UBE4B LEVELS DETERMINE THE EFFICACY OF EGFR AND STAT5 INHIBITORS IN TREATMENT RESISTANT NEUROBLASTOMA

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UBE4B LEVELS DETERMINE THE EFFICACY OF EGFR AND STAT5 INHIBITORS IN TREATMENT RESISTANT NEUROBLASTOMA

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UBE4B LEVELS DETERMINE THE EFFICACY OF EGFR AND STAT5 INHIBITORS IN TREATMENT RESISTANT NEUROBLASTOMA

A

DISSERTATION

Presented to the Faculty of

The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

David J. Savage, B. A.

Houston, Texas

August 2018
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Getting to the finish line and earning a Ph.D. is the hardest task I have ever undertaken, and there have been many points along the way when the finish line was very hard to see. My success is due in large part to a team of people who have consistently believed and me, and who have worked hard to help me get to this point.

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colleagues, and friends listed here have invested selflessly in me.
UBE4B LEVELS DETERMINE THE EFFICACY OF EGFR AND STAT5 INHIBITORS IN TREATMENT RESISTANT NEUROBLASTOMA

David J. Savage, B.A.
Advisory Professor: Andrew Bean, Ph.D.

Neuroblastoma is the most common malignancy in infants. Overexpression of the epidermal growth factor receptor (EGFR) in neuroblastoma tumors can result in enhanced EGFR signaling, uncontrolled proliferation, and may provide a mechanism for chemotherapy resistance. UBE4B, an E3/E4 ubiquitin ligase, ubiquitinates the EGFR and promotes its lysosomal degradation ultimately attenuating EGFR signaling. Interestingly, the UBE4B gene lies in a chromosomal region (1p36) whose loss is correlated with poor patient outcomes due to inefficient EGFR degradation and enhanced cell proliferation. We examined whether depletion of UBE4B in a chemoresistant neuroblastoma cell line would affect tumor responses to drugs that specifically target selected proteins that are upregulated in the absence of UBE4B. UBE4B depletion in a resistant neuroblastoma cell line resulted in a number of proteins whose levels were altered, including an increase in EGFR and STAT5a levels. We observed that treatment with Cetuximab, a therapeutic antibody targeting the EGFR, significantly inhibited the proliferation of neuroblastoma cells depleted of UBE4B. Addition of a STAT5 inhibitor potentiated the Cetuximab-induced inhibition of proliferation, reduced migration, and enhanced apoptosis in UBE4B-depleted neuroblastoma cells more than either drug treatment alone. Thus, screening resected patient tumors for 1p36 status and UBE4B levels may enable a novel treatment strategy in which selected patients who have low UBE4B-expressing tumors may benefit from simultaneously targeting multiple EGFR signaling pathways.
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CHAPTER 1 - Introduction

1.1 Characteristics, Incidence, and Prevalence of Neuroblastoma

Neuroblastoma is a malignant (1), embryonal tumor that arises from neural crest-derived cells of the sympathetic nervous system (2). It is the most frequently diagnosed tumor in the first year of life, and the most common extracranial solid tumor in children (3). The disease can spread from the head to the pelvis, but in a majority of cases the tumor arises from the adrenal medulla (Figure 1) (4, 5). Neuroblastoma represents about 8% of all childhood cancers (3), and more than 600 new cases of neuroblastoma are diagnosed in the United States annually (6). More than 70% of patients are diagnosed with neuroblastoma after metastasis has occurred (7).

Figure 1. Full-body CT scan demonstrating common locations for neuroblastoma

This figure demonstrates a computed tomography (CT) scan of a child showing an

The symptoms of neuroblastoma can be quite variable and are dependent upon the tumor site (8). Tumor compression on the spinal cord can lead to lower extremity paralysis, thoracic tumors can cause a Horner’s syndrome (anhidrosis, miosis, and partial ptosis) of the eye, liver tumors can cause abdominal swelling, metastasis to the bone marrow can cause hematologic disorders, and adrenal medullary tumors can cause abnormal catecholamine production (5). This pleiotropic set of symptoms and the rarity of the disease (3) can make it challenging to detect.

The long-term prognosis for neuroblastoma worsens with increased age of the patient at diagnosis (9). Patients who are diagnosed at ages older than 18 months, who either have a metastasis or who have tumors with unfavorable histology, or NMYC oncogene amplification, tend to have a worse prognosis (4, 10). In cases of neuroblastoma relapse, treatment options are limited since these tumor cells can rapidly develop resistance to conventional chemotherapeutics (11).

**1.2 Stratification of Neuroblastoma’s Severity**

Stratification of the severity of neuroblastoma requires assessing a number of factors. These include the stage of disease (12), age of the patient, whether or not the NMYC oncogene is amplified, histopathology of the tumor, and chromosome abnormalities (9, 13). One such common abnormality is a loss of heterozygosity (LOH) in the 1p chromosomal region (Figure
2), which is associated with a decreased response to treatment, a lower event-free, and a lower overall survival (14–16). Approximately one-third of neuroblastoma cases display deletions within the 1p36 chromosomal region (14–17). One gene within this region whose deletion has been shown to correlate with poor prognosis is the ubiquitin ligase UBE4B, which regulates growth receptor protein trafficking in the endocytic pathway (18, 19).
Deletions at the 1p36 chromosome site occur in one third of neuroblastomas. This is the site of the ubiquitin ligase, UBE4B, a regulator of growth factor degradation in the endocytic pathway.


Used with permission from Elsevier.
1.3 Conventional Treatment of Neuroblastoma and Long-term Prognosis

The treatment for neuroblastoma depends on the risk stratification of the patient. Patients are divided into low, intermediate, and high-risk groups (13). Therapy is multi-modal and may include observation, surgery, chemotherapy, radiotherapy, stem cell transplantation, differentiation therapy, and/or immunotherapy (8). While patients with non-metastatic, minimally invasive neuroblastoma may be disease-free following surgery alone, chemotherapy is used for patients with invasive or metastatic disease (20). Frontline chemotherapeutics for neuroblastoma are non-specific and affect non-cancerous cells as well (21). They function through either inducing DNA damage or interfering with the mitotic spindle to interrupt cell division (22). This induces cell death and acts in a greater degree on rapidly proliferating neuroblastoma cells, but it can affect normal cells, too (23). This explains many of the common late side effects of chemotherapy treatment such as hearing loss, pulmonary fibrosis, and cardiotoxicity (24).

Frontline chemotherapy, also called induction chemotherapy, for high risk neuroblastoma includes a combination of cisplatin, vincristine, carboplatin, etoposide, and cyclophosphamide (25). Each of these agents works by a different mechanism of action to kill cancer cells, but broadly they target either DNA replication or cell division to cause irreversible damage leading to apoptosis (23). Cisplatin, carboplatin (a cisplatin analog), and cyclophosphamide act by alkylating DNA bases. Subsequently, DNA repair enzymes cause DNA damage while attempting to repair the alkylation, which ultimately leads to cell death (22). Vincristine binds to the protein tubulin to interfere with cell division in mitosis (26). Etoposide is a topoisomerase II inhibitor that prevents DNA unwinding during replication,
leading to apoptotic cell death (27). A combination of drug agents in the frontline treatment phase delays the onset of selective chemotherapy resistance (28). Thus, using a combination of chemotherapy agents that act by different mechanisms to interfere with cell growth in the initial phase of treatment can increase the likelihood of a neuroblastoma patient becoming cancer free.

Beyond traditional cytotoxic chemotherapy, other treatments are used for high risk patients. They may be treated with myeloablation followed by autologous stem cell transplant for neuroblastoma that may have spread to the bone marrow (29). Subsequent possible treatments include forced cell differentiation with 13-cis-retinoic acid or targeted anti-GD2 antibody therapy which triggers cytotoxicity through an immune response (25). Retinoic acid is used to treat residual disease after myeloablative therapy, and acts to force growth arrest and terminal maturation of neural tissue (30). The anti-GD2 antibody works by binding to neuroblastoma cells, which overexpress the ganglioside GD2. This induces cell death through complement-mediated cytotoxicity and activation of the immune response (31). High risk neuroblastoma patients often require more complex treatment approaches to effectively treat their disease.

The long-term prognosis for neuroblastoma depends on a patient’s risk classification and tumor stage at the time of initial diagnosis (26). Patients in the low risk category can expect a 92% event-free survival and 96% probability of overall survival (32, 33). Patients at intermediate risk disease have an overall survival rate of 80% (34, 35), while patients with high risk disease have an event-free survival of less than 50% despite multimodal therapy (36, 37). Thus, while clinical outcomes are good for low and intermediate risk patients, new treatment strategies are needed for high risk patients to improve their outcomes.
1.4 Mechanisms of Neuroblastoma Chemotherapy Resistance

High risk patients with a tumor relapse have poor outcomes in part because of the development of chemotherapy resistance (4, 38). Some common cellular resistance mechanisms include decreased chemotherapy drug uptake (39), increased drug efflux (40), increased DNA damage repair (41), failure of cell death pathways (42), and increased growth factor receptor expression at the cell surface (Figure 3) (18). Amplification of the MYCN gene, a poor prognostic indicator in neuroblastoma, upregulates ABC transporters in cell membranes that promote efflux of chemotherapy drugs from cells (40, 43). Chemotherapy resistance may also be promoted by growth factor receptors, like the epidermal growth factor receptor (EGFR), which is increased in amounts in other neural cancers leading to conventional chemotherapy resistance by enhancing proliferation (44). EGFR protein levels are high in many in vitro neuroblastoma cell lines and patient tumor biopsies (1). There are many mechanisms of resistance in neuroblastoma that affect drug uptake and cellular response to chemotherapy, and increased growth factor-stimulated signaling may be another mechanism of resistance.
Figure 3. Common chemotherapy resistance mechanisms in cancer

Diagram of common mechanisms of chemotherapy resistance in cancer. Aside from increased growth factor signaling, these mechanisms lower the intracellular amount of drug or change the intended protein targets such that effects on cell division, DNA replication, or signaling pathways are not lethal. Modified from: El-Awady, Raafat et al. 2017. “The Role of Eukaryotic and Prokaryotic ABC Transporter Family in Failure of Chemotherapy.” Frontiers in Pharmacology 7(JAN):1–15.

1.5 EGFR and the Endocytic Pathway

The endocytic pathway internalizes membrane proteins and transports them for recycling, transit to other intracellular compartments, or degradation (45). It is an important regulator of EGFR-mediated signaling for the proliferation of neuroblastoma (46). EGFR may be internalized at the plasma membrane, trafficked through the endocytic pathway, and sorted, into a pathway that will result in its lysosomal degradation or will cause the receptor to be recycled for reuse (47). There are five ligands known to bind EGFR: epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin, epiregulin, and betacellulin
These ligands may have different fates during the trafficking process, but EGF binding enhances degradation of the receptor as a means of attenuating signaling (49).

Upon ligand binding, the EGFR dimerizes, which then triggers autophosphorylation of the tyrosine residues in the cytoplasmic tail of the receptor to initiate endocytosis. Binding of EGF or TGF-α specifically results in clathrin-mediated endocytosis (47). In this process, clathrin adapter protein 2 (AP2) protein complexes are recruited to the plasma membrane, which then recruit clathrin (50). As membrane invagination continues and clathrin polymerizes, AP2 stabilizes the coated pit. Next, the GTPase dynamin is required to induce fission of a deep clathrin-coated pit and its cargo from the plasma membrane, resulting in the formation of an endocytic vesicle (45). By the end of this initial step in the endocytic pathway, an early endosome is formed which can then be sorted to its destination.

