotting)	5% BSA in TBS-T
PBS	Media tech Inc, #21-040-CV
8X Sample loading buffer	Tris-Cl pH 6.8, 10%SDS, 30% glycerol, 0.1% bromophenol blue, 20% BME,
10XRunning buffer	250mM Tris base, 1.92M glycine, 1% SDS, pH 8.4
Stripping buffer	200mM Glycine. 0.1% SDS, 1% Tween-20, pH 2.2
Sucrose gradient cushion	2.5M sucrose in 20mM Tris
TBS-T	50 mM Tris, 150 mM NaCl, 0.1% triton, pH7.4
10X Transfer buffer	250mM Tris base, 1.92M glycine, pH 8.4
1X Transfer buffer	1X transfer buffer, 20% methanol

Table 2. 2 Primary Antibodies

Primary Antibody	Manufacturer	Catalog number	Dilution
Actin	Sigma Aldrich	A5441	1:10,000
ALK	Cell Signaling	3333	1:1000
Calnexin	Millipore	MAB3126	1:2000
Cathepsin B	Customized	N/A	1:2000-1:5000
EGFR	Cell Signaling	4267	1:1000
Grb-2	Cell Signaling	3972	1:1000
IGF-1R beta	Cell Signaling	3027	1:1000
IGF-1R beta (XP)	Cell Signaling	9750	1:500(IF), 1:100 (IP)
IRS-2	Cell Signaling	4502	1:1000
LAMP-1	Cell Signaling	3243	1:1000
LC3-B	Cell Signaling	3868	1000-1:3000
MAPK	Cell Signaling	9102	1:2000-1:5000
PKB	Cell Signaling	9272	1:2000-1:5000
p-IGF-1R beta	Cell Signaling	3024	1:500
p-MAPK	Cell Signaling	9101	1:1000
p-PKB	Cell Signaling	9271	1:1000
p-Shc A	Cell Signaling	2434	1:1000
RAS	Cell Signaling	3965	1:1000
Shc A	Cell Signaling	2432	1:1000
Shc C	BD transduction lab	610642	1:1000
TrkB	BD Transduction lab	610101	1:1000

Table 2.3 Secondary Antibodies

Antibody	Manufacturer	Catalog Number	Dilution
HRP conj. donkey anti sheep	Lifespan Bioscience	LS-C61141-1000	1:5000
HRP conj. goat anti mouse	EMD Millipore	AP106P	1:3000
HRP conj. goat anti rabbit	Amersham	NA934v	1:3000
Flour 568 goat anti rabbit	Invitrogen	A-11011	1:500

Chapter 3

RESULTS

Previous studies from our lab have shown that treatment of neuroblastoma cell lines SK-N-SH and IMR-32 with FYAD, an inhibitor of cathepsins B and L, causes cell cycle arrest and apoptosis in a dose dependent manner (Colella et al. 2010). Induction of apoptosis was specific to neuroblastoma cell lines, not being observed in other cancer cells and non-malignant fibroblastic cell lines (Colella et al. 2010). Apoptosis and cell cycle arrest was accompanied by accumulation of dense autophagic vesicles within inhibitor treated cells (Colella et al. 2010).

The molecular mechanism that leads to cell cycle arrest and apoptosis of neuroblastoma cells treated with inhibitors of lysosomal enzymes is not known. Lysosomes are known to play important roles in degradation of cell surface receptors, especially receptor tyrosine kinases. Receptor tyrosine kinases are key regulators of growth, proliferation and death of many cell types including cancer cells. The central hypothesis of this study is that cathepsin inhibitors disrupt receptor-mediated cell signaling and this leads to death of neuroblastoma cancer cells. In this study we explore the IGF-1 receptor signaling system as a model to determine how inhibition of lysosomal function can lead to death of tumor cells.

3.1 Uncleaved Cathepsin B and Cleaved LC-3 Accumulate in Inhibitor treated Neuroblastoma cells.

SK-N-SH cells were treated with vehicle control or protease inhibitors for 72 h as described in materials and methods. Whole cell lysates were collected and subject to immunoblotting. In untreated cells two major forms of cathepsin B exist, that correspond to a single-chain active form of the enzyme and the heavy chain of a cleaved from of the enzyme (Fig.3.1A). Inhibition of cathepsins B, L, and D resulted in a dramatic decrease in level of the cleaved form of cathepsin B in both cell lines (Fig. 3.1A) indicating that these enzymes are responsible for the processing of cathepsin B in these cells.

Accumulation of LC-3, a known marker of autophagy, indicates significant induction of autophagy. In untreated conditions the primary form of LC-3 corresponds to the uncleaved form that is cytosolic, whereas in treated cells the cleaved form that is incorporated into membranes of autophagic vesicles is predominant (Fig. 3.1B). Previous published data from Mason lab show accumulation of dense, multilayered vesicles that resembled autophagic vesicles within inhibitor treated neuroblastoma cells (Colella et al. 2010). Accumulation of autophagic vesicles was confirmed by immunofluorescent staining for LC-3 (Fig. 3.1C). In the vehicle treated cells LC-3 staining was diffuse with a few small LC-3 positive granules, consistent with a predominantly cytosolic location of protein. In inhibitor treated cells staining was punctuate with some donut-like structures, consistent with formation and accumulation of a high number of large autophagic vesicles (arrows, Fig. 3.1C).

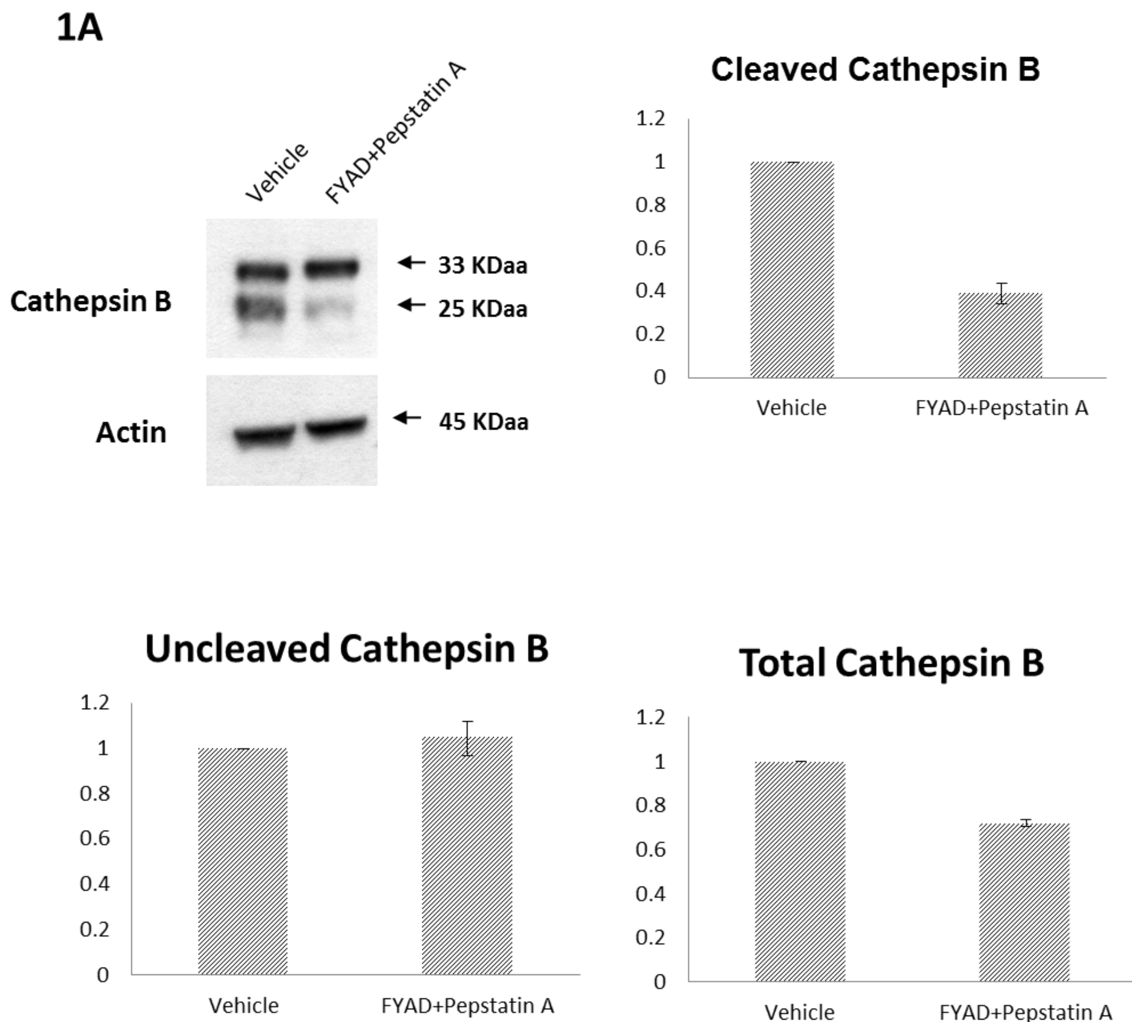


Figure 3.1A Effect of inhibitor treatment on processing of cathepsin B in neuroblastoma cells.

SK-N-SH cells were treated with protease inhibitors as described in materials and methods, then whole cell lysates were harvested and subject immunoblotting, followed by densitometric analysis and quantification. Cleaved cathepsin B decreased by 60% (p-value <0.0002), whereas levels of uncleaved cathepsin B did not change significantly. The ratio of cleaved to uncleaved enzyme decreased by 60% after inhibitor treatment (p-value<0.005).

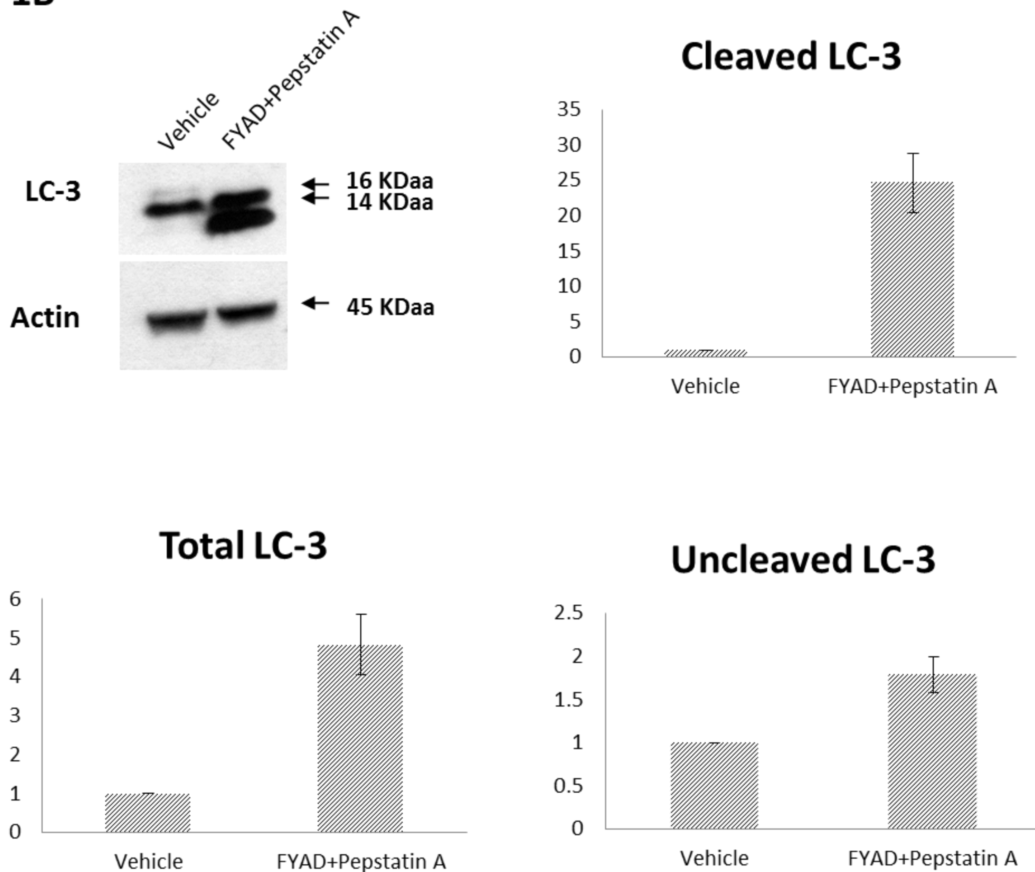


Figure 3.1B Accumulation of LC-3 in inhibitor treated neuroblastoma cells. SK-N-SH cells were treated with protease inhibitors as described in materials and methods, then whole cell lysates were harvested and subject to immunoblotting, followed by densitometric analysis and quantification. The cleaved form of LC-3 was at very low levels in untreated condition, but in inhibitor treated cells it showed a dramatic almost 25 fold increase (p-value<0.005). Total LC-3 showed a dramatic increases (p-value<0.02) whereas the unprocessed form showed a small but significant increase (p-value<0.001). The ratio cleaved to uncleaved increased 30 fold (p-value<5E-05).

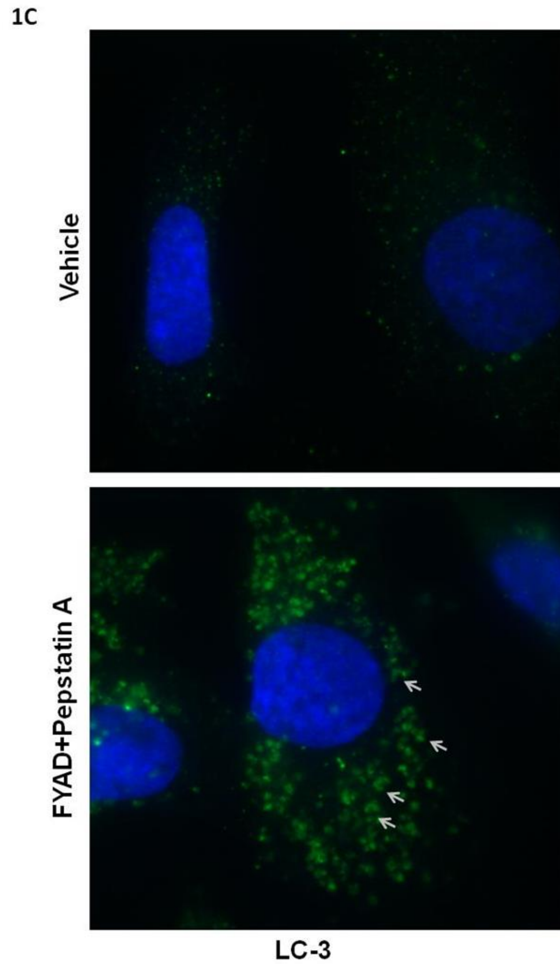


Figure 3.1C Accumulation of LC-3 positive vesicles within inhibitor treated neuroblastoma cells

Control and inhibitor treated SK-N-SH cells were subject to immunofluorescent staining against LC-3 (green) followed by confocal microscopic analysis. In untreated cells LC-3 showed a diffuse cytosolic distribution, with few small granules, whereas the inhibitor treated cells showed accumulation of a large number of large LC-3 positive granules many with crescent shape structures closing around fractions of cytosol (white arrows).

3.2 Two Fragments of IGF-1 ReceptorAappear in Inhibitor Treated Neuroblastoma cells.

The next step after confirming efficient inhibition of lysosomal enzymes and induction of autophagy was to determine whether inhibition had any effect on IGF-1 receptor turnover. An antibody against the cytosolic C-terminal end of the beta subunit of the receptor was used for western blot analysis of samples. Protease inhibitor treatment did not cause any significant change in the level of full length IGF-1 receptor in SK-N-SH cells, but resulted in the appearance of two fragments of IGF-1 receptor approximately 50 and 70 KDa (Fig. 3.2A). Other neuroblastoma cells lines, IMR-32, GM11027 and NB1691, also showed accumulation of fragments of the IGF1-R after treatment with protease inhibitors.

In these experiments, treatment with FYAD and pepstatin alone were compared with treatment with both inhibitors. Fragments only appeared when FYAD was added to cells and pepstatin resulted in appearance of an additional higher molecular weight band (Fig. 3.2B). These results demonstrate that the lysosomal proteases play a direct role in turnover of the IGF1-R in these cells.

In IMR-2 cells the effect of inhibitor treatment was less pronounced, but similar to that seen for SK-N-SH cells. In these cells cathepsin B exists primarily as the cleaved form, but inhibitor treatment result in increased levels of the unprocessed form of the mature enzyme. The processed form of LC-3 and fragments of IGF-1 receptor were also seen in inhibitor treated IMR-32 cells (Fig. 3.3).

