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Amyloid Precursor Protein (APP) Regulates G0/G1 Transition and Cell Growth

Anna Sobol

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LOYOLA UNIVERSITY CHICAGO

AMYLOID PRECURSOR PROTEIN (APP) REGULATES G<sub>0</sub>/G<sub>1</sub> TRANSITION AND CELL GROWTH

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR BIOLOGY

BY

ANNA SOBOL
CHICAGO, IL
AUGUST 2015
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To my Family
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<tr>
<td>--------------</td>
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<tr>
<td>4E-BP1</td>
<td>Eukaryotic translation initiation factor 4E-binding protein 1</td>
<td></td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
<td></td>
</tr>
<tr>
<td>ACL</td>
<td>Adenocarcinoma of the lung</td>
<td></td>
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<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase domain-containing protein</td>
<td></td>
</tr>
<tr>
<td>AHA</td>
<td>L-azidohomoalaine</td>
<td></td>
</tr>
<tr>
<td>AICD</td>
<td>Amyloid precursor protein intracellular domain</td>
<td></td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
<td></td>
</tr>
<tr>
<td>APLP</td>
<td>Amyloid precursor-like protein</td>
<td></td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
<td></td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma kinase</td>
<td></td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
<td></td>
</tr>
<tr>
<td>BACE</td>
<td>Beta-site APP cleaving enzyme</td>
<td></td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
<td></td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
<td></td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
<td></td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
<td></td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
<td></td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
<td></td>
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</tbody>
</table>
FBS  Fetal bovine serum
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GFP  Green fluorescent protein
GLUT-1  Glucose transporter 1
Grb2  Growth factor receptor-bound protein 2
GSI  \(\gamma\)-secretase inhibitor
HIF-1\(\alpha\)  Hypoxia inducible factor-1\(\alpha\)
HIFs  Hypoxia inducible factors
Hr  Hour
HRE  Hypoxia response elements
i.p.  Intraperitoneal
IGF-1  Insulin-like growth factor-1
IGF-1R  Insulin-like growth factor-1 receptor
LCC  Large-cell lung carcinoma
MAPK  Mitogen activated protein kinase
min  Minute
MKK  MAP kinase kinase
ml  Milliliter
mM  Millimolar
mTOR  Mechanistic target of rapamycin
mTORC-1  Mechanistic target of rapamycin (mTOR) complex 1
NCSTN  Nicastrin
xii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>Oct-4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDPK1</td>
<td>3-phosphoinositide dependent protein kinase 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5 trisphosphate</td>
</tr>
<tr>
<td>PRAS40</td>
<td>Proline-rich Akt substrate</td>
</tr>
<tr>
<td>PSEN</td>
<td>Presenilin</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>Raf</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>RAPTOR</td>
<td>Regulatory-associated protein of mTOR</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>REDD-1</td>
<td>Regulated in development and DNA damage responses 1</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small cell lung cancer</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>STYX</td>
<td>Serine threonine tyrosine interacting protein</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor A</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
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ABSTRACT

Non-small cell lung cancer (NSCLC) originates in the epithelia of the lung and persists as the leading cause of cancer-related death in the United States. Many studies have shown that hypoxia is prevalent in NSCLC tissue and negatively influences treatment outcome.

In previous studies, we investigated the efficacy of γ-secretase inhibitor (GSI) treatment in an orthotopic mouse NSCLC model. Analysis of hypoxic areas of tumors from GSI treated animals showed reappearance of 4E-BP1 phosphorylation at threonine 37/46 residues (T37/46). 4E-BP1 phosphorylation is primarily mediated by mechanistic target of rapamycin complex 1 (mTORC-1), a major cell integrator of growth factors stimulation, nutrient availability, and environmental cues.

In this study, we sought to identify a putative target of γ-secretase that could mediate this apparent reactivation of mTORC-1 activity in otherwise quiescent hypoxic NSCLC. We identified amyloid precursor protein (APP) as the substrate of γ-secretase that mediates this anabolic reactivation upon GSI treatment. APP is a highly pleiotropic protein implicated in a plethora of cellular functions, including malignant growth. siRNA-mediated depletion of APP in NSCLC cell lines cultured in hypoxia enhanced 4E-BP1’s phosphorylation at T37/46.
Our results indicated that depletion of APP caused 4E-BP1 phosphorylation pattern rearrangement by affecting various proteins including ERKs and the pseudophosphatase STYX, a novel APP target. Additionally, APP altered the recruitment of eIF4A RNA helicase to the translation initiation complex. These changes coincided with a prominent increase in the global protein synthesis rate.

Since cell growth and division are inherently linked processes in both normal and cancerous cells, we investigated if depletion of APP had any effect on cell proliferation. Surprisingly, APP depletion resulted in a cyclin C-dependent G₀ arrest of hypoxic NSCLC cells. This paradoxical uncoupling of cell growth and proliferation caused cellular abnormalities, including increased cell size and necrotic cell death. The observed changes were reversed by the overexpression of APP intracellular domain (AICD).

In conclusion, APP (via AICD) seems to mediate G₀/G₁ transitions and moderate cell growth rate. As inhibition of APP causes cell cycle arrest and necrosis, we propose that APP could be a novel therapeutic target for the eradication of hypoxic NSCLC.
CHAPTER ONE
INTRODUCTION

Novel approaches to target hypoxic non-small cell lung cancer (NSCLC) cells are urgently needed to improve the treatment of this deadly disease. NSCLC is the leading cause of cancer-related deaths in the US in both men and women, while low oxygen concentration, or hypoxia, was shown to confer resistance to radiotherapy or chemotherapy, promote tumor progression, provide a niche for cancer stem cells (CSC), and is linked to poor prognosis (Walsh et al., 2014; American Cancer Society, 2015).

LUNG CANCER

In the Western world, lung cancer is the leading cause of cancer-related deaths (Esposito et al., 2010). Projections for 2015 indicate that more people will die of lung cancer than of breast, prostate, and colorectal cancers combined (American Lung Association, 2015). The overall five-year survival rate of lung cancer patients is about 15% (all stages, American Lung Association, 2015). Age-adjusted death rate of lung cancer is higher in males than females.
It is also higher for black men compared to white men while the rates are the same for both black and white women (Centers for Disease Control and Prevention, 2014). Lung cancer is broadly subdivided into two major histologic subtypes: small cell lung cancer (SCLC) and non-small cell lung cancers (NSCLC). NSCLC is of epithelial origin and is considered less responsive to chemotherapy as compared to SCLC (Vescio et al., 1990). Although SCLC, which is of neuroendocrine origin, responds well to traditional cytotoxic therapies, it recurs quickly as chemotherapy-resistant. In this form, SCLC rapidly spreads to the entire chest and metastasizes very quickly. These features make SCLC one of the most aggressive known malignancies with a median survival after diagnosis of 11.8 months (Babakoohi et al., 2012). 70% of NSCLC cases are diagnosed at advanced stages (IIIB and IV). In these conditions, the five-year survival of NSCLC patients is about 2% (Govindan et al., 2006; Krug et al., 2008).

**Non-small cell lung cancer: Histology**

Approximately 85% of all lung cancer cases are NSCLC (National Cancer Institute, 2014). Although recently reclassified, NSCLC is traditionally subdivided into three main subtypes: adenocarcinoma of the lung (ACL), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). ACL, SCC, and LCC account for 40%, 25%, and 10% of lung cancer cases, respectively (National Cancer Institute, 2013).
The distinctive features of lung adenocarcinomas include histologic heterogeneity, glandular differentiation, and mucin production. These tumors are a mixture of different patterns including acinar, papillary, micropapillary, and bronchioalveolar (Travis et al., 2011). On the other hand, a key characteristic of squamous cell carcinoma is evidence of keratinization, including either intercellular bridges or squamous “keratin pearls” (Heighway and Betticher, 2004). Some lung tumors are poorly differentiated and cannot be classified as SCLC, ACL or SSC. If histological examination of these tumors shows no apparent features of squamous cells, neuroendocrine cells, columnar epithelium, and shows no signs of mucin secretion, then these tumors may be diagnosed as LCCs. Basically, LCC is a diagnosis of exclusion; the cells are not small, but they do not present a glandular-like organization or a squamous phenotype. Different variants of LCCs include clear cell carcinomas, large cell carcinomas with rhabdoid phenotype, and lymphoepithelioma-like carcinomas (Heighway and Betticher, 2004; National Cancer Institute, 2014).

ACL and LCC are located in peripheral areas of the lungs, while SCC typically arises in proximal bronchi (Schrump et al., 2008). Different localization indicates that these NSCLC subtypes may originate from distinct progenitor cells (Galluzzo and Bocchetta, 2011).
Cellular origin

Although the lung does not undergo a constant renewal of its epithelia, it can repair damage, even significant damage. Each lung compartment contains a limited number of progenitor cells that can undergo injury-induced proliferation and initiate the repopulation of the damaged areas. The multipotent progenitor cells located within the bronchial epithelium include cytokeratin 14-expressing basal cells and naphthalene-resistant Clara cell secretory protein (CCSP)-expressing cells (CE). Both cell populations appear necessary for the renewal of the bronchiolar epithelium upon injury (Wistuba et al., 1997; Hong et al., 2004). Most likely, these cells give rise to SCC, which generally originates in bronchi (Galluzzo and Bocchetta, 2011). ACL, which arises at the periphery of the lung, has been demonstrated to originate from progenitor cells localized to the bronchio-alveolar duct junctions (BADJ) (Giangreco et al., 2002). These bronchio-alveolar stem cells (BASCs) initiate renewal of damaged alveolar epithelium. BASCs are positive for prosurfactant apoprotein-C (SP-C), a T2 pneumocyte marker, and CCSP, a marker of Clara cells. In vivo studies indicate that BASCs genetically engineered with CRE-recombinase inducible oncogenes give rise to adenomas and adenocarcinomas, which are indistinguishable from human ACL (Kim et al., 2015). While the identity of LCC progenitor cells is still debated, experts in the field seem to agree that this malignancy can originate from BASCs (Hanna and Onaitis, 2013).
**Etiology**

90% of lung cancer deaths are due to active smoking (American Lung Association, 2015). At least 70 different chemicals present in cigarette smoke have been demonstrated to be carcinogenic (The United States Department of Health and Human Services, 2010). Some of the most harmful, cancer-causing chemicals include nicotine-derived nitrosamine ketone (NNK) and DNA damaging polycyclic aromatic hydrocarbons (PAH; Schrump et al., 2008). Cigarette-associated carcinogens were shown to activate various pro-survival signaling pathways including AKT and ERK, and to induce mutations within tumor suppressor genes like p53 (Belinsky et al., 1996; Tsurutani et al., 2005). Importantly, the risk of lung cancer depends on the duration and intensity of smoking, decreasing gradually after smoking cessation (Peto et al., 2000).

The role of genetic predisposition to lung cancer is still not clear. Nonetheless, some studies reported an association between polymorphisms involving several genes, such as ERCC2/XPD and CHRNA3, and lung cancer risk (Zhan et al., 2010; Xiao et al., 2014).

Environmental pollutants are increasingly implicated in NSCLC pathogenesis. Traditional environmental agents that are unequivocally considered to cause NSCLC are radon, certain types of coal, and asbestos (American Lung Association, 2015). Increasingly, the scientific community is considering other factors such as ground transportation or exhaust fumes as alternative causes of NSCLC (Pope et al., 1995; Larkin, 2000).
Numerous molecular driver mutations have been identified in NSCLC, and the best-identified examples are found in the most common histopathological type of NSCLC, adenocarcinoma. These include mutations of well-known oncogenes, such as KRAS, NRAS, ALK, MET, EGFR, PIK3CA, AKT1, BRAF, HER2, MEK1, RET, and ROS1. Tumor suppressor genes commonly lost in NSCLC include p16, p53, PTEN, and others (Schrump et al., 2008; Lovly et al., 2015). Some of these mutations can predict treatment outcomes. For example, specific EGFR mutations render some NSCLC sensitive to drugs such as erlotinib and gefitinib (Gazdar, 2009). However, no single mutation alone seems to represent a good indicator of prognosis (Schrump et al., 2008; Jackman et al., 2010).

**Diagnosis and treatment approaches**

About 70% of NSCLC cases are diagnosed at the stages IIIb and IV, involving regional (lymph nodes) and distant (mainly bone, liver, adrenals) metastasis (American Lung Association, 2015). The classic staging system used in NSCLC is called the Tumor (T, or primary tumor size and extent), Node (N, or lymph nodes involvement), Metastasis (M, or presence of distant metastases). This system is commonly referred as TNM Staging system and is summarized in Table 1 (refer to a table below; Schrump et al., 2008).

Since most of the NSCLC cases are diagnosed at advanced stage, there is an urgent need for early detection methods (National Cancer Institute, 2014). Some of the approaches, such as sputum cytology or chest x-rays, were evalu-
ated in the past, but were found to be ineffective for early detection of lung cancers (Schrump et al., 2008). Others, like low-dose helical computed tomography (CT) scans, are still evaluated (National Lung Screening Trial Research Team, 2011).
Table 1. Tumor, node, metastasis (TNM) staging system. The standard system used to stage NSCLC cases and assess the characteristics of primary tumor, but also proximal (nodal) and distant (other organs) metastasis. Modified from Schrump et al., 2008

<table>
<thead>
<tr>
<th>PRIMARY TUMOR</th>
<th>DESCRIPTION</th>
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<tbody>
<tr>
<td>TX</td>
<td>Main tumor cannot be measured</td>
</tr>
<tr>
<td>T0</td>
<td>No main tumor</td>
</tr>
<tr>
<td>T1-T4</td>
<td>Size and/or extent of the main tumor (Higher number corresponds to larger size or infiltration to nearby tissue). Example: T1 is a primary tumor of no more than 3 cm in diameter that does not infiltrate the pericardium, or the peritoneum.</td>
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<tr>
<th>REGIONAL LYMPH NODE INVOLVEMENT</th>
<th>DESCRIPTION</th>
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<tr>
<td>NX</td>
<td>Nodal involvement cannot be measured</td>
</tr>
<tr>
<td>N0</td>
<td>No nodal involvement</td>
</tr>
<tr>
<td>N1-N3</td>
<td>Number and location of lymph nodes that contain cancer (The more distal the node is from the primary tumor, the higher the “N” number)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>METASTATIC INVOLVEMENT</th>
<th>DESCRIPTION</th>
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<tbody>
<tr>
<td>MX</td>
<td>Metastases cannot be measured</td>
</tr>
<tr>
<td>M0</td>
<td>No metastases</td>
</tr>
<tr>
<td>M1</td>
<td>Metastases present (regardless of the size and location of the metastasis)</td>
</tr>
</tbody>
</table>
Currently, the standard of care for treatment of NSCLC patients is a combination of cytotoxic platinum based compounds, like cisplatin or carboplatin, and third-generation antineoplastic agents, including gemcitabine (a nucleoside analog) and paclitaxel (an inhibitor of mitosis; Rowinsky and Donehower, 1995; Schiller et al., 2002). The major disadvantage of these compounds is limited efficacy and high toxicity, which is a common health concern especially in elderly patients (Florea and Büsselberg, 2011). A new treatment approach is to target pathways that are essential for cancer cell survival, growth, metastasis, etc. One example is bevacizumab (inhibitor of vascular endothelial growth factor A), which is the only anti-angiogenic agent approved to use as the first-line therapy for advanced non-squamous NSCLC (Reck et al., 2009). However, bevacizumab treatment can only add about one month to the overall survival of NSCLC patients (Sandler et al., 2006; Lima et al., 2011). Other treatments, using EGFR and ALK inhibitors (erlotinib and crizotinib respectively) lead to resistance and tumor recurrence (Jackman et al., 2010; Doebele et al., 2012). Development of new drugs to target the underlying mechanisms of drug resistance is under investigation. Thus, new treatment strategies and molecular targets are urgently needed to improve the survival chances of NSCLC patients.

As for all solid tumors, the hypoxic tumor microenvironment represents the major obstacle for therapy. This is due to a number of features that characterize hypoxic NSCLC, which will be detailed in the next chapter.
TUMOR HYPOXIA

Hypoxic or low-oxygenated tumor microenvironment has been long recognized as a major obstacle for cancer therapy (Harrison et al., 2002). Even though the tumor responds to oxygen and nutrients deficits by inducing neoangiogenesis in surrounding tissues, this process is inefficient, abnormal, and unable to meet the oxygen demands of cancer cells (Liu et al., 1995). As the tumor grows, the distance between cancer cells and blood vessels increases and some cancer cells will inevitably have limited access to nutrients and oxygen (Figure 1). Importantly, delivery of anticancer chemotherapeutics to these distant tumor cells is also reduced, contributing to treatment resistance of hypoxic tumor tissue.