Internalized EGFRs follow an endocytic itinerary in which they pass through early endosomes and late endosomes/multivesicular bodies (MVB) prior to the lysosome for degradation. Ubiquitination of the cytoplasmic tail of EGFR allows recognition by the ESCRT machinery, a group of cytosolic protein complexes required for internalization from the endosomal membrane into intraluminal vesicles (ILVs), and subsequent degradation upon MVB-lysosome fusion (Figure 4) (51–58). Mono-ubiquitination of cargo at the plasma membrane can promote intracellular protein movement through the endocytic pathway, but it does not necessarily serve as a signal for lysosomal degradation (59). Conversely, polyubiquitination of cargo at the membrane of early endosomes is a necessary step for receptor internalization into ILVs and subsequent degradation by the lysosome. (50, 60). Regulation of EGFR ubiquitination and degradation can affect the total amount of EGFR that
resides on the cell surface (46). The endocytic pathway can modulate EGFR-mediated signaling by affecting the number of receptors on the surface of cells.

1.6 Ubiquitination and EGFR Degradation

Ubiquitination is a key regulator of EGFR degradation (53, 61). EGFR may be ubiquitinated on lysine residues by the E3 ubiquitin ligase Cbl at the plasma membrane (61) or by the E3/E4 ubiquitin ligase UBE4B at the endosomal membrane (53). Ubiquitination is a necessary step for lysosomal sorting in the endocytic pathway (46). A dominant negative of Cbl (62) or a depletion of UBE4B (53) can lead to a decrease of EGFR lysosomal degradation. Lysine residues 29, 48, and 63 are targets of mono-ubiquitination by the E3 ligase Cbl (61), whereas UBE4B can attach ubiquitin at those lysine residues as well as lysine residues 6, 11, 27, and 33 (63). Another key difference between Cbl and UBE4B is that UBE4B can act as an E4 ligase (63). E4 ligases are capable of ubiquitin chain assembly to mediate polyubiquitination (64). Furthermore, ubiquitination by UBE4B can be counteracted by the de-ubiquitinating enzyme USP8 (53). Ubiquitination is an essential step for endosomal sorting and lysosomal degradation of cargo in the endocytic pathway.

The amount of UBE4B in neuroblastoma cells correlates with patient survival. The gene for UBE4B resides at the 1p36 chromosome site, which is commonly deleted in one-third of neuroblastomas (Figure 2) (14, 19, 65). Patients with lower levels of UBE4B have a poor prognosis compared to patients with normal levels (18), even when stratified by disease stage (19). Furthermore, it has been suggested that decreased UBE4B levels are a predictor of relapse in low and intermediate risk pediatric patients (19). Thus, the levels of UBE4B correlate with
neuroblastoma patient outcomes, and understanding how decreased UBE4B levels contribute to poor prognosis through the proteins that it regulates may aid in finding new treatment approaches.

One way in which UBE4B may enhance the growth of neuroblastoma is through its regulation of EGFR. UBE4B protein levels in neuroblastoma patient biopsies are inversely proportional to EGFR protein levels (18). As the amount of cellular UBE4B decreases, the expression of EGFR increases and neuroblastoma cells may become more proliferative and resistant to conventional chemotherapy due to increased stimulation by pro-growth ligands in the environment (1). Conversely, cancer cells expressing high protein levels of UBE4B are less responsive to EGF-stimulated growth and EGFR-targeted anti-proliferative therapy (18). Transiently depleted UBE4B can produce a two-fold increase in whole cell EGFR in cancer cells in vitro (53). Therefore, low levels of UBE4B may enhance proliferation and worsen patient outcomes.

The UBE4B enzyme interacts with cargo proteins, like the epidermal growth factor receptor (EGFR), at the membrane of early endosomes to ubiquitinate them (53). The number of ubiquitin residues that are added determines whether modified cargo is degraded by the lysosome or recycled back to the cell’s surface (52). A decrease in the amount of cellular UBE4B can lead to decreased EGFR degradation due to decreased ubiquitination. Inactivation of the catalytic activity of UBE4B, which can lead to a dominant negative phenotype, has a similar effect of lessening EGFR degradation over time (53). Neuroblastoma cells overexpressing a catalytically inactive form of UBE4B, UBE4B_{P1140A}, decrease their degradation of EGFR, while neuroblastoma cells overexpressing UBE4B increase the amount of EGFR
that they degrade (53). Thus, it appears that when UBE4B is depleted or inactivated, an increased amount of EGFR can result.

Increased EGFR levels as a result of decreased amounts of UBE4B can lead to increased stimulation by growth factors (18) and increased pro-proliferative downstream signaling (53). The fact that stimulation by growth factors in vitro can enhance downstream signaling and cell growth provides some evidence that a portion of increased EGFR resides on the cellular surface. In cervical adenocarcinoma (HeLa) cells, transient depletion of UBE4B levels followed by stimulation with EGF leads to a significant rise in signaling in the pro-proliferative MAPK/ERK signaling pathway (53). Moreover, neuroblastoma cells that overexpress UBE4B have reduced proliferation in response serum and EGF (18). These data suggest that the mechanism by which low UBE4B levels result in worse patient outcomes may involve increased growth factor-stimulated proliferation due to increased EGFR levels.

Figure 4. Graphical depiction of EGFR traveling through the endocytic pathway

UBE4B interacting with EGFR (blue) at the endosomal membrane during trafficking in the endocytic pathway. The degree of ubiquitination determines cargo fate of either lysosomal degradation (Lyso) or plasma membrane recycling. Polyubiquitination of
the cytosolic-facing segment of the EGFR causes it to be internalized into intraluminal vesicles of the MVB/late endosome. Degradation of the receptor then occurs after fusion with the lysosome.

1.7 Mechanism of Cetuximab as a Treatment for Neuroblastoma

Cetuximab (mAb C225 or Erbitux) is a monoclonal antibody that specifically binds to the EGF receptor (66). It interacts with the extracellular domain of the receptor blocking the ligand binding site (67). Binding of Cetuximab to EGFR also stimulates receptor internalization (68). Like other cargo that travels through the endocytic pathway, the receptor is ultimately either degraded in the lysosome or recycled (47). Cetuximab binding also induces antibody-dependent cell-mediated cytotoxicity (69).

The benefit of Cetuximab is derived from blocking EGF binding to the EGFR and the drug’s effective reduction in growth factor receptors in a rapidly proliferating cancers (66, 70). The number of growth factor receptors has been shown to correlate with an increased rate of proliferation and resistance to chemotherapy agents that target cell division (71). Cetuximab has significant therapeutic benefit in treating colorectal cancer (72, 73) and cancers of the head and neck (74). In non-small cell lung cancer (NSCLC), Cetuximab is effective in patients with elevated levels of EGFR, regardless of whether those receptors have mutations (75). To date, however, Cetuximab has not been an effective treatment in neuroblastoma patients. In a phase 1 clinical trial involving treatment of Cetuximab and irinotecan in pediatric solid tumors (which included just two neuroblastomas), there was no sustained or complete response in the neuroblastoma patients to the combination therapy (76). The patients in that trial were not differentiated by the amount of EGFR in their tumors and the sample size was small. In the case of non-small lung cancer, the greatest response to Cetuximab was observed when patients
were strategically selected based on elevated levels of EGFR (77). Given the fact that one-third of neuroblastomas have deletions of the chromosomal location containing the UBE4B locus (14, 19), it may be possible to strategically select neuroblastoma patients with low levels of UBE4B for Cetuximab treatment.

1.8 Targeting Signaling Downstream of the EGF Receptor

EGFR transmits signals through activation of many cytosolic signaling cascades, including Ras/Raf (78), phosphatidylinositol 3-kinase/Akt (79), Jak/STAT (80), phospholipase C (81), and Nck/PAK signaling pathways (82). Each of these pathways have been implicated in cell proliferation, and their constitutive activation, independent of EGFR ligand binding, can blunt the effect of inhibitors that target that receptor (83). Consequently, combination therapies that target a pathway downstream of EGFR can increase the efficacy of EGFR-targeted therapy (84). This combination therapy approach has been used successfully in non-small cell lung cancer using a Jak-2 inhibitor in combination with Cetuximab. In pre-clinical trials, this combination overcame resistance, synergistically, when compared to Cetuximab treatment alone (85). Similarly, an increased anti-proliferative effect has been observed in head and neck squamous cell carcinomas treated with a combination of a Jak-2/STAT3 inhibitor and Cetuximab (86). It may be possible to simultaneously target EGFR and one member of a downstream signaling pathway in order to produce an anti-proliferative effect in neuroblastoma tumor cells.
1.9 Design of this Study

One of the goals of our research was to understand the role of UBE4B in regulating the levels of other proteins and mediating the chemotherapy response in neuroblastoma. I created a model neuroblastoma chemotherapy-resistant cell line where UBE4B was depleted to undetectable levels. This model was used to test several specific hypotheses:

1. Does stable depletion of UBE4B lead to increased EGFR in neuroblastoma?

2. Does this increase in EGFR make cells more sensitive to EGFR-targeted drug agents?

3. Does a depletion of UBE4B lead to other protein changes in neuroblastoma?

4. Can proteins that increase in amount due to UBE4B depletion be targeted to reduce neuroblastoma cell growth \textit{in vitro}?
Figure 5. Graphical depiction of UBE4B’s ubiquitination role in EGFR degradation

UBE4B ubiquitination determines the fate of endosomal cargo like EGFR that is trafficked through the endocytic pathway. Understanding which proteins are elevated as a result of a UBE4B depletion may lead to downstream targets for neuroblastoma patients with 1p36 deletions.

First, I used an in vitro metabolic activity assay to identify neuroblastoma cell lines that were either resistant or sensitive to conventional chemotherapy agents. I then used two resistant and sensitive cell lines to determine whether Cetuximab could be combined with the chemotherapeutic agent irinotecan, a topoisomerase inhibitor, to get a combined anti-proliferative effect in vitro, as has been tried in a clinical trial with pediatric solid tumors (76). Next, I created a model neuroblastoma cell line from the resistant SK-N-AS neuroblastoma cell line in which UBE4B levels were depleted in a stable fashion using shRNA. These cells were tested by immunoblot for increased protein levels of EGFR. They were then treated with
the EGFR-targeted antibody Cetuximab *in vitro* to study whether growth could be inhibited after UBE4B protein depletion.

High throughput screening was used to identify changes in amounts of other proteins as a result of UBE4B depletion. Drug targets for upregulated proteins were then investigated based on published pre-clinical and clinical data from other cancers. The two proteins selected for further interrogation were the EGFR and STAT5a, a signal transducing protein involved in EGFR-mediated signaling. I then used *in vitro* experiments to study whether the model neuroblastoma cells depleted of UBE4B became more sensitive to a STAT5 inhibitor by itself or in combination with the EGFR-inhibiting antibody Cetuximab. In addition, *in vitro* experiments to study the effect of these agents on the amount of neuroblastoma cell migration and apoptosis were performed.