3.3 Effect of Protease Inhibition on Downstream Adapter Proteins in the IGF-1R Signaling Pathway.

While it is clear that protease inhibition causes accumulation of fragments of the receptor, it is not clear how this could affect growth of cells, as levels of full-length receptor were not affected. To further understand alterations in IGF-1 receptor signaling pathway, I examined the effect of protease inhibition on adapter proteins that transmit IGF1 signaling events to downstream effector molecules. The primary adaptor proteins are Shc and IRS-1 and IRS-2, which directly bind to activated phosphorylated receptors. Western blot analysis

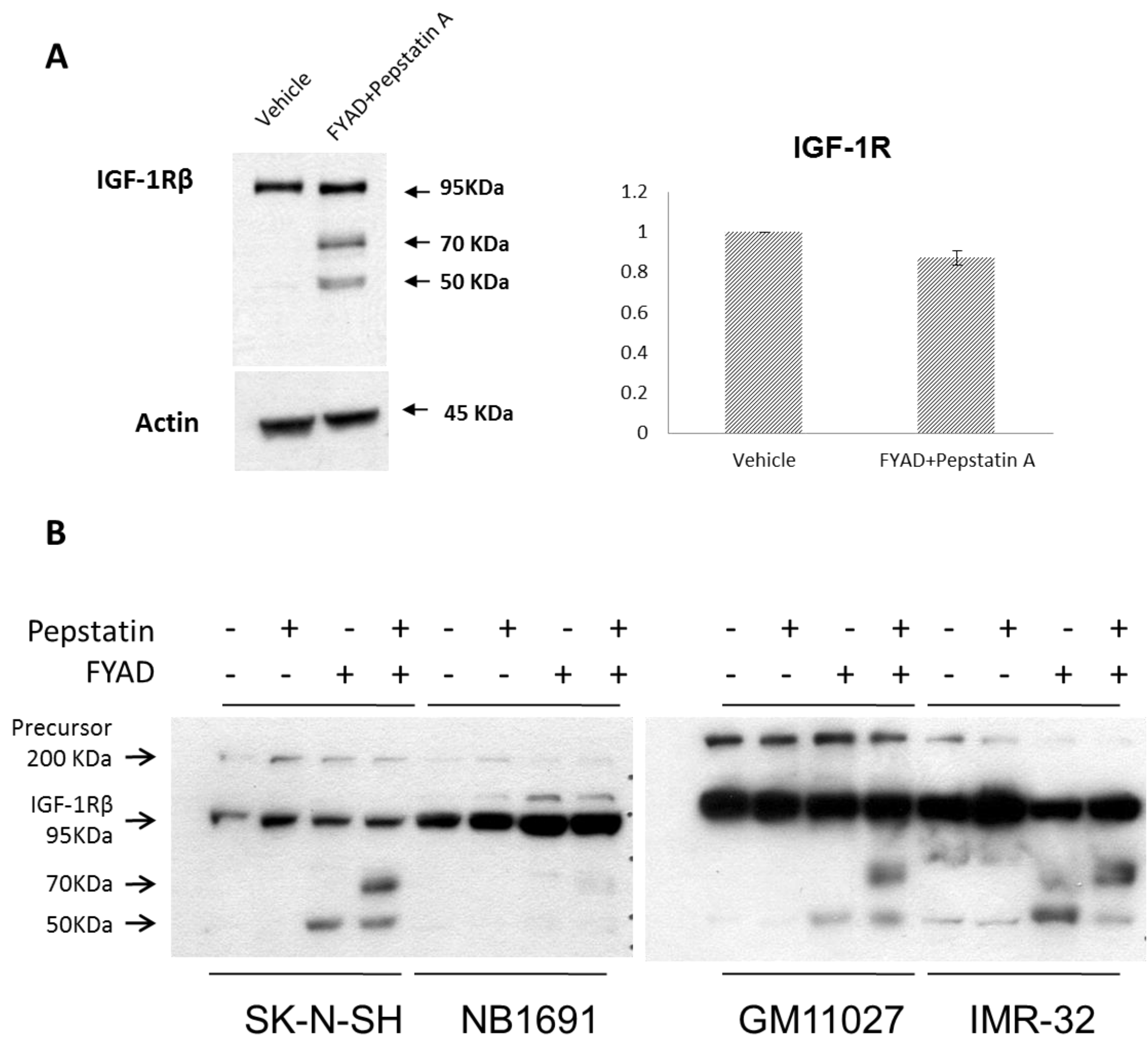


Figure 3.2 Effect of inhibitor treatment on IGF-1 receptor in neuroblastoma cells.

SK-N-SH cells were treated with protease inhibitors as described in materials and methods, then whole cell lysates were harvested and subject to immunoblotting with an antibody against the C-terminus of the IGF-1 receptor beta subunit, followed by densitometric analysis and quantification (A). The level of total receptor remained almost the same, but 2 C-terminal fragments of IGF-1 receptor appeared at 50 KDa and 70 KDa. Other neuroblastoma cell lines IMR-32, GM11027 and NB1691 were treated with combinations of FYAD and Pepstatin A (B). Fragments appeared only when FYAD was added to the cells. Pepstatin A treatment resulted in appearance of a larger fragment.

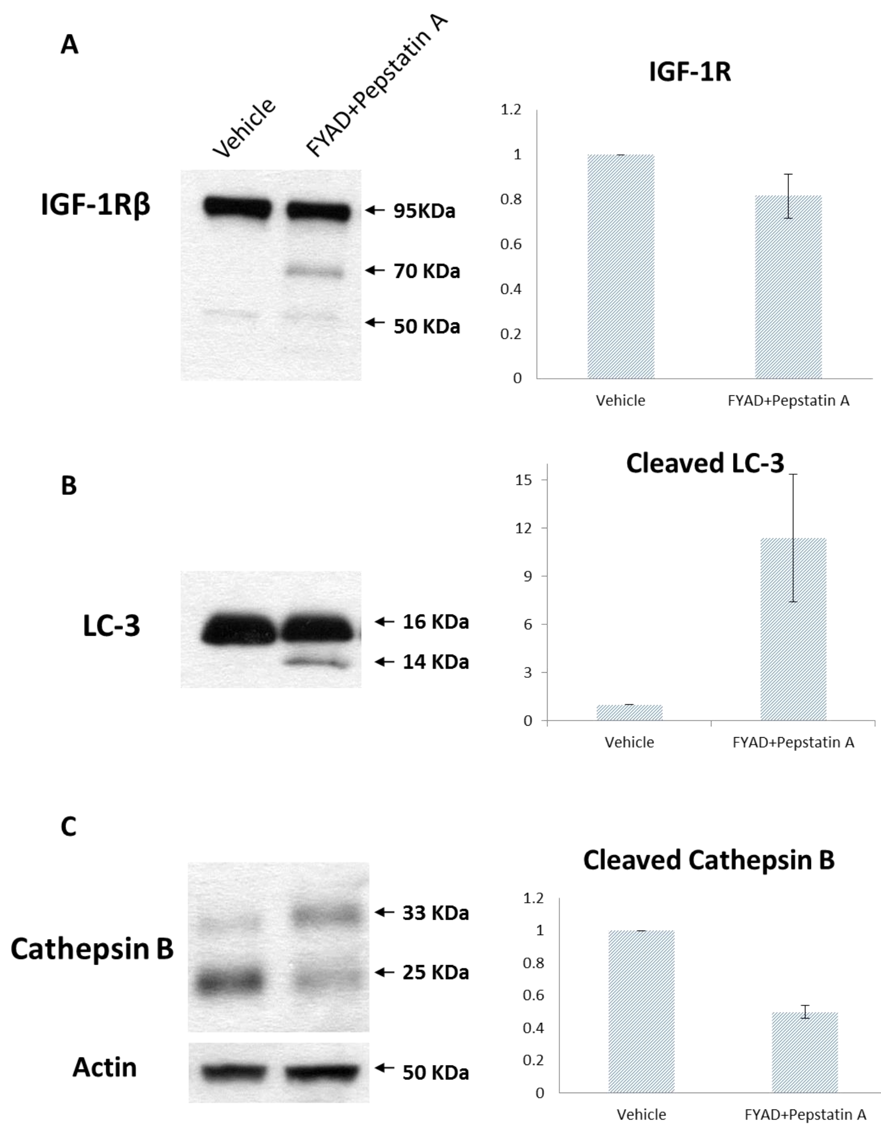
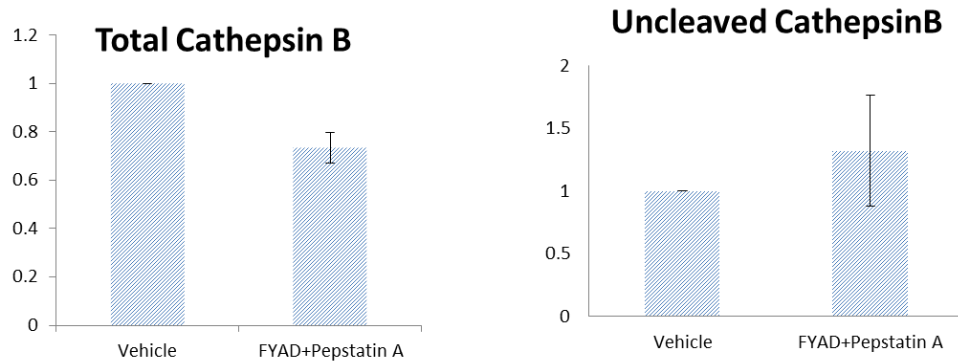


Figure 3.3 Effect of inhibitor treatment on IMR-32 neuroblastoma cell line. IMR-32 cells were treated with protease inhibitors as described in materials and methods, then whole cell lysates were harvested and subject to immunoblotting, followed by densitometric analysis and quantification. In inhibitor treated cells the level of full length IGF-1 receptor beta remained almost the same, and two fragments at 50 KDa and 70 KDa appeared (A). Cleaved LC-3 appeared in inhibitor treated cells (an almost 12 fold increase, $p\text{-value}<0.05$) (B). The cleaved form of cathepsin B showed an almost 50% decrease ($p\text{-value}<3E\text{-}05$) (C).

D



E

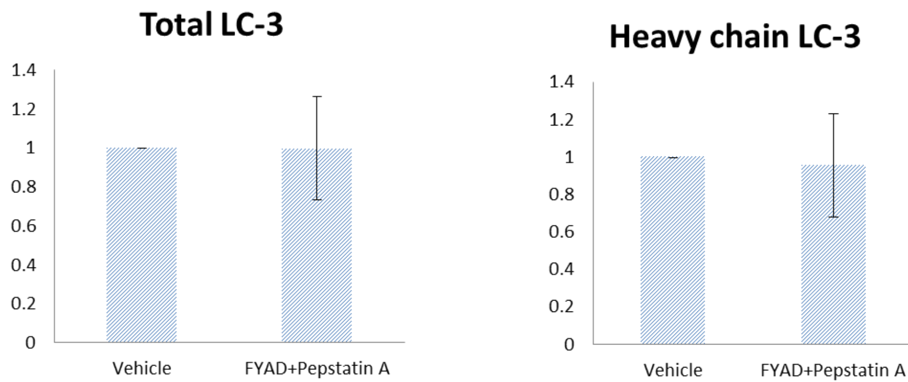


Figure 3.3 (continued) Further quantification of cathepsin B and LC-3 in IMR-32 cell.

The level of total cathepsin B showed a slight but significant decrease (p-value<0.01) (D). The uncleaved cathepsin B did not show a significant change (D). Total or uncleaved LC-3 remained almost the same (E). Although in these cells only a fraction of the LC-3 was processed after inhibitor treatment, levels were higher than in the control cells.

showed that SK-N-SH cells do not express IRS-1 protein (Figure 1S), so we focused on IRS-2 and Shc for these studies.

Neither total IRS-2 nor any of the Shc isoforms, p46, p52, p66, showed any significant changes on inhibitor treatment (Fig. 3.4A). Steady-state levels of RAS and Grb-2, which bridges between Shc and RAS, were also unchanged. Inhibitor treatment selectively leads to accumulation of activated, phosphorylated form of Shc (Fig. 3.4B).

Further quantification of phosphorylated Shc isoforms revealed that the predominant phosphorylated isoform that accumulates within inhibitor treated cells to an almost 4 fold level is the p52 Shc (Fig. 3.4B, C). This is consistent with its role as a signal transducer between IGF-1 receptor and the downstream pathway.

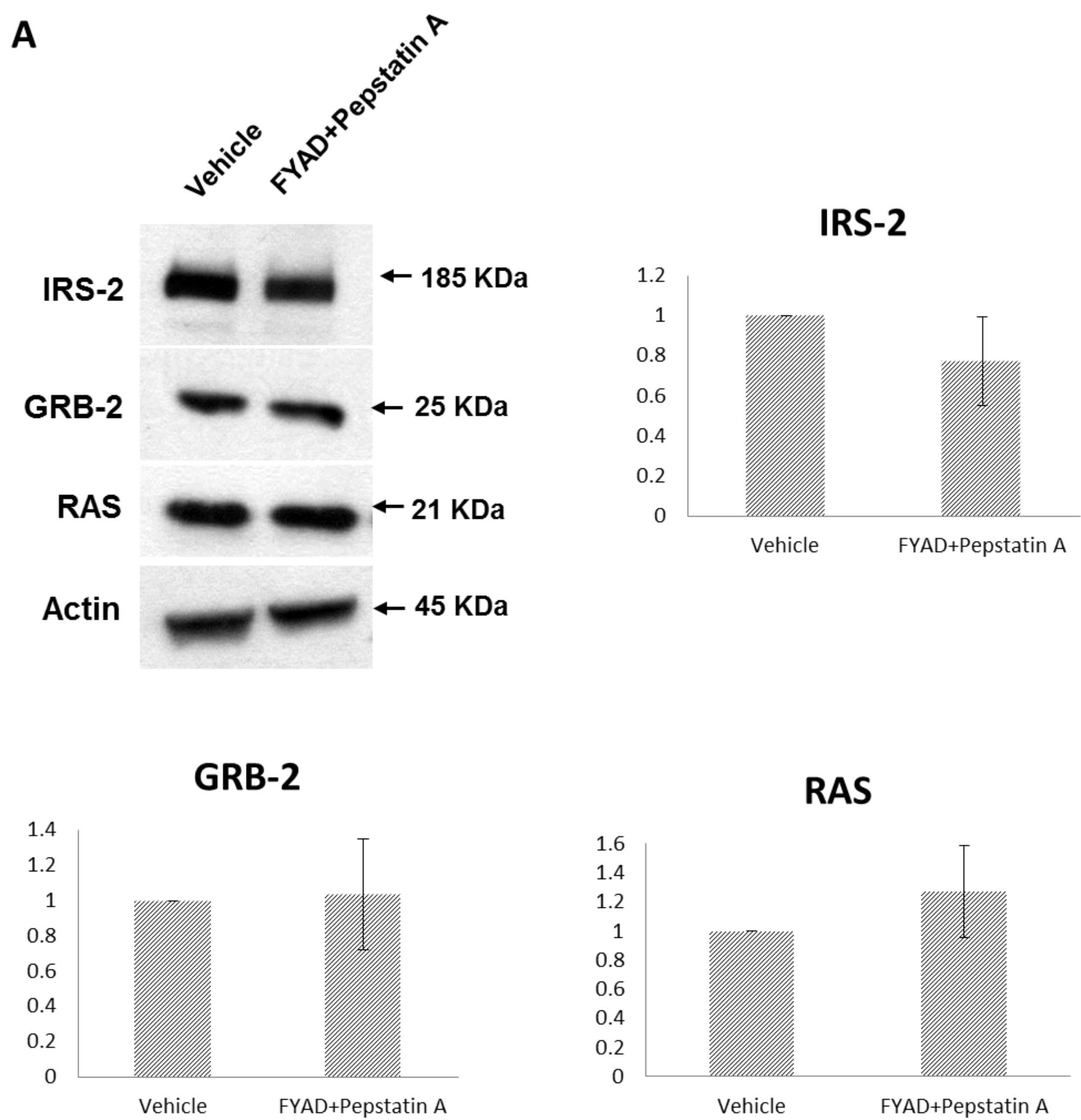


Figure 3.4A Effect of protease inhibition on effectors of the IGF-1R signaling pathway.

SK-N-SH cells were treated with protease inhibitors as described in materials and methods, then whole cell lysates were harvested and subject to immunoblotting, followed by densitometric analysis, normalization to actin, and quantification. IRS-2 Grb-2 and RAS did not show any changes in inhibitor treated cells.