Hypoxia regulates metabolic activity and proliferative potential of tumor cells. Cancer cells in a hypoxic microenvironment are under a metabolic checkpoint, mediated by the inhibition of cell growth regulator the mechanistic target of rapamycin complex 1 (mTORC-1) (see below). Cells within the hypoxic tumor mass are also quiescent (Busk and Horsman, 2013).
Figure 1. Tumor hypoxia. The microenvironment of solid tumors depends on their proximity to blood vessels. Areas in sufficient proximity to an arteriole have plenty of oxygen, nutrients, and buffering capability of human plasma. These cells are likely to proliferate and grow rapidly. The more distal tumor cells are to blood supply, the less anabolic and proliferative activities will be favored. In critical regions, oxygen, nutrients, and extracellular pH will no longer support growth or proliferation. These cells are quiescent and actively undergoing autophagy (green in the figure). If this situation is not ameliorated by increased blood flow to these areas, cancer cells will exhaust energy and undergo necrosis. This gradient of solutes distribution also applies to anticancer drugs. Modified from the OMCCCD website.
Hypoxia negatively influences both radiotherapy and chemotherapy. Lack of oxygen decreases the formation of DNA breaks mediated by ionizing radiation-induced oxidative radicals (Grey et al., 1953). It also increases resistance of cells to antibiotics that also induce DNA breaks, like bleomycin (Hahn, 1996). Hypoxia causes cell growth arrest, thus rendering cancer cells resistant to cell cycle selective chemotherapeutics, such as 5-fluorouracil (Goda et al., 2003). Cancer cells improve their chances of survival in hypoxic microenvironment by increasing resistance to apoptotic cell death using a variety of mechanisms. Thus, these cancer cells also become resistant to apoptosis promoting agents, like Etoposide (Hussein et al., 2006). Hypoxia is also known to promote epithelial-to-mesenchymal transition, thus increasing cancer invasiveness and metastasis (Pennacchietti et al., 2003; Hill et al., 2009; Chang et al., 2011). Because of profound alterations of the extracellular environment, hypoxic tumor tissue interferes with the activities of both the innate and acquired immune systems (Yotnda et al., 2010). Thus, hypoxia emerges as an important pro-survival factor that not only protects cancer cells from anti-cancer therapy but also favors a more aggressive phenotype. Not surprisingly, hypoxia is a well-recognized poor prognostic factor for cancer patients.
Molecular basis of tumor hypoxia

Hypoxia affects the biology of cancer cells by inducing changes in their gene expression pattern. These changes are mediated by the master transcriptional regulators Hypoxia Inducible Factors (HIFs). HIFs can bind to the promoters or enhancers of a wide variety of target genes encoding proteins essential for cell growth, cell proliferation/survival, metabolism, and angiogenesis (Table 2). Two closely related proteins, HIF-1 and HIF-2, were shown to possess endogenous transactivation activity, while HIF-3 acts as a dominant-negative regulator of HIF-1 (Makino et al., 2001). HIF-1 is an extensively studied, major regulator of the transition between normoxia and hypoxia (Semenza et al., 1998). HIF-1 consists of hypoxia inducible subunit α and a constitutively expressed subunit β (also known as aryl hydrocarbon receptor nuclear translocator, or ARNT). In normoxia HIF-1α is transcribed and expressed but rapidly degraded (Wang et al., 1995). The signal for degradation includes a hydroxylation of two proline residues and acetylation of a lysine residue within the oxygen-dependent degradation domain (ODDD). This is followed by the recruitment of the von Hippel-Lindau E3 ubiquitin ligase complex and subsequent polyubiquitination of HIF-1α. This polyubiquitination targets HIF-1α for 26S proteasome-mediated degradation (Masson et al., 2001). Additionally, hydroxylation of an asparagine residue within the C-terminal transactivation domain (C-TAD) inhibits the
interaction of HIF-1α with a co-activator CBP/p300, thus inhibiting its transactivation activity (Lando et al., 2002).

In hypoxia, the HIF-1α protein is stable; it translocates to the nucleus and associates with HIF-1β subunit. This complex binds the hypoxia response elements of target genes, recruits transcriptional co-activators, and consequently induces transcription (Lando et al., 2002). The list of HIF regulated genes is still growing and includes genes encoding regulators of glucose uptake and glycolytic enzymes (GLUT-1, PGK, ALDA) allowing cancer cells to adapt their metabolism to low oxygen concentration, genes related to angiogenesis (VEGF) promoting blood vessels growth, as well as genes increasing cancer aggressiveness and metastasis (CXCR4) (Semenza et al., 2003). Thus, HIF-1α not only helps cancer cells adopt to and survive in hypoxic environment but also promotes its progression.
Table 2. Examples of HIF targets, hypoxia regulated phenotypes, and drugs that target central signaling pathways governing such phenomena. HIFs regulate transcription of many genes responsible for a wide variety of cellular functions (Höckel et al., 2001; Schofield et al., 2004). Some examples of drugs that target these pathways are listed in this table.

<table>
<thead>
<tr>
<th>HIF’s Targets</th>
<th>Signaling Pathway</th>
<th>Inhibitors</th>
</tr>
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<tbody>
<tr>
<td>Vascular endothelial growth factor A, Vascular endothelial growth factor receptor 1, plasminogen activator inhibitor 1</td>
<td>Angiogenesis</td>
<td>Bevacizumab (Sandler et al., 2006)</td>
</tr>
<tr>
<td>Transferrin, Transferrin receptor</td>
<td>Transport</td>
<td>anti-TfR mAb (Hong et al., 2013)</td>
</tr>
<tr>
<td>Chemokine receptor CXCR4, cMet</td>
<td>Cell migration</td>
<td>Plerixafor (Uy et al., 2012)</td>
</tr>
<tr>
<td>Glucose transporter-1, Hexokinase-2, aldolase A</td>
<td>Energy metabolism</td>
<td>3-Bromopyruvate (Jae et al., 2009)</td>
</tr>
<tr>
<td>Insulin-like growth factor 2, Protransforming growth factor alpha</td>
<td>Survival</td>
<td>DX-2647 (Dransfield et al., 2010)</td>
</tr>
<tr>
<td>Notch, Oct4, Nanog</td>
<td>Stem cell homeostasis</td>
<td>GSI (Olsauskas-Kuprys et al., 2013)</td>
</tr>
</tbody>
</table>
Hypoxia and cancer stem cells

Hypoxia has been conclusively linked to stem cell status. Stem and progenitor cells are characterized by the induction of signaling pathways, which play pivotal roles during embryogenesis (e.g., Wnt, Hedgehog, Notch). Under hypoxia, there seems to be a central axis between Notch and Oct4. Notch receptors are well-established regulators of cell differentiation. Upon activation, Notch receptors are cleaved and produce an intracellular domain (ICN). This ICN then translocates to the nucleus and has transcriptional activity. It has been demonstrated that hypoxia inhibits myogenic satellite cell and neuronal stem cell differentiation in a Notch-dependent manner (Gustafsson et al., 2005). HIF-1α was shown to be recruited to the promoters of the Notch target genes and to interact with ICN. The interaction between HIF-1α and Notch signaling seems to be required for the maintenance of stem cells in an undifferentiated state. Additionally, HIF-2α binds the promoter of Oct-4 and increases its expression and activity. Oct-4 is an essential transcriptional regulator of stem cell self-renewal and maintenance. HIF-2α is thought to regulate stem cell identity via activation of Oct4 (Covello et al., 2006).

The inhibition of differentiation by hypoxia has important implications in cancer biology. Among HIF regulated genes, we can mention ATP-binding cassette transporters (ABC transporters including MDR1 or Bcrp/ABCG2), which
are expressed by stem cells and associated with chemoresistance (Tai et al., 2005; Comerford et al., 2002; Krishnamurthy et al., 2004).

Cancer stem cells (CSC) are thought to sustain cancer growth. They can either self-renew or give rise to cells that form a particular cancer type. Unfortunately, many therapeutic regimens do not lead to the eradication of cancer initiating cells (identified by some as cancer stem cells). The nature of NSCLC CSC is only recently being defined. A handful of recent reports have identified some surface markers associated with NSCLC CSC, including CD133 and CD44 (Alamgeer et al., 2013). Additionally, the expression and activity of the cytoplasmic enzyme aldehyde dehydrogenase (ALDH) was shown to correlate with stem-like characteristics in NSCLC cell lines (Alamgeer et al., 2013). These included the ability to self-renew, proliferate, and generate tumors in vivo (Jiang et al., 2009). The exclusion of membrane permeable dyes is attributed to the overexpression of ATP-binding transporters by stem-like cells (Bunting, 2002). Notably, a population of cells (“side population”, SP) that exclude a Hoechst 33342 dye was recently identified in NSCLC cell lines and tumors (Ho et al., 2007; Alamgeer et al., 2013). These cells were shown to possess stem-like characteristics, for example, the ability to regenerate SP and non-SP cell populations and resistance to multiple anti-cancer drugs (Ho et al., 2007). A recent study has indicated that inhibition of driver oncogenes (such as EGFR) in NSCLC enriches the population of ALDH+ cells that display stem cell-like properties. These cells seem dependent on Notch-3 signaling (Arasada et al., 2014). In conclusion, stem or progenitor cell identity seems to be mediated by a
number of factors that are provided within their niches. Since the ground-breaking study by Gustaffson et al., a wealth of data has been gathered establishing the link between hypoxia inducible factors (HIFs), Notch signaling, the maintenance of the undifferentiated state, and the survival of NSCLC under hypoxia (Gustafsson et al., 2005; Keith and Simon, 2007).

**NSCLC and hypoxia**

Different techniques have been used to assess hypoxic areas of NSCLC tumors. Some studies applied staining for hypoxia inducible markers like HIF-1\(\alpha\) or its downstream target glucose-transporter 1 (GLUT-1) (used as a hypoxia marker in our study; Jackson et al., 2010). GLUT-1 is often considered a more direct method to visualize hypoxic areas than HIF-1\(\alpha\), and its expression was shown to correlate with poor prognosis in numerous tumors, including NSCLC (Younes et al., 1997; Furudoi et al., 2001; Kawamura et al., 2001; Kang et al., 2002; Kunkel et al., 2003; Tohma et al., 2005). Tumor hypoxia can also be visualized with non-invasive imaging techniques using radiolabeled tracers like \(^{18}\text{F}\) fluoromisonidazole (FMISO) (Ziemer et al., 2003). In different studies of NSCLC patients, hypoxia was shown to be a predictive marker for tumor recurrence and treatment outcome (Jackson et al., 2010). Positron emission tomography (PET) imaging using fludeoxyglucose \((^{18}\text{F})\) of fifty-seven patients with NSCLC showed that the tracer distribution predicts the probability of postoperative recurrence of
these patients (Higashi et al., 2002). Another study, including fourteen patients with advanced NSCLC, showed that kinetics of FMISO as visualized by PET imaging is predictive of tumor recurrence after radiotherapy (Eschmann et al., 2005). In conclusion, hypoxia is a major problem in the treatment of NSCLC. Hypoxic NSCLC is poorly accessible to both radiotherapy and chemotherapy. Hypoxic pockets are most likely sheltering NSCLC CSC. My central hypothesis is that any successful therapeutic approach for NSCLC must aim at the eradication of hypoxic NSCLC microenvironment. This task is rendered even more challenging because of the aforementioned metabolic checkpoint/quiescence of hypoxic NSCLC cells. The vast majority of anticancer agents currently used or under development selectively target actively proliferating or metabolic active cells. These are exactly the antitheses of hypoxic NSCLC cells. One potentially novel strategy in targeting hypoxic NSCLC would be reactivating anabolic processes in dormant cells in an environment that cannot sustain these activities. These cells would rapidly exhaust their energy reserve and suffer a similar fate of cells in which the hypoxic condition cannot be relieved (necrotic cell death, see Figure 1). The cellular central hub where growth factor stimulation, energy levels, and nutrient availability are sensed and translated into cell fate choices is mechanistic target of rapamycin (mTOR) complex 1, or mTORC-1. The inherent linkage between growth and proliferation is achieved at this level (Laplante and Sabatini, 2009; Ruggero, 2013). To better understand these processes, a more detailed description of this complex and its activities is required.
mTOR PATHWAY IN THE REGULATION OF CELL GROWTH AND DIVISION

mTOR, a serine/threonine protein kinase, is one of the major regulators of cell growth and division in eukaryotic cells, from yeasts to humans. It was initially identified as a target of a drug rapamycin, which has immunosuppressant and anti-proliferative activities (Heitman et al., 1991). In mammalian cells, mTOR is a component of two large protein complexes: mTORC-1 and mTORC-2. The function and regulation of mTORC-1 is far better understood as compared to mTORC-2. Remarkably, mTORC-1 can sense the availability of oxygen, growth factors, and amino acids, as well as a plethora of stress stimuli. Essentially, mTORC-1 regulates cell homeostasis through its control of global protein and lipid synthesis, cell metabolism, and inhibition of autophagy (Figure 2).
Figure 2. mTOR signaling pathway. mTOR is a component of both mTORC-1 and mTORC-2 complex. Growth factors and nutrients availability as well as stress and oxygen levels regulate the activity of mTORC-1. In response to these stimuli, mTORC-1 affects lipids and protein synthesis rates as well as autophagy. The above schematic has been modified from Laplante and Sabatini, 2012. For details, see text.
Upstream regulators of mTORC-1

mTORC-1 is a complex of multiple proteins with separate functions. mTOR is a serine/threonine kinase. It specifically associates with regulatory-associated protein of mTOR (RAPTOR), which determines substrate specificity of the mTORC-1 complex (Asnaghi, et al., 2014). RAPTOR recognizes its substrates by binding to TOS motifs (Schalm et al., 2003). Another central mTORC-1 component is the structural protein mammalian lethal with SEC13 protein 8 (mLST8). Besides these core components, other proteins can associate to the mTORC-1 complex. Proline-rich AKT-substrate of 40 kDal (PRAS40) is thought to be an mTORC-1 inhibitory protein whose properties are lost or greatly diminished upon AKT phosphorylation (Laplante and Sabatini, 2009). Another loosely associated protein is ras homologue enriched in brain (Rheb). This protein is necessary for mTORC-1 activity when it is bound to GTP. The mTORC-1 complex is considered to intrinsically sense amino acids availability (especially leucine and asparagine; Avruch et al., 2006). However, this sensing capability is completely lost in the absence of Rheb, along with any kinase activity. The major cellular inhibitor of mTORC-1 is the tuberous sclerosis complex (TSC), a large heterodimer of TSC-1 (hamartin) and TSC-2 (tuberin). The main functions of TSC are to promote hydrolysis of GTP bound to Rheb and convert the complex into Rheb-GDP (Laplante and Sabatini, 2012).
In response to growth factor stimulation, such as insulin, the PI3K/AKT and Ras/ERK1/2 pathways inactivate TSC1/2 and activate mTOR kinase (Manning et al., 2002; Ma et al., 2005).

The tyrosine kinase growth factor receptors activate Phosphoinositide 3-kinase (PI3K), which phosphorylate phosphoinositides and generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 recruits AKT to membrane compartments (including the plasma membrane) where it is first phosphorylated by phosphoinositide dependent kinase 1 (PDK1) at T308 (Peltier et al., 2007). mTORC-2 further phosphorylates AKT at S473 (Sarbassov et al., 2005). In general, AKT phosphorylated at T308 is mostly responsible for AKT functions on mTORC-1, while fully phosphorylated AKT (at both T308 and S473) is involved in the regulation of CREB, FOXO transcription factors, and hyperactivation of pro-survival, anti-apoptotic proteins (Peltier et al., 2007; Rafalski and Brunet, 2011, Laplante and Sabatini, 2012). AKT is a central regulator of mTORC-1. It inhibits TSC2 by phosphorylating it at multiple sites, thus activating mTORC-1 (Zhang et al., 2003). It also phosphorylates and inactivates the proline-rich AKT substrate 40 (PRAS40) at T246. PRAS40 is an additional mTORC-1 inhibitor (Sancak et al., 2007).

ERK1/2 have also be shown to phosphorylate TSC2 but at different sites than AKT. This phosphorylation inhibits the Rheb-GAP activity of the TSC1/TSC2 complex and is thought to promote TSC1-TSC2 dissociation (Ma et al., 2005). Thus, the ERK1/2 pathway activates mTORC-1.
While amino acid levels can enhance mTORC-1 activity via Rag GTPases (Kim et al., 2008) chronic stress leads to repression of mTORC-1 activity.

Low energy status or low oxygen content (hypoxia) causes activation of adenosine monophosphate-activated protein kinase (AMPK) that activates TSC2, inhibiting mTORC-1 (Gwinn et al., 2005). AMPK not only exerts its function via the regulation of TSC, but also through the phosphorylation of RAPTOR, compromising mTORC-1’s function (Gwinn et al., 2008). Hypoxia also regulates activity of mTORC-1 via upregulation of REDD1, which then activates TSC1/2 (DeYoung et al., 2008). DNA damage increases the expression of TSC2 and, consequently, inhibits mTORC-1 (Feng et al., 2005).

**Downstream targets of mTORC-1**

mTORC-1 regulates cell growth via multiple pathways. It positively regulates protein synthesis via phosphorylation of 4E-BP1 (mainly at threonine 37/46, T37/46). 4E-BP1 is a central repressor of protein synthesis where both the PI3K/AKT and the Ras/MEK signaling pathways converge to promote tumorigenicity (She et al., 2010). 4E-BP1 binds the eukaryotic initiation factor eIF4E and prevents the formation of the eukaryotic initiation of translation complex (collectively known as eIF4F; Parsyan et al., 2010). Phosphorylation of 4E-BP1 at T37/46, followed by the phosphorylation at threonine 70 (T70) and serine 65 (S65), allows eIF4E binding to eIF4G initiating protein synthesis (Sonenberg and Hinnebusch, 2009; see below).
mTORC1 also phosphorylates S6K (at threonine 389), a serine/threonine protein kinase that regulates protein synthesis, insulin sensitivity, and cell cycle (Ma and Blenis, 2009; Shin et al., 2011). S6K targets many components important for protein synthesis regulation. These include eEF2K, TIF1A and eIF4B (Sonenberg and Hinnebusch, 2009).

Regulation of lipid synthesis by mTORC-1 is mediated by lipin 1 and sterol regulatory element-binding protein 1/2 (SREB1/2) which transcriptionally activates genes related to fatty acid and cholesterol synthesis (Porstmann et al., 2008; Peterson et al., 2011).

mTORC-1 also contributes to cell growth by inhibition of autophagy. It phosphorylates and suppresses a protein complex responsible for this degradation pathway (ULK1/Atg13/FIP200) (Jung, 2009). Finally, mTORC-1 can also inhibit lysosomes biogenesis (Lee et al., 2009; Settembre et al., 2011).

**mTORC-1 and cell cycle progression**

mTORC-1 coordinates cell growth and cell division during G₁ to S transition. This is not surprising as anabolic processes are also activated during this period. Every cell has to clear critical checkpoints before committing to proliferation and for progression through the cell cycle. Early in the G₁ phase, the cell clears a growth-factor dependent restriction point (R). Mammalian cells pass this restriction point 3 to 4 hours post-G₁ entry (Zetterberg and Larsson, 1985). However, there is another checkpoint in G₁ phase. It is the late-G₁ metabolic check-
point, which is reminiscent of the $G_1$ checkpoint known in yeast as START. It is a nutrient-dependent “cell growth” checkpoint that heavily relies on mTORC-1 functions (Fingar and Blenis, 2004; Foster et al., 2010). The existence of this checkpoint was suggested more than 30 years ago. In one experiment, mouse fibroblasts were starved of amino acids or treated with mTORC-1 inhibitor rapamycin. These cells arrested at the $G_1$ phase just like upon growth factor withdrawal. However, replenishing cells arrested at START with amino acids caused these cells to re-enter the cell cycle much faster than cells forced into quiescence after growth factors deprivation (Yen and Pardee, 1978). In another experiment, Stiles et al. noticed that even if the exposure of cells to platelet-derived growth factor (PDGF) is sufficient for cell cycle commitment, progression through the $G_1$ phase required continuous insulin-like growth factor-1 (IGF1) treatment (Stiles et al., 1979). IGF1 activates mTORC-1 via PI3K/mTOR signaling pathway. These and other experimental data strongly support the existence of the mTORC-1 dependent metabolic checkpoint.