The results reported here show that a model neuroblastoma cell line containing low levels of UBE4B has elevated amounts of EGFR and STAT5a, which makes it sensitive to growth inhibition by EGFR- and STAT5-targeted drug agents. This approach for inhibiting neuroblastoma’s growth may be effective for the one-third of neuroblastoma patients who have a 1p36 depletion leading to lower levels of UBE4B in their tumors.
CHAPTER 2 – Materials and Methods

2.1 Tissue Culture

The SK-N-AS, SK-N-BE(2), CHP134, LAN5, CHLA20, and SK-N-SH human neuroblastoma cell lines used in this study have been previously utilized and described by our laboratory (87). Cells were cultured in RPMI media (Corning Life Sciences, Tweksbury, MA) supplemented with 10% fetal bovine serum and 1% L-glutamine. TLA-HEK293T cells used for lentivirus production (Thermo Fisher Scientific, Waltham, MA) were cultured in DMEM with 10% fetal bovine serum at 37°C with 5% CO₂ and passaged to maintain 20-80% confluence.

2.2 Chemotherapy Agents

The chemotherapy agents topotecan, irinotecan, doxorubicin, temozolomide, and cisplatin were obtained from Sigma (St. Louis, MO). Topotecan was dissolved in PBS to make a 0.5 mM stock, irinotecan was dissolved in DMSO (Sigma) to make a 50 mM stock, doxorubicin was dissolved in PBS to make a 6.9 mM stock, temozolomide was dissolved in DMSO to make a 50 mM stock, and cisplatin was dissolved in PBS to make a 3 mM stock. Cetuximab (Erbitux, Eli Lilly, Indianapolis, IN) with a stock concentration of 2 mg/mL was a generous gift of the University of Texas MD Anderson Cancer Center pharmacy. The STAT5 inhibitor SH-4-54 (ApexBio Technology, Houston, TX) was purchased as a 10 mM stock.
solution in DMSO. Drug dilutions for experiments were prepared in cell culture media from these stocks just prior to cell treatment.

2.3 Lentiviral Depletion of UBE4B in Neuroblastoma Cells

UBE4B-shRNA was obtained from Sigma’s Mission shRNA library. See table for sequences (Table 1). Lentiviral packaging plasmids pMD2.g, pRSV-Rev, and pMDLg/pRRE, and scrambled shRNA sequences were a gift of Dr. Sara Prijic (UTHealth McGovern Medical School, Houston, TX). Plasmid DNA was purified (Qiagen, Germantown, MD) and Lipofectamine 3000 (Thermo Fisher Scientific) was used to transfect HEK 293T cells (60% confluent at the time of transfection) using the following amounts of DNA in each reaction: 2.24 µg shDNA or shScrambled, 5.6 µg pMDLg/pRRE, 2.8 µg pRSV-Rev, and 3.36 µg pMD2.g. Twenty four hours following HEK 293T cell transfection, the media was changed to normal media and cells were incubated for an additional 48 hrs. After 48 hours, supernatant was collect to obtain virus. Target neuroblastoma cells were plated at 80% confluence and infected using the viral-rich media that had been mixed with 1 µg polybrene (Sigma) per 1 mL of media and filtered through a 0.45-micron PVDF membrane (Thermo Fisher Scientific). The filtered virus was incubated at room temperature for 10 min and transferred onto the target cells. The target cells were incubated overnight with virus, after which the media was changed to virus-free media. Two days after infection, the media was supplemented with 1 µg puromycin per 1 mL of media to select for infected cells. Cells were selected until untreated control cells completed died, which typically took approximately 4 days. A Western blot was used to confirm UBE4B levels.
<table>
<thead>
<tr>
<th>Sigma ID #</th>
<th>UBE4B shDNA Sequence</th>
</tr>
</thead>
</table>
| TRCN0000338295 | CCGGGCCCCATTTGCGTATATCTCGAGATA  
TAGCGACGGAACCTAGGCTTTTTG |
| TRCN0000007548 | CCGGGCAGGGATCAAATCCACAATAACTCGAGT  
ATTGTGGATTTGATCCCTGCTTTTTT |
| TRCN0000350907 | CCGGGCAGGGATCAAATCCACAATAACTCGAGTA  
TTGTGGATTTGATCCCTGCTTTTTG |
| TRCN0000338354 | CCGGAAGTGTTCAAGCAGATATTTCTCGAGAA  
ATATCTGCTTGAACACTTCTTTTTG |

Table 1. shDNA sequences used for depleting UBE4B in neuroblastoma cell lines.

2.4 Western Protein Blotting

Cell pellets were collected by scraping cells or using 5 mM EDTA (Millipore Sigma, Billerica, MA) to release them from their culture dishes. Cells were centrifuged at 1500 \( x \) \( g \) for 10 min, resuspended in a small volume of RIPA (1% Triton X-100, 6.1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40) buffer with a protease inhibitor cocktail (10 mM leupeptin, 1 \( \mu \)g/\( \mu \)L pepstatin, 0.3 mM aprotinin, and 1.74 \( \mu \)g/\( \mu \)L PMSF), lysed using sonication (5 pulses of 1 second at output control 3, Branson Sonifier 250, VWR Scientific), and centrifuged at 2000 \( x \) \( g \) to separate membranes from cytosolic protein. Lysate was kept on ice throughout the lysis process. The amount of protein in each sample was quantified (BCA protein assay, Thermo Fisher, Walham, MA) according to the manufacturer’s instructions. Samples of lysate (20 \( \mu \)g) were mixed with 6x loading buffer (50 mM Tris-HCl at pH 6.8, 4%
SDS, 60% glycerol, 0.6% bromophenol blue, and 30% β-mercaptoethanol), boiled for 5 min at 100°C, and separated using polyacrylamide gel electrophoresis (SDS-PAGE, 5% stacking, 10% separating). Proteins were transferred to nitrocellulose membranes in a buffer of tris, glycine, and methanol (100 V and 500 mAmp) for an hour. The resultant membrane was stained with Ponceau S (Sigma). Next the blot was blocked for an hour at room temperature with either BSA (1% in PBS) or nonfat dried milk (5% in PBS), depending on the antibody. The blots were incubated overnight at 4°C with either anti-actin antibody (Sigma, A2066), anti-UBE4B antibody (Abcam), anti-EGFR antibody (Invitrogen, Carlsbad, CA), or anti-STAT5 antibody (Cell Signaling, Danvers, MA). Membranes were subsequently washed three times with PBS-T and then incubated in a goat anti-rabbit secondary antibody (1:5000 for EGFR, 1:2000 for UBE4B, 1:5000 for actin, and 1:2000 for STAT5). Membranes were washed three times and developed using chemiluminescence (SuperSignal West Pico, Thermo Fischer Scientific) and visualized using x-ray film.

2.5 MTT (MethylThiazolyldiphenyl-Tetrazolium bromide) Cell Metabolic Activity Assays

Human neuroblastoma cells were diluted 1:1 with trypan blue and counted with the Countess automated cell counter (Invitrogen, Carlsbad, CA). Cells were then diluted in RPMI media to enable plating 7.5e3 cells per well on a 96-well plate with a multichannel pipette and incubated for 24 hours to allow adherence. On the second day, the media from each well was aspirated and replace with 100 µL of complete RPMI media containing either a chemotherapy agent or the appropriate vehicle. For experiments where only a 72-hour time point was taken, the treated cells were incubated for three days at 37°C with 5% CO₂. In experiments involving
Cetuximab, the drug was added fresh to the cells daily. For experiments involving daily measurements, cells were plated in three sets so that daily MTT assays could be performed. MTT measures mitochondrial metabolic activity and is a surrogate measure for cell viability in response to a drug challenge. A stock solution of 5 mg/mL MTT (Sigma) in PBS was first prepared and 10 µL of MTT were added to each 100 µL of media (final concentration 0.5 mg/mL). The plate was protected from light and incubated for 4 hours prior to removal of the media and non-reduced MTT, and 100 µL of DMSO was added to each well, incubated for a further 10 min, and the absorbance (560 nm) of each well was measured. The background absorbance of media only with the MTT reagent was measured and subtracted. Samples assessed at 72 hours only were compared to the absorbance of control-treated cells. Samples assessed daily were compared to the absorbance of cells at Day 0 before a drug or inhibitor was added. The significance of drug treatments in depletion cells was assessed with a two-way ANOVA using a Dunnett's multiple comparisons test. All statistical analysis was performed with GraphPad Prism software version 7 (La Jolla, CA).

For the IC$_{50}$ growth inhibition experiments in Figure 6, we first assessed the literature for a published IC$_{50}$ values for our model neuroblastoma cell lines and chemotherapy agents. We examined the concentrations of selected chemotherapeutics required to inhibit the proliferation of various neuroblastoma cell lines. Calculation of IC$_{50}$ values was done using a Boltzmann best fit regression. Only data with a Pearson correlation coefficient $\geq 0.8$ and at least three cell viability measurements between 20% and 80% were used for analysis. Significance of the IC$_{50}$ results was assessed with a one-way ANOVA with a Tukey multiple comparison test.
2.6 Cleaved Caspase Assay

Wild type, scrambled, and shUBE4B SK-N-AS cells were counted with the Countess automated cell counter and then plated at a density of 4e5 cells in 96-well plates and allowed to adhere overnight. On the second day, the media was aspirated from these cells and replaced with 100 µL of media alone, media with 5 µM SH-4-54, media with 2 µM Cetuximab, or media containing a combination of 5 µM SH-4-54 and 2 µM Cetuximab. CellEvent Caspase 3/7 Green Detection Reagent (Invitrogen, Carlsbad, CA) was added to the media of all four treatment groups at a final concentration of 3 µM, and the cells were incubated for 48 hrs at 37°C with 5% CO₂ protected from light. Next, the cells were imaged using first phase contrast microscopy and then fluorescent microscopy using a GFP filter. The percentage of green-fluorescing cells to total cells was then calculated. Significance between treatment groups was determined with a two-way ANOVA with a Dunnett multiple comparison test using GraphPad Prism.

2.7 Transwell Migration Assay

Cells were seeded in six well plates at a density of 3e5 cells per well and incubated overnight at 37°C with 5% CO₂ to adhere. On Day 2, the cells were treated vehicle (DMSO and PBS) and media alone, media with 5 µM SH-4-54, media with 2 µM Cetuximab, or media containing a combination of 5 µM SH-4-54 and 2 µM Cetuximab. The cells were incubated for an additional 24 hours. On the third day, the cells were separated from the dish with trypsin and counted using the Countess automated cell counter. Next, EGF was added to RPMI for a
concentration of 100 ng/mL, and 750 μL was added to wells of a 12 well plate. Falcon Cell Culture inserts (Corning Life Sciences, Tweksbury, MA) with 8 μM pores were submerged in the wells. Finally, 1.5e5 of the pre-treated SK-N-AS cells were diluted in serum-free RPMI media and added to the cell culture insert. The number of cells added was based on the number of viable cells from the Countess cell counting procedure so that an equal number of living cells was added to each insert. The cells were allowed to incubate overnight 37°C with 5% CO₂.

The following day the cells were washed and fixed for microscopy. First, the non-invasively cells were scrubbed from the top surface of the membrane using a cotton-tipped swab moistened with media for the first scrub and PBS for the second scrub. Next, the membranes were fixed with 100% methanol for 5 min. The membranes were then rinsed in water for 1 min and stained with a 1 μg/mL DAPI in PBS with 0.1% Tween for 20 min at room temperature protected from light. The membranes were then washed again in water for 1 min and inverted to dry in room air. Lastly, a fine-tipped scalpel was used to cut the membranes from the insert. The membranes were place bottom-side-up on a glass microscope slide. FluroSave reagent (Millipore Sigma, Billerica, MA) was added dropwise to the membranes. They were then covered with glass coverslips and then sealed with nail polish.