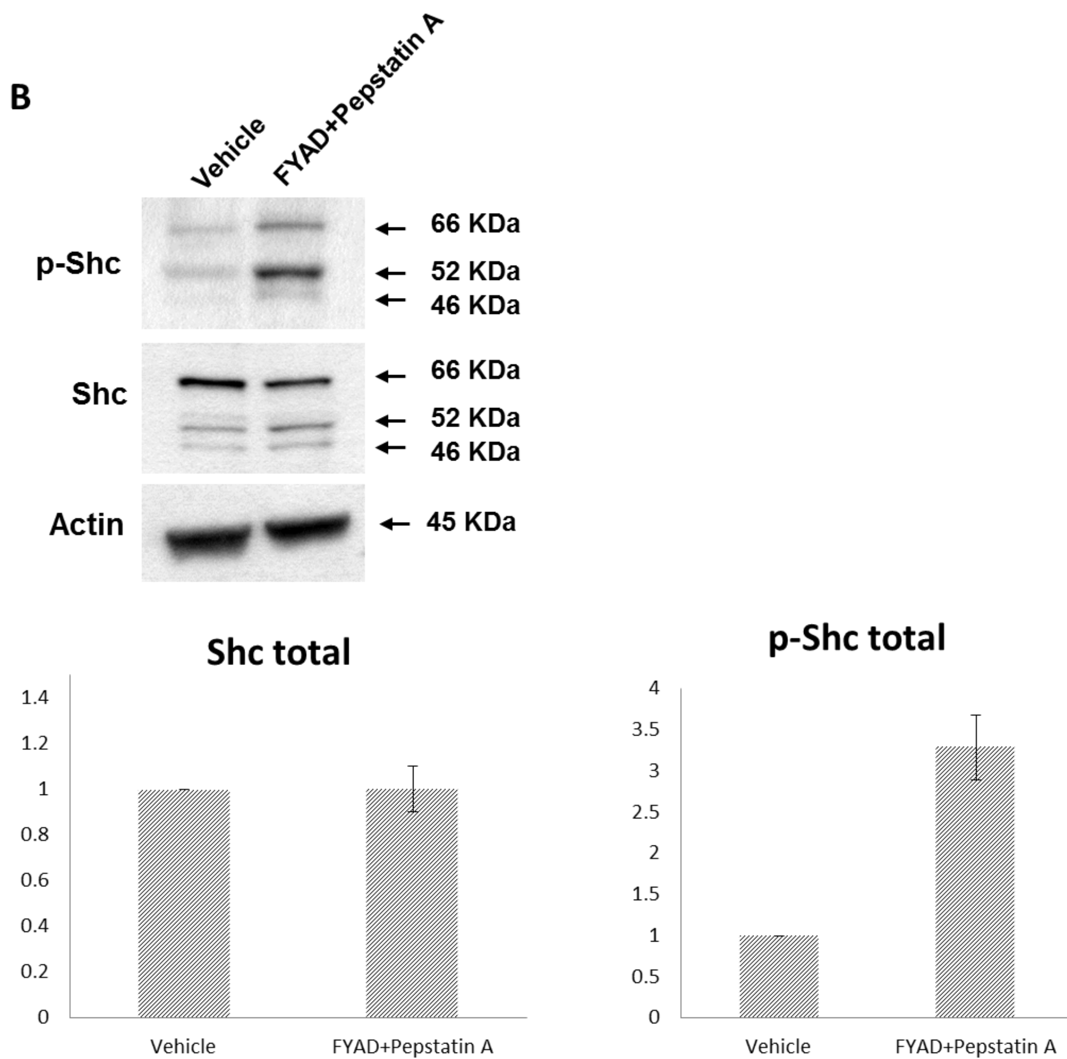


Figure 3.4B Effect of protease inhibition on effectors of the IGF-1R signaling pathway.

SK-N-SH cells were treated with protease inhibitors as described in materials and methods, then whole cell lysates were harvested and subject to immunoblotting, followed by densitometric analysis, normalization to actin, and quantification. Total Shc did not show any significant change, whereas p-Shc showed a more than 3 fold increase (p-value<0.005).

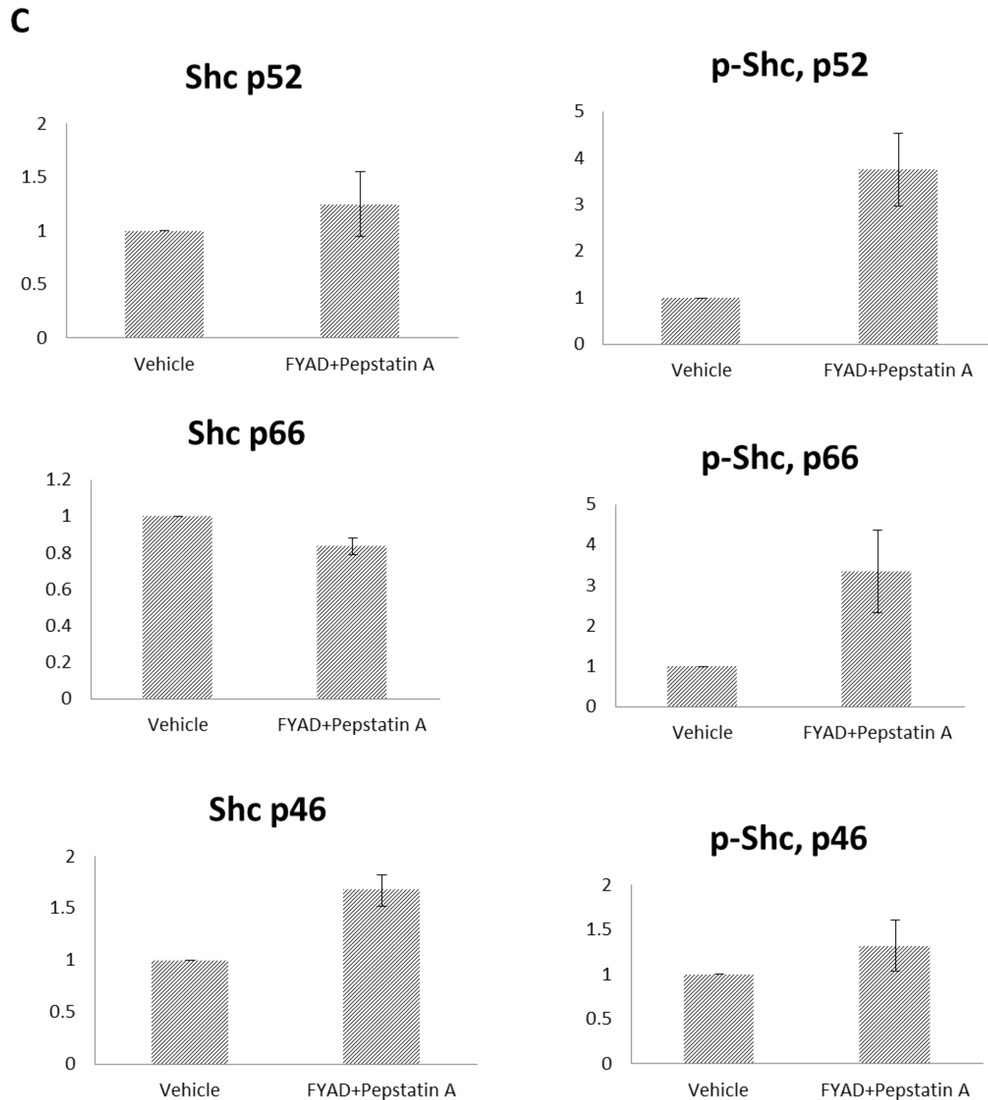


Figure 3.4C Effect of protease inhibition on effectors of the IGF-1R signaling pathway.

Further quantification of Shc protein isoforms in inhibitor treated cells. Phosphorylated proteins were normalized to total protein. Protein levels of p52 and p66 Shc did not show any major changes. Phosphorylated p52 showed a 4 fold increase (p-value<0.05). The increase in phosphorylated p66 was not statistically significant. Total p46 protein showed a significant 1.7 fold increase (p-value<0.02) but the slight increase in its phosphorylated form was not statistically significant.

3.4 Effect of Protease Inhibition on Effectors of the IGF-1R Signaling Pathway.

Changes in steady-state levels of activated Shc indicate that IGF1 signaling might be impacted by protease inhibition. Total levels of MAPK were not significantly affected by protease inhibition in either SK-N-SH or IMR-32 cells. However, steady-state levels of p-MAPK were decreased by a factor of almost 5 in both cell lines (Fig. 3.4. D and 3. 5C). Effects of protease inhibition on activation of PKB were less pronounced and although there was a significant decrease of p-PKB in SK-N-SH cells, total levels of PKB were similarly decreased (Fig. 3. 4. D and 3. 5 C).

IMR-32 cells showed a similar pattern with most proteins tested (Fig. 3.5 A, B, C). The only protein that showed a slightly different pattern was total MAPK with a significant 1.42 fold increase (Fig. 3.5. C). Phosphorylated forms of p46 and p66 Shc were not detectable in IMR-32 cells. p52 was detectable and similar to SK-N-SH cells showed a significant, dramatic increase (Fig. 3.5. B).

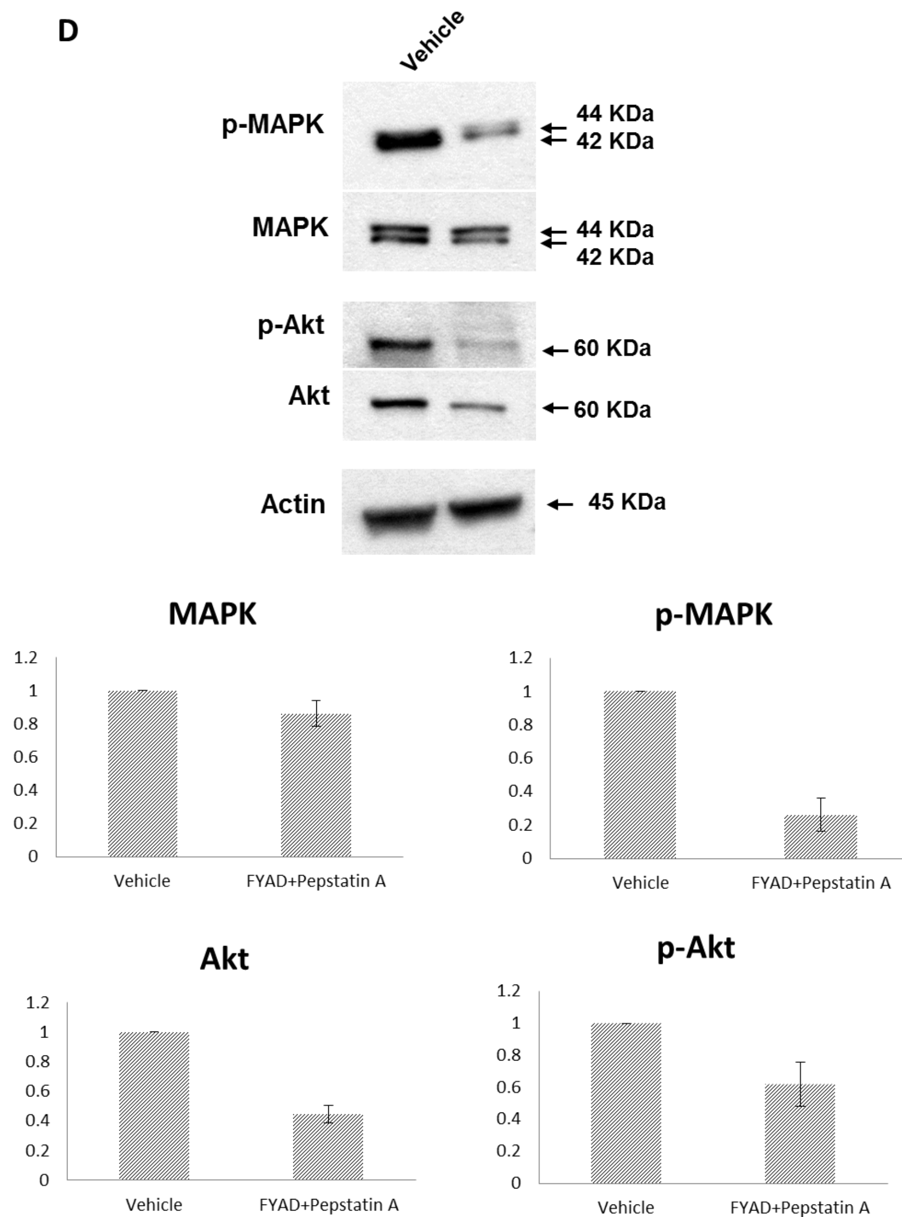


Figure 3.4D Effect of protease inhibition on effectors of the IGF-1R signaling pathway in SK-N-SH neuroblastoma cell line.

Total MAPK did not change, but p-MAPK showed more than 3 fold decrease (p-value<0.002). Total Akt decreased about 50% (p-value<0.001), p-Akt showed some variation, with an overall 40% fold decrease which was not statistically significant.

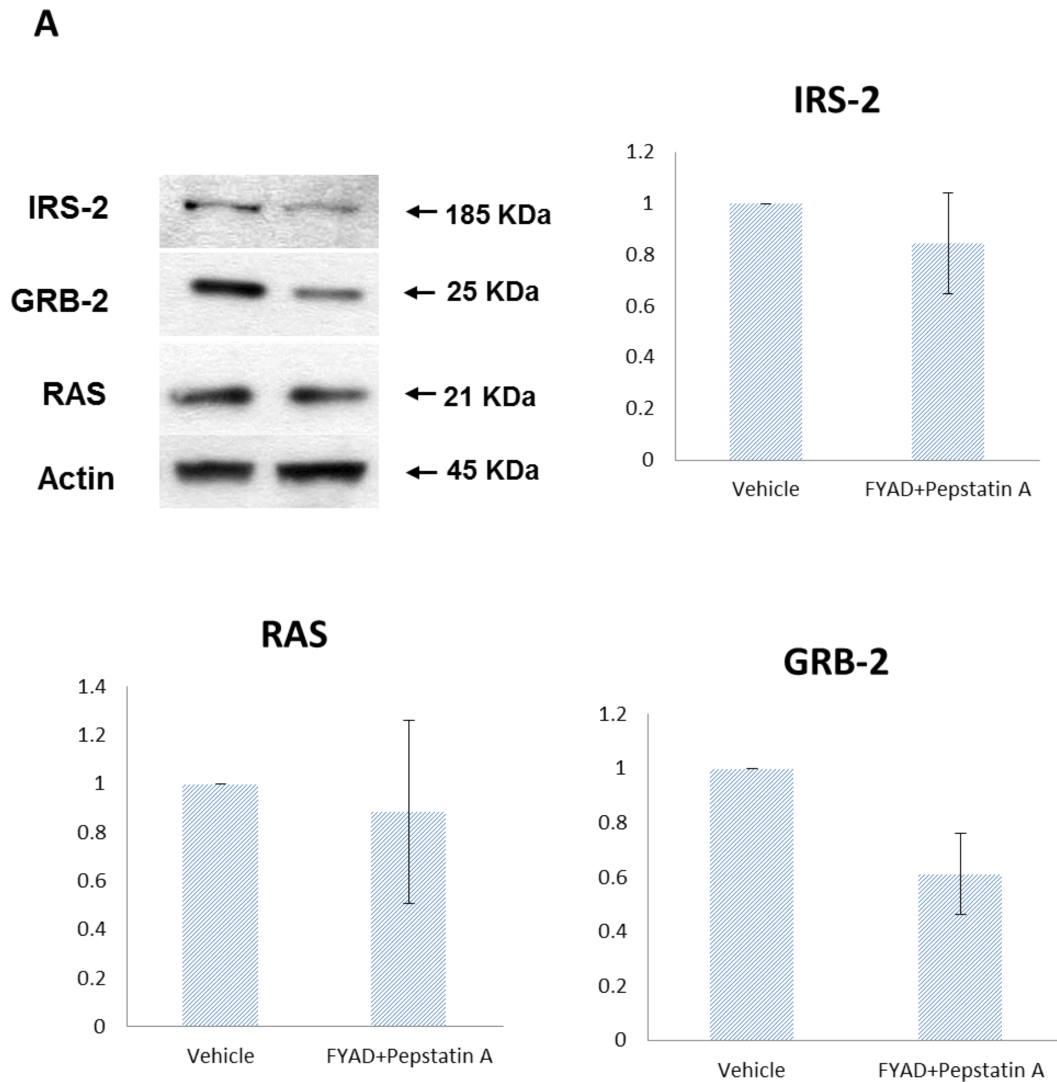


Figure 3.5A Effect of protease inhibition on effectors of the IGF-1R signaling pathway in IMR-32 neuroblastoma cells.

IMR-32 cells were treated with protease inhibitors as described in materials and methods, then whole cell lysates were harvested and subject to immunoblotting, followed by densitometric analysis, normalization to actin, and quantification. IRS-2 and RAS did not show any changes in inhibitor treated cells. Grb-2 showed a small decrease to 61% of baseline value (p-value<0.05).