As mTORC-1 signaling regulates essential cellular processes, its deregulation is associated with different human disorders including cancer, obesity, diabetes, cardiovascular disease, and aging (Laplante and Sabatini, 2012). Mutations within the mTORC-1 signaling pathway that activate mTORC-1 can force cells to bypass the metabolic checkpoint; these hereditary syndromes are characterized by tumor-like growths in different organs. This is best represented in Cowden syndrome and tuberous sclerosis complex (Brook-Carter et al., 1994; Liaw et al., 1997), disorders characterized by the uncontrolled growth of
Hamartomas in multiple organs. On the other hand, downregulation of components of the PI3K/mTOR pathway (for example Igf-1 and Igf-2 null mutations or AKT2 and AKT3 mutations) results in organ hypoplasia and a growth deficiency in mice (Liu et al., 1993; Dummler et al., 2006).

One of the best-characterized pathways regulated by mTORC-1 is protein synthesis. Its deregulation was implicated in different human pathological conditions including cancer (Le Quesne et al., 2010). A large plethora of empirical evidence support the notion that deregulated protein synthesis can lead to malignant transformation (Ruggero and Pandolfi, 2003). Aberrant expression of initiation factors (especially eIF4E, which is upregulated in the vast majority of malignancies; De Benedetti and Graff, 2004), mutations of ribosomal proteins, and translation ancillary factors lead to so-called ribosomopathies that seem to sustain the malignant phenotype (Ruggero, 2013). Protein translation is one of the most complex mechanisms in eukaryotic cells. Most of its regulation takes place at the stage of the pre-initiation complex formation on the mRNA cap. This process is universally considered the rate-limiting step in protein translation.

**INITIATION OF PROTEIN SYNTHESIS AND ITS REGULATION**

Protein synthesis is an essential biological process that allows the conversion of information encoded by the genetic code into protein molecules with a variety of cellular activities. It is one of the most complex and highly regulated processes consisting of three major stages: initiation, elongation, and termination.
(Figure 3). Initiation involves the interactions between translation initiation proteins, initiator tRNA (transfer RNA), and small ribosomal subunit 40S on mRNA (messenger RNA). After start codon recognition, the large ribosomal subunit 60S joins to form a translation elongation competent complex 80S. Translation terminates at a stop codon and results in dissociation of the newly synthesized polypeptide from tRNA and ribosomal subunits from mRNA.

Translation initiation is the protein synthesis step where most regulation occurs (Sonenberg and Hinnebusch, 2009; Spriggs et al., 2010). Regulation during elongation is mostly restricted to phosphorylation of eEF2, resulting in protein synthesis arrest after lethal cellular injuries (Hizli et al., 2013). Notably, any defects during translation elongation cause the ribosomes to stall on substrate mRNAs (Buchan and Stansfield, 2007; Lykke-Andersen and Bennett, 2014). This ribosomal pausing during elongation was, for example, associated with the defects in the protein folding mechanism (Liu et al., 2013). The cellular responses to translational pausing often result in frame shifting mutation and aberrant mRNA production, or ribosomes drop off and mRNA decay (Buchan and Stansfield, 2007). The endonucleolytic cleavage of mRNAs near the site where ribosomes stall is referred to as “no-go decay” (Doma and Parker, 2005).

Some translational regulation takes place during termination. However, since termination is mechanistically linked to initiation, it appears that modulation of termination mostly affects protein synthesis initiation (see below).
**Figure 3. Eukaryotic translation initiation.** Simplified mechanism of protein synthesis focusing on the cap-dependent translation initiation with indicated action of inhibitors used in this project.
The mechanism of eukaryotic translation initiation

Translation initiation of the vast majority of eukaryotic mRNAs is cap-dependent, although cap-independent mechanisms of initiation also exist (reviewed below). The m7GpppN (where N stands for any nucleotide) structure called a cap is located at the 5’ end of cellular mRNAs. Translation initiation involves recruitment of a 43S pre-initiation complex to the cap, mediated by eukaryotic translation initiation factors (eIFs). The pre-initiation complex includes the initiating methionyl-tRNA (Met-tRNA\textsuperscript{Met}), GTP-bound factor eIF2, and small ribosomal subunit 40S. The formation of the pre-initiation complex is mediated by factors: eIF3, eIF1, eIF1A, and eIF5. eIF3 is a multisubunit factor that contains between 11 and 13 subunits (eIF3a - eIF3m) (Hinnebusch, 2006). eIF3 facilitates the binding of eIF2- Met-tRNA\textsuperscript{Met} and the 40S ribosomal subunit. It also mediates the binding between the 43S pre-initiation complex and mRNA via its interaction with the scaffold protein eIF4G, which is present within the eIF4F complex. eIF3 also plays a role in scanning for an initiation codon and in post-translational dissociation of ribosomal subunits. eIF1 and eIF1A also regulate the scanning mechanism.

Initiation codon recognition results in the codon-anticodon base pairing and formation of the 48S complex. eIF5 is a GTPase-activating protein (GAP) that promotes GTP hydrolysis on eIF2 after recognition of an initiation codon. Conversely, the GDP to GTP transition on eIF2 is mediated by the GTP exchange factor (GEF) eIF2B (Jackson et al., 2010). The affinity of GDP-bound
eIF2 to Met-tRNA$^{\text{Met}}$ is drastically reduced and it is partially lost from the 48S complex. Joining of the large ribosomal subunit 60S with GTP-bound eIF5B promotes complete displacement of eIF2 and release of the other factors (eIF1, eIF3, eIF4B or H, eIF4F and eIF5). Hydrolysis of eIF5B-bound GTP promotes the dissociation of eIF1A and GDP-bound to eIF5B itself, and the formation of translation elongation competent 80S ribosome (Jackson et al., 2010).

**eIF4F complex**

As mentioned above, the eIF4F complex mediates binding between the pre-initiation complex and mRNA. It consists of a cap-binding protein eIF4E, ATP-dependent RNA helicase eIF4A, and a large scaffolding protein eIF4G. Binding of eIF4E and eIF4A to eIF4G is anti-cooperative (LeFebvre, 2006). eIF4G additionally binds: eIF3 (attached to a ribosomal subunit), poly(A) binding protein PABP (which binds the poly(A) tail of mRNA) and Mnk1/2 or MAPK-interacting serine/threonine kinase (which phosphorylates eIF4E). eIF4E binding to the cap is secured between two tryptophan residues in a concave surface of eIF4E and is further stabilized by interaction with the proximal nucleotide of the cap. Binding of eIF4G strengthens the binding of eIF4E to mRNA (von der Haar et al., 2004). Importantly, the simultaneous interaction between eIF4G, eIF4E, and PABP allows for the circularization of mRNA, making a “closed loop” (Pestova et al., 2007). It has been suggested that it increases the efficiency of translation initiation by stabilizing the initiation complex for efficient and fast recycling of
the components of translation initiation machinery after protein synthesis terminates (Gallie, 1991).

Eukaryotic mRNAs possess secondary structures with varying levels of complexity. Unwinding of these structures is mediated by the DEAD-box RNA helicase eIF4A, which requires ATP and is aided by the ancillary factors eIF4B, eIF4H, and even eIF4G. The interaction of eIF4A with eIF4G, and eIF4B and/or eIF4H, allows eIF4A to undergo conformational changes, increasing its ligand binding affinity, processivity, and rate of ATP hydrolysis. eIF4A is a founding member of the DEAD-box helicases family whose name comes from the presence of the ATP binding motif containing the D-E-A-D (asp-glu-ala-asp) sequence. Interestingly, eIF4A is the most abundant translation initiation factor and its expression in yeast is five times higher than ribosomes (von der Haar and McCarthy, 2002). There are three mammalian eIF4A proteins: eIF4AI (DDX2a), eIF4AII (DDX2b), and eIF4AIII (DDX48). The eIF4AI and eIF4AII both play a role in translation initiation. The eIF4AIII, on the other hand, is involved in pre-mRNA splicing and mRNA localization (Andreou and Klostermeier, 2012). eIF4AI is by far the most studied protein of the family. Its helicase core consists of well-characterized motifs that bind both ATP and RNA. This helicase core changes between an open and closed conformation allowing for the unwinding of RNA.

The activity of eIF4A is regulated by eIF4G, eIF4B, and eIF4H. There are two binding sites for eIF4A within the eIF4G protein (HEAT1 and HEAT2 domains). The N’ terminus HEAT domain aligns the DEAD-box helicase motif of eIF4A and promotes its helicase activity while the C’ terminal HEAT2 plays a
regulatory function (Marintchev et al., 2009). The eIF4B, and to a lower extent its homolog eIF4H, increases the helicase activity of eIF4A. Additionally, eIF4B co-operates with eIF4G in increasing the efficiency with which ATP hydrolysis by eIF4A is coupled to RNA unwinding (Ozes et al., 2011). It is also suggested that eIF4B and eIF4H may increase the processivity of eIF4A by preventing the mRNA from re-annealing (Marintchev et al., 2009).

**Internal ribosome entry site-mediated mechanisms of translation initiation**

The alternative mechanism of translation initiation in eukaryotes involves the recruitment of ribosomes to internal ribosome entry sites (IRES) and is independent of recognition of the mRNA 5' cap. This alternative mode of translation initiation receives increasing attention because of its importance in pathological stress conditions when cap-dependent translation is compromised (Komar and Hatzoglou, 2011). IRES are special RNA sequences located upstream of the initiation codon. They were initially found in viral genomes and, soon after, in some mRNAs of the eukaryotic host cell. Human cells use this mode of translation initiation under conditions when cap-dependent translation is inhibited like during mitosis and cell differentiation. It is also used to translate mRNAs with highly structured 5' UTR, not compatible with the canonical scanning mode. As expected this initiation mechanism is also used in stress conditions including endoplasmic reticulum (ER) stress, hypoxia, and reduced nutrient availability (Komar and Hatzoglou, 2011). It does not require many traditional translation initiation factors
like eIF4E or eIF4G. However, eIF4A is required for the initiation of both cap- and IRES-dependent translation (Mokrejs et al., 2010). The IRES-transacting factors (ITAFs) are also important in this translation initiation process (Komar and Hatzoglou, 2011). Even though IRES-dependent protein synthesis is considered an alternative way to translate eukaryotic mRNA, it seems that it may be more common than originally thought. Still, more studies are required to determine the full scope of the cellular IRES-dependent translation.

**Regulation of eukaryotic translation initiation**

Initiation of protein synthesis is regulated at different levels. Firstly, the unwinding of the secondary structure of mRNA increases the accessibility of the cap for translation factors and allows efficient mRNA translation. As outlined above, it is an ATP dependent mechanism and eIF4A helicase along with other proteins are catalyzing this process. Secondly, the cap is recognized by eIF4E only when eIF4E is freed from its repressing binding proteins (4E-BPs or eIF4E binding proteins). This dissociation between eIF4E and 4E-BP1 is triggered by full phosphorylation of 4E-BP at four residues T37/46, T70, and S65. These sites are phosphorylated by mTORC-1 and other kinases, including ERKs and CDK1 (Herbert et al., 2002; Villalonga et al., 2009). Finally, many different signaling pathways modify and regulate the activity and accessibility of translation initiation factors. For example, Mnk 1/2 kinase (recruited by eIF4G) phosphorylates eIF4E. This phosphorylation event seems to be dispensable in normal cells, but plays a
role in carcinogenesis (Proud, 2014). On the other hand, the phosphorylation of eIF4B positively regulates eukaryotic translation in response to a wide variety of extracellular signals like insulin and phorbol esters. The eIF4B protein is hyper-phosphorylated by members of the AGC protein kinase family including p70S6K and p90 ribosomal S6 kinase (van Gorp et al., 2009).

New levels of translation regulation are still being discovered, enriching our knowledge about this complex process. In the work I am presenting here, we have found a novel role of Amyloid Precursor Protein or APP in the regulation of protein synthesis in NSCLC cells, with a detailed description of this protein reported below.

**APP SIGNALING PATHWAY**

The human Amyloid beta precursor protein (APP) was first cloned in 1987 from neuronal tissue. Researchers were looking for a precursor of a protein sub-unit present within the cerebral amyloid plaques in Alzheimer’s disease and Down’s syndrome patients (Kang et al., 1987). They identified a 695 amino acid long APP protein (APP695). The APP gene is localized on chromosome 21. There are 10 different splice variants of APP that were found in mammalian and non-mammalian organisms (Muller and Zheng, 2012). The major isoforms are APP695, with expression restricted to neuronal tissue, APP770, and APP751. These latter isoforms are present in non-neuronal tissues of different origin (immune system, muscle, lung, kidney etc.). After being translated, APP undergoes
posttranslational modifications that include phosphorylation, N- or O-glycosylation, and sulfation.

**Proteolytic cleavage of APP**

APP is a type I transmembrane glycoprotein that undergoes sequential cleavage events (Haass et al., 2012). APP cleavage normally proceeds via the non-amyloidogenic pathway without amyloid deposits. In pathological conditions and throughout aging, APP is cleaved following the amyloidogenic pathway, which results in amyloid plaque formation (both pathways are reported in Figure 4). The prevalence of the cleavage pathway depends on many variables such as mutational status of APP or its cleavage enzymes, and age. First, cleavage occurs within the extracellular domain and is catalyzed by either \( \alpha \)-secretase (in non-amyloidogenic pathway) or \( \beta \)-secretase (in amyloidogenic pathway). It produces the large soluble fragments APP\( \alpha \) and APP\( \beta \) respectively leaving behind membrane bound C terminal fragments (APP-CTF\( \alpha \) and APP-CTF\( \beta \)). These carboxyl-terminal fragments are further processed by a large \( \gamma \)-secretase complex and produce APP intracellular domain (AICD) and either a 3-kDa peptide (from APP-CTF\( \alpha \)) or A\( \beta \) (from APP-CTF\( \beta \)). The 3-kDa peptide is also deposited in the cerebral tissue of the Alzheimer’s disease and Down’s syndrome patients, just like A\( \beta \) (Lalowski et al., 1996; Tekirian et al., 1998). The main aspartyl protease responsible for the \( \beta \) cleavage is called BACE1 (\( \beta \)-site APP cleaving enzyme).
(Luo et al., 2001). Many enzymes from the ADAM (a disintegrin and metallopro- 
tease domain) family were suggested to cleave APP and produce APP-CTFα, 
but it seems that ADAM10 is the major α-secretase involved in APP processing 
(Kuhn et al., 2010). γ-secretase, on the other hand, is a large protein complex. 
The major components of the γ-secretase complex include presenilin, nicastrin, 
APH-1 (anterior pharynx-defective 1), and PEN-2 (presenilin enhancer 2). Presenilin 
is responsible for the enzymatic activity of γ-secretase. A “Regulated intra- 
membrane proteolysis” model explains how exactly γ-secretase cleaves APP 
(Takami et al., 2009). It starts at the C terminus of the substrate and proceeds 
towards the N terminus of the transmembrane domain. It is a sequential multistep 
process and its cleavage sites within APP are named: γ, ε and ζ (Lichtenthaler et 
al., 2011).
Figure 4. Proteolytic cleavage of APP. APP can be processed via two pathways. First, cleavage is mediated by either α- or β-secretase (from the ADAM and BACE family) and results in the production of soluble fragment α (sAPPα) or β (sAPPβ) respectively. In both pathways, the carboxyl-terminal fragments (CTF) are cleaved by a large complex γ-secretase. This cleavage produces APP intracellular domain (AICD) and either short peptide p3 or Aβ.
Many different factors can regulate APP’s cleavage, one of them being hypoxia. It has been reported that the incidence of Alzheimer’s disease increases after stroke and cerebral ischemia (hypoxia induced by the insufficient blood flow to the brain). Furthermore, it has been demonstrated that hypoxia increases the cleavage of APP by the upregulation of APP cleavage enzymes. Specifically, hypoxia induced factor 1 (HIF-1) was shown to up-regulate both β- and γ-secretases (Wang et al., 2006; Zhang et al., 2007).

**Pleiotropic functions of APP**

APP belongs to a gene family that, in mammals, also includes two APP-like proteins, APLP1 and APLP2. Both, APP and APLP2 are ubiquitously expressed, while expression of APLP1 is restricted to neuronal tissue (Slunt et al., 1994). The sequence of all proteins is homologous within the E1 and E2 extracellular domains and intracellular tail (Figure 5; Müller and Zheng, 2012). Single or combined mice knockouts of the members of the APP family showed that they are all functionally different (Aydin et al., 2012). The knockout of APP in mice does not result in embryonic lethality, but causes some interesting phenotypic changes like reduction of the body mass by 15-20%, decreased brain weight, and impaired locomotor activity (Zheng et al., 1995).