The glass slides were protected from light in the fridge at 4°C. The membranes were later imaged with 10x magnification on a fluorescent microscope. The instrument was set for DAPI with an excitation wavelength of 358 nm and an emission wavelength of 461 nm. Four 10x fields on each membrane were counted, and then the average was taken per condition to compute the number of cells per high powered field (HPF). The experiment was performed in
triplicate and significance was determined with a two-way ANOVA with a Dunnett multiple comparison test using GraphPad Prism.
CHAPTER 3 – Characterization of Antiproliferative Response to Chemotherapy in Neuroblastoma

3.1 Rationale

We wanted to identify an appropriate model neuroblastoma cell line that would be resistant to chemotherapy treatment in vitro. This line would become the model for future experiments where UBE4B was depleted. We studied the growth inhibitory effect of five conventional chemotherapy agents and Cetuximab, a monoclonal antibody that specifically targets the EGFR, toward seven neuroblastoma cell lines in vitro. Given the different molecular profiles of these lines, it was not clear how each would respond to an in vitro chemotherapy challenge. We wanted to identify lines that were especially resistant or sensitive to chemotherapy in order to select a resistant line for later experiments. We also wanted to use a cell line with a 1p deletion and possibly higher levels of EGFR to assess whether Cetuximab combined with a conventional agent that targets cell division might be able to inhibit neuroblastoma’s growth.

3.2 SK-N-AS and SK-N-BE(2) are Resistant and LAN5 and CHP134 are Sensitive Neuroblastoma Cell Lines

The relative sensitivity or resistance of commonly used neuroblastoma cell lines to chemotherapeutics has been previously described (88), however, cross-lab variability because of cell passage number, culture conditions, drug formulation variability, and assay variability
can affect these results (89). Therefore, we first examined the sensitivity of seven neuroblastoma cell lines (Table 2) to five chemotherapeutics in MTT cell metabolic activity assays. The IC₅₀ (concentration of drug that inhibits proliferation by 50%) was calculated for each drug in each cell line. SK-N-AS and SK-N-BE(2) cell lines were the most resistant, and LAN5 and CHP134 cells lines were the most sensitive to chemotherapeutics (Figure 6). With the exception of doxorubicin, the IC₅₀ for SK-N-AS was significantly higher for each chemotherapy drug when compared with many of the other cell lines tested.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Sensitive or Resistant</th>
<th>Source</th>
<th>Primary site</th>
<th>Met site</th>
<th>1p del.</th>
<th>1p trans.</th>
<th>N-myc amp</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-SH</td>
<td></td>
<td>4 yr, female</td>
<td>thorax</td>
<td>bone marrow</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>NGP</td>
<td></td>
<td>2 yr 6 mo,</td>
<td>unknown</td>
<td>bone marrow, lung</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>R</td>
<td>8 yr, female</td>
<td>adrenal</td>
<td>bone marrow</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CHP-134</td>
<td>S</td>
<td>1 yr 1 m,</td>
<td>adrenal</td>
<td>lymph nodes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LA-N-5</td>
<td></td>
<td>5 mo, male</td>
<td>unknown</td>
<td>bone marrow</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CHLA-20</td>
<td>R</td>
<td>2 yr, female</td>
<td></td>
<td></td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK-N-BE(2)</td>
<td>R</td>
<td>2 yr 2 mo,</td>
<td>unknown</td>
<td>bone marrow</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 2. Molecular characteristics of the neuroblastoma cell lines used in this study.

All cell lines except SK-N-SH had either a 1p deletion or translocation. The 1p36 site specifically is the location of UBE4B. Additionally, the lines NGP, CHP-134, LAN5, and SK-N-BE(2) had N-myc amplifications which are a bad prognostic indicator in patients. Finally, the lines SK-N-AS, CHLA-20, and SK-N-BE(2) have been described in the literature as being resistant (R), while CHP-134 has been described as sensitive (88).
Figure 6. IC\textsubscript{50}s of model neuroblastoma cell lines to chemotherapy agents

The inhibitory concentration required for 50% inhibition of proliferation (IC\textsubscript{50}) in vitro at 72 hours varies across seven neuroblastoma cell lines using five chemotherapy agents with varying mechanisms of action. We identified SK-N-AS as being more resistant because these cells required higher concentrations of chemotherapeutics for inhibition with all drugs except cisplatin. We identified LAN5 and CHP134 as more sensitive cell lines because lower drug doses were needed to inhibit proliferation. Graphs show the average of at least three independent trials ± the SD.
3.3 Cetuximab and Irinotecan Combination Treatment Increases the Efficacy of Low Doses of Irinotecan

EGFR has been reported to be overexpressed in proliferating neuroblastoma, especially resistant lines taken from relapsed patients (1). After establishing the resistant and sensitive cell lines in our panel, we next studied whether Cetuximab, a monoclonal antibody that specifically targets EGFR, in combination with irinotecan, which interferes with DNA replication, would inhibit proliferation. Cetuximab has had limited efficacy toward neuroblastoma in patients (76, 90), but there is sparse in vitro data published to describe the response of neuroblastomas to the anti-proliferative effect of Cetuximab. EGFR has been reported to be elevated in resistant neuroblastomas (1, 2), so we examined whether this combination of Cetuximab and irinotecan would be effective in the resistant lines SK-N-AS and SK-N-BE(2) when compared with the more sensitive LAN5 and CHP134. The results showed no significant change in the IC₅₀s between irinotecan monotherapy and combination therapy at 72 hours (Figure 7). However, the combination did significantly improve the efficacy of low doses of irinotecan in all lines except SK-N-BE(2). Cetuximab alone did not have much of an effect on proliferation and neither did the lowest dose of irinotecan, but the combination showed a significant change (Figure 8). This suggests that Cetuximab may not be effective at inhibiting growth of wild type cells, but it can enhance the cytotoxic efficacy of low concentrations of irinotecan.
Figure 7. Combined treatment of wild type SK-N-AS can increase the anti-proliferative efficacy of low concentrations of irinotecan when measured at 72 hours.

Cetuximab was combined with increasing concentrations of irinotecan to test for synergy between the two agents in two resistant lines SK-N-AS, SK-N-BE(2) (a, b) and two sensitive lines LAN5, CHP134 (c, d). Proliferation was assessed at 72 hours using an MTT assay for mitochondrial activity.
Figure 8. Comparison of low dose irinotecan combined with Cetuximab sensitive and resistant neuroblastoma cell lines

In a post-hoc analysis, the effectiveness of the lowest concentration of irinotecan was compared with Cetuximab and the combination of both drugs for each cell line (a - d) revealing that while the IC₅₀ did not appreciably change, the efficacy of lower irinotecan doses did increase when combined with Cetuximab. All graphs represent the means ± standard deviation of at least three independent trials.

3.4 Conclusions

These experiments indicate that of all the neuroblastoma cell lines tested, SK-N-AS and SK-N-BE(2) are two of the most resistant lines to the five chemotherapy agents examined. This was one reason for selecting SK-N-AS for future experiments of this study as a model
cell line that is resistant to treatment. In addition, it does not appear that the addition of Cetuximab, an EGFR inhibitor, significantly changes the IC₅₀ for irinotecan in the four cell lines that were tested in vitro, although addition of Cetuximab to irinotecan did appear to enhance the growth inhibitory effect of low doses of irinotecan.
CHAPTER 4 – Anti-proliferative Effect of EGFR Targeted Therapy

4.1 Rationale

We wanted to study whether a depletion of UBE4B levels in a treatment resistant neuroblastoma cell line could make them more sensitive to growth inhibition with a drug agent that targets EGFR. Transient UBE4B depletion (53) results in a two-fold increase in the levels of EGFR in HeLa cells. Neuroblastoma cells overexpressing UBE4B were less sensitive to the anti-proliferative effects of Cetuximab (18). Our hypothesis was that stable depletion of UBE4B levels in a model neuroblastoma cell line would increase EGFR levels. This would introduce more targets to binding of the EGFR-targeted antibody Cetuximab and make those cells prone to growth inhibition by Cetuximab.

4.2 Depletion of UBE4B in SK-N-AS Leads to Increased EGFR Levels and Increased Anti-Proliferative Response to Cetuximab

Since UBE4B promotes the degradation of the EGFR (53), we hypothesized that resistant neuroblastoma cells depleted of UBE4B might become more sensitive to EGFR inhibition. UBE4B was depleted in SK-N-AS cells using a lentiviral-delivered shRNA against UBE4B followed by antibiotic selection. After one week of selection, we observed nearly undetectable levels of UBE4B in SK-N-AS cells (Figure 9). Since EGFR levels and downstream MAPK/ERK signaling are regulated by UBE4B (19, 53), we examined EGFR levels in the UBE4B-depleted SK-N-AS cells and observed a two-fold increase in EGFR levels (Figure 9), similar to what we have previously observed after acute depletion (53). Increased
EGFR levels promote cell proliferation in neuroblastoma (2) and lead to poor patient outcomes (18, 91). We hypothesized that elevated EGFR expression would enable anti-EGFR antibody (66) therapeutic efficacy. Treatment with increasing concentrations of Cetuximab significantly inhibited the proliferation of UBE4B-depleted SK-N-AS cells but did not inhibit the proliferation of parental cells (Figure 10). These data suggest that the elevated EGFR levels produced by UBE4B depletion may provide additional targets for Cetuximab allowing the drug to inhibit proliferation.
Depletion of UBE4B to undetectable levels using shRNA in the SK-N-AS neuroblastoma cell line leads to a two-fold increase in EGFR. Quantifications in are the averages of three independent trials ± the SD.
Figure 10. Growth inhibitory response to the EGFR-targeted antibody Cetuximab in neuroblastoma SK-N-AS with stable UBE4B expression

Treatment of model SK-N-AS neuroblastoma cells bearing a stable depletion of UBE4B (shUBE4B) with 1 $\mu$M and 2 $\mu$M of Cetuximab, a drug specific to EGFR, led to a significant drop in growth over 72 hours as measured by an MTT assay.

4.3 Conclusions

These experiments demonstrated that it is possible to deplete UBE4B to undetectable levels using a lentiviral shRNA delivery system resulting in a concurrent two-fold increase in EGFR protein levels. In vitro MTT assays revealed that Cetuximab (1 and 2 $\mu$M) can significantly inhibit the growth of UBE4B-depleted SK-N-AS cells a decrease not observed in
parental cells or those transduced with a scrambled shRNA suggesting that the change in response is a result of UBE4B depletion.
CHAPTER 5 – Downstream Protein Changes as a Result of UBE4B Depletion

5.1 Rationale

There is an inverse correlation between UBE4B and EGFR levels in neuroblastoma (18, 19, 53). The increase in EGFR levels that occurs following transient UBE4B depletion may result from decreased ubiquitination of EGFR resulting in decreased EGFR degradation. We examined whether other proteins might be increased or decreased in amount as a result of UBE4B depletion. We stably depleted SK-N-AS cells and assessed lysates using the Reverse Phase Protein Array (RPPA) method in which the levels of 305 cancer-related proteins were examined.