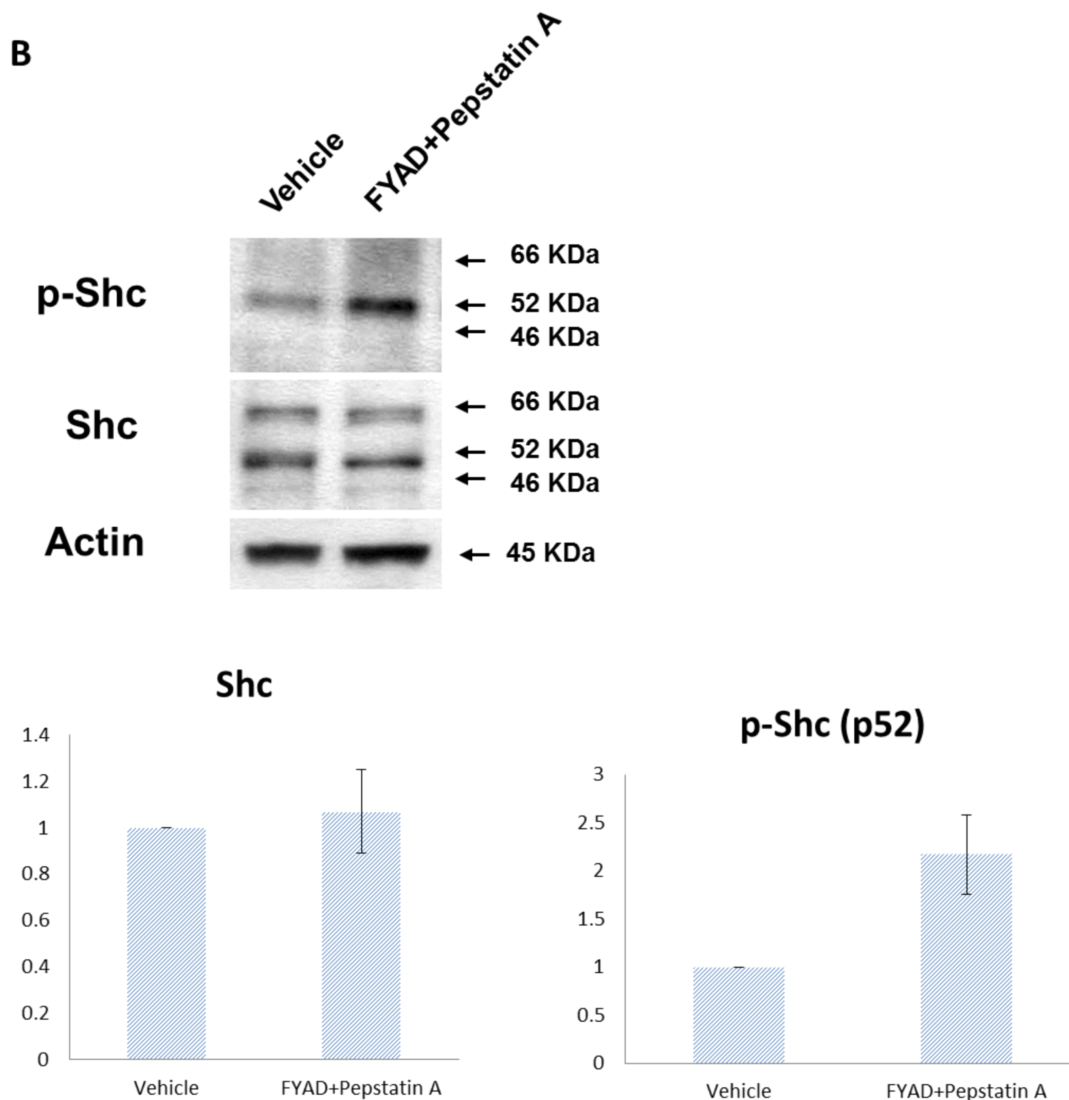


Figure 3.5B Effect of protease inhibition on effectors of the IGF-1R signaling pathway in IMR-32 neuroblastoma cell line.
IMR-32 cells were treated with protease inhibitors as described in materials and methods. Whole cell lysates were harvested and subject to immunoblotting, followed by densitometric analysis, normalization to actin, and quantification. Total Shc did not show any significant change, whereas p-Shc showed a more than 2 fold increase (p-value<0.05). The only phosphorylated Shc isoform that was detectable was p52. Phosphorylated p46 and p66 were not detected in treated or untreated conditions.

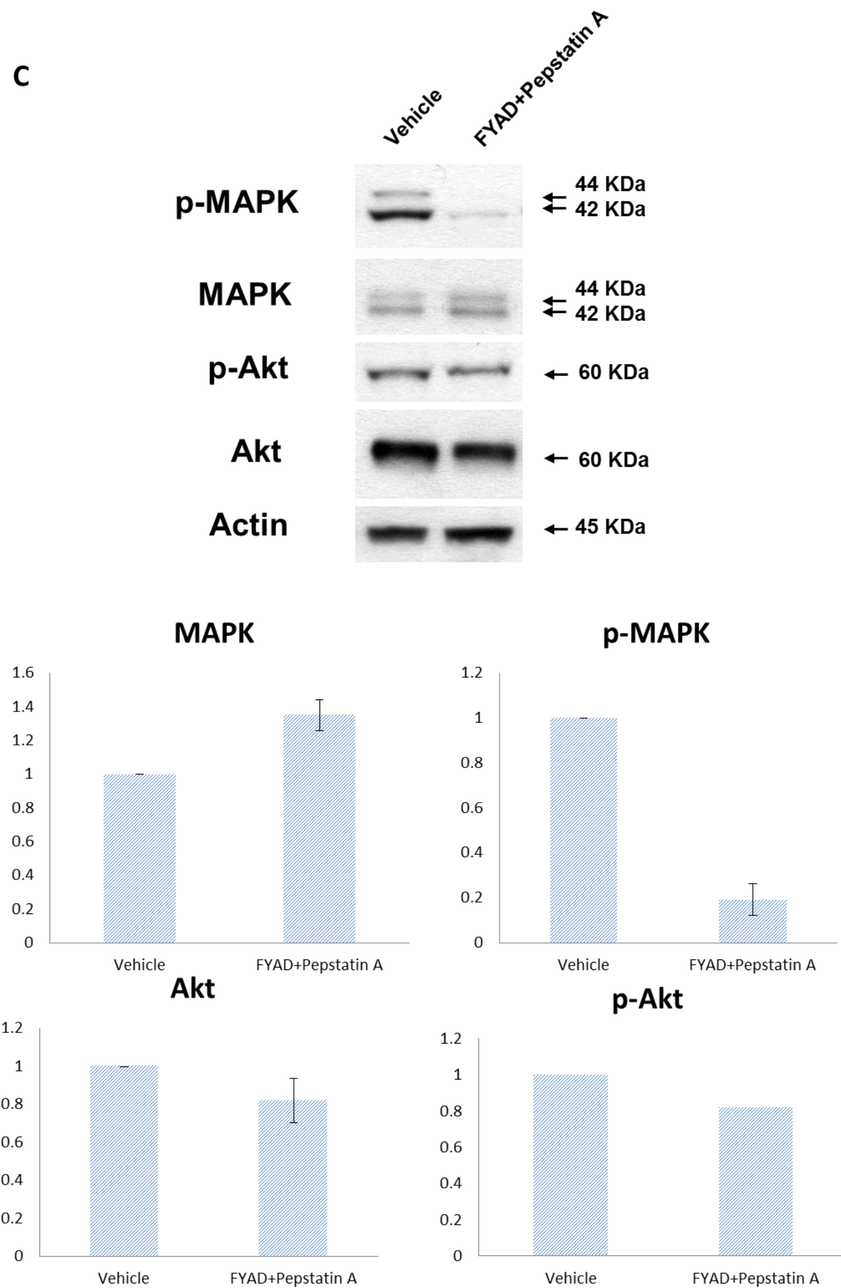


Figure 3.5C Effect of protease inhibition on effectors of the IGF-1R signaling pathway in IMR-32 neuroblastoma cell line.

Total MAPK showed a small but significant increase (p-value<0.05). P-MAPK showed a dramatic, significant decrease (p-value<5E-05). Total PKB or p-PKB did not change significantly.

While reduced activation of MAPK is consistent with reduced growth and survival of inhibitor-treated cells, it is counter intuitive to find that this is accompanied by increased levels of pShc, which would be expected to lead to activation of MAPK.

3.5 Effect of IGF-1 on its Receptor in Inhibitor Treated Human Neuroblastoma Cells.

SK-N-SH cells were treated with protease inhibitors for 72 h and then serum starved to deplete IGF-1 and then the effect of IGF-1 treatment on receptor activation examined, as described in materials and methods. After the immunoprecipitation step and blotting for total IGF-1 receptor beta subunit, the fragments of the receptor seen in early studies were absent. These fragments were discovered in the pellet that was not solubilized by the Triton-X-100 immunoprecipitation extraction buffer. Immunoblotting shows full-length receptor is solubilized by the Triton extraction buffer and is phosphorylated in response to IGF-1 treatment. Inhibitor treatment had a negligible effect on activation of the full-length receptor by IGF-1 (Fig. 3. 6). The fragments of IGF-1R were found in the Triton insoluble fraction of inhibitor treated SK-N-SH cells. Western blot analysis revealed that these fragments are phosphorylated and that IGF-1 does not enhance this phosphorylation (Fig. 3.6).

3.6 Induction of MAPK and PKB Phosphorylation by IGF-1 Treatment in Inhibitor Treated Human Neuroblastoma Cells.

Although steady-state levels of activated MAPK were reduced in inhibitor treated cells, added IGF-1 was still able to stimulate receptor activation in inhibitor-treated cells indicating that the IGF-1 signaling pathway may still be intact. SK-N-SH cells were treated with protease inhibitors for 72 h followed by serum starvation and IGF-1 treatment, as described in materials and methods. In vehicle treated cells, both MAPK and PKB were phosphorylated upon treatment with IGF-1. However, in inhibitor treated cells induction of MAPK activation was inhibited by 60% (Fig. 3.7). By contrast, phosphorylation of PKB was not significantly affected (Fig. 3.7).

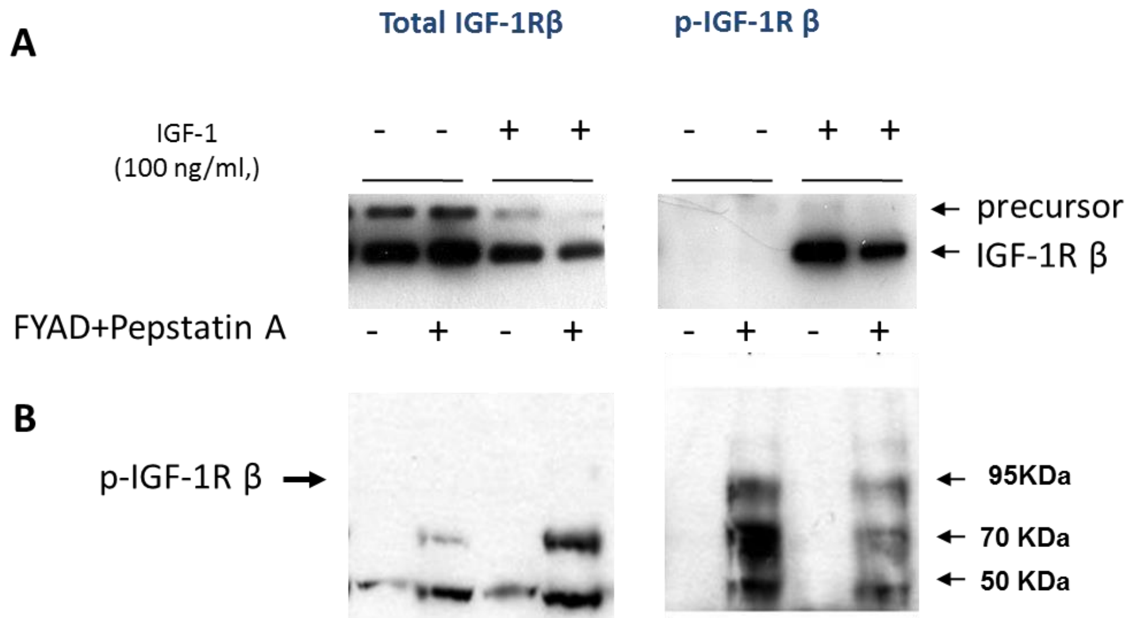


Figure 3.6 Phosphorylation of full length IGF-1 receptor and its fragments in inhibitor treated neuroblastoma cells.

SK-N-SH cells were treated with inhibitors for 72 hours, with the last 3 hours serum deprivation, then treated with IGF-1, as described in materials and methods. Cells were then harvested and subject to immunoprecipitation using anti-IGF-1R beta antibody. The immunocomplexes were then recovered and separated by SDS/PAGE analysis, followed by immunoblotting with anti-phospho-IGF-1R antibody. Phosphorylation of full length IGF-1R was not affected by protease inhibitor treatment (A). Fragments of IGF-1R were recovered from Triton insoluble fraction of cells, as described in materials and methods, followed by direct immunoblotting against total or phosho-IGF-1 receptor (B). Fragments of IGF-1R were present in inhibitor treated cells in non-inducible phosphorylated state.

3.7 Subcellular distribution of components of the IGF-1 receptor signaling pathway in inhibitor treated cells.

The discovery of fragments of the IGF1 receptor in the Triton insoluble fraction indicated that this fraction might represent the autophagic vacuole fraction of cells. This fraction was enriched for the cleaved form of LC-3, whereas the lysosomal enzyme cathepsin B was mostly solubilized by Triton (Fig. 3.8). IRS-2 and total and activated forms of MAPK were also primarily solubilized by Triton. As found for the phosphorylated fragments of the IGF1-R, phosphorylated Shc was primarily located in the Triton insoluble pellet (Fig.3.8). This was accompanied by some Shc protein although the majority of the Shc protein was solubilized by Triton.

To better understand the subcellular localization of fragments of IGF-1R, cleaved LC-3 and phospho-Shc A in inhibitor treated cells, a Percoll density gradient was used to fractionate cell components based on density, as described in materials and methods. Fourteen fractions were collected separated. The two most dense fractions were composed primarily of Percoll and sucrose, and could not be resuspended for western blot analysis. Representative blots of fractions are shown in figure 3.9. The full-length IGF-1 receptor was clearly separated from its fragments in the inhibitor treated cells, with the fragments being in the more dense fractions. LC-3 showed a similar distribution, with the uncleaved form co-localizing with the full-length receptor and the cleaved form co-localizing with the fragments of IGF1-R in the dense fractions. IRS-2 and RAS also co-localized with uncleaved LC-3 and IGF1-R in the lighter fractions, consistent with a cytosolic and/or plasma membrane location

of these proteins. There is no significant change in the location of IRS-2 or RAS in treated cells.

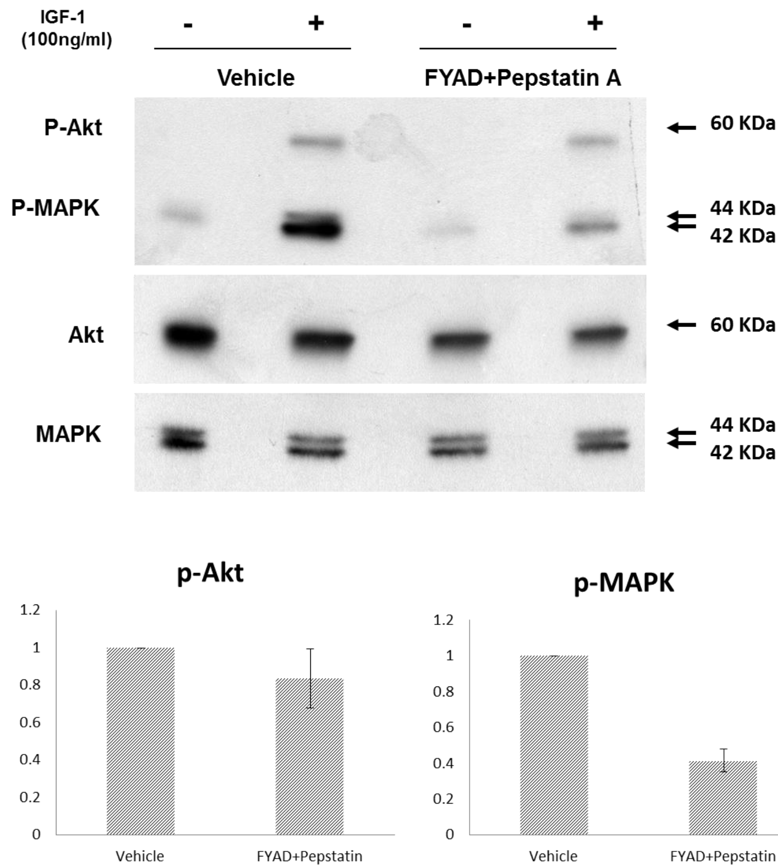


Figure 3.7 IGF-1 induced activation of MAPK and PKB in inhibitor treated SK-N-SH cells.

SK-N-SH cells were treated with protease inhibitors for 72 hours, the last 3 hours in absence of serum, and then treated with IGF-1, as described in materials and methods. Cells were harvested and subject to immunoblotting, followed by densitometry and statistical analysis. In vehicle treated cells, both MAPK and PKB were phosphorylated upon treatment with IGF-1. However in inhibitor treated cells, induction of MAPK activation was decreased to 40% ($p\text{-value}<0.001$). By contrast phosphorylation of PKB was not significantly affected.