Literature about APP is extensive. APP seems to play a role in numerous neuronal and synaptic processes, but was also shown to contribute to malignant growth in cells of different origin (see below; Müller and Zheng, 2012). It is still
debated whether APP is a receptor or a ligand. Two studies implicated that Aβ may act as a ligand for APP but also glycoprotein F-spondin and multi-functional protein Netrin-1 were shown to interact with a membrane tethered APP (Lorenzo et al., 2000; Lu et al., 2003; Ho et al., 2004; Lourenco et al., 2009). Thus, they can potentially function as APP’s ligands as well. APP has also been implicated in the regulation of proliferation, differentiation, synaptic activity, iron transport, growth of neurons, and cell adhesion (Figure 5; Dawkins et al., 2014). The extracellular domain of APP was implicated in cell and synaptic adhesion, shown to bind extracellular matrix proteins and proteoglycans, and was proposed to interact with a synaptic adhesion complex neurexin (Small et al., 1999; Norstrom et al., 2010). Interestingly, APPsα was suggested to have neuroprotective properties in cells of different origins like fibroblasts, keratinocytes, and neurons (Saitoh et al., 1989; Araki et al., 1991; Young-Pearse et al., 2008). APP was shown to influence many different signaling pathways. For example, Aβ oligomers were shown to activate AKT signaling by inhibiting its association with PDK (Nizzari et al., 2007). Additionally, Aβ was also shown to hyperactivate mTOR by mediating the phosphorylation of the mTOR inhibitor, PRAS40 (Caccamo et al., 2011). Another adaptor protein that binds the YENPTY motif within the cytoplasmic domain of APP is the growth factor receptor-bound protein 2 (GRB2). It binds phosphorylated tyrosine residue of APP via its SH2 domain. This promotes the phosphorylation of Erk1/2 and activation of MAPK pathway, a major regulator of proliferation (Nizzari et al., 2007).
Figure 5. Pleiotropic functions of APP. APP’s domain composition is presented on the left and corresponding functions are indicated on the right. APP is a transmembrane protein that contains extracellular portion (Ectodomain 1 and 2), transmembrane and cytoplasmic domain. Greek letters symbolize the proteolytic cleavage sites for α- and β-secretases and three sites of γ-secretase cleavage.
AICD

The cytoplasmic domain of APP has also been extensively studied and implicated in important intracellular signaling pathways like signal transduction, gene regulation, and trafficking. The approximately 6-kDa sized C terminus of APP or AICD contains a YENPTY motif that is conserved in all APP family members. Normally, AICD is very unstable but binding of an adaptor protein Fe65 (through its phosphotyrosine binding domain) stabilizes AICD (Kimberly et al., 2001). AICD along with Fe65 can translocate to the nucleus and interact with a histone acetyltransferase Tip60 or Tat-interactive protein (Gao and Pimplikar, 2001). This results in transcriptional regulation of various genes. Many different transcriptional targets of AICD have been identified, for example APP, BACE-1, aquaporin-1, and NEP (Figure 6). Besides Fe65, AICD can bind about 20 independent cellular partners and has been implicated in a variety of cellular processes, including cytoskeleton organization (Beckett, 2012). Transcriptional activity of AICD was also confirmed by the finding that AICD interacts with a component of the eukaryotic transcriptional mediator complex MED12 (Turner et al., 2011; Xu et al., 2011).
Figure 6. AICD and its function. Some of the binding partners of AICD include Fe65, Tip60 and MED12. They localize to the nucleus and regulate transcription of genes involved in different pathways. Some of them are indicated in the table.
APP in cancer research

One of the first studies on the role of APP in human tumors was performed in colon cancer. It was shown that soluble APP fragments promote proliferation of colon carcinoma cells. It has been suggested that it may be mediated via APP’s regulation of ERKs (Meng et al., 2001). Hansel et al. also showed that increased expression and cleavage of APP in pancreatic cancer promotes cell proliferation (Hansel et al., 2003). Additionally, APP was found to be upregulated in thyroid and parathyroid tumors and to regulate cell differentiation (Haven et al., 2004; Krause et al., 2008). APP is also involved in the regulation of the androgen-dependent growth of prostate cancer. (Takayama et al., 2009). Recently, studies confirmed that APP responds to androgen signaling and promotes proliferation of breast cancer cells (Takagi et al., 2013). Overall, APP has been found to be upregulated in human tumors of different origin and to regulate proliferation and differentiation of cancer cells. Whether this is mainly due to the regulation of ERK signaling or if other signaling pathways are also involved remain to be established.
Studies in an orthotopic NSCLC mice model revealed that a treatment with an inhibitor of γ-secretase (GSI) induced cell death and phosphorylation of 4E-BP1 (p4E-BP1) at T37/46 in hypoxic NSCLC cells. 4E-BP1, a cell growth marker, is one of the downstream targets of the mTORC-1 complex. The initial hypothesis of this dissertation was that γ-secretase treatment could reactivate mTORC-1 activity in a quiescent hypoxic tumor environment. To pursue our hypothesis, we have first investigated if GSI has the same effect on NSCLC cell lines. All experiments were performed in hypoxic environment: 0.5% O₂, 5.0% CO₂, and 94.5% N₂. These conditions do not restrict the growth of cancer cells and in these conditions the O₂ consumption rate and ATP synthesis of mitochondria are not compromised (Höckel and Vaupel, 2001). We showed that γ-secretase treatment increases p4E-BP1 at T37/46 also in vitro. However, instead of focusing on γ-secretase, which regulates many cellular processes, we decided to look for a putative target of this enzyme that could mediate the observed phenotype. We further showed that depletion of APP, a well-established γ-secretase cleavage target (McCarthy et al., 2009), mirrors the enhanced phosphorylation at T37/46. Overexpression of APP intracellular domain (AICD), a transcription regulator, had an opposite effect. To find transcriptional target(s) of APP that could mediate the change on p4E-BP1, we have used genome-wide, discovery-driven approaches, such as Illumina gene expression and antibody arrays. This allowed us to identify
a novel target of APP, the pseudophosphatase STYX. Depletion of STYX enhanced p4E-BP1 at T37/476. Further studies showed that APP causes extensive rearrangement of 4E-BP1 phosphorylation. We also showed that APP affects translation initiation complex formation. Considering the changes on p4E-BP1 and translation initiation complex, we hypothesized that APP may be involved in the regulation of protein synthesis rate. Fluorescence-based experiments with methionine surrogate confirmed our hypothesis. We also investigated if APP can regulate both mechanisms of translation initiation (cap- and IRES-dependent) using a bicistronic vector from which two luciferase genes are translated via two different modes. Since mTORC-1 is a major regulator of protein synthesis, our further focus was on the potential involvement of the mTORC-1 complex in the observed phenotype. This allowed us to discard our initial hypothesis. The observed change on protein synthesis upon APP depletion seemed to be independent of mTORC-1 activity.

Since, cell growth and cell cycle are interdependent processes, we further hypothesized that APP depletion may affect cell cycle progression. Cell cycle analysis using different techniques showed that APP promotes G₀ to G₁ progression. We identified cyclin C as a novel APP’s target that is involved in the regulation of G₀ to G₁ cell cycle transitions. The dissociation between cell growth and cell cycle progression, mediated by APP depletion, resulted in increased cell size and cell death of hypoxic NSCLC cells.
In summary, we showed that APP is necessary for cell growth and cell cycle progression in hypoxic NSCLC cells. We also suggest that APP may be a valid target in hypoxic NSCLC treatment.
CHAPTER TWO
MATERIALS AND METHODS

Cell culture

NSCLC cell lines (H1299, A549, H1437, and H1650) and human lung fibroblasts (WI-38) were purchased from ATCC (Manassas, VA). NSCLC cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The mesothelioma cell line (Me16) was a gift from Dr. Harvey I. Pass (New York University) and an immortal human keratinocyte cell culture (HaCaT) was a gift from Dr. Mitchel Denning (Loyola University Chicago). Both Me16 and WI-38 were grown in DMEM supplemented with 10% FBS. Media and FBS were purchased from Life Technologies (Grand Island, NY) except for EMEM, which was purchased from ATCC.

All cell lines were fingerprinted using the GenePrint fluorescent STR system (Promega, Madison, WI). We also screened cell cultures for mycoplasma contamination using the MycoSensor qPCR assay kit (Stratagene, La Jolla, CA). Cells were cultured under hypoxic conditions (0.5% O₂, 5.0% CO₂, 94.5% N₂) in a Coy CleanSpot glove-box incubator (Coy Laboratory Products, Grass Lake Charter Township, MI).
Animal work

The Loyola Institutional Animal Care and User Committee at the Loyola University Chicago Medical Center approved all animal work. Five-week-old female NOD.CB17-Prkdcscid/J mice (Jackson Laboratories, Bar Harbor, ME) were injected via lateral tail vein with $2.5 \times 10^6$ cells in 100 µl of sterile saline solution. Injected cell lines included A549 and H1437, which were transduced with a lentivirus expressing luciferase (ViraPowerTM T-Rex Lentiviral expression system, Grand Island, NY). During the studies, animals were housed in a pathogen-free animal facility at Loyola University Medical Center. Mice were monitored daily until they reached one of the end points (observed: dyspnea or irritability). At the time of euthanasia, human cells comprised 93% ± 0.8% of the total lungs of the mice (Eliasz et al., 2010). Tumor burden was assessed using bioluminescence imaging (Xenogen Vivo-Vision IVIS 100 In Vivo Imaging System, Caliper Life Science, Hopkinton, MA) and, after euthanasia, by quantitative polymerase chain reaction (PCR) determination of human to mouse GAPDH ratio.

The γ-secretase inhibitor (GSI) MRK-003 was administered by oral gavaging at a dose of 100 mg/kg in 0.05% methylcellulose 3 days a week. After euthanasia, lungs, livers, and brains were harvested and flash frozen for molecular analyses. 8 µm thick slides of frozen lungs were used in immunofluorescence experiments for the marker of hypoxia GLUT-1, for proliferation marker Ki67, and phosphorylation of 4E-BP1 at T37/46, a marker of
active protein synthesis. Fixation was performed with 3.7% paraformaldehyde in PBS for 15 minutes, followed by permeabilization with 50 mM NH₄Cl in PBS containing 0.1% Triton X-100 for 15 minutes. 5% BSA in PBS (0.05% Triton X-100) was used as a blocker and antibodies were diluted in 1% BSA in PBS supplemented with 0.05% Triton X-100. The fluorescently labeled secondary antibodies used in this work were goat anti-mouse or goat anti-rabbit Alexa Fluor 488 (or 568) (Life Technologies). All antibodies were used at the concentrations recommended by the manufacturer.

In vivo bromodeoxyuridine (BrdU) incorporation was performed according to the manufacturer instruction (FITC BrdU Flow kit, BD Pharmingen, San Jose, CA). In situ TUNEL assay was performed using the manufacturer's protocol (refer to Liang et al., 2012). Images were acquired with an AX80 microscope (Olympus, Center Valley, PA, USA).

Reagents

The γ-secretase inhibitor MRK-003 was a generous gift from Merck & Co. (Whitehouse Station, NJ). 5 µM MRK-003 was used in the in vitro experiments unless otherwise stated in figure legends. Torin 1 (1-[4-[4-(1-Oxopropyl)-1-piperazinyl]-3-(trifluoromethyl)phenyl]-9-(3-quinolinyl)-benzo[h]-1,6-naphthyridin-2(1H)-one) (Tocris Bioscience, Bristol, UK) was used at a final concentration of 250 nM. Roscovitine (Cell Signaling, Danvers, MA) was used at a final concentration of 20 µM. UO126 (Selleck Chemicals, Houston, TX) was
used at a final concentration of 10 μM. Silvestrol (Medchemexpress LLC, Princeton, NJ) was used at a final concentration of 40 nM and 100 nM. Homoharringtonine (Tocris Bioscience) was used at a final concentration of 100 nM. All of the above chemicals were dissolved in DMSO (Sigma-Aldrich, Atlanta, GA). Insulin (Sigma-Aldrich) was diluted in sterile PBS and used at the specified concentrations. MG132 (C2211; Sigma-Aldrich) was dissolved in DMSO and used at a final concentration of 30 μM. Propidium Iodide (PI; 81845; Sigma-Aldrich) was dissolved at 1 mg/ml in distilled water and used at the specified concentrations. Acridine Orange (AO; Life Technologies Corp.; Carlsbad, CA) was dissolved in distilled water at a 10 mg/ml concentration and used as specified below.

**Plasmids**

pcDNA3-RLUC-POLIRES-FLUC expressing both Renilla and firefly luciferase was a gift from Nahum Sonenberg (McGill University, Montreal). The schematic of the vector is presented in Figure 7. The Renilla luciferase mRNA is translated in a cap-dependent fashion, while the firefly luciferase mRNA is translated through the poliovirus internal ribosomal entry sequence (IRES).

Bioluminescence was normalized for the expression of plasmid DNA using luciferase specific primers and Q-PCR. pCDF1-MCS1-EF1-cop GFP expressing 59 aa long APP C-terminal fragment (AICD) was a gift from Dr. Xiao Z.C. (Institute of Molecular and Cell Biology, Singapore); pCAX expressing full
length APP (isoform 695 aa long) was purchased from Addgene (Cambridge, MA). We used the empty plasmids as transfection controls.
Figure 7. Schematic representation of the bicistronic vector used for assessment of the cap- and internal ribosome entry site (IRES)-mediated translation. The *Renilla reniformis*, Poliovirus IRES and firefly luciferase (Luc) genes are cloned in the pcDNA3 vector backbone. *Renilla* and firefly luciferase gene expression is driven by the CMV promoter upstream of the *Renilla* luciferase gene (Addgene plasmid # 45642; Poulin et al., 1998).
Transfection experiments

Most of the transfection experiments were performed using electroporation. Briefly, after trypsinization, cells were washed with 10 ml of Opti-MEM (serum free) to remove residual serum and improve transfection efficiency. After counting, 3 million cells in 600 µl of Opti-MEM were transferred to 0.4 cm-wide electroporation cuvettes (Bio-Rad, Los Angeles, CA). For transfection experiments, 6 µL of 200 nM of siRNA or 4 µg of plasmid was used per cuvette. Cells were transfected by GenePulser Xcell (Bio-Rad) under the following settings: 300 V for 25 msec with only 1 total pulse. Following transfection, cells were immediately resuspended in 7 ml of media supplemented with 10% FBS and 1 million cells were plated per 10 cm diameter tissue cultures dishes. Cells were allowed to recover overnight in normoxic conditions at 37°C and the following day all plates were transferred into the hypoxia glovebox. Unless otherwise stated, most experiments were performed 48 hours after transfection, when the effects of the transfections were experimentally determined to be at their peak. In some experiments, transfection of plasmid DNA was done using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol.

For the APP depletion experiments, we tested three different siRNA against APP from: Santa Cruz Biotechnologies, Cat no: sc-29677 (Santa Cruz, CA) and two from Qiagen, siRNA9 Cat no: SI02780281 and siRNA10 Cat no: SI02780288 (Valencia, CA). The majority of experiments were performed with
SI02780288, which targets the 3’ terminus of all ten APP isoforms, and which yielded the most reproducible results. Landmark experiments (for example, the *in vitro* BrdU incorporation assay) were confirmed by using two shRNAs against APP from Sigma-Aldrich, Cat no: TRCN0000011043 and TRCN0000006707. We confirmed efficient APP downregulation in each experiment by Western blot analysis.

**Protein analysis**

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting, cells were washed with PBS and trypsinized. Cell pellets were washed with ice-cold PBS twice and resuspended in RIPA lysis buffer on ice (0.1% SDS, 1% NP-40, 200 µM PMSF, 0.5% sodium deoxycholate, 0.2 mM sodium vanadate, 50 mM sodium fluoride, protease inhibitor cocktail [Sigma-Aldrich], and phosphatase inhibitor cocktail [Sigma-Aldrich] in PBS). Lysates were sonicated and insoluble material was removed by 15 minutes centrifugation at 14,000 rpm at 4°C. Protein concentration was determined using Bradford assays. Specific amount of proteins (experimentally determined for the antibody used, ranging from 25 to 100 µg) were mixed with a Laemmli sample buffer (for 2X: 100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% (wt/vol) bromophenol blue and 20% glycerol), heated at 95°C for 5 minutes, and loaded on SDS-PAGE gels. The molecular weight reference PageRuler Plus Prestained Protein Ladder from Thermo Scientific was used. After electrophoresis proteins were
electrotransferred onto Hybond-C Extra nitrocellulose membrane (Amersham Biosciences) overnight at 4°C. As a blocking agent, a non-fat, dry milk dissolved in TBS-T buffer (1X TBS with 0.1% Tween-20) was used. For primary antibody dilution, either blocking solution or 5% (wt/vol) bovine serum albumin (BSA, this blocking solution was used for Cell Signaling antibodies) in TBS-T were used. The length of the primary antibody incubations was determined according to the manufacturer’s recommendations. Secondary antibodies were diluted in blocking solution and incubated for 45 minutes at room temperature (RT). Washes between the incubations were done with freshly prepared TBS-T buffer. Proteins were visualized using chemiluminescent reagent ECL (SuperSignal West Dura Extended Duration Substrate, Prod# 34076, Thermo Scientific) and exposed to x-ray films (Denville Scientific Inc.). At the end of the experiments, membranes were stained with Ponceau S solution (Sigma-Aldrich) to confirm equal loading in each lane.

For immunoprecipitation experiments, cell lysates were incubated with an antibody at the recommended concentration overnight at 4°C. Protein A/G Magnetic Beads (Thermo Fisher Scientific Inc., Waltman, MA) were washed in TBS-0.1% Tween, and incubated with samples for 1h at room temperature with rotation. Elution was performed in 2X sample buffer followed by SDS-PAGE and Western Blotting.

For immunofluorescent microscopy, 30,000 transfected cells were plated in 4-well glass slides (Chamber Slide, Lab-TekII, Rochester, NY). 48h after transfection, cells were rinsed with PBS, fixed in 3.7% paraformaldehyde
(Electron Microscopy Sciences, Haltfield, PA) for 15 minutes, washed three times with PBS, and permeabilized with 50 mM NH₄Cl in PBS with 0.1% Triton-X. Blocking was performed in 5% BSA (Thermo Fisher Scientific Inc., Rockford, IL) in PBS with 0.05% Triton-X (DMSO, Sigma-Aldrich, St. Louis, MO) and antibodies were diluted in 1% BSA in PBS with 0.05% Triton-X for a final concentration recommended by the manufacturers. Fluorochromes used were Alexa Fluor 488 and Alexa Fluor 568. The Golgi apparatus was visualized using rhodamine labeled wheat germ agglutinin (Vector Laboratories, Burlingame, CA). As a mounting medium, we used ProLong Gold Antifade Mountant with DAPI (Life Technologies Corp., Carlsbad, CA) that also stained nuclei. Images were acquired at room temperature using EVOS FL Cell Imaging System (Life Technologies Corp.).