5.2 Depletion of UBE4B Leads to a Two-fold Increase in STAT5a

We observed 57 proteins to be increased by two-fold or more (Figure 11a) and 26 proteins decreased by 50% or more (Figure 11b). RPPA analysis confirmed a two-fold increase in EGFR, consistent with our data (Figure 9) and earlier studies (53). Among the proteins that were increased in amount, there was a mixture of proteins involved in proliferation and growth inhibition (Table 3). Among the proteins that were decreased in amount, many were pro-proliferative in nature (Table 4). We confirmed these increases in STAT5a protein levels using immunoblotting (Figure 12), and we found that it remained increased two-fold. These data suggest that EGFR and STAT5a levels increased specifically because of UBE4B protein depletion in the model SK-N-AS neuroblastoma cell line.
Figure 11. Reverse Phase Protein Analysis (RPPA) of increased and decreased proteins subsequent to UBE4B depletion in neuroblastoma SK-N-AS

Reverse phase protein analysis was used to assay 305 proteins increased or decreased in amount as a result of a UBE4B depletion the neuroblastoma SK-N-AS. This subset of that data shows proteins that are either increased two-fold or more (a) or decreased 50% or more (b) relative to the wild type and scrambled versions of that cell line. The red arrows indicate EGFR and STAT5a, the two proteins that were studied here.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Pathway</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-3-3-ζ</td>
<td>Promotes cell proliferation, adhesion and survival, and it inhibits apoptosis in multiple cancers. (92)</td>
<td></td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>BiP-GRP78</td>
<td>Endoplasmic reticulum chaperone protein necessary for cancer survival (93)</td>
<td></td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>p27-Kip-1</td>
<td>Negative regulator of apoptosis; not regulated by MYCN in neuroblastoma (94)</td>
<td></td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>Bid</td>
<td>Promotes apoptosis and can be used for survival prediction in colon cancer (95)</td>
<td></td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Bim</td>
<td>Induces apoptosis and anoikis (96)</td>
<td>AKT/PKB, blocks β-catenin</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>PEA-15</td>
<td>Positive regulation of apoptosis (97)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atg3</td>
<td>E2-like enzyme that is essential for promoting autophagy. It is degraded in the presence of chemotherapy agents like etoposide (98)</td>
<td></td>
<td>Autophagy</td>
</tr>
<tr>
<td>WIPI1</td>
<td>Also known as ATG18 and it promotes autophagy (99)</td>
<td></td>
<td>Autophagy</td>
</tr>
<tr>
<td>CD171</td>
<td>Cell adhesion marker that has characteristic expression in many cancers (100)</td>
<td></td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>CD44</td>
<td>Mediates cell-cell and cell-matrix adhesion and is expressed in tumors that are epithelial in origin. It can be used as a prognostic marker. (101)</td>
<td></td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>Collagen-VI</td>
<td>Cell adhesion protein that is highly expressed in a number of cancers (102)</td>
<td></td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Calcium-dependent adhesion molecule. Its dysregulation leads to tumor progression (103)</td>
<td></td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>N-Cadherin</td>
<td>Calcium-dependent adhesion molecule that is elevated in amounts in tumor tissues (104)</td>
<td></td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>Stat5a</td>
<td>Signal transduction and activation of transcription (STAT) is activated by EGFR. (105)</td>
<td>Jak/STAT</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Box</td>
<td>Subunit of an E3 ubiquitin ligase. Its dysregulation leads to human malignancy. (106)</td>
<td>JNK</td>
<td>E3 ligase</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>Mediates the regulation of protein translation by hormones, growth factors, and other stimuli that signal through the MAPK and AKT pathways. Loss of 4E-BP1 function induces epithelial-mesenchymal transition (EMT) and increases metastatic capability of cancer cells by translational activation of Snail. (107)</td>
<td>AKT and MAPK/ERK</td>
<td>Metastasis</td>
</tr>
<tr>
<td>EMA</td>
<td>Epithelial membrane antigen is a glycoprotein and its altered distribution in cells in a prognostic marker in breast cancer. (108)</td>
<td></td>
<td>Metastasis</td>
</tr>
<tr>
<td>TWIST</td>
<td>Transcriptional regulator of cell migration (109)</td>
<td></td>
<td>Migration</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Process</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>NDRG1_pT346</td>
<td>Stress-responsive protein involved in hormone responses, cell growth, and differentiation (110)</td>
<td>Migration inhibitor</td>
<td></td>
</tr>
<tr>
<td>YB1_pS102</td>
<td>Y-Box protein 1 mediates pre-mRNA alternative splicing regulation. It is a promoter of proliferation (111)</td>
<td>Proliferation</td>
<td></td>
</tr>
<tr>
<td>CD29</td>
<td>Integrin protein that is marker of metastasis in breast cancer (112)</td>
<td>Pro-migration</td>
<td></td>
</tr>
<tr>
<td>Hes1</td>
<td>Transcriptional repressor that is involved in cell migration by promoting epithelial mesenchymal transition (EMT). It mediates crosstalk between Jak/STAT and Notch signaling through Stat3. (113)</td>
<td>Jak/STAT, Notch</td>
<td></td>
</tr>
<tr>
<td>MMP2</td>
<td>Matrix metalloproteinase-2 promotes cell migration(114)</td>
<td>Pro-migration</td>
<td></td>
</tr>
<tr>
<td>VHL-EPPK1</td>
<td>Epiplakin 1 (EPPK1) is a cytoskeletal linker protein that connects to intermediate filaments and controls their reorganization in response to stress (115)</td>
<td>Pro-migration</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>Intermediate filament that is overexpressed in epithelial cancers (116)</td>
<td>SMAD</td>
<td></td>
</tr>
<tr>
<td>Cox-IV</td>
<td>Cytochrome oxidase that has elevated levels in colorectal cancer (117)</td>
<td>Proliferation</td>
<td></td>
</tr>
<tr>
<td>Cyclophilin n-F</td>
<td>Accelerates folding of proteins and counteracts p53 regulation of cancer growth (118)</td>
<td>Proliferation</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>Growth factor receptor that is increased in amount in many cancers (119)</td>
<td>AKT/PI3K, MAPK/ERK, Jak/STAT</td>
<td></td>
</tr>
<tr>
<td>Histone-H3</td>
<td>The H3 variant of histones, which are involved in transcription regulation, DNA repair, DNA replication and chromosomal stability, has been shown to common in malignant pediatric brain cancers. (120)</td>
<td>Proliferation</td>
<td></td>
</tr>
<tr>
<td>IGFBP2</td>
<td>IGF binding protein 2 inhibition has been shown to limit tumor proliferation. It potentiates EGFR-STAT3 signaling. (121),(122)</td>
<td>STAT</td>
<td></td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor is implicated in the tumorigenesis, angiogenesis, and metastasis of many cancer phenotypes (123)</td>
<td>MAPK/ERK, AKT/PI3K</td>
<td></td>
</tr>
<tr>
<td>MSI2</td>
<td>RNA binding protein that promotes TGF-β signaling and non-squamous cell lung cancer metastasis (124)</td>
<td>TGF-β</td>
<td></td>
</tr>
<tr>
<td>PI3K-p110-a</td>
<td>This is a specific mutation in Phosphoinositide-3-kinase (PI3K) signaling.</td>
<td>PI3K/ AKT</td>
<td></td>
</tr>
<tr>
<td>PRAS40_pT246</td>
<td>Proline-rich AKT substrate and subunit of mTORC1, which regulates cell growth and survival in response to nutrient and hormonal signals. (125)</td>
<td>PI3K/ AKT</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Function and Importance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TFAM</strong></td>
<td>Binds to the mitochondrial light strand promoter and functions in mitochondrial transcription regulation. Its knockdown lessens proliferation (109)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stathmin-1</strong></td>
<td>Microtubule destabilizer that plays an important role in cell cycle progression, segregation of chromosomes, clonogenicity, cell motility and survival. Its overexpression has been reported in malignant hematopoietic cells. (126)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DJ1</strong></td>
<td>Negative regulator of apoptosis and that increases breast cancer cell invasion. (127)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cox2</strong></td>
<td>Highly expressed in many cancers. It converts prostaglandin H2 to make PGE2 and promotes proliferation and apoptosis (128)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IGFRb</strong></td>
<td>Receptor tyrosine kinase which mediates the pleiotropic actions of insulin (129)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Insulin receptor β signaling</strong></td>
<td>Insulin receptor β signaling may be highly stimulated and therefore targeted in certain cancers. (130)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Creb</strong></td>
<td>Nuclear transcription factor that mediates response to growth factors. It is involved in tumor initiation, progression and metastasis. (131)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ENY2</strong></td>
<td>Coordinates activity of deubiquitinating enzymes and its dysregulation can promote cancer (132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MERIT40_pS29</strong></td>
<td>Part of the BRCA-1 complex. Its activation through phosphorylation mediates DNA repair after cancer treatment with doxorubicin. (133)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>14-3-3-β</strong></td>
<td>Blocks the nuclear translocation of the phosphorylated form (by AKT1) of SRPK2. It is a tumor suppressor. (134)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>53BP1</strong></td>
<td>Involved in cell cycle checkpoint and DNA repair activities. It is a known tumor suppressor in breast cancer. (135)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p16INK4a</strong></td>
<td>Negative regulator of proliferation and acts as a tumor suppressor (136)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pdcd4</strong></td>
<td>Inhibits translation initiation and cap-dependent translation and functions as a tumor suppressor (137)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Smad4</strong></td>
<td>Tumor suppressor that may be inactivated in many cancers (138)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SOD2</strong></td>
<td>The superoxide dismutase 2 destroys superoxide radicals. Polymorphisms may increase cancer risk. (139)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TAZ</strong></td>
<td>Restricts proliferation and promotes apoptosis (140)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
UBAC1  | E3 ligase that causes proteasome-mediated degradation of NF-κB. It is also known as KPC1. (141) | E3 ligase | Tumor suppressor
--- | --- | --- | ---
YAP_pS127 | Restricts proliferation and promotes apoptosis. It overlaps with TAZ (140) | Hippo & Wnt | Tumor suppressor
Caveolin-1 | May act as a scaffolding protein within caveolar membranes and is associated with tumor progression. Depletion leads to altered Jak/STAT, JNK, and Src signaling. (142) | Jak/STAT, JNK, Src | Tumorigenic
EIF4E | Translation initiation factor. Its phosphorylation promotes tumorigenesis. (143) | MAPK and AKT/PI3K | Tumorigenic
EIF4E_pS209 | Phosphorylation site for EIF4E (143) | MAPK and AKT/PI3K | Tumorigenic
PAI-1 | Plasminogen activator inhibitor 1 is a negative regulator of cell adhesion. It has a pro-tumorigenic role in cancer by promoting angiogenesis and tumor cell survival. (144) | | Tumorigenic
PKC-δ_pS664 | Calcium-independent, phospholipid- and diacylglycerol (DAG)-dependent serine/threonine-protein kinase. This mutation promotes tumorigenesis in pancreatic cancer. (145) | PKC | Tumorigenic (when mutated)

Table 3. Description of proteins increased two-fold or more subsequent to UBE4B depletion in neuroblastoma SK-N-AS