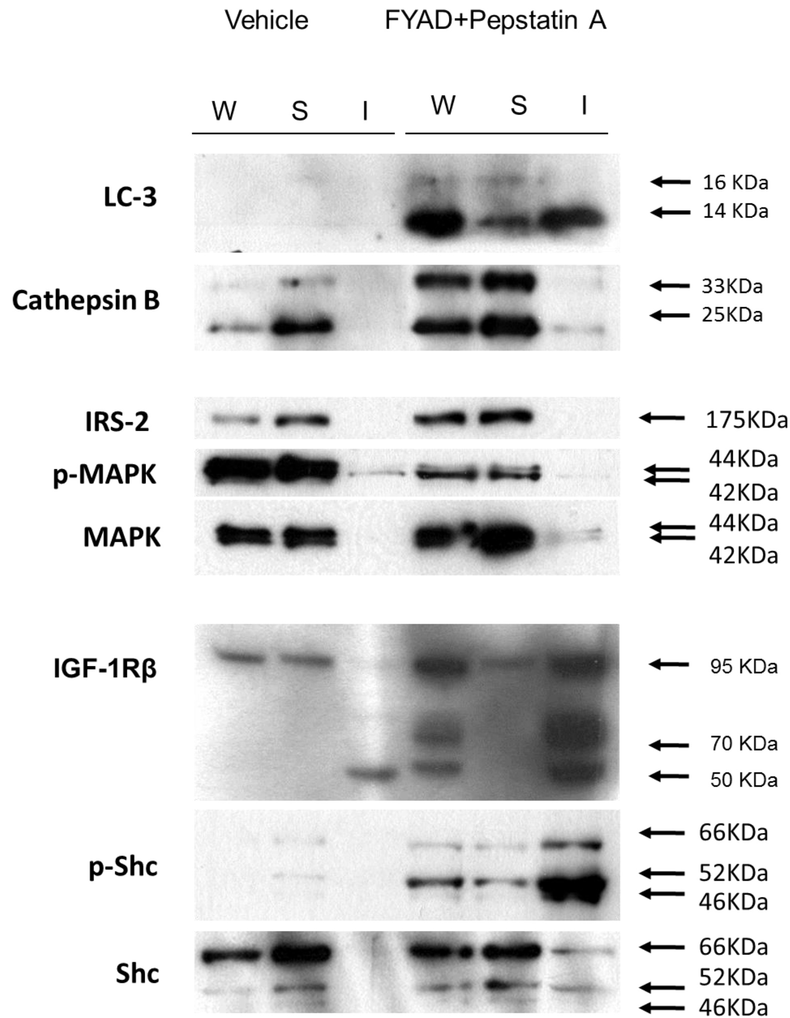


Figure 3.8 Distribution of components of the IGF-1 receptor signaling pathway in Triton soluble and insoluble fraction of neuroblastoma cells.

SK-N-SH cells were treated with protease inhibitors followed by whole cell lysate preparation (W) or Triton fractionation (S: Triton soluble. I: Triton insoluble), as described in materials and methods. The collected fractions were then subject to immunoblotting. The cleaved LC-3 was enriched in the Triton insoluble fraction, whereas the lysosomal enzyme cathepsin B was mostly solubilized by Triton. IRS-2 and total and activated forms of MAPK were also primarily solubilized by Triton. As found for the phosphorylated fragments of the IGF1-R, phosphorylated Shc was primarily located in the Triton insoluble pellet. The majority of the Shc was solubilized by Triton.

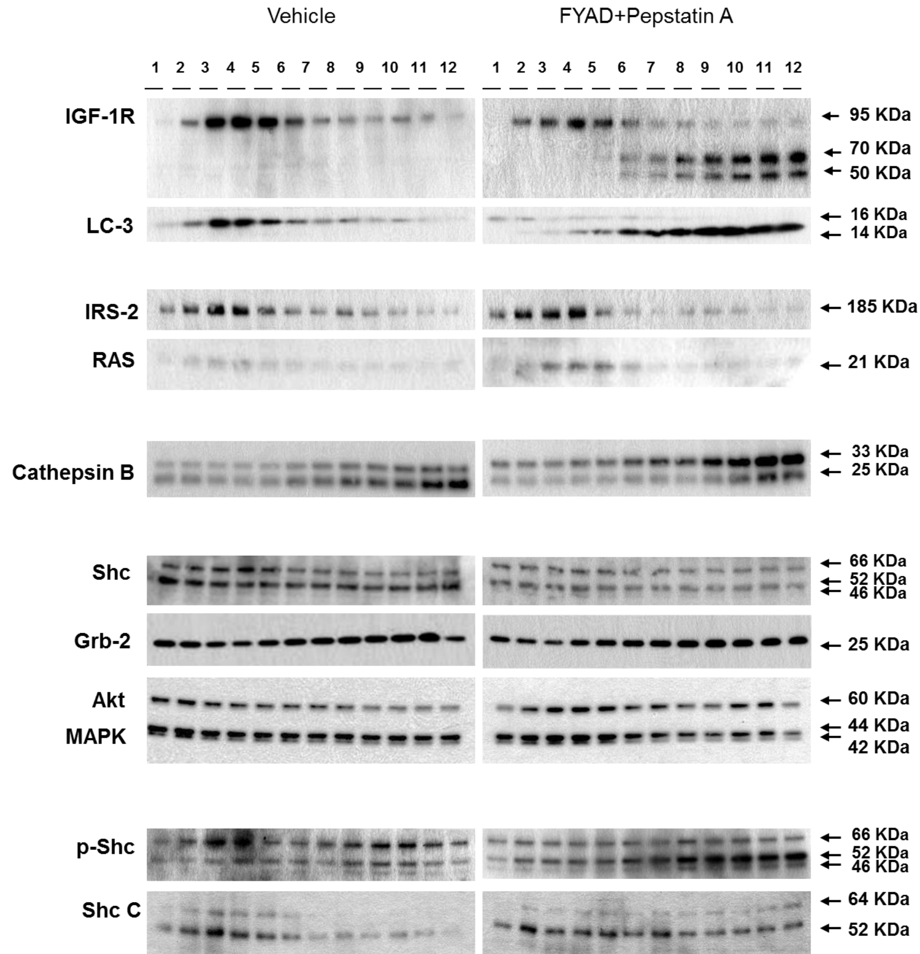


Figure 3.9 Percoll gradient density fractionation of components of IGF-1 signaling cascade in inhibitor treated neuroblastoma cells.
SK-N-SH cells were treated with protease inhibitors, then harvested and homogenized, as described in materials and methods. The homogenized cell suspensions were run on a Percoll density gradient, and 12 fractions were collected, and subject to immunoblot analysis. Uncleaved LC-3 and full-length IGF-1 receptor localize to lighter fractions of the density gradient. The fragments of the IGF-1 receptor and cleaved LC-3 localized to heavier fractions. IRS-2 and Ras localize to the same fraction as IGF-1 receptor without any change with inhibitor treatment. The lysosomal enzyme cathepsin B localizes to denser vesicles. Shc, Grb-2, PKB and MAPK are evenly distributed in the gradient. P-Shc is primarily localized to the denser fraction of inhibitor treated cells. Shc C showed a shift from lighter fractions to heavier fractions in inhibitor treated cells.

Cathepsin B was spread throughout the gradient but was more concentrated in the dense fractions, as would be expected for a lysosomal protein. In inhibitor treated cells, there is decreased concentration of cleaved cathepsin B, and a dramatic increase in the un-cleaved form which is more concentrated in the more dense fractions. Shc, Grb-2, PKB and MAPK are distributed throughout the gradient, possibly due to weak interactions with membranes of organelles with different densities. However, inhibitor treatment does not significantly alter this distribution. By contrast, the neuronal-specific adapter protein, Shc C, is primarily located with soluble/plasma membrane proteins in untreated cells, but shows a significant shift towards more dense fractions in inhibitor treated cells. Similarly, phosphorylated p52 Shc is primarily located in dense fractions in inhibitor treated cells.

Co-migration in Percoll gradients only indicates that proteins are located in subcellular sites that have similar densities and does not necessarily indicate that proteins are in the same sub-cellular compartments. To more closely examine the co-localization of LC-3 and IGF1-R we used Structured Illumination Microscopy (SIM). Cells were treated with protease inhibitors or vehicle control for 18 hours and subject to immunofluorescent labeling, as described in materials and methods. IGF-1 receptor showed endoplasmic reticulum and Golgi like distribution pattern in both treated and untreated groups (Fig. 3.10.). LC-3 showed low intensity, diffuse staining in the cytosol and a few very small granules in the vehicle treated cells (Fig. 3.10.). However, in response to treatment, a great accumulation of several, large LC-3 positive granules were observed. Sequential Z sectioning as well as 3D reconstruction

was used to determine the relative localization of LC-3 and IGF-1 receptor. The level of resolution of this instrumentation allowed us to demonstrate images where IGF-1R is enclosed within LC-3 positive vesicles in the inhibitor treated cells (Fig. 3.10). This phenomena was not observed in vehicle treated cells, where large, LC-3 positive vesicles are not present (Fig. 10).

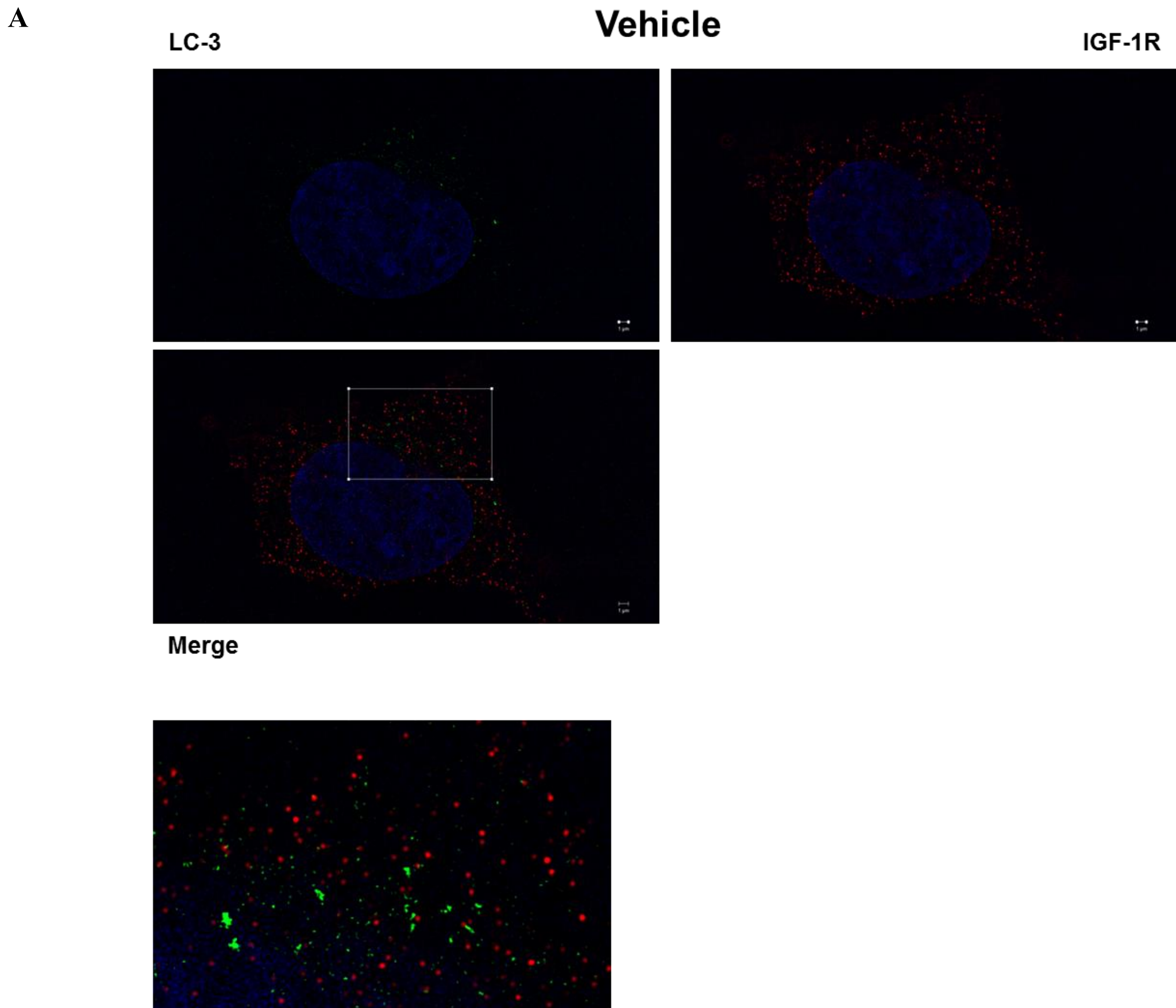


Figure 3.10 Localization of IGF-1 receptor and LC-3 in neuroblastoma cell lines.

SK-N-SH cells were treated with the vehicle control (A) or FYAD and Pepstatin A (B) following by immunefluorescent staining against IGF-1 receptor (red) and LC-3B (green) and SIM imaging, as described in materials and methods. IGF-1 receptor showed a punctate staining pattern in both vehicle and inhibitor treated cells. The vehicle treated cells showed diffuse cytosolic pattern for LC-3, with few small granules (box) (A).

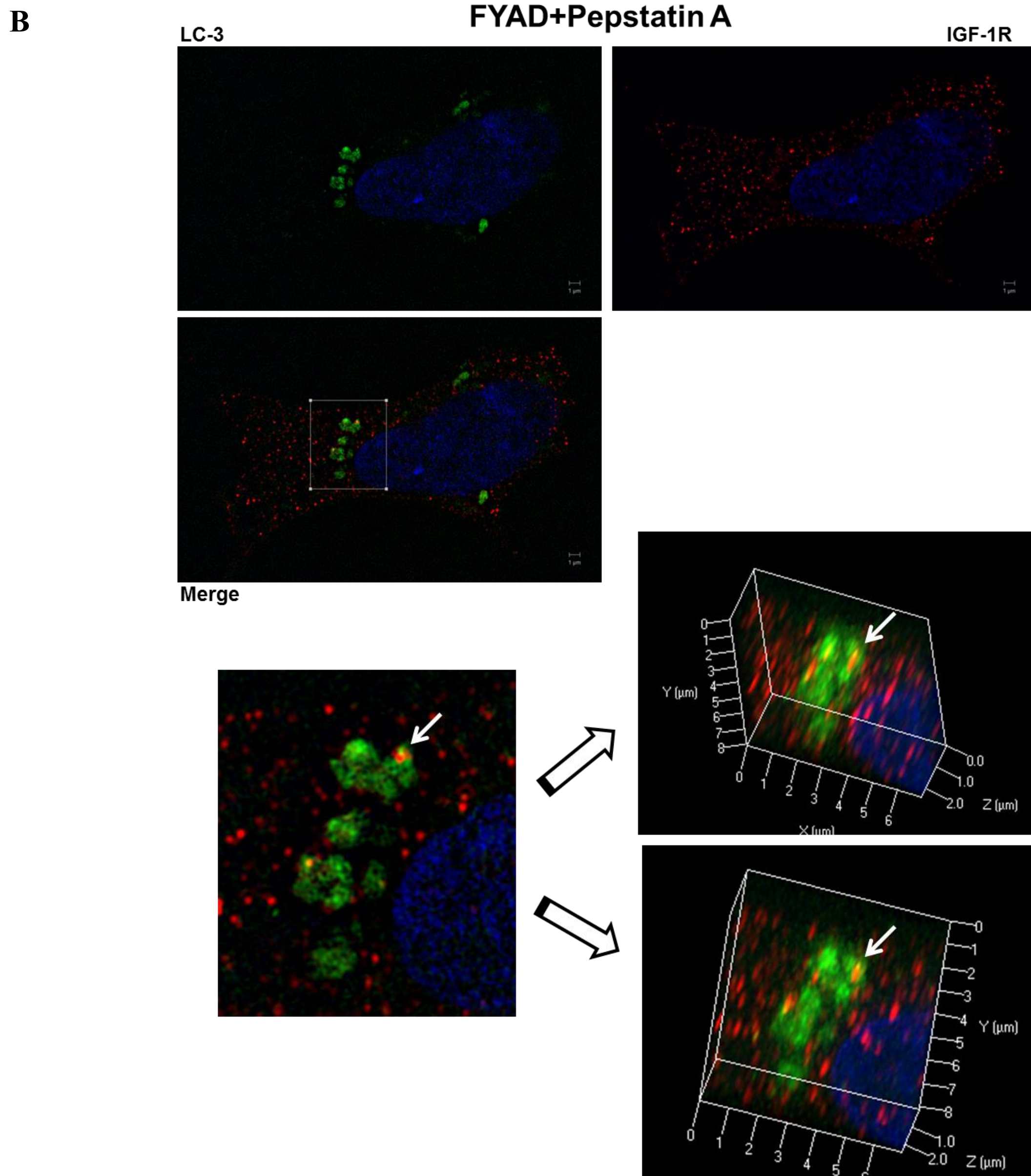


Figure 3.10 (continued) Localization of IGF-1 receptor and LC-3 in inhibitor treated neuroblastoma cell lines.

SK-N-SH cells were treated with FYAD and Pepstatin A (B) following by immunefluorescent staining against IGF-1 receptor (red) and LC-3B (green) and SIM imaging. Several large LC-3 granules appeared in inhibitor treated cells. Three-dimensional modeling revealed sequestration of some IGF-1 receptor in LC-3 positive vesicles (box and 3 dimensional analysis).

Chapter 4

DISCUSSION

4.1 Lysosomal Processing of the IGF-1 Receptor

In previous studies we have shown that inhibition of lysosomal proteases cathepsins B, L and D induces selective apoptosis of neuroblastoma cells without affecting normal or other cancer cells lines. Induction of apoptosis was preceded by accumulation of autophagic vesicles within inhibitor treated cells, suggestive of a role for autophagy in inducing cell death.