Cell Cycle and Cell Cycle Phospho Antibody Microarray were purchased from Full Moon BioSystems (Sunnyvale, CA). Experiments were performed 48 hours after transfection of H1299 and A549 cell lines with either control siRNA or siRNA against APP. For cell lysis, protein extraction, labeling, conjugation, and detection we used the Antibody Array Assay Kit (Full Moon BioSystems) and followed the manufacturer’s protocol. Briefly, extracted cells were biotinylated, conjugated to the antibody array, and detected using Cy3-Streptavidin. The arrays were scanned and signal quantified by Full Moon BioSystems.
Cap binding assay

Cells transfected with either control siRNA or siRNA against APP were collected by trypsinization 48 and 72 hours after transfection, washed with ice-cold PBS, and frozen as cell pellets. At the time of the experiment, cell pellets were resuspended in 300 µl of cap binding buffer (20 mm Hepes, pH 7.2, 1 mm EDTA, 100 mm KCl, 10% (v/v) glycerol, 7 mm 2-mercaptoethanol, 50 mm β-glycerophosphate, 50 mm NaF, 200 µM PMSF, protease inhibitor cocktail [Sigma-Aldrich] and phosphatase inhibitor cocktail [Sigma-Aldrich]). The cell suspension was subjected to three consecutive freeze-thaw cycles and cleared by centrifugation at 14,000 rpm at 4°C for 15 minutes (Villalonga et al., 2009). To capture cap-binding proteins, we used m7-GTP-Sepharose 4B beads (GE Healthcare, Cleveland, OH) and as a negative control, we used unmodified Sepharose 4B beads (Sigma-Aldrich). Lysates (corresponding to 100 µg of total protein as determined by Bradford assay) were incubated with 30 µL of beads in 500 µL overnight at 4°C. The next day, beads were washed three times with cap-binding buffer. Proteins associated with the cap were eluted using 40 µL Laemmli buffer, loaded on SDS PAGE, and blotted onto nitrocellulose membranes. Proteins were identified by Western Blotting using antibodies against the eukaryotic translation initiation factors specified in figures and figure legends.
Global protein synthesis rate

We assess the rate of global protein synthesis using the Click-iT AHA Protein Synthesis kit (Invitrogen, Grand Island, NY) according to the manufacturer’s instruction. 48 or 72 hours after transfection media was changed to methionine-free medium (RPMI, no methionine GIBCO, Life Technologies Corp., Grand Island, NY) for 1 hour. Then, cells were incubated with the methionine analog Click-iT AHA (L-Azidohomoalanine) for 3 hours at a final concentration of 50 µM. Fixation and permeabilization of cell membranes were performed according to the manufacturer’s instruction. The incorporation of AHA into the nascent proteins was detected with an alkyne-containing fluorescent dye (Alexa Fluor® 488 Alkyne) at a final concentration of 5 µM. The incorporation of AHA for the detection of nascent protein was analyzed by flow cytometry using a BD FACS Canto II instrument (Becton Dickinson, San Jose, CA) measuring 20,000 events for each sample. Flow cytometry data was analyzed by FlowJo software. AHA incorporation was quantified using geometric mean fluorescence calculated by the software.

For the experiments with Torin-1, we used the aforementioned protocol with the exception that fresh Torin-1 was added when media was changed. For silvestrol or homoharringtonine, cells were incubated with methionine-free media supplemented with 1% FBS overnight. The next morning, cells were treated with AHA along with silvestrol, homoharringtonine (at the concentrations specified above), or DMSO control for 20, 30, or 40 minutes.
Cell viability, cell cycle, and cell proliferation assays

For cell viability, 10^6 cells transfected with siRNAs were washed twice in PBS and resuspended in 1 ml of PBS containing 20 µl of 1 mg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO). Cells were then analyzed by flow cytometry as mentioned previously.

For cell cycle analysis, cells were washed with 2 ml of 5% bovine calf serum (BCS). Fixation was performed in 70% ethanol and then cells were incubated with 250 µl of 10 µg/ml RNase A (Thermo Fisher Scientific Inc., Rockford, IL) at 37°C for 15 minutes followed by 5 minutes at room temperature. 250 µl of 100 µg/ml propidium iodide was added and samples were incubated for at least 1 hour prior to flow cytometry analysis.

To assess DNA synthesis, we used a bromodeoxyuridine (BrdU) incorporation (FITC BrdU Flow kit, BD Pharmingen, San Diego, CA)/7-aminoactinomycin D (7-AAD, Sigma-Aldrich, St. Louis, MO) staining kit following the manufacturer instructions. After transfection with nucleic acids, cells were incubated with BrdU at a final concentration of 10 µM for 4 hours.

The incorporation of propidium iodide or BrdU was analyzed by flow cytometry using a BD FACS Canto II instrument (Becton Dickinson, San Jose, CA).

Flow cytometry data was analyzed by FlowJo software. For propidium iodide staining, the percentage of cells in different cell cycle phases was calculated using the Watson pragmatic model. For BrdU incorporation, a manual
gating of cells was used to calculate the percentage of cells in each phase of the cell cycle.

**Acridine orange staining**

Acridine Orange (AO, Life Technologies Corp., Grand Island, NY) was used to stain both DNA and RNA. After transfection of nucleic acids, cells were resuspended in culture medium at the density of $10^6$/ml. 0.2 ml of cell suspension was used for further staining. 4 ml of permeabilizing solution (0.1% Triton X-100, 80 mM HCl, 150 mM NaCl) was added to the cell suspension and 1.2 ml of ice-cold, 1 mg/ml AO staining solution was added within 15 seconds. \\

Samples were analyzed using both the BD FACS Canto II instrument (Becton Dickinson, San Jose, CA) and Amnis Image Stream X (EMD Millipore Corporation, Billerica, MA).

Flow cytometry data was analyzed by FlowJo software. A manual gating of cells was used to calculate the percentage of cells in each phase of the cell cycle.

**Gene expression analysis**

Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen, Valencia, CA). The RNA concentration was measured with a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., Rockford, IL). 1 µg of total
RNA was used for cDNA synthesis, which was performed using the iScript Reverse Transcription Supermix RT-qPCR (Bio-Rad Laboratories, Hercules, CA).

Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7300 thermal cycler (Applied Biosystems). Reactions containing water instead of template and non–reverse transcription reactions served as negative controls. Human ribosomal protein RPL13A, β-actin, and β-tubulin were used as internal reference genes.

A549 and H1299 cells were transfected with siRNAs as described above. 48h after transfection, total RNA was used for the Illumina Gene Expression Micro-array (HumanHT12, 48,000 probes, RefSeq plus EST). Samples that were used included: untransfected A549 and H1299 cells and cells transfected with either siRNA or siRNA against APP from three independent transfection experiments. Gene expression analysis was performed at the University of Chicago Genomics Core facility.

Results were analyzed using Genome Studio software (iLLumina). We focused on genes in which expression either increased or decreased at least two fold in both cell lines. Results were confirmed with quantitative real-time PCR.
Statistical analysis

We performed statistical analysis of our data using Student’s t test. Values were considered statistically significant at $p < 0.05$ in 2-tailed tests.
Table 3. List of siRNAs used in this dissertation work.
Adapted from Sobol et al., 2015

<table>
<thead>
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Table 4. List of antibodies used in this dissertation work.
Adapted from Sobol et al., 2015

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| APP Rev Transcript Variant: 4 | 5'-ACATGGCCCGTTAAAAGATG-3' | NM_001136016.3 | 201-182 |
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CHAPTER THREE

RESULTS

NSCLC is a terminal illness with an urgent need for new treatment approaches. Hypoxic areas within lung cancer are associated with poor prognosis and tumor recurrence (Jackson et al., 2010). Hypoxia renders tumors resistant to both chemo- and radiotherapy. It is essential for tumor maintenance, promotes cancer invasiveness, and metastasis (Hill et al., 2009). Previous data from our laboratory have shown that Notch-1 signaling is required for the survival of hypoxic NSCLC cells. Notch-1 induces the expression of insulin-like growth factor 1 receptor (IGF-1R), its ligands IGF-1 and IGF-2, and inhibits phosphatase and tensin (PTEN) homolog expression. This leads to the activation of the pro-survival AKT signaling pathway (Graziani et al., 2008; Eliasz et al., 2010). Canonical Notch-1 signaling is initiated by a number of proteolytic cleavage events and culminates in the release of the Notch intracellular domain (Notch\textsuperscript{IC}), catalyzed by a large intramembranous protease complex named \( \gamma \)-secretase. To target hypoxic NSCLC cells, we inhibited Notch-1 signaling using a \( \gamma \)-secretase inhibitor (GSI) in an orthotropic model of NSCLC. GSI treatment prolonged the median survival of tumor bearing mice as compared to vehicle treated controls.
It also decreased overall markers of tumor hypoxia, and reduced tumor metastasis to both brain and liver (Liang et al., 2012). We verified cancer cell death in hypoxic NSCLC tissue using a variety of techniques, including TUNEL assay. We used glucose transporter 1 (GLUT-1) as a marker of hypoxia. Staining of frozen lung sections showed cell death in hypoxic areas of tumors from GSI treated animals (Figure 8 A-D). Notably, DNA fragmentation, visualized with TUNEL assay, was detected outside of the cells. We also observed another characteristic of these cancer cells that we could not explain at that stage: cells exposed to GSI appeared to be larger as compared to controls (Figure 8; C and D compared to A and B). We thought, at first, that this could have been an experimental artifact. Further experiments suggested possible explanations of these occurrences (see below).
Figure 8. GSI treatment causes cell death of hypoxic NSCLC cells. Top: vehicle treated mice. Bottom: GSI treated mice (100 mg/kg of MRK-003 in 0.05% methylcellulose 3 days a week).
A and C: bright field images.
B and D: immunofluorescent staining of 8 μm sections of frozen lungs (representative experiments are shown).
Green: immunofluorescent staining of GLUT-1 (marker of hypoxia).
Red: in situ TUNEL assay (DNA fragmentation, a marker of cell death).
Adapted from Liang et al., 2012.
γ-SECRETASE INHIBITION MODIFIES 4E-BP1 PHOSPHORYLATION

Additional analysis of lung tissues revealed that cancer cells in hypoxic tumor areas do not proliferate, as visualized by two independent assays: *in vivo* BrdU incorporation assay and Ki67 staining. GLUT-1 positive cells stain negative for the proliferative markers, BrdU and Ki67 (Figure 9A). We further assessed the metabolic properties of these cells by the activation of mTORC-1 complex. The mTORC-1 complex is a major regulator of cell growth and proliferation that controls anabolic processes of eukaryotic cells (Laplante and Sabatini, 2012). To assess mTORC-1 activation, we monitored the phosphorylation status of one of its major downstream targets, 4E-BP1, at threonines (T) 37/46 (T37/46). 4E-BP1 is a repressor of protein synthesis and phosphorylation at T37/46 of 4E-BP1 is required for cap-dependent translation initiation (Villalonga et al., 2009). We did not detect phosphorylation of T37/46 in quiescent cancer cells within hypoxic regions of tumors from vehicle treated animals (two representative tumor tissues, Figure 9B). However, we observed co-localization of the hypoxia marker GLUT-1 and T37/46 phosphorylation in tumors from GSI treated animals (two representative tumor tissues, Figure 9C).
Figure 9. γ-secretase inhibition enhances 4E-BP1 phosphorylation at T37/46.

A. NSCLC cells in hypoxic tumor regions are quiescent. Co-immunofluorescence of the hypoxia marker GLUT-1 (red) and proliferation markers: Ki67 (top: green) or BrdU in vivo incorporation (bottom: green).

B-C. Lung sections of two representative NSCLC bearing mice treated with vehicle (B) or GSI (C). Co-immunofluorescence of the hypoxia marker GLUT-1 (red) and phosphorylated T37/46 4E-BP1 (green). Co-localization of GLUT-1 and phosphorylated T37/46b is visible only upon GSI treatment (yellow). Bright field images: bf, immunofluorescent staining: fl.

D. Western blot analysis of the specified proteins and phosphoproteins in total cell lysate obtained from the indicated NSCLC cell lines upon treatment with vehicle (DMSO, c) or GSI.

E. mRNA levels of 4E-BP1 as determined by Q-PCR in cells treated with DMSO(c) or GSI for 48h. Columns represent averages of three independent experiments, bars represent S.D. The mRNA abundance for cells treated with DMSO (c) was arbitrarily set to 1.

F. Left: Western blot analysis of the specified proteins and phosphoproteins in total cell lysates obtained from NSCLC cell line H1299 after transfection with either a control siRNA (c), or siRNAs against preselinin-1 and nicastrin (siPres/Nic); Right: Western blot analysis of the specified proteins confirming downregulation efficiency. Similar results were obtained in other NSCLC cell lines A549 and H1437.

Adapted from Sobol et al., 2015 [1].
Our initial hypothesis was that GSI treatment could reactivate mTORC-1 in hypoxic NSCLC cells. Since mTORC-1 regulates cell growth, this reactivation could have explained the initially observed cell enlargement and death (Figure 8). Active anabolic processes in the nutrient-deprived, hypoxic tumor microenvironment should rapidly exhaust energy resources leading to cell necrosis. In other terms, inappropriate mTORC-1 reactivation in cells, which should be under a metabolic checkpoint, could lead to energy exhaustion and cell death.

We sought to confirm our findings in vitro. Because of its intrinsic variability, mimicking tissue hypoxia in vitro is probably impossible (Palmer et al., 2010). We decided to culture our cells in complete medium, but preserving hypoxia inducible factors (HIF1α, 2α and 3α), because they collectively regulate a significant portion of the transcriptome under hypoxic conditions (Pawlus and Hu, 2013). To achieve this, we cultured our cells in a 0.5% O2, 5.0% CO2, and 94.5% N2 atmosphere. Under these conditions, mitochondrial function is not compromised; hence, these conditions cannot be considered as “deep hypoxia” (Höckel and Vaupel, 2001). Higher oxygen concentrations did not consistently preserve HIFs expression (not shown). Similar to what was observed in vivo, treatment of various NSCLC cell lines with GSI enhanced 4E-BP1 phosphorylation at T37/46 (Figure 9D). Three bands of total 4E-BP1 protein correspond to 4E-BP1 phosphorylated at the following residues: T37/46, T37/46 plus threonine 70 (T70), and T37/46 plus T70 and serine 65 (S65) (Gingras et al.,
Merging of these bands, observed in A549, H1299 and H1650 cell lines, could suggest a loss of 4E-BP1 phosphorylation at S65 and/or T70 (Figure 9D). The total protein level of 4E-BP1 in H1437 cells increased upon GSI treatment, but mRNA levels remained the same (Figure 9D, E). We did not focus our attention on this phenomenon since γ-secretase cleaves more than 90 cellular substrates. Moreover, some GSI are able to diminish proteasome activity independently of γ-secretase inhibition (Han et al., 2009). Thus, to confirm that the observed change on 4E-BP1 phosphorylation can be attributed specifically to γ-secretase inhibition, and not to off-target effects of the drug, we downregulated two central components of the γ-secretase complex: presenilin and nicastrin. These proteins are essential for catalytic activity and substrate recognition of γ-secretases (Herreman et al., 2003). Depletion of these proteins reproduced the increase in 4E-BP1 phosphorylation at T37/46 observed upon GSI treatment without affecting the expression of 4E-BP1 (Figure 9F). This supported the idea that the observed change at T37/46 was due to the inhibition of γ-secretase activity and not to off-target effects of the drug treatment. As mentioned above, the γ-secretase complex cleaves and activates more than 90 different cellular proteins with a variety of functions (Haapasalo and Kovacs, 2011). Therefore, instead of investigating the role of γ-secretase itself, we decided to identify a specific γ-secretase target that could mediate the observed change on 4E-BP1 phosphorylation.
AMYLOID PRECURSOR PROTEIN (APP) IS THE $\gamma$-SECRETASE SUBSTRATE THAT ALTERS THE 4E-BP1 PHOSPHORYLATION STATUS

Initially, the rationale of using GSI was to target Notch signaling, which is known to regulate the prosurvival AKT pathway (upstream activator of mTORC-1) in hypoxic NSCLC (Eliasz et al., 2010). However, downregulation of all four Notch receptors in separate experiments or forced expression of each Notch receptor intracellular domains did not alter the phosphorylation status of 4E-BP1 significantly (results not shown).

Next, we focused on Amyloid Precursor Protein or APP. APP is one of the most well-known and extensively studied cleavage substrates of $\gamma$-secretase (De Strooper et al., 2012). APP is known to play some roles in cell proliferation through the regulation of different signal transduction pathways, including the mitogen-activated protein kinase cascade (Nizzari et al., 2007). We hypothesized that depletion of APP could potentially alter the phosphorylation status of 4E-BP1. siRNA-mediated downregulation of APP increased 4E-BP1 phosphorylation at T37/46 in different NSCLC cell lines cultured under hypoxia (Figure 10A). The total levels of 4E-BP1 were unchanged. For each experiment shown here, we assessed the efficiency of APP’s downregulation at the mRNA and protein levels. A representative experiment is shown in Figure 10 (B, C). The siRNAs and shRNAs used in this study are specified in Materials and Methods. Generally, we performed pilot experiments using a siRNA targeting the shared 3’-end of all 10
APP splice variants and confirmed experiments using one or more alternative siRNA or shRNA.

To confirm that APP signaling had a role in the regulation of 4E-BP1 phosphorylation at T37/46, we overexpressed the APP C-terminal domain, or AICD, and obtained opposite results compared to APP depletion (Figure 10A). These results strengthened our hypothesis that APP could represent a putative γ-secretase substrate that regulates 4E-BP1 phosphorylation.

We wanted to further confirm that APP is active in our NSCLC cell lines. Activation of APP depends on its proteolytic processing and culminates with the cleavage by γ-secretase (Xu et al., 2011). An indication of active APP signaling is, therefore, its cleavage. To confirm that APP is cleaved in NSCLC cell lines, we treated them with GSI and assessed the accumulation of the total APP protein. The γ-secretase cleavage product, AICD, is only approximately 6-kDa in size and its detection is highly difficult, especially because available antibodies against it are inefficient. For this reason, we sought evidence of inhibited cleavage by monitoring the accumulation of full-length APP protein upon GSI administration. Treatment with GSI led to a dose-dependent increase in total APP levels (Figure 10D). This accumulation of full-length APP provided positive evidence that γ-secretase cleaves APP in NSCLC cell lines and that APP signaling is active in our cells. Multiple bands, observed by Western blot analysis, at high molecular weight, correspond to both isoforms of APP (which our
antibody is expected to detect) and post-translationally modified APP protein (e.g., the products of N- and O-glycosylation).