The proteins that were elevated two-fold or more in the RPPA analysis of SK-N-AS cells depleted of UBE4B were analyzed to determine their role in the cell and associations with known signaling pathways.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Pathway</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPKα</td>
<td>Catalytic subunit of AMP-activated protein kinase (AMPK). Regulates cellular metabolism and is associated with cell growth, metabolism, and autophagy. Downregulation in gastric cancer is associated with poor prognosis. (146)</td>
<td>AMPK</td>
<td>Autophagy</td>
</tr>
<tr>
<td>Cyclin-B1</td>
<td>Essential for the control of the cell cycle at the G2/M (mitosis) transition. High levels are associated with poor prognosis in breast cancer. (147)</td>
<td></td>
<td>Cell cycle regulator</td>
</tr>
<tr>
<td>PLK1</td>
<td>Serine/threonine-protein kinase and positive regulation of ubiquitin protein ligase activity. (148)</td>
<td></td>
<td>Cell division regulator</td>
</tr>
<tr>
<td>DUSP4</td>
<td>Regulates mitogenic signal transduction by dephosphorylating both threonine and tyrosine residues on the MAP kinases ERK1 and ERK2. (149)</td>
<td>MAPK/ERK</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Raptor</td>
<td>Regulates mTORC1 activity. Upregulation makes cells less sensitive to PI3K/AKT pathway inhibition (150).</td>
<td>PI3K/AKT</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>mTOR</td>
<td>Serine/threonine protein kinase which is a central regulator of cellular metabolism, growth and survival (151).</td>
<td>PI3K/AKT</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>PKCa</td>
<td>Calcium-activated, phospholipid- and diacylglycerol (DAG)-dependent serine/threonine-protein kinase important in cell signaling. (152)</td>
<td>PKC</td>
<td>Cell signaling, pro-proliferation</td>
</tr>
<tr>
<td>BAP1</td>
<td>Specifically mediates deubiquitination of histone H2A and regulates cell growth. (153)</td>
<td></td>
<td>Deubiquitination and tumor suppressor</td>
</tr>
<tr>
<td>ADAR1</td>
<td>Catalyzes the hydrolytic deamination of adenosine to inosine in double-stranded RNA (dsRNA). This is referred to as A-to-I RNA editing. It is associated with tumor-infiltrative lymphocytes in triple negative breast cancer. (154)</td>
<td></td>
<td>Pro-migration</td>
</tr>
<tr>
<td>Rictor</td>
<td>Subunit of mTORC2 that functions upstream of Rho GTPases to enhance cell migration. Overexpression yields poor prognosis in colon cancer. (155)</td>
<td>PI3K/AKT</td>
<td>Pro-migration</td>
</tr>
<tr>
<td>TRIM25</td>
<td>E3 ubiquitin ligase that enhances cell growth and proliferation in cancer by modulating p53 signaling. (156)</td>
<td></td>
<td>Pro-proliferation; E3 ubiquitin ligase</td>
</tr>
<tr>
<td>Protein</td>
<td>Description</td>
<td>Phosphorylation</td>
<td>Signaling Pathway</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>----------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>A-Raf</td>
<td>Isoform of Raf which is involved in cancer proliferation, migration, and invasion through involvement in the MAPK pathway.</td>
<td>MAPK/ERK</td>
<td>Pro-proliferation</td>
</tr>
<tr>
<td>eIF4G</td>
<td>Commonly found in breast cancer and increases translation for proteins required for proliferation and survival.</td>
<td></td>
<td>Pro-proliferation</td>
</tr>
<tr>
<td>S6</td>
<td>Ribosomal protein that regulates cell growth and proliferation. Reduced phosphorylation can increase sensitivity to MEK inhibition.</td>
<td>PI3K/AKT</td>
<td>Pro-proliferation</td>
</tr>
<tr>
<td>Src_pY527</td>
<td>Non-receptor protein tyrosine kinase that is involved in the regulation of cell growth and survival, apoptosis, cell-cell adhesion, cytoskeleton remodeling, and differentiation. Overexpressed in a number of human cancers.</td>
<td></td>
<td>Pro-proliferation</td>
</tr>
<tr>
<td>Stat3</td>
<td>Signal transducer and transcription activator that mediates cellular responses to interleukins, KITLG/SCF, LEP and other growth factors.</td>
<td></td>
<td>Pro-proliferation</td>
</tr>
<tr>
<td>TFRC</td>
<td>Cellular uptake of iron occurs via receptor-mediated endocytosis of this ligand-occupied transferrin receptor into specialized endosomes. Promotes proliferation when overexpressed.</td>
<td></td>
<td>Pro-proliferation</td>
</tr>
<tr>
<td>FAK_pY397</td>
<td>Non-receptor protein-tyrosine kinase that plays an essential role in regulating cell migration, adhesion, spreading, reorganization of the actin cytoskeleton, formation and disassembly of focal adhesions and cell protrusions, cell cycle progression, cell proliferation and apoptosis.</td>
<td></td>
<td>Pro-proliferation and migration</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>Key downstream component of the canonical Wnt signaling pathway.</td>
<td>WNT</td>
<td>Proto-oncogene</td>
</tr>
<tr>
<td>eEF2</td>
<td>Tumor-associated antigen that is overexpressed in many types of cancer.</td>
<td></td>
<td>Tumor antigen</td>
</tr>
<tr>
<td>PTEN</td>
<td>Tumor suppressor</td>
<td>PI3K/AKT</td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>TSC1</td>
<td>TSC2, inhibits the nutrient-mediated or growth factor-stimulated phosphorylation of S6K1 and EIF4EBP1 by negatively regulating mTORC1 signaling.</td>
<td>PI3K/AKT</td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>Tuberin TSC2</td>
<td>In complex with TSC1, this tumor suppressor inhibits the nutrient-mediated or growth factor-stimulated phosphorylation of S6K1 and EIF4EBP1 by negatively regulating mTORC1 signaling.</td>
<td>PI3K/AKT</td>
<td>Tumor suppressor</td>
</tr>
</tbody>
</table>
FASN | Fatty acid synthetase whose overexpression is common in many types of cancer. (168) | Tumor marker

Table 4. Description of proteins decreased 50% or more subsequent to UBE4B depletion in neuroblastoma SK-N-AS

The proteins that were decreased by 50% or more in the RPPA analysis for SK-N-AS cells depleted of UBE4B were analyzed to determine their role in the cell and associations with known signaling pathways.

Figure 12. Western blot and quantitation of changes in STAT5 in neuroblastoma SK-N-AS with UBE4B depletion

One protein of interest, STAT5a, increased two-fold in the RPPA dataset and this increase was then confirmed with quantitative Western immunoblotting.
5.3 Conclusions

I observed that depletion of UBE4B protein in SK-N-AS cells results in increases and decreases in the levels of a number of proteins. EGFR and STAT5a levels both increased two-fold. These data suggest that these two pro-proliferative proteins can be targeted individually or in combination in order to inhibit the growth of the model neuroblastoma cells in vitro.
CHAPTER 6 – Growth Inhibitory Effect of Combined EGFR and STAT5 Targeted Therapy

6.1 Rationale

We wanted to test whether targeting EGFR and STAT5a pharmacologically could inhibit growth of the model SK-N-AS neuroblastoma cell line depleted of UBE4B in vitro. The levels of both of these proteins were increased two-fold in the RPPA analysis and immunoblot of cell lysates. We also found evidence in the literature of studies that had targeted either EGFR (18, 91) or STAT5 (169) in neuroblastoma. Our hypothesis was that dual inhibition of both EGFR and STAT5 would reduce growth in vitro and increase apoptotic cell death. Dual inhibition might also prevent migration in vitro, which is an early step in metastasis (170).

6.2 Depletion of UBE4B Enhances the Anti-Proliferative and Pro-Apoptotic Effect of Cetuximab and the STAT5 inhibitor SH-4-54

STAT5a is a member of the Jak/STAT signaling pathway that can be activated by EGFR (171). STAT5a levels are increased in proliferative cancers, and its constitutive activation can contribute to resistance to EGFR-targeted therapies (83). STAT5a has also been targeted with chemotherapeutics as either monotherapy or combination therapy in other cancers like leukemia (84, 172) and prostate cancer (173). STAT5 is a part of Jak/STAT signaling of EGFR (80), and directly inhibiting signaling pathways of EGFR can partially
prevent signal transduction even when the EGF ligand is bound (174). Therefore, it may be beneficial to specifically target both EGFR and STAT5a to reduce cell proliferation and resistance to targeted therapies. We hypothesized that simultaneous inhibition of EGFR and STAT5a might be more effective at inhibiting SK-N-AS proliferation than either agent alone. The rationale for this was that in some cases signaling would be blocked by competitive inhibition of EGF binding because of Cetuximab, whereas in other cases the ligand would still bind, but signaling would still be partially inhibited by STAT5 inhibition.

The combination of a STAT5a inhibitor SH-4-54 (5 μM) with Cetuximab (2 μM) has a significant anti-proliferative effect in UBE4B-depleted SK-N-AS neuroblastoma cells (Figure 13). This antiproliferative effect was not observed in control cells transfected with a scrambled shRNA or in parental cells. These data suggest that combined inhibition of EGFR and STAT5a inhibits SK-N-AS cell growth because of the depletion of UBE4B. We saw signs of cell detachment and morphology changes in vitro with microscopy when measuring cell growth (data not shown). Additionally, the fraction of metabolically viable cells began to decrease at 72 hours as measured by MTT assays. These facts suggest that combined EGFR/STAT5 inhibition may be producing apoptosis. Moreover, the addition of SH-5-54 appears to potentiate the effect of Cetuximab (Figure 14). The anti-proliferative effect observed with 2 μM Cetuximab is enhanced by the addition of SH-5-54.
Figure 13. Anti-proliferative effect of combination EGFR and STAT5 inhibition in neuroblastoma SK-N-AS with UBE4B depletion.

The proliferation of wild type, scrambled, and UBE4B-depleted SK-N-AS neuroblastoma cells was measured in the presence of erlotinib, an EGFR tyrosine kinase inhibitor (c), Cetuximab (a), the STAT5 inhibitor SH-4-54 (f), and a combination of either the STAT5 inhibitor with erlotinib (d) or STAT5 inhibitor with Cetuximab (b) using an MTT mitochondrial activity assay for 72 hours. Measurements were taken daily and normalized to day zero, the time when the drugs were initially added to cells in vitro. Data points for each graph represent the mean ± the SD from three independent trials.
Figure 14. Demonstration of potentiation between Cetuximab alone and Cetuximab with a STAT5 inhibitor in inhibiting the proliferation of neuroblastoma

The combination of 2 µM Cetuximab with 5 µM of SH-4-54, a STAT5 inhibitor, shows a potentiation effect (175). Cetuximab alone causes a significant decrease in proliferation in model SK-N-AS cells with a stable depletion of UBE4B. STAT5 inhibition alone does not show a significant anti-proliferative effect. The combination of the STAT5 inhibitor SH-4-54 and Cetuximab is greater for all neuroblastoma SK-N-AS lines that we tested. In the model SK-N-AS line with a stable depletion of UBE4B (shUBE4B), there is a significantly greater anti-proliferative effect than in the wild type or control cells.