In this study, I discovered that two fragments of the IGF-1 receptor β chain accumulate in inhibitor treated cells (Fig 3.2). This indicates that these fragments are substrates for cathepsins B, L and D and that other enzymes, yet to be identified, process the IGF-1 receptor prior to complete digestion by these lysosomal proteases. Pepstatin A alone does not induce appearance of any fragments. A lighter 50 KDa fragment appears after cells are treated with FYAD alone, while an additional higher molecular weight fragment appears with combination treatment of both FYAD and Pepstatin A. This indicates that cathepsin D is not essential for the turnover of the IGF-1 receptor, but can process the beta subunit to a 50 KDa fragment in the absence of cathepsin B and L activity. The molecular weight of the 50 KDa fragment corresponds to cytosolic and transmembrane region of the receptor. It has been proposed that the proteosome is responsible for turnover of the IGF-1 receptor [Carelli

et al.2006, Girnita et al. 2003], but in this study I found no evidence of higher molecular weight ubiquitinated forms of the receptor. The cleavage site that gives the 70 KDa fragment corresponds to a region in the extracellular domain of the receptor, indicating that the initial processing is by either another lysosomal protease or a cell surface/extracellular protease as this domain is not accessible to the proteosome. These results emphasize the important role of lysosomes in degradation of IGF-1 receptor in neuroblastoma cell lines.

In this study I have focused on the role of cathepsins in regulation of IGF-1 signaling and autophagy in neuroblastoma cells. IGF-1 signaling has well established roles in cancer. The IGF-1 receptor is over-expressed in many cancer cell lines and tumor specimens, and IGF-1 signaling enhances survival and stimulates proliferation of cancer cells [reviewed in Pollak et al. 2008 and 2012]. IGF-1 signaling also has important roles in several stages of development and function nervous system, including growth, proliferation, and differentiation of neuronal cells. Although the IGF-1 receptor is expressed in most neuronal tissues, it is expressed at lower levels in the mature nervous system when proliferation is minimal [reviewed in Torres 2012, O'Kusky et al. 2012]. In neuroblastoma, IGF-1 receptor is usually expressed at high levels, and has an important regulatory role in proliferation and survival of the tumor cells [Martin et al 1992, and 1993, Singleton et al. 1996].

IGF-1 exerts its biological functions by activating downstream pathways first by binding to and activating IGF-1 receptor, then recruiting and activating adaptor proteins Shc and IRS to the activated, phosphorylated receptor, resulting in further

recruitment and activation of downstream cascades including effector proteins MAPK and PKB (Fig. 1.3) and alteration in gene and protein expression and activity ending in stimulation of survival and proliferation.

I discovered that p-Shc A and p-IGF1R fragments accumulate within LC-3 positive autophagic vesicles of inhibitor treated cells (Fig. 3.9, 3.10). This sequestration was accompanied by attenuation of MAPK activation by IGF-1 treatment (Fig 3.7) The sequestration of p-Shc A (and potentially more importantly p-ShcC) uncouples IGF-1 signaling from downstream cascades, resulting in the inability of IGF-1 signaling to induce growth and survival promoting effects for neuroblastoma cells.

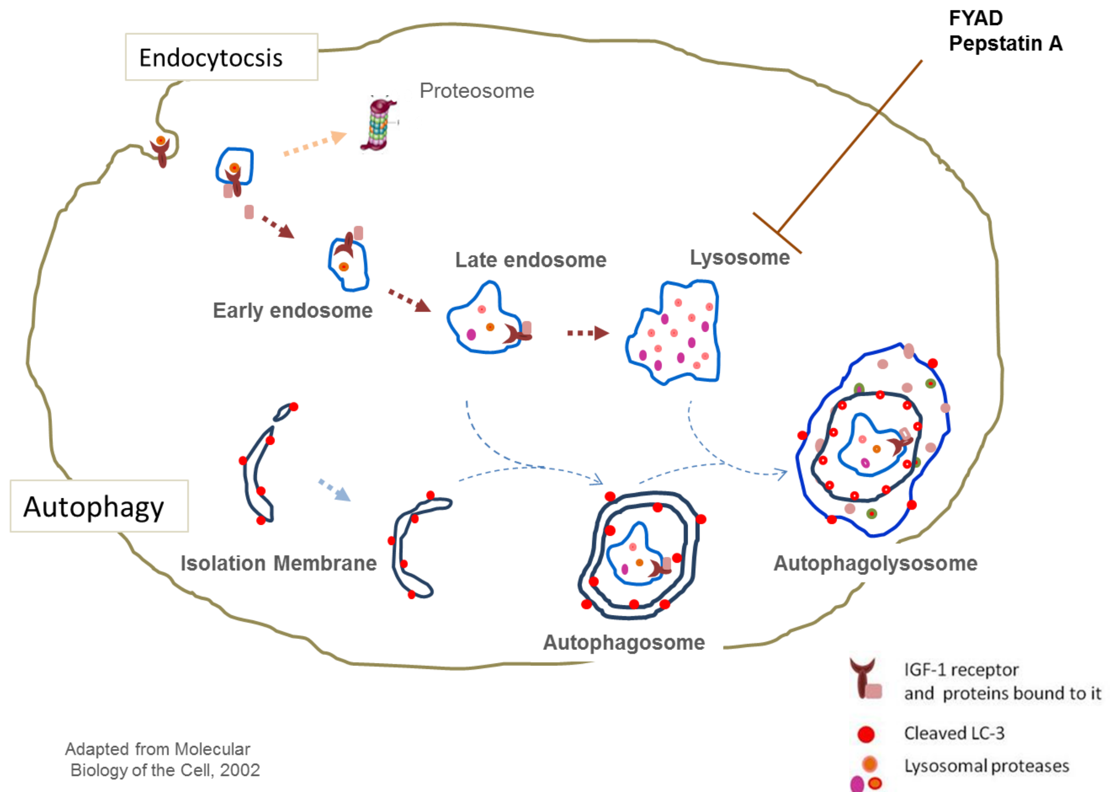


Figure 4.1 Regulation of IGF-1 signaling by autophagy in neuroblastoma cells.

Activated IGF-1 receptor is internalized into endosomes. If endosomes fuse with lysosomes, the C-terminal portion of the receptor will remain on the cytosolic face of the lysosomes and not be accessible to the cathepsins. However, if the endosome is sequestered in an autophagosome, receptor (and Shc) will be removed from the cytosol to block downstream cell signaling. Fusion of the autophagosome with a lysosome will allow the formation of an autophagolysosome and, after lipase-mediated dissolution of the inner membrane, the C-terminal portion of the receptor can be degraded by the cathepsins. The experimental data presented in this work support the autophagic mechanism of degradation of the IGF1 receptor and Shc.

4.2 Biology of Shc proteins and Their Role in Neuroblastoma

During the course of my investigations, I discovered a unique accumulation of Shc proteins in inhibitor treated cells. This unexpected observation requires a more careful discussion of the role of these proteins.

Shc A [Src homolog or collagen homolog] (also known as Shc) is an adaptor protein first discovered in 1992 [Pelicci et al, 1992]. The Shc A gene on chromosome 1q21 encodes 3 products, p66, p52, and p46. P66 is produced by alternative splicing, and p52, and p46 differ in initiation of translation site [Pelicci et al. 1992, Segatto et al. 1993, Ravichandran 2001].

4.2.1 Structure and function of Shc protein isoforms

All three isoforms contain SH2 [Src homolog], CH1 [collagen homolog] and PTB [phosphotyrosine binding] domains. The SH2 and PTB domains recognize and bind to the phosphotyrosine residues in other proteins, while the CH1 domain contains the tyrosine residues that get phosphorylated and recognized by SH2 domains in adaptor protein Grb-2 [Ravichandran et al. 2001]. In spite of structural similarities, the different Shc proteins have different functions. When Shc p66 is phosphorylated it binds to Grb-2, but does not activate Ras or downstream effectors [Bonfini et al. 1996, Pinton et al. 2008]. The primary role of p66 is in regulation of oxidative stress [Stevenson et al. 1998, Orsini et al. 2006] and longevity. Cytosolic p66 translocates to the mitochondria upon oxidative stress [Orsini et al. 2006] where it binds to cytochrome C and acts as an oxidoreductase to shuttle electrons from cytochrome C to

oxygen and produce reactive oxygen species [Gorgio et al, 2005]. My results are consistent with p66 not having a role in IGF1 signaling as, unlike p52, it does not accumulate in dense organelles of inhibitor treated cells in a phosphorylated form.

Both p52 and p46 get phosphorylated upon growth factor stimulation; however, the efficiency of phosphorylation varies depending on cell type and growth factor [Segatto et al. 1993, Okada et al. 1995, Ravichandran 2001]. Due to sequence differences in the PTB domain, p46 is thought to be less efficient than p52 in binding to phosphoproteins and consequently is less likely to play a major role in signaling [Trub et al, 1995, Ravichandran 2001]. p46 is translocated to the mitochondrial matrix by a mitochondrial translocation signaling motif that is not present in p52 or p66 Shc [Ventura et al, 2004]. p52 is the primary form of Shc that activates Grb2, Ras and other downstream effector molecules, and is consequently the critical Shc isoforms that promotes cell proliferation.

4.2.2 Other mammalian homologs of Shc may play unique roles in the nervous system

Shc A was examined in detail in this study because it is well established as an IGF-1 adapter protein and consequently best suited to explore the role of these proteins in cathepsin inhibitor induced death of neuroblastoma cells. Mammals have two closely related proteins, Shc B [Sli/SCK] and Shc C[Rai/N-Shc] [O'Bryan et al. 1996, Pelicci et al. 1996, Nakamura et al. 1998] that may be more relevant to neuronal cell death. Shc A is ubiquitously expressed in most organs except the adult nervous

system, whereas Shc B and Shc C are restricted to the neuronal system [Sakai et al. 2000]. In the nervous system, Shc A expression is most significant during development and dramatically decreases at birth, whereas the expression pattern of Shc C follows the opposite of this pattern [Sakai et al. 2000], indicating different roles of the two proteins in development and function of nervous system. Studies in PC-12 cells have shown that Shc C has a lower affinity for Grb-2, and is less efficient in Ras dependent MAPK activation [Nakamura et al. 2002]. Thus in the mature nervous system, IGF-1 signaling may be uncoupled from downstream cell survival and proliferation signals and may play other roles. Nevertheless, Shc C mRNA levels are elevated in most neuroblastoma cell lines, especially those with N-myc or ALK amplification, and are also elevated in tumor samples from patients with very poor prognosis [Terui et al. 2005, Miyake et al. 2009]. High levels of phosphorylated Shc C [but not IRS-2 or PLC-gamma], have been observed in stable complexes with ALK in neuroblastoma cell lines and tissue samples with ALK amplification [Miyake et al. 2002]. Despite its reduced efficiency in Ras dependent MAPK activation, the role of Shc C in survival, proliferation, and motility of neuroblastoma is dependent on its ability to bind to Grb-2 and activate MAPK and PKB [Miyake et al. 2005, Pelicci et al. 2002]. My results show that cathepsin inhibitor treatment causes a shift of Shc C from a low density cytosolic location to a more dense autophagic location (Fig 3.9). This shift was more pronounced than seen for Shc A. I was not able to determine whether Shc C was phosphorylated due to lack of phospho-Shc C specific antibody. Shc C immunoprecipitation followed by phosphoprotein blotting was also not possible

due to the insolubility of the autophagic fraction. However, by analogy to results with Shc A, it is likely that the sequestered forms are phosphorylated.

4.2.3 Significance of targeting Shc proteins in neuroblastoma

Shc proteins act as adaptor proteins for several receptor tyrosine kinases, and consequently restricting activated Shc function can prevent signaling from multiple receptors simultaneously. One of the major drawbacks of therapeutic strategies to inhibit signaling from a single receptor tyrosine kinase is the induction of activation of other receptor tyrosine kinases as a compensatory mechanism to promote continued tumor cell growth. Targeting of Shc would block function of multiple receptors and consequently may prevent these compensatory effects and provide a better therapeutic approach for cancer treatment.

Although Shc plays a pivotal role in cell signaling, it has not been examined as a target for therapeutic interventions because strategies for targeting intracellular binding proteins are very challenging. Nevertheless, interventions that selectively interfere with the function of Shc, especially Shc C, may be highly efficient in selectively targeting neuroblastoma cells without affecting other rapidly dividing cells within a child's body. This should produce fewer side effects than current therapies that affect all rapidly dividing cells. Targeting Shc C may be even more beneficial to patients with N-MYC and ALK amplifications as prognosis for these patients is very poor.

4.2.4 Inhibition of cathepsins results in selective sequestration of p-Shc A and prevention of its function

By specifically inhibiting cathepsins B, L and D I was able to induce sequestration and accumulation of activated p-Shc A within autophagic vesicles of inhibitor treated neuroblastoma cells. Furthermore, as discussed in 4.2.2, ShcC also shifts toward these vesicles. The sequestration of Shc was confirmed by Triton fractionation (Fig. 3. 8) and Percoll gradient separation (Fig. 3. 9.). P-Shc A did not co-segregate with full length IGF-1 receptor (Fig. 3. 9.), but co-localized with fragments of IGF-1R, and cleaved LC-3, supportive of its localization within autophagic vesicles. Within these dense multilayered vesicles, p-Shc A and fragments of IGF-1R and are disconnected from the downstream signaling cascade. They are protected from the action of cytosolic phosphatases and cannot activate the downstream effecter protein MAPK, thereby preventing growth promoting functions of IGF-1 receptor. Although not all of the Shc was sequestered within autophagic vacuoles, downstream activation of MAPK was significantly impaired. The activation of PKB was less affected, consistent with the observation that cathepsin inhibition did not result in sequestration of IRS-2 or block phosphorylation of the intact IGF-1 receptor. However, it should be noted that suitable antibodies for detection of phospo-IRS-2 are not available. Therefore, it is formally possible p-IRS-2 is present in the insoluble autophagic fraction.

4.3 IGF-1 Signaling is an Important Target for Developing Novel Cancer Therapeutics

Due to its proposed critical role in cancer progression, the IGF-1 receptor has been proposed for developing targeted therapies to treat cancer. A range of antibodies have been developed to specifically inhibit the function of the IGF-1 receptor. Although they vary in their IgG subtype and half life in the circulation, all of them bind to the ligand binding domain of the receptor and prevent its normal function. Treating patients with these antibodies may be associated with increased risk of hyperinsulinemia and hyperglycemia and high levels of circulating IGF-1 due to the body's response to decreased IGF-1 function [reviewed in Pollak et al. 2008, Gualberto, et al. 2009]. IGF-1 also has an important role in regulating the physiology of pancreatic beta cells, where reduced function would result in hyperglycemia [Kulkarni et al.2002]. Due to the inability of the antibodies to penetrate through blood brain barrier, no serious concern exists regarding potential side effects on the central nervous system.

Initial phase I and II clinical trials and pre-clinical animal studies with several antibodies against IGF-1 receptor were promising, showing low toxicity, good tolerance, and improved patient outcome [reviewed in Pollak 2008, Gualberto et al. 2009]. Phase II and III clinical trials are still in progress, and although some phase II trials show little toxicity with good responses [Olmos et al. 2010, Atzori et al. 2011,] several phase III trials have failed due to lack of efficacy and or associated with significant metabolic toxicity, mainly hyperglycemia [Yee et al. 2012, Pollak et al.

2012, also see clinical www.trials.gov]. Despite these setbacks, the promising data from preclinical and phase I and II clinical trials emphasize the importance of IGF-1 receptor as a target for cancer therapy. Further understanding into predictive biomarkers, resistance mechanisms, and combination therapy options, may provide better insight to subsets of patients and therapy designs that would benefit from targeting IGF-1 receptor.

An alternative approach to blocking IGF-1 receptor signaling is to inhibit the tyrosine kinase activity of the receptor. Despite concerns about the effect of such inhibitors on the tyrosine kinases of other receptors, especially the insulin receptor [Pollak 2008, Garcia et al. 2004, Haluska et al. 2006, Ji et al. 2007], recent preclinical and clinical trials suggest that these agents are less toxic than previously thought [Pollak 2012, Dool et al. 2011]. These promising results with tyrosine kinase inhibitors indicate that despite the potential for side effects, targeting more than one receptor tyrosine kinases may be therapeutically advantageous.

Antibody or small molecule inhibitors of the IGF1 receptor appear to be effective against neuroblastoma, both *in vitro* and *in vivo* [You et al. 2013, Kolb et al. 2010, Geoerger et al. 2010, Houghton et al. 2010, Kolb et al. 2008, Huang et al. 2009, Maloney et al. 2003]. Studies have not yet progressed to clinical trials in children.

The limitations of current therapies to target IGF-1 signaling include down-regulation of all effects of the receptor and potential development or resistance by activation of alternative signaling pathways. My results show that cathepsin inhibition can eliminate or diminish both of these limitations by selectively blocking Shc-

mediated signaling while affecting such signaling from a range of receptors that transduce signals via Shc.