Serine 65 (S65) and threonine 70 (T70) are two additional, major phosphorylation residues of 4E-BP1, which determine the extent of eIF4E inhibition. We assessed their phosphorylation status upon APP depletion. We reproducibly observed that phosphorylation of S65 decreased after APP downregulation in different NSCLC cell lines, while APP depletion had negligible effect on T70 phosphorylation (Figure 10E).
Figure 10. APP is the γ-secretase substrate that alters the 4E-BP1 phosphorylation pattern.

A. Western blot analysis of the specified proteins and phosphoproteins in total cell lysates obtained from the indicated NSCLC cell lines after transfection with either a control siRNA (c) or siRNA to APP (siAPP); and control plasmid (c) or a plasmid encoding APP intracellular domain (AICD).

B. Q-PCR of APP mRNA levels on cells transfected with a control siRNA or with siAPP. Columns represent the average of four independent experiments (one in each cell line A549, H1299, H1437 and H1650); bars represent S.D. The mRNA abundance for cells transfected with control siRNA (c) was arbitrarily set to 1 at 48 hours after transfection.

C. Representative Western blot analysis of APP protein levels from the lysate obtained from H1299 cells at the specified time-points after siRNA transfection as described in B. Virtually identical results were obtained in all cells tested. The remaining band after APP depletion (not visible in all our Western blots) may be unspecific or correspond to the APP homolog protein APLP-2.

D. Western blot analysis of the specified proteins and phosphoproteins in total cell lysates obtained from cell line H1299 after exposure to the indicated concentrations of GSI. As a reference, cells transfected with a control siRNA or siAPP cells were treated with vehicle (DMSO).

E. Western blot analysis of the specified proteins and phosphoproteins in total cell lysates obtained from cell line H1299 48 hours after transfection, either with a control siRNA or a siRNA to APP. Similar results were obtained in multiple experiments and in cell line A549.

Adapted from Sobol et al., 2015 [1].
We hypothesized that one of the known signaling pathways downstream of APP could be involved in the regulation of 4E-BP1 phosphorylation. It has been demonstrated that APP signaling contributes to the activation of MAPK and AKT pathways. Activation of MAPK is mediated by the interaction of GRB2 and the C terminus of APP (Nizzari et al., 2007). AKT, on the other hand, is activated by oligomers of βeta-amyloid, one of the possible cleavage products of APP (Lee et al., 2009). Activation of both of these pathways results in the inactivation of tuberous sclerosis 1 and 2 (TSC1/TSC2), upstream inhibitors of mTORC-1.

Thus, either pathway could mediate the change of 4E-BP1 phosphorylation downstream of APP. 24, 48, and 72 hours after APP depletion, MAPK activation was reduced as determined by measurement of decreased phosphorylation of ERK at T185/187 (Figure 11A). In the same experiments, phosphorylation of AKT (at either T308 or S473 residues) was unchanged (Figure 11B, C). While the T37/46 residue of 4E-BP1 is mainly phosphorylated by mTORC-1, the phosphorylation of S65 and T70 may be mediated by ERKs and cyclin-dependent kinase 1 (CDK-1; Heesom et al., 2001; Herbert et al., 2002). To ascertain whether ERKs or CDK-1 could have contributed to the altered phosphorylation pattern of 4E-BP1 observed upon APP depletion, we inhibited MAPKK (the upstream activator of ERKs) with UO126 and CDK-1 with Roscovitine. We confirmed the efficiency of these pharmacological inhibitors by assessing the phosphorylation status of ERKs and phosphorylation of a downstream target of CDK-1, wee 1. Phosphorylation of ERKs and wee 1 was
reduced after treatment with UO126 and Roscovitine, respectively (Figure 11D, E). However, neither ERKs nor CDK-1 inhibition could recapitulate the phosphorylation pattern of 4E-BP1 resulting from APP downregulation (Figure 11D, E). We only observed a decreased phosphorylation of the S65 and T70 residues upon administration of either UO126 or Roscovitine, but no effect on T37/46 was detected. This suggested that APP might alter the 4E-BP1 phosphorylation pattern (at least in part) through its effects on ERK’s activation, but other molecular players awaited identification. Nonetheless, the empirical evidence collected thus far allowed us to exclude AKT involvement in these phenomena.
Figure 11. APP regulates ERKs activity but does not affect AKT phosphorylation status.

A-C. Western blot analysis of the indicated proteins and phosphoproteins in total cell lysates obtained from H1299 cells after transfection with a control siRNA (c) or siRNA against APP. Similar results were obtained in cell lines A549 and H1437.

D. Western blot analysis of the indicated proteins and phosphoproteins in total cell lysates obtained from H1299 cells after 48 hours exposure to vehicle (c) or roscovitine (Rosc.).

E. Same as (D) but cells were treated with UO126 for 24 hours.

Adapted from Sobol et al., 2015 [1].
Screening for potential genes expression alterations caused by APP depletion

As previously mentioned, 4E-BP1 phosphorylation at T37/46 was reduced upon overexpression of AICD (Figure 10A). AICD is known to interact with Fe65, histone acetyltransferase Tip60, or eukaryotic transcriptional mediator complex MED12, among other proteins, to transcriptionally regulate expression of a large number of genes (Gao and Pimplikar, 2001; Turner et al., 2011; XU et al., 2011). We hypothesized that a putative transcriptional target of APP (still unidentified) could mediate the observed change on 4E-BP1 phosphorylation at T37/46. To identify these targets of APP, we performed a genome-wide RNA expression analysis. We transfected two NSCLC cell lines (H1299 and A549) with either control siRNA or siRNA against APP and subjected them, along with mock transfected cells, to the Illumina HumanHT12 array (48,000 probes, RefSeq plus EST). The experiment was performed three times in independent fashion. We looked for genes, with expression that would decrease at least by half or increase at least two fold after APP depletion in both cell lines. Among the vast number of genes with altered expression, we identified 17 genes in which expression was altered in both cell lines and followed a similar pattern (e.g., either upregulated or downregulated in both cell lines). This subset was first identified with the mRNA arrays and the results were confirmed using Q-PCR (Figure 12A, B).
Figure 12. Expression levels of 17 genes changed after APP downregulation in two NSCLC cell lines.
A. Genes with expression level changes upon APP depletion in two NSCLC cell lines (H1299 and A549) as determined by the Illumina gene array experiments. This table summarizes the ratio of expression between siAPP and control transfected cells. Purple highlights indicate those genes that were downregulated with commercially available siRNAs in follow-up experiments.
B. mRNA levels measured using Q-PCR; the experiments were performed on total RNA extracted from A549 and H1299 cells transfected with either a control siRNA or a siRNA to APP. Columns represent averages; bars represent S.D. The mRNA abundance for cells transfected with control siRNA (c) was arbitrarily set to 1 at 48 hours after transfection.
Adapted from Sobol et al., 2015 [1].
STYX is the downstream target of APP regulating 4E-BP1 phosphorylation at T37/46

We used siRNAs that were commercially available and downregulated 14 genes in which expression changed upon APP depletion in a similar fashion in both H1299 and A549. We also decided to artificially downregulate those genes whose expression was increased upon APP depletion under the assumption that if these genes could have potentially affected 4E-BP1 phosphorylation, their modified expression level would have resulted in altered T37/46 phosphorylation in some way. First, we confirmed downregulation efficiency with gene-specific primers and Q-PCR (data not shown). Next, we assessed if downregulation of any of the genes could affect phosphorylation status of 4E-BP1 measuring the phosphorylation status of T37/46 by Western blot analysis. Out of all 14 genes that we downregulated, only one gene affected the phosphorylation status of 4E-BP1. This gene encodes the pseudophosphatase STYX. We depleted STYX in NSCLC cell lines using two independent siRNAs. Due to the lack of antibodies capable of detecting endogenous levels of STYX at the time of the study, we confirmed the downregulation efficiency at the mRNA level (Figure 13A). siRNA-mediated downregulation of STYX increased 4E-BP1 phosphorylation at T37/46. Notably, it did not affect the phosphorylation status of two other residues of 4E-BP1 (Figure 13B). These results seemed to support the idea that STYX is a transcriptional target of APP directly involved in the regulation of 4E-BP1
phosphorylation at T37/46. To confirm this, we overexpressed the full-length APP and found that mRNA levels of STYX increase in three different NSCLC cell lines (Figure 13C). Additionally, we observed that when downregulating either APP or STYX alone, similar increases in 4E-BP1 phosphorylation at T37/46 were measured. However, when we co-transfected cells with both siRNA against APP and STYX, we observed an additive effect on phosphorylation of T37/47 (Figure 13D, compare lanes 1 through 4). Transfection of AICD alone decreased 4E-BP1 phosphorylation at T37/46 (lanes 5 and 6), but co-transfection of cells with siRNA against STYX and AICD partially reversed this change (lanes 7 and 8).

In conclusion, the results supported the idea that the increase in 4E-BP1 phosphorylation at T37/46, seen upon APP depletion, is regulated (either directly or indirectly) by STYX, which seems to be a bona fide APP transcriptional target.
Figure 13. STYX regulates 4E-BP1 phosphorylation at T37/46.
A. STYX mRNA in H1299 cells transfected with a control siRNA (c) or with the indicated siRNAs to STYX (siSTYX2, siSTYX3) as assessed by Q-PCR. Columns represent averages of three independent experiments while bars represent S.D. The mRNA abundance for cells transfected with control siRNA (c) was arbitrarily set to 1 at 48 hours after transfection. We did not assess the STYX protein levels because an antibody against endogenous STYX was unavailable at the time of the study.
B. Western blot analysis of the indicated proteins and phosphoproteins 48 hours after transfection of the indicated siRNAs.
C. Q-PCR of STYX mRNA in cells transfected with the control plasmid pCAX (c) or with APP 695 cloned in pCAX (APP695). Columns represent averages of independent experiments performed in A549, H1299, and H1650 cells; bars represent S.D. The mRNA abundance for cells transfected with control siRNA (c) was arbitrarily set to 1 at 48 hours after transfection.
D. Western blot analysis of the indicated proteins and phosphoproteins in H1299 48 hours after transfection with the indicated nucleic acids. Lanes:
1, cells transfected with siRNA control (c);
2, cells transfected with siRNA to APP (siAPP);
3, cells transfected with siRNA to APP and siRNA to STYX (siAPP+siSTYX);
4, cells transfected with siRNA to STYX (siSTYX);
5, cells transfected with control plasmid (pc);
6, cells transfected with a plasmid encoding AICD (AICD);
7, cells transfected with control plasmid and siRNA to STYX (pc+siSTYX);
8, cells transfected with a plasmid encoding AICD and with siRNA against STYX (AICD+siSTYX).
Adapted from Sobol et al., 2015 [1].
APP DEPLETION ALTERS THE COMPOSITION OF THE TRANSLATION INITIATION COMPLEX eIF4F

Hyper-phosphorylated 4E-BP1 is thought to release the cap binding protein eIF4E, allowing the formation of the translation initiation complex (eIF4F) on the 5’ end of mRNA (referred to as cap; Jackson et al., 2010). We asked if the 4E-BP1 phosphorylation pattern observed upon APP depletion could affect the formation/composition of the eIF4F complex. We addressed this question in pull-down assays using the cap analog, 7-methylguanosine, conjugated to Sepharose resin. We analyzed the binding of translation initiation factors to the cap analog upon siRNA-mediated APP depletion. We specifically looked at the recruitment of the cap binding protein eIF4E, the ATP-dependent RNA helicase eIF4A, the scaffolding protein eIF4G, and also the binding of 4E-BP1. The binding of 4E-BP1 to the cap decreased after APP downregulation (Figure 14A). Reduced 4E-BP1 to the cap is generally considered an indication of enhanced rate of cap-dependent protein synthesis (Thoreen et al., 2012). We also detected a significant increase in the recruitment of eIF4A to the cap in APP depleted cells (Figure 14A). The fact that increased recruitment of eIF4A to the initiation complex was not accompanied by an increase of other translation initiation factors is not surprising. It has been demonstrated that the binding of eIF4E and eIF4A to eIF4G is anti-cooperative and that eIF4A alone can enhance the stability of translation initiation complexes (LeFebvre, 2006).
Additional evidence that APP depletion affects the activity and/or recruitment of eIF4A helicase to the eIF4F complex

eIF4A, a helicase that unwinds secondary structures of mRNA, is essential for the initiation of translation. To investigate a potential role of APP in the regulation of eIF4A recruitment to the cap, we used commercially available inhibitors of eukaryotic protein synthesis. One example is silvestrol, which prevents the binding of eIF4A to the initiation complexes and thus inhibits translation initiation (Liu et al., 2012). We measured the effect of silvestrol treatment on the protein synthesis rate in APP-depleted cells. The rate of protein synthesis was measured by incorporation of the methionine surrogate, L-azidohomoalanine (AHA), which was analyzed by flow cytometry. AHA was added to cells along with inhibitors. In the case of complete initiation inhibition, AHA is incorporated only into polypeptide chains translated from already pre-initiated mRNAs. If initiation is not completely inhibited, additional AHA incorporation into newly synthesized proteins may be measured. Interestingly, APP depleted cells seemed resistant to both partially and completely inhibitory concentrations of silvestrol (Figure 14 B, C). The incorporation of AHA by these cells was greater than in control siRNA transfected cells treated with silvestrol. The efficiency of the inhibitors in decreasing protein synthesis rate was confirmed by AHA incorporation in DMSO treated cells (Figure 14D).
These experiments supported the hypothesis that APP depletion may alter the eIF4F composition by altering eIF4A activity and/or recruitment to the eIF4F complex. The fact that APP regulates protein synthesis initiation and not elongation was confirmed by a control experiment in which APP-depleted cells were treated with homoharringtonine, which blocks translation elongation (Tujebajeva et al., 1989; Kantarjian et al., 2001). The incorporation of AHA in cells treated with homoharringtonine was inhibited regardless of the absence or presence of APP. Cells showed only autofluorescence similar to what was observed in cells not exposed to AHA (Figure 14B). Collectively, the experiments presented here supported our hypothesis that APP regulates protein synthesis at the level of translation initiation, most likely by the regulation of eIF4A recruitment.
**Figure 14.** Downregulation of APP results in rearrangements of the translation initiation complex on the mRNA cap.

**A.** Representative Western blot analysis of the indicated proteins in total cell lysates (cell lys.) and in 7-methylguanosine(m7G)-Sepharose pulled-down obtained from H1299 cells transfected with either a control siRNA (c) or a siRNA against APP (siAPP). Seph. Beads corresponds to Sepharose beads alone (the negative control in the pull-down assay). Similar results were obtained in multiple experiments and in cell line A549.

**B.** Click-iT AHA (L-azidohomoalaine) incorporation assay at the indicated time-points of cells transfected with either a control siRNA (c) or a siRNA against APP and exposed to 100nM silvestrol or homoharringtonine (homohar.). Cells were exposed to antibiotics alongside AHA. Geometric means of AHA incorporation histograms are presented as acquired by the FlowJo software.

**C.** As in (B) but cells were exposed to 40nM silvestrol.

**D.** As in (B), but cells were treated with vehicle only (DMSO). This is an example of a control experiment (no inhibitor treatment) that was performed alongside the experiments with silvestrol or homoharringtonine. The graphs summarize three experiments performed in A549 cells. Similar results were obtained in H1299 cells. Bars represent S.D.

Adapted from Sobol et al., 2015 [1].
APP REGULATES THE RATE OF PROTEIN SYNTHESIS

Increased phosphorylation of 4E-BP1 and enhanced recruitment of eIF4A to the cap, both observed upon APP depletion, led to the hypothesis that APP may play a role in the regulation of protein synthesis. Using the AHA incorporation assay, we showed that the rate of protein synthesis increases significantly upon downregulation of APP (Figure 15A, B). The increase in protein synthesis rate was observed not only in different NSCLC cell lines but also in a mesothelioma cell line (ME16), in immortalized human keratinocytes (HaCat), and in primary human lung fibroblasts (WI-38) (Figure 15C).

To determine whether a change on protein synthesis observed upon APP depletion is physiologically relevant, we compared it to the increase of protein synthesis observed in cells that were serum-starved overnight and then stimulated with insulin (Figure 15D). Insulin is a potent activator of protein synthesis (Proud, 2006). It increases the rate of translation by activating the AKT/mTOR signaling pathway. It also activates translation initiation factor eIF2B allowing for the recycling of eIF2 to GTP-bound eIF2, which, along with methionyl-transfer RNA and 40S ribosomal subunit, forms a ternary complex required for translation initiation (Proud, 2006). We first cultured cells overnight in media supplemented with 1% fetal bovine serum (FBS). The next day, we stimulated these cells with different concentrations of insulin and performed AHA incorporation assays. Notably, the increase of AHA incorporation upon APP depletion was comparable to the increase observed after insulin stimulation as
assessed by the FlowJo software (Figure 15D). The magnitude of the increase supports the global nature of the translation regulation by APP.
Figure 15. APP regulates protein synthesis

A. Representative histogram of flow cytometry analysis of the methionine analog AHA (L-azidohomoalanine) incorporation in A549 cells transfected with either a control siRNA (c, red) or siRNA against APP (siRNA, blue).

B. The average geometric mean of AHA incorporation in NSCLC cell lines A549, H1299, and H1650 as quantified by FlowJo (flow cytometry analysis software). Columns represent averages, bars represent S.D.

C. Depletion of APP results in the increase of protein synthesis in cells of different origin. Percentage increase in the geometric mean of AHA incorporation. Average with S.D. is presented for three independent experiments in specified cell lines.

D. APP-mediated increase in protein synthesis is comparable to the effect of insulin stimulation. AHA incorporation of H1299 cells treated with vehicle DMSO (c, red) or stimulated with either 10 nM (blue) or 100 nM (green) of insulin for two hours. Negative control, no AHA (black, dotted line).