We next examined whether the combination of Cetuximab and SH-4-54 affected apoptosis in UBE4B-depleted cells. UBE4B-depleted SK-N-AS cells, parental cells, or cells transfected with a scrambled shRNA were treated with Cetuximab and SH-4-54 alone or in combination. We measured the number of cells that were fluorescently labeled indicating caspase 3 and 7 activation and calculated the ratio of fluorescently-labeled cells to the total number of cells (Figure 15). There was an increase from about 1.5% GFP+ cells (cells treated
with vehicle alone) to 5% GFP+ cells (scrambled cells) or 7.5% GFP+ in wild type SK-N-AS when they were treated with a combination of Cetuximab and SH-4-54. The model SK-N-AS depleted of UBE4B increased significantly from 2.5% GFP+ cells with vehicle treatment to nearly 13% with combination treatment. Treatment with SH-4-54 and Cetuximab alone showed 4.7% and 5.1% increases, respectively, in cleaved caspases indicating that these agents have some toxic effect by themselves. These data suggest that the addition of SH-4-54 potentiates the antiproliferative effect of Cetuximab in neuroblastoma SK-N-AS cells, causing greater cell death than either agent alone.
Figure 15. Pro-apoptotic effect of combination EGFR and STAT5 inhibition in neuroblastoma SK-N-AS with UBE4B Depletion

Cetuximab combined with STAT5 inhibition causes apoptotic cell death at 48 hours, particularly in cells depleted of UBE4B. Wild type SK-N-AS neuroblastoma cells or...
SK-N-AS cells with stable expression of a resistance plasmid with a scrambled insert or an shUBE4B insert were treated with 5 µM STAT5 inhibitor, 2 µM of Cetuximab, or a combination of the two. (a) Caspase-3/7 activity was assessed with the CellEvent green detection reagent and phase contrast microscopy was used to quantify cell number. (b) The ratio of green-expressing cells to total number of cells was significantly greater in UBE4B depleted cells co-treated with the STAT inhibitor and Cetuximab.

6.3 Combination Treatment with Cetuximab and the STAT5 Inhibitor SH-4-54 Inhibits Cell Migration In Vitro

One of the challenges in treating stage 4 neuroblastoma is that the disease has often metastasized prior to diagnosis (7). Cell migration is an initial step in the metastatic process (170), that can be assessed in vitro. We have shown that the combination of EGFR and STAT5a inhibition reduces growth and increases apoptosis. Since EGFR activation can underlie migration (176), we used a transwell migration model to assess whether EGFR and STAT5a inhibition might affect tumor cell migration.

UBE4B-depleted SK-N-AS cells and control cells (parental cells and those transfected with a scrambled shRNA) were pre-treated for 24 hours with a combination of Cetuximab (2 µM) and SH-4-54 (5 µM). We observed that Cetuximab and SH-4-54 slightly decreased migration for parental, scrambled, and UBE4B-depleted cells (Figure 16). However, the combination of Cetuximab and SH-4-54 significantly reduced transwell migration in UBE4B-depleted SK-N-AS cells when compared to the effect of the combination in parental and scrambled control cells (Figure 16). The effect of the combined inhibitors in the depletion line was also significantly less compared to either STAT5 or EGFR inhibition alone. These data
suggest that in neuroblastoma cells with low levels of UBE4B, the combination of EGFR and STAT5 inhibitors may reduce tumor cell migration.

Migration of UBE4B-depleted SK-N-AS neuroblastoma cells is impaired when treated with a combination of the STAT5 inhibitor SH-4-54 and the EGFR inhibitor Cetuximab. Cells were pretreated with 24 hours with PBS/DMSO vehicle, the STAT5 inhibitor, Cetuximab, or a drug combination mixed in complete media. Cells

Figure 16. Anti-migratory effect of combination EGFR and STAT5 inhibition in neuroblastoma SK-N-AS with UBE4B Depletion
were then counted and an equal amount of 1.5e5 viable cells was added to the top surface of transwell membranes. Cells were allowed to grow and migrate for 24 additional hours in the presence of drug at 37°C. Membranes were then washed, fixed, and stained with DAPI to visualize migratory cells (a) which were then quantified by counting cells visible in a high-power field at 10x magnification and averaging at least three fields per condition. The quantitation (b) represents the average of three trials ± the SD.

6.4 Conclusions

We observed that EGFR and STAT5a inhibitors can produce growth inhibitory effects over a three-day time course in UBE4B-depleted SK-N-AS cells. Growth inhibition was observed with Cetuximab, but not erlotinib, a tyrosine kinase inhibitor. Addition of a STAT5 inhibitor potentiated the growth inhibitory effect of Cetuximab. In fact, in vitro experiments suggest that the combination of EGFR and STAT5 inhibitors increased apoptotic cell death of all cell lines, and impaired cellular migration. The inhibition of migration is greater in the UBE4B-depleted SK-N-AS cells than in parental cells or those transduced with a scrambled shRNA. A potential limitation of the cell migration assay was that measurements for migration were not blinded, which could have introduced bias. It would be ideal to re-score the saved DAPI-stained transwell membranes from the cell migration experiments using a blinded observer. This would provide additional validity to these results that suggest an anti-migratory effect of EGFR and STAT5 inhibition that is greater when UBE4B is depleted. Regardless, these data suggest that combined inhibition of EGFR and one of its downstream signaling partners, STAT5, may have a greater inhibitory effect on growth and cell migration in cells with low levels of UBE4B.
CHAPTER 7 – Discussion and Future Directions

7.1 Overview

High-risk neuroblastoma is associated with frequent relapses and tumors that are resistant to treatment (177) and children with refractory or recurrent neuroblastoma have very poor survival rates (4, 178, 179). We have previously observed that low UBE4B expression is associated with poor outcomes in patients with neuroblastoma (18). UBE4B is required for efficient degradation of the EGFR, and UBE4B protein levels are inversely related to EGFR protein levels (18), signaling (53), and cellular differentiation (19) in patient tumor samples. We screened a chemoresistant neuroblastoma cell line for intracellular proteins whose levels are altered when UBE4B is depleted. Interestingly, STAT5a was among the proteins whose levels were increased and it is also linked to EGFR-mediated proliferative signaling. We found that in contrast to inhibiting EGFR and STAT5 individually, targeting both EGFR and STAT5 potentiated the inhibition of cell proliferation and migration, and promoted apoptosis in UBE4B-depleted SK-N-AS cells. These data suggest that multiplexed targeting of EGFR signaling may be an effective strategy for inhibition of growth when tumors have low levels of UBE4B. This may not be an effective approach, however, for patients with normal levels of UBE4B. Instead it should be viewed as a form of personalized cancer therapy for a particular patient subset: patients whose tumors express low amounts of UBE4B.
7.2 SK-N-AS as a Resistant Model Cell Line

The cell line SK-N-AS was determined to be more resistant to many of the conventional chemotherapy agents that we tested. SK-N-AS has been reported to be a resistant cell line (88) and it was originally cultured from a patient with metastasis to the bone (180). Like most of the cell lines that we tested (Table 2), it also has a deletion of the 1p chromosome site, which is the location of UBE4B (65). Low UBE4B protein levels correlate with poor prognosis (18, 19), and this is consistent with the resistant behavior of that neuroblastoma cell line in our study.

The parental SK-N-AS line we used had detectable levels of UBE4B. Neuroblastoma is a very heterogeneous cancer (181) with variable expression of UBE4B throughout a single tumor (19). This heterogeneity is also another contributor to the variable chemotherapy response in neuroblastoma (181). After depletion of UBE4B, the doubling of EGFR protein levels seen here was consistent with earlier in vitro experiments in HeLa cervical adenocarcinoma cells using silencing RNA toward UBE4B (53). This supports the model suggesting that UBE4B ubiquitinates the EGFR at the endosomal membrane and promotes receptor degradation (53).

7.3 Cetuximab Enhances the Growth Inhibition of Low Doses of Irinotecan In Vitro

We thought that a combination of irinotecan, a topoisomerase inhibitor, with the anti-EGFR antibody Cetuximab might lower the IC₅₀ necessary for irinotecan to inhibit SK-N-AS proliferation. SK-N-AS cells were more resistant to irinotecan than any other cell line we
examined, and upregulation of EGFR-mediated signaling has been shown to mediate irinotecan resistance (182). Additionally, in clinical literature, the combination of irinotecan and Cetuximab is specifically indicated for colorectal cancer that has become refractory to irinotecan (183). However, I observed that the IC$_{50}$ achieved with irinotecan alone did not change significantly with the addition of Cetuximab. This lack of effect parallels a phase I clinical trial where irinotecan and Cetuximab were used in combination in pediatric solid tumors with no therapeutic benefit (76). This might be explained in part by the high cellular heterogeneity in neuroblastoma (181) leading to inconsistent response to treatments that are effective in other cancers.

One observation of note with the irinotecan and Cetuximab combination data, was that the anti-proliferative effect of the lowest doses of irinotecan did seem to be enhanced by the addition of Cetuximab. This may be because the inhibition of proliferative signaling pathways caused by EGFR binding by Cetuximab (66) is sufficient to amplify the effects of an agent like irinotecan which interferes with DNA replication. Clinically, this combination might be used to enhance the efficacy of smaller doses of irinotecan to limit the toxicities caused by this drug in children (184).

7.4 UBE4B Depletion Increase Levels of Signaling Proteins (EGFR and STAT5a)

Our study identified many proteins that are increased or decreased in amount following UBE4B depletion in neuroblastoma cells. Many of the upregulated proteins like SMAD4 (138) and PEA-15 (185), a tumor suppressor and a positive regulator of apoptosis respectively, are consistent with a cell inhibiting proliferation. However, other upregulated proteins like EGFR
and Signal Transducer and Activator of Transcription 5a (STAT5) are consistent with a hyperproliferative state (186). A thorough review of the roles, pathways, and functions of proteins that increased or decreased in amount are provided in Table 3 and Table 4 in Chapter 5. Of the proteins increased in amount that were identified for the model SK-N-AS cells depleted of UBE4B, there were several proteins associated with EGFR signal transduction pathways like MAP kinase/ERK, Akt/PI3K, and Jak/STAT (187). We subsequently looked for ways to co-target EGFR and one of its downstream signaling pathways pharmacologically to test the hypothesis that a combination of agents that would inhibit multiple aspects of EGFR signaling might provide enhanced antiproliferative responses.

EGFR and STAT5a both increased two-fold in UBE4B-depleted SK-N-AS cells. The regulation of EGFR trafficking by UBE4B has been described (53). The ubiquitination of STAT5a is a requisite step for its degradation, but the identity of that E3 ligase has not yet been established (188). One possibility for the link between STAT5a and UBE4B is that UBE4B ubiquitinates STAT5a to promote its degradation. Alternatively, UBE4B may lead to the ubiquitination and degradation of another protein which is a regulator of STAT5a. In either case, a decrease in UBE4B levels would lead to an increase in STAT5a levels. There was one other E3 ligase that was downregulated in our RPPA analysis, TRIM25, but we found no evidence of a demonstrated link between UBE4B and TRIM25, or between TRIM25 and STAT5a. Therefore, there is reason to believe that reduced ubiquitination of STAT5a might explain why it increased here, but the exact mechanism will require future investigation.

STAT5 is a member of the Jak/STAT signaling and it has been targeted with specific drugs as treatments for breast cancer (186), prostate cancer (173), and leukemia (189). STAT5 levels are increased in proliferating tumors (190), and the Jak/STAT pathway is one of the
signaling cascades that may be activated by EGFR (171). Constitutive activation of STAT5 can contribute to resistance to EGFR-targeted therapies (83). There are also parallel examples of Jak/STAT inhibition combined with EGFR-targeted therapy in lung (85) and skin malignancies (174) to successfully inhibit cell growth. This led us to ask whether chemotherapeutic targeting of both EGFR and STAT5 could significantly decrease proliferation in model cells with a stable depletion of UBE4B.