4.4 Inhibiting Autophagy Induces Neuroblastoma Cell Death

Neuroblastoma arises from neuroblastic cells that usually mature to neuronal cells, so both cancer cells and mature neurons share similarities in homeostatic pathways. Mature neuronal cells have long dendrites and axons and high volumes of cytoplasm. Accumulation of damaged proteins and organelles can ultimately lead to neuronal cell death because, unlike proliferating cells, mature neuronal cells cannot rely on dilution of toxic compounds by cell division. Mutations that lead to inactive lysosomal proteins that are involved in lysosomal function and autophagy pathways have severe effects in brain and neuronal cells, and can lead to neurodegenerative disorders, such as Alzhiemers [reviewed in Nixon et al. 2013]. Such sensitivity might argue against treatments that target the lysosomal or autophagic pathway due to concerns of neurotoxicity. However, toxic effects can take many years to develop and consequently neurotoxicity may not be an issue during the timeframe of a therapeutic regimen that targets these pathways. Neuroblastoma cancer cells are much more sensitive to cathepsin inhibition than other cells, indicating that protein turnover and autophagy may be particularly important for proliferating cells of neuronal origin. Thus, targeting lysosomal function may be an efficient way to help eradicate neuroblastoma.

4.4.1 Inhibition of autophagy prevents cancer cell dormancy

Several links exist between autophagy and cancer cell dormancy. Autophagy is activated in response to starvation or growth factor deprivation. It regulates cell size and growth and enables cells to recycle their damaged proteins and organelles to reuse them as bioenergetic resources. This is particularly important in established tumors, with poor vascularization and hypoxic microenvironment which is deficient in nutrients and growth factors, and stress induced by chemo agent and radiation. The similarities between cancer cell dormancy and quiescent stages of the yeast and *C. elegans* life cycle raised the possibility of similarity in molecular mechanism involved in these processes. In the context of cancer, the autophagy molecular pathway crosstalks with several pathways that are involved in dormancy, e.g. lack of mitogenic signaling, blockade of integrin signaling and trail induced apoptosis [reviewed in Ghiso et al. 2013]. A few recent studies have taken a closer look at the potential role of autophagy in cancer cell dormancy. A series of studies focused on ARHI (ras homologue member), a protein that plays an important role in induction of autophagy, angiogenesis and cell migration [Lu et al. 2013, Lyu et al. 2013]. It is down regulated in about 60% of ovarian cancers. Over expression in ovarian cancer cells resulted in tumor regression and formation of dormant tumors [Lu et al. 2008, Amaravadi 2008]. These tumors where able to re-grow upon termination of ARHI over expression. Treating ARHI over expressing tumors with lysosomal inhibitor hydroxychloroquine reduced the re-growth of tumor, suggestive of an important role of autophagy in survival of cancer cells.

Another study treating gastrointestinal stromal tumor (GIST) cells [Gupta et al. 2010] with imitanib, a tyrosine kinase inhibitor, kept tumor cells in a quiescent non-proliferative state, but did not cause tumor cell death. The tumors reverted back to their proliferative state upon withdrawal of imitanib. This study showed that imitanib induced autophagy in treated cells, both *in vivo* and *in vitro*. Knockdown of Atg genes or inhibition of lysosomal function with chloroquine induced death of dormant cells, showing that induction of autophagy was essential for cells to enter and stay in a dormant state. Thus, co-treatment of tumors with anti-malarial drugs such as chloroquine may sensitize tumors to anticancer drugs.

The inhibitors used in my study specifically inhibit function of cathepsins B, L and D. They do not bind to any other known molecule within the cells [Colella et al. 2010]. Unlike chloroquine and its derivatives, the cathepsin inhibitors have very specific targets, and potentially will have fewer side effects in the clinic. Cathepsin gene knockout and inhibition studies indicate that inhibitors will selectivity target rapidly proliferating cells of neuronal origin and have little effect on other cells and tissues [Felbor et al. 2002, Stypman et al. 2002,Reinhekel et al. 2001, Roth et al. 2000,]. FYAD and pepstatin block the digestion of the content of autophagosomes by lysosomal enzymes. This results in accumulation of substrates of the lysosomal digestion pathway within double layered membranes of autophagic vesicles. Discovering the accumulation of phosphorylated fragments of IGF-1 receptor further demonstrates that this receptor is degraded through the lysosomal pathway in neuroblastoma cell lines. p-Shc A was also concentrated in autophagic vesicles. IRS-2,

the other key adaptor protein that binds to activated IGF-1R, did not show a similar co-localization pattern, suggestive of a possible selective mechanism for turnover of p-Shc A.

Simultaneous inhibition of IGF-1 signaling and possibly other RTKs, to block autophagy vesicle turnover, and prevent formation of cancer dormant cells, would provide a powerful synergistic approach to selectively eradicate neuroblastoma tumors with minimal effects on other normal tissues within a child's body. My results have shown that cathepsin inhibition can affect all of these key pathways and consequently may be a valuable therapy alone or in combination with other therapies that target one or more of these pathways.

4.4.2 Combination of chemotherapy with autophagy inhibitors

There is renewed interest in pursuing therapeutic approaches that target autophagy in combination with conventional chemotherapy for several cancer types. Several recent studies show anti-tumor activity for inhibitors of autophagy or lysosome function, as single agents or in combination with chemotherapeutic agents. When used in combination with chemotherapeutic agents, they show a synergistically higher level of cancer cell death and a much lower recurrence rate [Rosenfeld et al. 2014, Rangwala et al. 2014, Mahalingam et al. 2014, Ji et al. 2014, Liu et al. 2014, Bokobza et al. 2014, Selvakumaran et al. 2013, Donohue et al. 2013, Shen et al. 2013, Wu et al. 2013, Chang et al. 2013, Seitz, et al. 2013, McAfee et al. 2012, Carew et al. 2011 and 2012, Amaravadi et al. 2007 and 2009]. The same trend is observed in

autophagy deficient, Atg knockout cell lines and mouse models, confirming inhibition of autophagy as the mechanism that leads to enhanced cytotoxicity [Wei et al. 2011 and 2013].

Pre-clinical trials are ongoing to determine whether therapies combined with autophagy inhibitors such as 3-methyladenine (3-MA) (autophagy inhibitor through class III PI3K), bafilomysin A1 (vacuolar ATPase inhibitor), monensin (lysosomal pH disruptor), and pepstatin A (cathepsin D inhibitor), can improve efficacy of chemotherapies [reviewed in Cheong et al. 2012]. Chloroquine and its hydroxylated derivative hydroxychloroquine are in clinical trial in combination with traditional chemotherapies [Nguyen et al. 2014, Racoma et al. 2013, Seitz et al. 2013, Verschooten et al. 2012, Geng et al. 2010]. These weak bases diffuse into the lysosome, get protonated in the acidic environment, become entrapped and accumulate. This raises the pH and decreases lysosomal function [Poole et al. 1981]. Both compounds are currently used as anti-malarial drugs and therefore have known toxicity profiles. Trial results show that they enhance toxicity of chemotherapeutic agents [Macintosh et al. 2013, Shen et al. 2013, , Donohue et al. 2013, Shimizu et al. 2012, Carew et al. 2011 and 2012, McAfee et al. 2012, Amaravadi 2007]. Similar but more potent derivatives of chloroquine have been synthesized and tested in pre-clinical studies, emphasizing importance of using more potent lysosomal inhibitors [Amaravadi et al. 2012]. All of the lysosomal inhibitors mentioned above have broad non-specific effects on other cellular functions that rely on pH differences in cellular compartments, including receptor signaling, protein trafficking and intracellular

organelle sorting. In our studies we only target the proteases so would expect limited side effects. Furthermore, unpublished *in vivo* data from our lab (Fig. 8S) shows FYAD has does not cross blood-brain barrier and has minimal effects on the central nervous system.

4.5 Conclusions

In this investigation I examined the IGF-1 signaling pathway as a model to study the role of autophagy in regulation of receptor tyrosine kinase signaling. It was revealed that inhibition of turnover of autophagic vesicles results in a dramatic decrease in activation of Shc mediated signaling from the IGF-1 receptor, blocking the growth promoting effects of IGF-1.

From my work, it is clear that the cytoplasmic tail of the IGF-1 receptor is degraded in autophagic vacuoles by lysosomal proteases. When these proteases are blocked, the cytoplasmic portion of the receptor accumulates as a phosphorylated form, indication that sequestration in autophagic vacuoles and degradation by lysosomal proteases is a key mechanism for down-regulation of IGF-1 signaling. Thus, lysosomes are the point of crosstalk between autophagy and receptor tyrosine kinase pathways.

My results show that autophagy regulates the IGF-1 receptor signaling cascade by sequestration of activated internalized receptor and the active adaptor protein Shc within autophagic vesicles, thereby limiting their access to downstream cytosolic components of the cascade. Since autophagy is a global mechanism, and Shc is a key

transducer of signaling of many receptor tyrosine kinases, it is anticipated that a similar effect will be observed for other receptor tyrosine kinases.

Cathepsin inhibition did not affect IRS-2 or signaling through PKB, indicating that treatment of cancers with these inhibitors may not affect the anti-apoptotic effects of IGF-1 signaling that protect non-proliferating cells.

My studies provide the first model system to show a direct interaction and regulation between IGF-1 receptor signaling and autophagy. IGF-1 signaling has a well established role in cancer and autophagy has a well established role in neuronal cells and the results of this study highlight the importance of simultaneously targeting both pathways to enhance efficiency of cancer treatments, as well as inhibiting dormancy and relapse of cancer.

4.6 Future Directions

My studies have uncovered a mechanism by which autophagy is linked to receptor tyrosine kinase signaling. While the model system has focused on the IGF-1 signaling pathway, other receptor tyrosine kinase pathways are likely to be more relevant to uncontrolled growth of neuroblastoma. The neuronal specific Shc C appears to be more severely affected and other receptor kinases that have specific roles in neuroblastoma such as ALK and TrkB may be key targets that result in the enhanced sensitivity of neuroblastoma to cathepsin inhibition. As suitably sensitive reagents become available to study trafficking and activation of these receptors, the key roles of these neuronal-specific proteins will be revealed.

It is likely that other tumors derived from neuronal progenitor cells such as glioblastoma and even melanoma will be sensitive to cathepsin inhibition, increasing the potential impact of any drugs developed to treat cancers. A major challenge to developing new treatments for cancers of childhood is the limited numbers of patients relative to the more common adult cancers. Prospects for developing cathepsin inhibitors are enhanced by studies of other diseases, especially osteoporosis and Chagas disease, that also target cathepsins. If these programs targeting larger patient populations are successful, it may be possible to translate the results to improvement of treatments for neuroblastoma.

A major challenge in developing treatments that target the lysosomal proteases is that enzyme concentrations are very high in lysosomes. Thus, *in vivo* efficiency of enzyme inhibition will need to be optimized. While 100% enzyme inhibition is easily achieved in cell culture, this is not likely *in vivo*. The identification of the consequences of cathepsin inhibition found in this study will help guide combination therapies that will improve *in vivo* efficacy of neuroblastoma treatment.

Chapter 5

SUPPLEMENTAL DATA

5.1 Expression of IRS-1 and IRS-2 in Cancer Cell Lines.

Expression of IRS-1 and 2 is variable among different cell lines. SK-N-SH and two control cell lines - a prostate cancer cell line, PC-3, and a breast cancer cell line, MCF-7, were examined for expression of IRS-1 and 2. MCF-7 cells express detectable levels of both IRS-1 and 2. SK-N-SH and PC-3 cells only express IRS-2. As expression of IRS-2 is robust in SK-N-SH cells, this adapter protein was used for further examinations in this project (Fig.5.1).

5.2 Effect of Temperature on Triton Solubility of Fragments of IGF-1 Receptor.

Ice cold buffer systems containing Triton-X-100 have been used to extract lipid rafts and cell surface receptors associated with them [Radeva et al. 2004]. These lipid rafts are soluble in detergents at room temperature. To determine whether temperature affects solubility of IGF-1 receptor in Triton X-100, I performed the extraction protocol both on ice and at room temperature. The fragments of IGF-1 receptor and a portion of full length receptor were not soluble in the Triton X-100 extraction buffer at either temperature, indicating that the fragments are probably not

in lipid rafts (Fig.5.2). Furthermore, the Triton insoluble fraction did not float, but precipitated, further supporting a location different from a lipid raft.

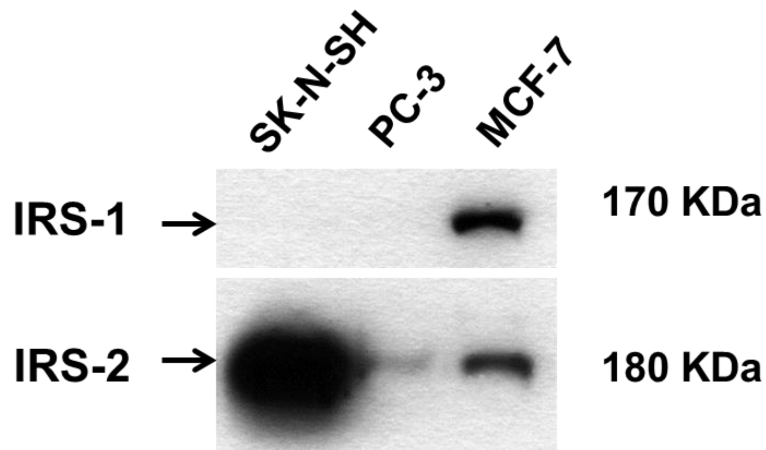


Figure 5.1 Expression of IRS-1, 2 in cancer cells lines.
SK-N-SH, PC-3 and MCF-7 cells were maintained in normal, untreated conditions. Equal amounts of cell protein (10 µg) were loaded into each lane and separated by SDS/PAGE prior to immunoblotting. Both IRS-2 and IRS-1 were detected in MCF-7 cells. The neuroblastoma cell line SK-N-SH express large amounts of IRS-2, but no IRS-1.

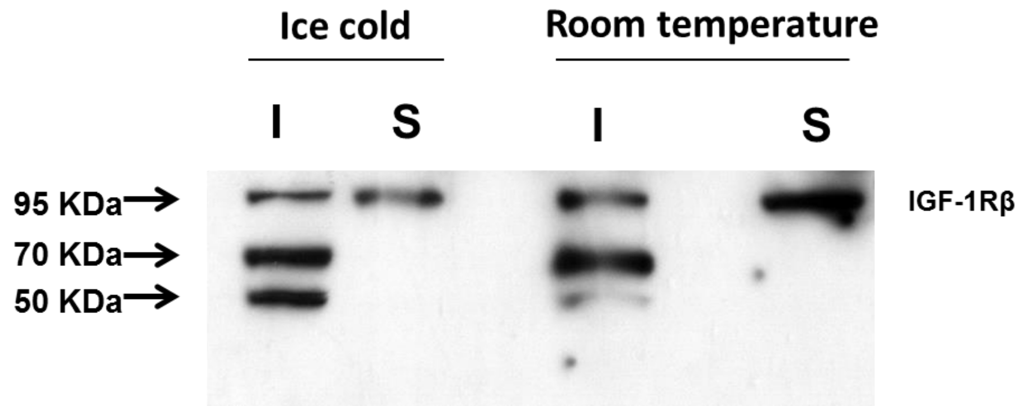


Figure 5.2 Solubiliztaion of IGF-1 receptor and its fragments in the cold and at room temperature inTriton lysis buffer.
SK-N-SH cells were treated with inhibitors for 72 hours followed by Triton extraction on ice or at room temperature. Triton soluble (S) and insoluble (I) fractions were extracted as described in materials and methods. No difference was observed between the two temperatures. At both temperatures fragments of IGF-1 receptor and a portion of the full length receptor localized to the Triton insoluble fraction.

5.3 Effect of Protease Inhibition on Other Receptor Tyrosine Kinases in Neuroblastoma Cells.

Lysosomes are a major degradation path for many receptor tyrosine kinases. While my primary focus was on the effect of protease inhibition on the IGF-1 signaling pathway, I considered that other pathways might also be affected that lead to death of cancer cells and neuroblastoma.

I examined the effect of protease inhibition on the EGF receptor, as it has an established role in several cancers. SK-N-SH and IMR-32 cells were treated with inhibitors for 72 hours followed by immunoblot analysis using an antibody against the C-terminal end of the receptor. The level of full length EGF receptor showed a slight but significant increase in both cell lines (Fig.5.3). Over exposure of the blots of SK-N-SH cells showed that several fragments of EGF receptor appear in inhibitor treated cells, but at a much lower level than full length receptor (Fig.5.3). Some of the receptor also accumulates in the Triton insoluble fraction, consistent with sequestration in autophagosomes, as shown for the IGF-1 receptor. Although we could not clearly detect fragments of the EGF receptor by over exposure of these blots, we did detect proteolytic fragments in the dense fractions of Percoll gradients (data not shown). This suggests that lysosomes also play a role in the autophagic degradation of EGF receptor. The low level of fragments could be due to the proteases playing important roles in the initial processing of the EGFR, unlike the IGF1-R.