Adapted from Sobol et al., 2015 [1].
Eukaryotic mRNAs can be translated via cap or internal ribosome entry sequence (IRES) dependent mechanisms. Importantly, eIF4A was shown to be required for both modes of translation initiation (Komar and Hatzoglou, 2011). Since, we observed that APP depletion interferes with eIF4A activity/recruitment, we hypothesized that APP may regulate both modes of translation initiation. To test this hypothesis, we co-transfected NSCLC cells with either a control siRNA or siRNA against APP and a bicistronic vector which allows for cap dependent translation of Renilla luciferase and cap independent (IRES) translation of firefly luciferase. Bioluminescence results showed that APP depletion resulted in enhanced Renilla and firefly luciferase activities (Figure 16A). We confirmed our hypothesis and concluded that APP regulates both cap- and IRES- dependent protein synthesis in dividing cells of different origin.

The effect on protein synthesis observed upon APP depletion was reversed by the overexpression of AICD, which is known to interact with more than 20 cellular proteins and to regulate transcription of many different targets (Figure 16 B; Beckett et al., 2012).
Figure 16. APP depletion increases cap- and IRES-dependent translation initiation. AICD overexpression results in a decrease of protein synthesis. 

A. The average bioluminescence of cells co-transfected with either a control siRNA (c) or siRNA against APP (siAPP) and a bicistronic vector encoding Renilla luciferase (representative of cap dependent translation) or firefly luciferase (representative of IRES dependent translation). The vector contains poliovirus IRES (see Material and Methods for details). Average of 9 experiments is presented. Bioluminescence was normalized for the expression of the plasmid DNA using luciferase specific primers and Q-PCR.

B. AHA incorporation in H1299 cells transfected with a control plasmid or the plasmid expressing AICD. Cells transfected with DNA show overall lower AHA incorporation as compared to cells transfected with siRNAs. Adapted from Sobol et al., 2015 [1].
Regulation of protein synthesis by APP does not depend on mTOR signaling

Phosphorylation of 4E-BP1 at T37/46 is catalyzed by the mTORC-1 complex, a major regulator of protein synthesis (Proud, 2006). In response to intra- and extracellular cues, mTORC-1 regulates translation initiation (Jackson et al., 2010). It is a checkpoint where decision the cell makes a decision whether or not it should engage in anabolic processes. Considering our initial hypothesis that mTORC-1 activity could be reactivated by GSI treatment and that deletion of APP resulted in increased 4E-BP1 phosphorylation at T37/46, we further hypothesized that mTORC-1 could also be involved in the regulation of protein synthesis by APP. However, most of the experimental evidence presented below suggested that regulation of protein synthesis by APP is independent of mTOR signaling, which consequently allowed us to reject our hypothesis.

First, the phosphorylation of 4E-BP1 observed upon APP depletion seems to be mediated by downregulation of the pseudophosphatase STYX (Figure 13). Second, APP depletion had no effects on AKT activation, as we did not observe any changes on T308 nor S473 phosphorylation (Figure 11B, C). Third, we measured the phosphorylation status of one of the canonical mTORC-1 downstream targets, S6 kinase (p70 S6K), which modulates protein synthesis via phosphorylation of multiple targets (eIF4B, PDCD4, CBP80, eEF2A, etc.; Laplante and Sabatini, 2012). We observed no change in phosphorylation of p70
S6K and S6 protein after APP depletion (Figure 17A). Finally, to further investigate the role of mTORC-1 in the regulation of protein synthesis by APP, we used a commercially available, specific inhibitor of mTOR kinase’s catalytic activity, Torin-1 (Thoreen et al., 2012). 48h exposure of NSCLC cells to Torin-1 suppressed mTORC-1 activation, as seen by abolished phosphorylation of two mTORC-1 targets: 4E-BP1 and S6K (Figure 17B, left). Torin-1 treatment also abolished protein synthesis, as seen by inhibited AHA incorporation (Figure 17B, right). At this stage, about a third of Torin-1 treated cells underwent cell death. Interestingly, even under these harsh inhibitory conditions, the protein synthesis rate of APP depleted cells exposed to Torin-1 increased as compared to Torin-1 treated cells transfected with a control siRNA (Figure 17C). For these reasons, we concluded that APP's effect on protein synthesis is mostly independent on mTORC-1 activity.
Figure 17. Regulation of protein synthesis by APP is independent of mTOR signaling.

A. Western blot analysis of the indicated proteins and phosphoproteins in total cell lysates obtained from H1299 cells transfected with either indicated siRNAs or plasmid DNA.

B. Treatment with Torin-1 suppresses mTORC-1 activity and inhibits protein synthesis rate. Left: Western blot analysis of the indicated proteins and phosphoproteins in total cell lysates obtained from H1299 cells treated with vehicle DMSO (c) or Torin-1. Right: AHA incorporation in H1299 cells treated with DMSO (c) or Torin-1.

C. Incorporation of AHA in H1299 cells transfected with a control siRNA and exposed to DMSO (red), transfected with siRNA against APP and exposed to DMSO (blue), transfected with a control siRNA and exposed to Torin-1 (light green), and transfected with siRNA against APP and exposed to Torin-1 (light brown).

D. Percentage increase in the AHA incorporation as assessed by geometric mean acquired with the FlowJo software. Average with S.D. is presented for three independent experiments in specified cell lines.

Adapted from Sobol et al., 2015 [1].
Finally, APP has a close homolog, APLP-2, with which it shares not only sequence homology but also tissue-specific distribution pattern (Aydin et al., 2012). We investigated whether the observed phenotype of protein synthesis is specific to APP or if APLP-2 may also be involved in the regulation of translation. We transfected cells with siRNAs against either APP or APLP-2. Utilizing Q-PCR, we showed that downregulation of APP does not affect the mRNA levels of APLP-2 and confirmed downregulation efficiency in cells transfected with siRNA against APLP-2 (Figure 19A). Notably, unlike depletion of APP, the downregulation of APLP-2 gene did not affect AHA incorporation as compared to relevant controls (Figure 19B).
Figure 18. APP’s homolog APLP2 does not play a role in regulation of protein synthesis rate.
A. mRNA levels obtained with Q-PCR experiments and performed on total RNA extracted from H1299 cells transfected with indicated siRNAs. Columns represent averages; bars represent S.D. The mRNA abundance for cells transfected with control siRNA (c) was arbitrarily set to 1.
B. AHA incorporation in H1299 cells 48h after transfection with a control siRNA (c) and siRNA against APP (siAPP, left) or siRNA against APLP2 (siAPLP2, right).
Adapted from Sobol et al., 2015 [1].
In conclusion, APP inhibits protein synthesis via regulation of different signaling pathways like ERKs and STYX. It also regulates the recruitment of eIF4A to the initiation complex, thus affecting both cap- and IRES-dependent translation initiation. Based on the results from several experiments, we concluded that the regulation of protein synthesis by APP is mTORC-1 independent. Finally, considering the pleiotropic effect of the APP protein, we cannot exclude additional downstream effectors of APP signaling may also be involved in the observed phenotype.

APP DEPLETION LEADS TO THE ARREST OF NSCLC CELLS IN G₀ PHASE OF THE CELL CYCLE

We have elucidated that APP regulates translation initiation. Protein synthesis is one of the most important processes undertaken by a cell and is closely associated with cell growth and proliferation. It has been accepted that the rate of protein synthesis increases linearly during cell division (Baxter and Stanners, 1978). Progression through the cell cycle depends on many factors, including the availability of nutrients and growth factors. As an example, Receptor Tyrosine Kinases (TKR) can transduce growth factor stimulation to activate both MAPK and PI3K/AKT/mTOR pathways, which consequently lead to the activation of protein synthesis (Laplante and Sabatini, 2012). During the G₁ phase of the cell cycle, cells have to clear the dominant, nutrient-dependent
restriction point that corresponds to START in yeast (Fingar and Blenis, 2004). Thus, it appears that at this point during cell division the major synchronization between cell growth and cell cycle progression takes place. This synchronization is very important for cell homeostasis, as inhibition of protein synthesis leads to cell death while excessive protein translation results in deregulated proliferation and tumor-like outgrowth (Lindqvist et al., 2012). Recent discoveries also showed that genetic changes affecting the translation machinery are associated with cancer susceptibility, with this phenomena described as “ribosomopathies” (Ruggero, 2013). Thus, translational rate and cell growth are two interdependent cellular processes.

Since we have noticed that protein synthesis increases significantly upon APP depletion, we hypothesized that APP could affect cell cycle progression and cell growth. We transfected NSCLC cells with either a control siRNA or siRNA against APP and stained DNA of permeabilized cells with propidium iodide. Flow cytometry analysis revealed the accumulation of cells in the G₀/G₁ phase of the cell cycle upon APP depletion (Figure 20B).
Figure 19. APP depletion causes G₀/G₁ arrest and decreases cell proliferation.
A. Representative Western blot analysis of the indicated proteins in total cell lysates obtained from NSCLC cells transfected with either a control siRNA (c) or siRNA against APP (siAPP).
B. Propidium iodide (PI) cell cycle staining of NSCLC cells (representative of three experiments in: top panel, A549; middle panel H1299; bottom panel, H1437) transfected with a control siRNA (c, left) and with a siRNA against APP (siAPP, right). Numbers represent percentage of cells in each phase of the cell cycle as determined by FlowJo analysis using the Watson pragmatic model. The observed changes were reproducible and statistically significant.
Adapted from Sobol et al., 2015 [2].
To analyze cell proliferation, we used bromodeoxyuridine (BrdU) incorporation assays. Total DNA was stained with 7-aminoactinomycin D (7-AAD) and analysis was made using FACS. This assay revealed that APP’s loss of function reduces the percentage of proliferating cells (Figure 21). Decreased proliferation and cell cycle arrest observed upon APP depletion were reproducible in all NSCLC studied and in normal lung fibroblasts, although to a lower extend (Figure 21).
Figure 20. APP depletion reduces the fraction of cells that replicate DNA. Bromodeoxyuridine (BrdU) incorporation followed by flow cytometry analysis of indicated cells transfected with a control siRNA (left) and a siRNA against APP (right). Representative of three independent experiments is shown. The observed changes were reproducible in cells of different origin and were statistically significant.
Adapted from Sobol et al., 2015 [2].
Furthermore, we wanted to determine whether APP depletion causes accumulation of cells in either $G_0$ or $G_1$ phase of the cell cycle. To discriminate between the two, we used the acridine orange (AO) staining, which differentiates between the dsDNA and ssRNA content of the cells (Darzynkiewicz et al., 2004). We assessed that cells transfected with siRNA against APP had a 13.8±3.5 fold increase in the $G_0$ fraction compared to cells transfected with a control siRNA (Figure 22A). AO staining does not accurately set a boundary between cells in S and $G_2/M$ phases. However, it allowed us to identify a new cell population that was present within APP depleted cells. This population consisted of cells that stained negatively for both DNA and RNA content but with a size similar to cells in $G_0$ or $G_1$ phase of the cell cycle. We named this population “sub-$G_0$”. To analyze the “sub-$G_0$” cells in more detail, we used an Amnis Image StreamX imaging flow cytometer. Analysis of images of these cells demonstrated that they lack nuclei. It appears that these cells could be necrotic (Figure 22B).

In conclusion, APP seems to synchronize protein synthesis rate and cell cycle progression and its loss results in what appears to be necrotic cell death.
**Figure 21. APP depletion causes G₀ arrest and cell necrosis.**

A. Acridine orange (AO) staining of A549 cells transfected with a control siRNA (c, left) or siRNA against APP (siAPP, right).

B. Amnis Image StreamX imaging flow cytometer analysis of siRNA against APP-transfected H1299 cells. Left: gating of cell populations; Right: bright field and fluorescence images of representative cells from each cell population. Original magnification 40X. Same results were obtained in A549 and H1650 cells.

Adapted from Sobol et al., 2015 [2].
**APP mediated G₀ arrest involves cyclin C destabilization**

We wanted to clarify which downstream target(s) of APP may be involved in the observed cell cycle arrest. We already confirmed that loss of APP results in the inhibition of MAPK pathway (Figure 11A), a well-established regulator of cell proliferation (Zhang and Hui, 2002). Inhibition of ERKs but also cyclin dependent kinases (CDK), which regulate cell cycle progression, could contribute to the changes observed upon APP downregulation. Thus, we inhibited the MAPK pathway using UO126 and CDK-1/CDK-2 using roscovitine (Schutte et al., 1997). However, treatment of hypoxic NSCLC cells with the aforementioned inhibitors did not recapitulate the cell cycle distribution observed after APP depletion. Treatment with roscovitine resulted in a G₂/M arrest, while treatment with UO126 resulted in the arrest of cells in G₁ phase of the cell cycle with no apparent accumulation of cells in the G₀ phase (Figure 23A-D).
Figure 22. Inhibition of CDKs and ERKs does not recapitulate cell cycle distribution observed upon APP depletion.

A. Western blot analysis of the indicated proteins and phosphoproteins in total cell lysates obtained from H1299 cells treated with a vehicle control (c, DMSO) or roscovitine (Rosc.) Cdk1 mediated phosphorylation of wee1 served as positive control for the roscovitine treatment.

B. PI staining of H1299 cells treated as in (A).

C. Western blot analysis of the indicated proteins and phosphoproteins in total cell lysates obtained from H1437 cells treated with a vehicle control (DMSO, c) or UO126. Phosphorylation of ERK served as positive control for the UO126 treatment.

D. PI staining of H1437 cells treated as in (C).

E: AO staining of H1437 treated as in (C). Please note that neither UO126 nor roscovitine treatment generated the "sub-G₀" population of necrotic cells.

Adapted from Sobol et al., 2015 [2].
To find possible mediator(s) of G₀ arrest induced by APP depletion, we used commercially available antibody arrays, including a large number of proteins and phosphoproteins known to regulate cell cycle. We looked for changes caused by APP downregulation common to two NSCLC cell lines (H1299 and A549). The only apparent changes that have been observed included a modest decrease in cyclin E and a 2.5 fold decrease in cyclin C protein expression levels. We investigated the protein and mRNA levels of both cyclins by Western blot and Q-PCR analysis in cells after APP depletion. Cyclin E was reduced at both protein and mRNA level 48 hours after siRNA-mediated APP downregulation. On the other hand, while mRNA levels of cyclin C remained the same, its protein levels were reduced at every time point after siRNA to APP transfection, with these outcomes reproducible in different cell lines (Figure 24A, B). Cyclin C protein expression changes are consistent with the timing of APP depletion-induced cell cycle arrest. We hypothesized that cyclin C is a target of APP involved in the G₀/G₁ arrest.

Cyclin C is known to bind CDK8 and contribute to the phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II (Rickert et al., 1996). More importantly, a second pool of cyclin C binds CDK3 and controls G₀ to G₁ progression. Cyclin C-bound CDK3 phosphorylates the retinoblastoma protein (pRb) at S807/811 allowing cells to exit G₀ phase of the cell cycle (Ren and Rollins, 2004). It has also been determined that cyclin C (at least in yeast) is a short-lived protein and is possibly degraded via the ubiquitin–proteasome system
(Hautbergue and Goguel, 1999). Notably, we only observed changes in protein and not mRNA levels of cyclin C upon APP depletion. This implies that APP may regulate cell cycle progression via destabilization of cyclin C protein levels. To test this hypothesis, we immunoprecipitated cyclin C from cells transfected with either a control siRNA or siRNA against APP and blotted for cyclin C and ubiquitin. We performed this experiment in the presence of proteasomal inhibitor, MG132. We investigated if total levels and ubiquitination of cyclin C could be affected by APP levels and if APP can mediate cyclin C degradation via the proteasome mediated mechanism. In the absence of MG132, only about 25% of cyclin C was immunoprecipitated from cells depleted of APP as compared to control cells (Figure 24C). We also observed a more intense smear when blotting these immunoprecipitates for ubiquitin as compared to the relevant control (Figure 24D). When transfected cells were treated with MG132, equal amounts of cyclin C were immunoprecipitated from both control and APP depleted cells (Figure 24E). The change in the migration of Cyclin C upon MG132 treatment resulted from ubiquitin moieties added to cyclin C. We expected to find bands at higher molecular weight. However, this situation is extremely common when immunoprecipitating any protein and then blotting for the same protein. This phenomenon has been attributed to “ubiquitin depletion” caused by prolonged MG132 exposure (Kim et al., 2011). In conclusion, we have found that APP regulates the stability of cyclin-C via polyubiquitination and proteasome mediated degradation.
**Figure 23.** APP depletion causes proteasome-mediated cyclin C degradation.

A. Q-PCR analysis of the indicated mRNAs in A549, H1299 and H1650 transfected with a control siRNA (c) or with a siRNA against APP (siAPP). Columns represent averages, bars S.D. The mRNA abundance for cells transfected with control siRNA (c) was arbitrarily set to 1.

B. Western blot analysis of the indicated proteins and phosphoproteins in total cell lysates obtained from H1299 cells transfected with control siRNA (c) or siRNA against APP (siAPP) at the indicated time-points. Similar results were obtained in A549, H1650 and H1437 cells.

C. Immunoprecipitation of cyclin C in H1299 transfected with a control siRNA (c) or siRNA to APP (siAPP). IgG; irrelevant immunoglobulin G.

D. Same as (C), but membrane was blotted with an antibody against ubiquitin.

E. Same as (C), but H1299 cells were treated with the proteasomal inhibitor MG132 for 24 hours prior to immunoprecipitation.

Adapted from Sobol et al., 2015 [2].
Depletion of cyclin C reproduces the cell cycle distribution observed upon APP depletion. Overexpression of AICD reverses this phenotype

We assessed if APP depletion affects the phosphorylation status of pRb. We performed experiments with a control siRNA or siRNAs against either APP or cyclin C. Depletion of both APP and cyclin C resulted in a similar decrease in phosphorylation of the S807/811 residues (Figure 25A). Moreover, when blotting for cyclin C, we noticed the disappearance of a band corresponding to the cyclin C isoform associated with the G₀ exit in yeast (Hautbergue and Goguel, 1999).