There are seven proteins in the STAT family, including STAT1, STAT2, STAT3, STAT4, STAT5a, and STAT5b (171). The proteins STAT3, STAT5a, and STAT5b are thought to have a role specifically in the development of cancer, while STAT1 has an opposite effect (190), and all transmit EGFR signals through the Jak/STAT pathway (191, 192). This signaling enhances proliferation, cell migration, and angiogenesis (193). STAT5a and STAT5b are highly similar isoforms of STAT5 that share 93% sequence homology (194, 195). Our RPPA data for the neuroblastoma SK-N-AS cell line depleted of UBE4B showed that there was a two-fold increase in STAT5 protein levels, and a 90% decrease in STAT3. In addition, phosphorylated STAT3 decreased by 56% when compared with parental and scrambled control lysates. STAT3 and STAT5 are good targets for inhibition to inhibit cancer’s growth (105), especially in hematologic malignancies (196). However, because of the large overlap between STAT3 and STAT5 in signal transmission, STAT5 inhibition may not be sufficient to decrease proliferation if STAT3 protein levels are high (171). In the case of our model SK-N-AS cell line, the lower levels of STAT3 and activated, phosphorylated STAT3 increase the opportunity for a STAT5 inhibitor to reduce neuroblastoma’s growth in vitro.

Our data suggest that inhibition of STAT5 by SH-4-54 is not highly effective by itself in inhibiting neuroblastoma cell proliferation in vitro regardless of whether UBE4B is depleted.
The dopamine antagonist pimozide, that also has STAT5 inhibitory activity, has been reported to have some anti-proliferative effect on SK-N-AS (169). In addition, STAT5 and STAT3 have very similar roles in promoting signals for cancer proliferation (190), and there are several examples of STAT3 inhibitors used as a single agent (197) or in combination with a Jak inhibitor to successfully reduce neuroblastoma proliferation in vitro and tumor growth in vivo (198). Even though STAT3 levels were lower in these model cells depleted of UBE4B, these examples provided an indication that STAT5 inhibition might be able to inhibit neuroblastoma cell growth. Doing so, however, might require a combination with another antiproliferative agent.

7.5 UBE4B Depletion Enhances Sensitivity to Cetuximab, an anti-EGFR antibody

Our data demonstrate that the chemotherapy resistant neuroblastoma cell line SK-N-AS is more sensitive to Cetuximab-mediated EGFR inhibition than the tyrosine kinase inhibitor erlotinib. The growth inhibitory effect of Cetuximab suggests that at least some of the non-degraded EGFR is located on the cell surface where it can be targeted by Cetuximab. Furthermore, the maximum Cetuximab dose used in a clinical trial with children having solid tumors was 250 mg/m² (76), which equates to a serum concentration of less than 1 µM based on the average body mass and blood volume of an 18-month-old child (199). Thus, the Cetuximab concentrations used in this study may be in the therapeutic range for patients whose tumor cytology indicates low protein levels of UBE4B.

Erlotinib is a small molecule that binds to the intracellular tyrosine kinase domain of EGFR to prevent enzymatic function (200). It was used here as a control and an alternative
method of targeting EGFR. Our past cell growth assays with this drug agent have shown it to be ineffective slowing the growth of the neuroblastoma SK-N-AS line (data not shown). However, other studies have reported a 4 µM IC₅₀ for erlotinib in wild type SK-N-AS neuroblastoma cells in vitro (91), and a phase I clinical trial with erlotinib has shown efficacy in patients (201). It is unclear why erlotinib was not effective at inhibiting growth in this study. Gefitinib, which also targets the tyrosine kinase activity of EGFR, is not effective at inhibiting neuroblastoma proliferation (202). One explanation for the non-response to erlotinib may be that STAT5 activation specifically reduces sensitivity to erlotinib in other cancers (83). However, this still does not explain the similar behavior between wild type and UBE4B depleted cells since the non-depleted cells would be expected to have a growth inhibitory effect. It will be worth repeating this experiment with a new preparation of erlotinib and low passage SK-N-AS cells in the future to determine whether SK-N-AS is in fact non-responsive to erlotinib. Neuroblastoma is very heterogeneous, which affects the chemotherapy response (181), and long durations of cell passages in vitro could generate a cell population that is non-responsive to erlotinib.

7.6 Combination of STAT5 and EGFR Inhibition Reduces Neuroblastoma Growth, Promotes Apoptosis, and Reduces Migration In Vitro

Our data suggest that combined EGFR and STAT5 inhibition results in decreased neuroblastoma cell growth and increased apoptotic cell death. This finding parallels a study in chronic myelogenous leukemia (CML), that indicated high STAT5 levels mediated resistance to a small molecule tyrosine kinase inhibitors (189), and another study suggesting that EGFR
blockade combined with Jak/STAT pathway inhibition was highly effective in epidermoid carcinoma (174). This combination therapy targets the EGFR to prevent ligand binding, reduce Jak/STAT signaling, and promote receptor degradation (Figure 17) (190). Cetuximab is unlikely to saturate all of the EGFR on a cell’s surface in vitro because of competitive inhibition with the ligand EGF (203), and small amounts of antibody actually reach tumors in vivo (204, 205). Therefore, dual inhibition of the receptor and one of its pathways may potentiate the effect of Cetuximab to achieve a more robust antiproliferative effect in vitro (Figure 15) (175). Cells expressing greater levels of EGFR, potentially on the cell surface, would have more targets for Cetuximab binding to promote cell death. Thus, this targets a pro-proliferative phenotype of increased levels of EGFR to provide an additional treatment approach for neuroblastoma.
Figure 17. Combined EGFR and STAT5 blockade potentiates the effect of Cetuximab

Cetuximab, an EGFR-specific antibody, binds to the EGFR on the surface of neuroblastoma to prevent ligand binding and induce receptor degradation. SH-4-54, a STAT5 inhibitor, blocks one of the pathways of EGFR proliferative signaling. The combined effect was observed to be greater than either agent alone.

In addition to halting cell growth, there was evidence that the combination therapy also caused cell death. We observed decreased cell viability with the MTT assay at 72 hours when compared to the 24- and 48-hour time points, and microscopic examination suggested that cells were less adherent and dense at longer time points (data not shown). STAT5 normally activates anti-apoptotic pathways in cancer, and other studies have used a STAT5 null cell line to significantly enhance the apoptosis induction by a tyrosine kinase inhibitor (189). I found a significant increase in apoptosis when cells we treated with a combination of the STAT5
inhibitor SH-4-54 and the EGFR inhibitor Cetuximab, but there was no statistical difference between UBE4B depleted neuroblastoma cells and the control cells. EGFR inhibition with Cetuximab can cause cell death by autophagy in addition to apoptosis, especially when there are high levels of the autophagy-related gene (Atg) proteins (206). In the RPPA dataset, we saw a four-fold increase in Atg3 in SK-N-AS cells depleted of UBE4B (Figure 11). This may indicate that death in cells treated with Cetuximab and the STAT5 inhibitor SH-4-54 is occurring as a result of autophagy more so than apoptosis, especially in cells depleted of UBE4B.

Migratory behavior of tumor cells is one of the early signs of metastatic spread of cancer (170, 207), and metastasis to distant sites in the body is a hallmark of more advanced neuroblastoma disease (12). The epidermal growth factor receptor is also a key driver of wound healing on the skin, which is a migratory behavior of cells (208). We found that in UBE4B-depleted neuroblastoma cells, treatment with EGFR and STAT5 inhibitors reduced transwell migration of the cancer cells in vitro. EGFR inhibition in glioma cells reduces migration in a similar manner (209). Given that about 70% of neuroblastoma patients have experienced metastatic disease at the time of initial diagnosis (7), this is an exciting finding requiring further examination. One pitfall to our present data is that it may not have appropriately taken into account cell death caused by the combination treatment. Pre-treated cells were counted with trypan blue, and the number of cells added to the transwell insert for migration experiments was based off of the number of live cells measured by that reagent. Trypan blue would not take into account the number of cells on the verge of death, however, so there may have been cells that failed to migrated because of death alone. An alternative method to both count the cells and assess viability before the migration assay would be to use flow cytometry with cell sorting.
(FACS). In summary, these data indicate that in neuroblastoma cells expressing low amounts of UBE4B, a combination of a STAT5 inhibitor and Cetuximab can inhibit proliferation, induce cell death, and prevent cell migration that could lead to metastasis.

7.7 Summary and Implications for Treatment

We have observed that depletion of UBE4B can lead to increases in the levels of proteins involved in proliferation, including EGFR and STAT5a. EGFR inhibition using Cetuximab has growth inhibitory effects in UBE4B-depleted neuroblastoma cells at lower concentrations than has been seen previously in wild type cells (18). Finally, EGFR and STAT5 dual inhibition reduced proliferation, induced apoptosis, and slowed cell migration in UBE4B-depleted neuroblastoma cells more than either agent alone.

Patients with refractory neuroblastomas often have fewer chemotherapeutic options. Their care team must rely on salvage chemotherapy, repeat autologous stem cell transplantation, radiotherapy, or immunotherapy to save the patient’s life (179, 210). The novel combination of EGFR and STAT5 inhibition described here may provide an additional option for refractory patients with a 1p36 deletion and lower levels of UBE4B. Given that surgical resection is the common first step for many neuroblastomas, it would be possible to measure UBE4B protein levels in patient tumors to determine whether they would be appropriate candidates.
7.8 Future Directions

Orthotopic Animal Experiments

One of the most critical areas for further exploration for this project will be to perform an *in vivo* experiment to test the antiproliferative effect of a combination of Cetuximab and the STAT5 inhibitor SH-4-54 *in vivo*. An orthotopic xenograft model of neuroblastoma would be an appropriate model (211). Introduction of luciferase would enable tumor size tracking and modification of the UBE4B-depleted SK-N-AS cells to express luciferase would allow examination of tumor responses to EGFR and STAT5 treatments.

Given the anti-proliferative, pro-apoptotic, and anti-migratory effect seen in the model neuroblastoma line in response to a combination treatment, we hypothesize that *in vivo* tumors grown from neuroblastoma cells with a depletion of UBE4B and then treated with this combination will have less tumor volume over a 5-week experiment, and they may also have lower incidence of metastasis. In this experiment we would monitor tumor volume with IVIS as well as body weight and survival time. Following sacrifice we would weigh the excised tumors and perform immunoblotting for levels if UBE4B, EGFR, STAT5 as well as histologic analysis for CD31 to assess angiogenesis (212).

Neuroblastoma patients with a deletion at the 1p36 chromosomal location would potentially be responsive to EGFR and STAT5 inhibition as a form of treatment. About one-third of neuroblastoma patients have this deletion in their cancer cells (14, 19). UBE4B levels could be tested in surgically resected tumors. Lower levels of UBE4B might indicate using this novel treatment approach, whereas it might be contraindicated in patients with tumors containing high levels of UBE4B. This kind of customized therapy for cancer patients is the
emerging wave of the future (213). Even though neuroblastoma is a relatively rare cancer and the number of patients treated in this manner would be potentially small, it could improve survival outcomes for certain high-risk patients. For those patients and their families, this could be incredibly important.

Assessment of Additional EGFR and Signaling Pathway Combined Treatments

We focused on STAT inhibition here because of the elevated STAT5 and decreased STAT3 protein levels, which the literature indicated would make these model cells ideal for treatment with an EGFR and STAT inhibitor (171). Despite this, there are several examples in the literature where the AKT/PI3K pathway (214) or the MAPK/ERK (215) pathway have been targeted in neuroblastoma. Future studies could assess the growth inhibitory effects of those agents in these model cells when combined with Cetuximab.
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