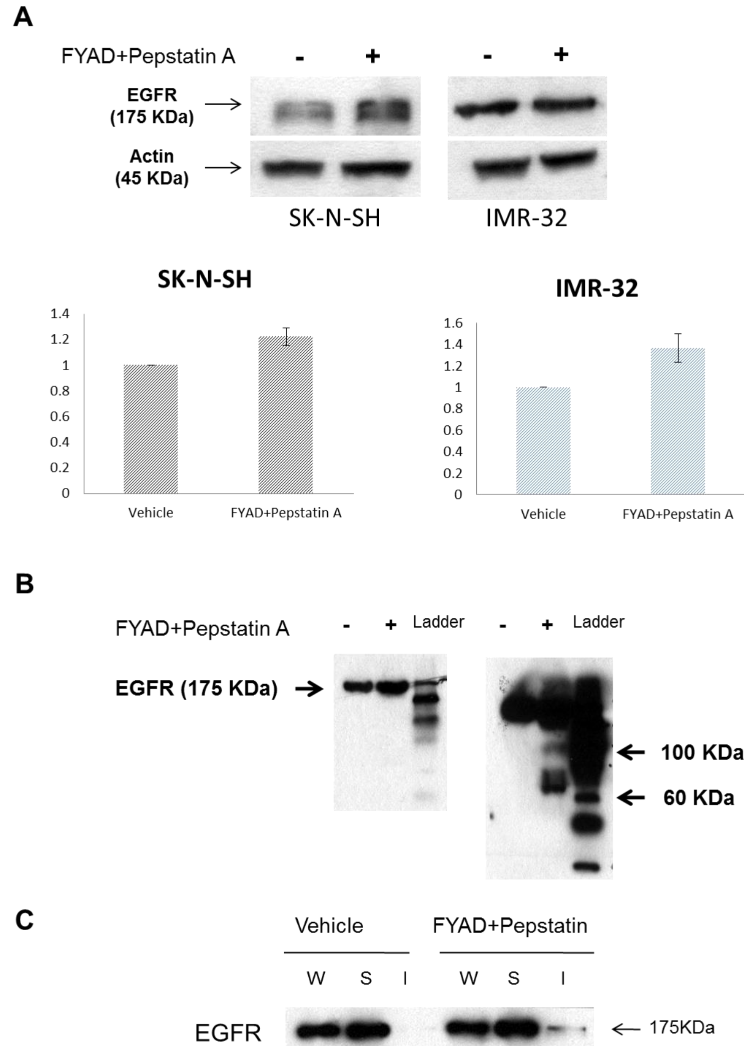


Figure 5.3 **Effect of cathepsin inhibition on EGFR.**
SK-N-SH and IMR-32 cells were treated with protease inhibitors and harvested for subsequent immunoblot analysis. In both cell lines full length receptor showed a small but statistically significant increase due to protease inhibition (p-value<0.05 for both cell lines) (A). When the blots from SK-N-SH were over exposed, several fragments of EGF receptor were detected in the extracts from inhibitor treated cells. The right panel is a higher exposure of the same blot, to show the fragments (B). Similarly treated cells were extracted as Triton soluble and insoluble fractions, W: whole lysate, S: soluble fraction, I: insoluble fraction (C). A portion of the full length receptor was detected in the Triton insoluble fraction in the inhibitor treated cells.

We next examined TrkB and ALK, two receptor tyrosine kinases that have important roles in high risk neuroblastoma. We did not observe any detectable level of either receptor in SK-N-SH cells, whereas IMR-32 cells express both. IMR-32 cells were treated for 72 hours with inhibitors, then harvested for immunoblot analysis and quantification.

ALK and TrkB did not show any detectable fragments in inhibitor treated cells (Fig.5.4). The level of both ALK and TrkB did not show any changes in inhibitor treated cells (Fig.5.4).

5.4 Effect of Cathepsin Inhibition on Induction of MAPK and PKB Phosphorylation by EGF in Human Neuroblastoma Cells.

SK-N-SH cells were treated with protease inhibitors for 72 h followed by serum starvation and EGF treatment. Although EGF was still able to stimulate receptor activation in inhibitor-treated cells the level of activation of MAPK was significantly impaired compared to control cells (Fig.5.5). By contrast, phosphorylation of PKB was not significantly affected (Fig.5.5).

5.5 Effect of Cathepsin Inhibitor Treatment on Phosphorylation of IGF-1 Receptor and its Fragments by IGF-1.

I showed that cathepsin inhibition did not affect IGF-1 mediated activation of its receptor (Fig.3.6). The transient nature of this activation is clearly shown in figure 6S. Activation of the receptor is optimal 5 min after addition of IGF-1 and is similar in

both vehicle and inhibitor treated cells. This result also confirms that the receptor and fragments trapped in autophagosomes in inhibitor treated cells cannot be further activated by IGF-1.

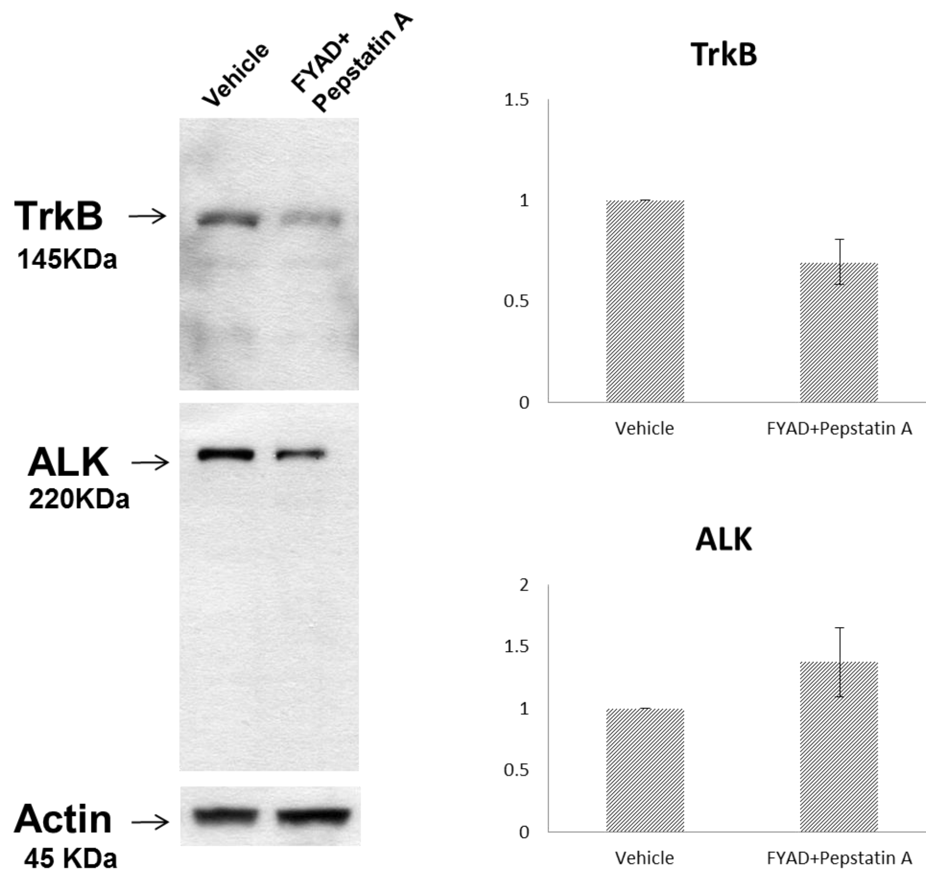
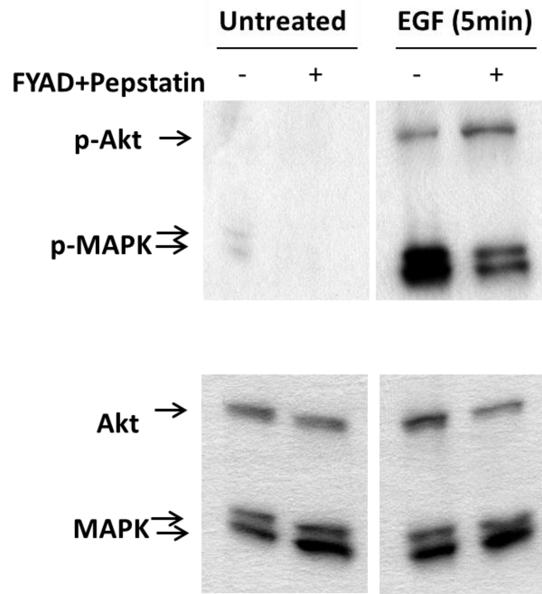


Figure 5.4 Effect of inhibition of cathepsins on ALK and TrkB in IMR-32 cells.
IMR-32 cells were examined for expression of ALK and TrkB. Cells were treated with inhibitors as described in materials and methods and whole lysates were subject to western blot analysis and quantification. Neither of the proteins showed any detectable proteolytic fragments appear in inhibitor treated cells. The level of full length receptor did not show any significant changes.



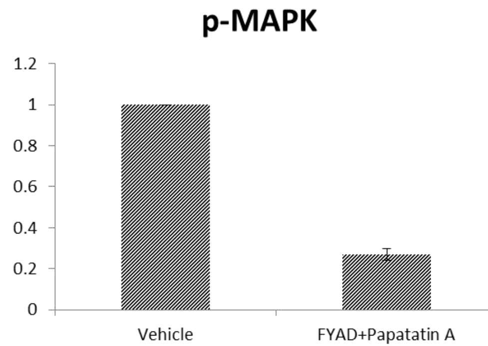


Figure 5.5 Effect of cathepsin inhibition on activation of MAPK and PKB by EGF in SK-N-SH cells.

SK-N-SH cells were treated with protease inhibitors for 72 hours, the last 3 hours in absence of serum, and then treated with EGF as described in materials and methods. Cells were harvested and subject to immunoblotting, followed by densitometry and statistical analysis. In vehicle treated cells, both MAPK and PKB were phosphorylated upon treatment with EGF. However in inhibitor treated cells, induction of MAPK activation was greatly diminished. By contrast phosphorylation of PKB was not significantly affected.

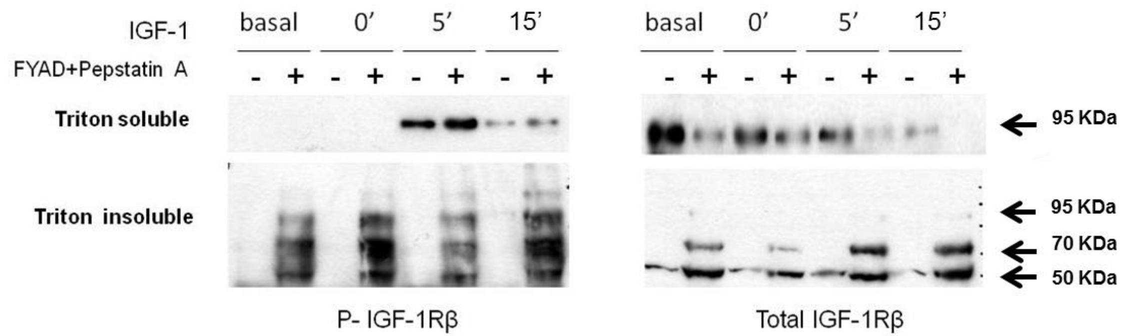


Figure 5.6 Time course of phosphorylation of IGF-1 receptor and its fragments by IGF-1 in control and inhibitor treated neuroblastoma cells.

SK-N-SH cells were treated with inhibitors for 72 hours, with the last 6 hours serum deprivation. Cells were then treated with IGF-1, as described in materials and methods. Cells were harvested and subject to immunoprecipitation after solubilization in Triton X-100 buffer using anti-IGF-1R beta antibody. The immunocomplexes were then recovered and separated by SDS/PAGE, followed by immunoblotting. Fragments of IGF-1R were recovered from Triton insoluble fraction of cells, as described in materials and methods, followed by direct immunoblotting against total or phosho-IGF-1 receptor. The highest level of phosphorylation of IGF-1 receptor was observed 5 min after IGF-1 treatment, and greatly diminished after 15 min of treatment. Inhibitor treatment had no detectable effect on timing or extent of IGF-1 receptor phosphorylation. IGF-1 receptor and its fragments in the Triton insoluble fraction were in a constant non-inducible phosphorylated state.

5.6 Effect of Short-term Inhibition of Proteases on Activation of MAPK and PKB.

All the previous work in this project was done after long term inhibition of lysosomal proteases for 48-72 hours. When cathepsins are only inhibited for 3 hours in serum deprived conditions, activation of MAPK and PKB was not affected (Fig.5.7). Three hours is sufficient time for FYAD and pepstatin to inhibit cathepsins B, D and L in cells, indicating that short-term inhibition of cathepsins is not sufficient to impair IGF-1 signaling.

5.7 *In vivo* effect of FYAD on Inhibition of Cathepsin B in Mouse Tissues.

In vivo, FYAD blocks the growth of neuroblastoma tumors but does not appear to affect growth in normal animals (Colella et al 2010). As seen in cells, inhibitor treatment of mice does increase relative levels of the unprocessed form of cathepsin B in liver and kidney (Fig.5.8). It also increases total levels of cathepsin B in these tissues, but does not increase levels in brain. This is probably because FYAD does not efficiently pass through the blood brain barrier.

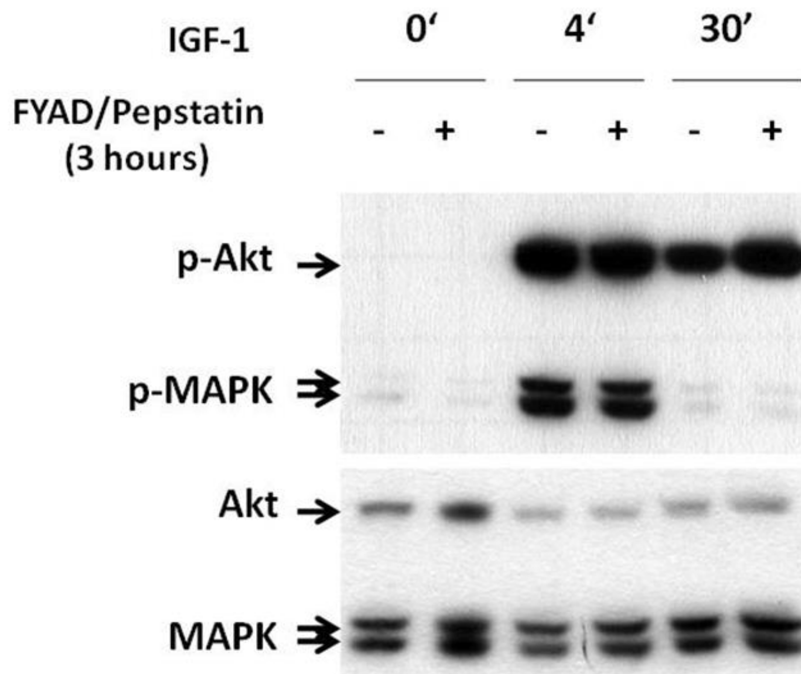
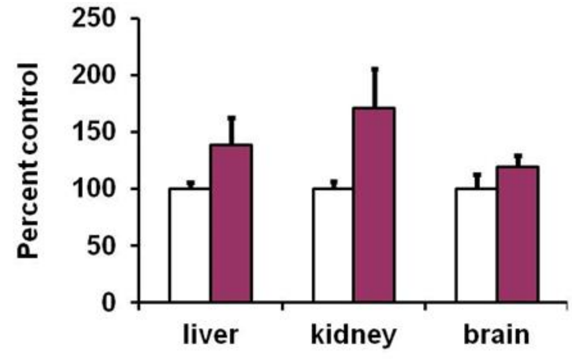


Figure 5.7 Activation of MAPK and PKB in response to short term inhibition of cathepsins.
SK-N-SH cells were treated with inhibitors in serum deprived condition and then treated with IGF-1 and harvested at indicated time points, followed by immunoblot analysis. MAPK and PKB were activated by IGF-1. The peak of MAPK was 5 min after treatment, and was almost completely diminished after 30 min. PKB was activated after 5 min and remained active after 30 minutes. The activation of MAPK did not change in inhibitor treated cells. PKB remained active for at least 30 minutes.



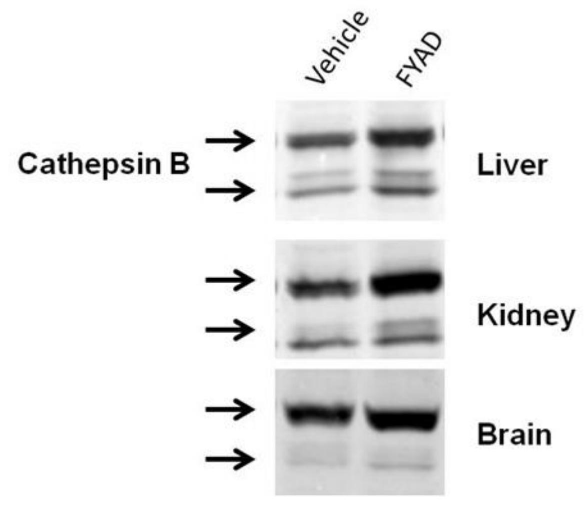


Figure 5.8 ***In vivo* effect of FYAD on inhibition of cathepsin B in mouse tissues.**

Balb/c mice were treated with 500 micrograms FYAD or vehicle control intraperitoneally once per day for 5 days. 6 h after the last injection mice were sacrificed and tissues harvested. Western blots of cathepsin B were quantified using FITC conjugated secondary antibody and scanned by Typhoon imager. Total levels of cathepsin B were determined and expressed as mean and standard deviation, normalized to untreated controls. Top, quantitation of cathepsin B in tissues from FYAD treated mice (solid bars) and untreated controls (open bars). In liver and kidney, total levels of cathepsin B increased but levels were unaltered in brain. Representative blots are shown below.

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