Using AO staining we investigated whether siRNA-mediated depletion of cyclin C caused a similar cell cycle distribution as seen after APP downregulation. Both G₀/G₁ arrest and, more importantly, the appearance of the “sub-G₀” population were observed in cyclin C depleted cells as compared to the relevant controls (Figure 25B). These effects were not as strong as with APP depletion. We investigated if cyclin C may regulate protein synthesis rate like APP. However, we found that cyclin C levels does not affect protein translation (not shown). Thus, cyclin C seems to produce some sort of uncoupling of cell cycle progression and cell growth (e.g., unaltered rate of protein synthesis paralleled by cell cycle arrest). However, this uncoupling is not as severe as after APP downregulation, which causes cell cycle arrest and an increase in the rate of protein synthesis. This may explain why the percentage of cell death is not as high in cyclin C versus APP depleted cells.
It seems that AICD is involved in the regulation of protein synthesis rate. Thus, we investigated if this APP domain may also be involved in the regulation of cell cycle progression. Interestingly, overexpression of AICD increased the percentage of proliferating cells and decreased the fraction of necrotic cells as visualized by the acridine orange assay (Figure 25C).
Figure 24. Cyclin C depletion mimics and AICD reverses the phenotype observed on cell cycle upon APP downregulation.

A. Western blot analysis of the indicated proteins and phosphoproteins in H1299 cells transfected with a control siRNA (c), with siRNA against APP (siAPP), or with siRNA against cyclin C (sicyclin-c). Similar results were obtained in A549 and H1650 cells.

B. AO staining of A549 cells after transfection with a control siRNA (c), or with a siRNA against cyclin C (siCycl.C).

C. AO staining of A549 cells transfected with a control plasmid (pCont) or with a plasmid expressing AICD. Similar results were obtained in H1299 cells.

Adapted from Sobol et al., 2015 [2].
APP DEPLETION RESULTS IN THE INCREASED CELL SIZE OF HYPOXIC NSCLC CELLS

APP depletion increases protein synthesis rate and promotes cell cycle arrest. We tested the effect that this uncoupling could have on cell growth and cell viability. Interestingly, the size of APP depleted cells were larger compared to the control cells. We measured a 113-130% increase of cell size 72 hours after siRNA transfection, as determined by flow cytometry (Figure 26A). It also appeared that the integrity of the cell membrane of APP depleted cells was compromised as visualized by PI staining of previously non-permeabilized cells, grown in hypoxia and nutrients’ restriction. After APP downregulation, larger population of cells stained positive for PI (Figure 26B).
Figure 25. The size of APP depleted cells increases and these cells undergo necrotic cell death.

A. Forward light scattering flow cytometry analysis of H1299 cells 72 hours after transfection with either a control siRNA (dotted line) or siRNA against APP (siAPP, solid line). Similar results were obtained in A549, H1650 and H1437 cells.

B. Flow cytometry analysis of PI staining of non-permeabilized A549 cells 72 hours after transfection with either a control siRNA (left) or siRNA against APP (siAPP, right). Cells were grown in reduced serum media (10% FBS media was diluted 1/40 in PBS). Similar results were obtained in H1299 and H1650 cells. Adapted from Sobol et al., 2015 [2].
Overall, we showed that loss of APP function results in enhanced cell growth and $G_0$ arrest of NSCLC cells. This uncoupling between protein synthesis rate and cell cycle progression led to increased cell size and necrotic cell death. We believe that this paradoxical uncoupling between growth and proliferation makes sense for a protein that regulates $G_0/G_1$ transition, when rates of high cell growth are probably unnecessary and possibly detrimental in yet uncommitted hypoxic NSCLC cells (see Discussion).
CHAPTER FOUR
DISSCUSSION

We have demonstrated that loss of APP function produces a number of seemingly paradoxical effects. APP depletion leads to G₀ arrest and enhanced rate of global protein synthesis (both cap- and IRES-dependent). This unique dissociation between proliferation and growth causes cellular abnormalities, such as increased cell size of NSCLC cells cultured in hypoxic conditions, and ultimately to necrotic cell death. Our data indicate that APP seems to be required for G₀/G₁ transition by preserving cyclin C levels. It also modulates protein synthesis rate via pleiotropic effects on different signaling molecules. These include (but may not be limited to) ERK kinases and STYX pseudophosphatase that together alter phosphorylation status of 4E-BP1, an inhibitor of translation. Additionally, regulation of eIF4A function and/or its recruitment to the cap seems to be a major contributing factor to the observed changes on the rate of protein synthesis upon APP depletion (a schematic summary of these phenomena is reported in Figure 26).
Figure 26. Summary of the findings of this dissertation. APP (through its intracellular domain activity, which, at least in part, includes transcriptional regulation) controls G₀/G₁ transition and the rate of global protein synthesis. Experimental evidence suggests that APP modifies translation initiation via pleiotropic effects including (but not necessarily limited to) regulation of ERKs, STYX, and eIF4A function and/or its recruitment to the 5’ end of mRNA. On the other hand, the APP/AICD promotes transition to the G₁ phase of the cell cycle by the regulation of Cyclin C protein levels.
APP REGULATION OF PROTEIN SYNTHESIS IN NSCLC CELLS

The RAS/ERK and PI3K/AKT/mTOR signaling pathways, which regulate proliferation and cell growth, have been recently shown to converge at the translation initiation step, specifically on 4E-BP1 phosphorylation status and its ability to inhibit eIF4E (She, 2010). It has been shown that 4E-BP1 phosphorylation and cap-dependent protein synthesis depend mainly on AKT signaling in malignant cells with hyperactive PI3K/AKT. However, malignant transformation also requires the deregulation of RAS/ERK pathway. In all cancer cells tested, 4E-BP1 is a target of both pathways and inhibition of one pathway alone is not sufficient to inhibit 4E-BP1 phosphorylation, cap-dependent translation, cell growth, proliferation, and oncogenicity in vivo (She, 2010). Since our data supports a role for APP in the regulation of cell cycle entry and protein synthesis rate, it is not surprising that APP (via ERKs and STYX) ultimately affects 4E-BP1 phosphorylation status. This appears to be the point at which the PI3K/AKT and RAS/ERK pathways converge, promoting both growth and proliferation of malignant cells (She, 2010).

APP depletion resulted in increased phosphorylation at T37/46 and decreased phosphorylation at S65 residues of 4E-BP1. ERKs are known to regulate S65 and T70 phosphorylation. We found that the pseudophosphatase STYX regulates phosphorylation at T37/46. This is an entirely novel role for STYX. Although sharing sequence and structural similarities with genuine phosphatases, STYX is considered a pseudophosphatase because it is catalytically inactive. In-
stead of cysteine in the catalytic site, required for dephosphorylation, STYX has a glycine residue (Wishart et al., 1995). Even though they lack catalytic activity, pseudophosphatases are known to regulate different signaling pathways (Tonks, 2009). STYX itself was shown to be required for spermatogenesis and STYX knockout male mice are infertile. STYX binds a testicular RNA-binding phosphoprotein CRHSP-24 that is important during differentiation of spermatids into spermatozoa (Wishart and Dixon, 2002). Using a computational modeling based approach, STYX was also found to interact with MAPK signaling pathway. STYX regulates the nuclear export of ERK1/2 kinases, inhibits their activation, and reduces differentiation of PC12 cells (Reiterer et al., 2013). Proteins containing STYX (phosphoSerine, phosphoThreonine, or phosphoTyrosine) domain probably evolved from the PTP (protein tyrosine phosphatase) family (Wishart and Dixon, 1998). It has been suggested that they retain the ability to bind the phosphorylated substrates of proteins with which they interact. The binding of pseudophosphatases to phosphorylated substrates alters, among other properties, their cellular localization, substrate selectivity, and binding partner selection. This binding also gives these catalytically inactive proteins the ability to signal (Tonks, 2009). A parallel between pseudophosphatases and 14-3-3 proteins can be made. The latter also play important roles in vital cellular processes without possessing specific catalytic activities. 14-3-3 proteins bind as dimers to phosphorylated polypeptides altering their functions substantially (Fu et al., 2000). The exact mechanism by which STYX can affect 4E-BP1 phosphorylation at T37/46 but it could bind to these sites directly, thus preventing their de-phosphorylation. Al-
ternatively, STYX could alter the substrate specificity of ERKs and direct them towards T37/46 (besides S65 and T70). Similar types of interactions have been described before. For example, it has been suggested that a pseudophosphatase TAB1 (TAK1 [transforming growth factor-β-activated kinase 1]-binding protein 1), which is a subunit of TAK1, has no catalytic activity but modulates the accessibility of phosphorylation residues on TAK1 substrates (Conner et al., 2006). Also, a pseudophosphatase is involved in cell cycle regulation and Cyclin-CDK (cyclin dependent kinase) interactions. Cyclins are well known to alter substrate specificity of the CDKs they associate with or to change their intracellular localization (Miller and Cross, 2001; Moore et al., 2003). For example, substrate preference differs whether CDK2 is bound to either cyclin E or cyclin A (Schulman et al., 1998). Alternatively, the mTOR kinase, which is phosphorylated on a variety of residues, could be a target of STYX. Rapamycin, an example of a molecule which can allosterically alter mTOR activity independently from mTORC-1 classic regulation, binds to the FRB domain of mTOR, alongside FKBP12, thus inhibiting mTORC-1 independently of stimulatory signals (Laplante and Sabatini, 2009).

We have been unable to test these hypotheses because efficient antibodies against human STYX are just now becoming available. This could explain why the scientific literature concerning human STYX includes less than a handful of papers. To summarize, the hypotheses that STYX affects substrate specificity of ERKs or mTOR should be investigated in future experiments and may have far-reaching implications. Notably, we have shown that it regulates phosphorylation of 4E-BP1 under conditions which should prevent it. This implies that STYX inhi-
bition alone could resume anabolic processes in tumor environments that are incompatible with them. Therefore, inhibiting STYX may have a role in the promotion of necrosis of hypoxic tumor cells.

We propose that a major contribution to the APP mediated increase in the rate of protein synthesis is due to the increased recruitment of eIF4A in pre-initiation complexes. Additionally, APP depleted cells showed resistance to the treatment with silvestrol, a translation initiation inhibitor, which prevents binding of eIF4A to the eIF4F initiation complex (Liu et al., 2012). eIF4A, a DEAD-box helicase that catalyzes the ATP-dependent unwinding of RNA duplexes (Korneeva et al., 2001; for details see section 1.4.2), is essential for both cap-dependent and IRES-dependent translation initiation (Komar and Hatzoglou, 2011). The fact that APP regulates both modes of translation initiation further strengthen the case that eIF4A may be a downstream target of APP responsible for the observed phenotype. The exact mechanism by which APP/AICD regulates eIF4A activity needs to be determined in future studies. However, it is well known that the majority of cellular mRNAs harbor long 5' untranslated regions (UTR) that form complex tertiary structures. An increased recruitment of the initiation factor responsible for the unwinding of these structures most likely renders the initiation complex more efficient in scanning 5' UTRs, therefore rendering initiation (the rate-limiting step in protein synthesis) more efficient. Since regulation of translation fundamentally affects the biology of the cell, the role of APP in translation initiation regulation warrants further investigation. For example, poly-some profiling (Faye et al., 2014) could be implemented to determine if APP de-
pletion in hypoxic NSCLC cells correlates with changes in translation of eukaryotic mRNAs on a global level or only within a subset of eukaryotic mRNAs. These mRNAs could be related to a malignant phenotype or to survival in hypoxic environment. It is increasingly recognized that “qualitative” regulation of protein synthesis plays a fundamental role in these processes (Ruggero, 2013). eIF4A is being considered as a therapeutic target for cancer treatment (Grzmil and Hemsings, 2012). However, the eIF4A inhibitors developed thus far have proven overly toxic and their introduction in clinical settings is lagging.

**APP REGULATION OF G\(_0\)/G\(_1\) TRANSITIONS**

APP appears to promote cell cycle entry by regulating cyclin C steady-state expression levels. Cyclin C exists in the cell in two distinct pools: one fraction associates with CDK8 and another associating with CDk3. In this first pool, cyclin C associates with CDk8, promoting phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II. This Cyclin C/CDK8 association was found to further associate with RNA polymerase II both *in vitro* and *in vivo* (Rickert et al., 1996), profoundly affecting the general properties of the polymerase. The second pool of cyclin C (which is extremely short-lived; Ren and Rollins, 2004) associates with CDK3 and is required for G\(_1\) entry (Rickert et al., 1996; Ren and Rollins, 2004). We propose that APP selectively regulates the stability of this second pool of cyclin C. Firstly, western blot analysis of cyclin C upon downregulation of APP showed a band corresponding to a cyclin C isoform
that binds CDK3 and regulates cell cycle in yeast (Hautbergue and Goguel, 1999) is lost. Secondly, if the activity of RNA polymerase II, a major transcriptional regulator (Hahn, 2004), would change in our system due to interference by APP on the pool of cyclin C with which it associates with, we would have measured dramatic changes in gene expression in our Illumina arrays, most likely in both cell lines tested. This hypothesis can be rejected because we observed only 17 genes with expression which was either increased 2 fold or decreased at least by half after downregulation of APP in both NSCLC cell lines. The question that still awaits clarification is how APP/AICD signaling selectively regulates Cyclin C levels. The pool of cyclin C that associates with CDK3 is phosphorylated at specific sites, while CDK8-associated cyclin C is not (Hautbergue and Goguel, 1999). We speculate that AICD may transcriptionally regulate a component of the ubiquitin proteasome pathway involved in Cyclin C degradation (e.g., a specific E3 ubiquitin ligase that recognizes a specific pattern of phosphorylation on cyclin C). Alternatively, AICD may directly interact with CDK3-associated cyclin C.

Importantly, does APP act as a regulator of cell growth and proliferation only in a nutrient-deprived, hypoxic tumor environment or does it still contribute to malignant transformation independently of the microenvironment? There is sufficient literature in the field to propose hypotheses. APP is overexpressed in different types of tumors (Meng et al., 2001; Hansel et al., 2003; Haven et al., 2004; Krause et al., 2008; Takayama et al., 2009). These reports would support an oncogenic role for APP independently of the tumor environment. However, the cleavage of APP (hence, APP signaling) is drastically increased under hypoxia
(Wang et al., 2006; Zhang et al., 2007). For this reason, we support the hypothesis that cancer cells in hypoxic microenvironment depend on APP signaling for their survival. Increased APP activity in malignant cells under these conditions is expected to suppress a process with high energy consumption like protein synthesis to avoid cell death due to energy exhaustion. One should bear in mind that, under hypoxia, cancer cells are under a metabolic checkpoint and are actively engaging in autophagy (Hu et al., 2012). Therefore, in hypoxic tumor regions NSCLC cells may be more dependent on APP signaling compared to the same cells located in different environmental conditions. This dependence on APP signaling could be referred to as an “oncogene addiction” (Weinstein and Joe, 2008), which in this case, is specific to the hypoxic tumor environment. Regulation of cell growth and cell cycle progression could also be a physiological role of APP, which is known to produce pleiotropic effects in cells of different origin (Muller and Zheng, 2012).

We did not expect to see cell cycle arrest upon APP depletion because the same genetic manipulation led to increased cell growth. Growth and proliferation are inherently linked processes and their complete dissociation appeared paradoxical. This apparent contradiction can be explained by taking into consideration the following traditional observations in cell biology. G₀ exit (and G₁ entry) is promoted by growth factor stimulation. This event leads to a very rapid activation of the PI3K/AKT and Ras/ERKs pathways. Therefore, one of the earliest phenomena that should be observed upon G₁ entry should be a “burst” in global protein synthesis. This phenomenon has never been observed but takes place
gradually through G₁ progression (Alberts, 2002). This means that a signaling pathway is inhibiting mTORC-1 activity early in G₁ and this pathway must follow regulatory networks completely independent from the known mTORC-1 regulatory signals. We propose that APP is centrally involved in this finely tuned process. APP, which promotes cell cycle progression (G₀/G₁), will also limit the rate of a highly energy-consuming process like protein synthesis in cells that are uncommitted to proliferation. Cells exiting the G₀ phase are not fully committed to the cell division because they still need to clear important checkpoints, which take place in G₁ (Fingar and Blenis, 2004; Foster et al., 2010). The Ras/Raf/MEK/ERK and the PI3K/PTEN/AKT/mTOR signaling cascades control the restriction point and “cell growth” checkpoint, respectively, during the G₁ phase of the cell cycle (Foster et al., 2010). The nutrient dependent “cell growth” checkpoint, that is equivalent to START in yeast, depends on mTOR activity (Foster et al., 2010). Activation of the PI3K/AKT/mTORC-1 pathway, upon stimulation of cells with serum, leads to the inhibition of TGF-β signaling, indirectly suppressing the cyclin E-CDK2 complex required for the transition from G₁ to S phase (Song et al., 2006; Gadir et al., 2008). At this stage, mTORC-1 signaling may become predominant over APP signaling.

Essentially, we propose that APP controls transitioning of cells from G₀ to G₁ phase of the cell cycle while moderating protein synthesis in a stage when cell fate has yet to be determined, through an mTORC-1 independent mechanism.

Uncoupling of cell growth and cell cycle progression (observed upon APP depletion) has been described before but it seems to result in cell death. For ex-
ample, overexpression of p16 (INK4A), an inhibitor of cyclin dependent kinases regulating G₁ to S phase transition, led to arrest of cells in G₁ with a normal protein synthesis rate. This uncoupling resulted in apoptotic cell death (Ausserlechner et al., 2005). In our cells, APP depletion caused cell cycle arrest (at the G₀/G₁ phase) and increased protein synthesis rate. This complete dissociation between growth and proliferation led to cell enlargement and necrotic cell death. Just like APP, myc and PI3K signaling pathways control cell growth and cell cycle progression. However, besides the regulation of protein synthesis rate, both pathways also regulate neolipogenesis (Laplante and Sabatini, 2009). If APP regulates only protein synthesis, increases in cell size without concomitant synthesis of major membrane components would be detrimental for cell membrane integrity and the fate of the cell (Dang, 2011; Yecies et al., 2011). If this is the case, then necrosis that occurs due to the loss of APP function seems to be unplanned. While many studies implicat the role of specific signaling pathways in the regulation of necrotic cell death (Vanden Berghe et al., 2014), it seems that in our system necrosis is a result of compromised cell membrane integrity, occurring spontaneously.
APP MAY PARTICIPATE IN AN EARLY CELL SIZE CHECKPOINT

Evidence indicates that some control mechanisms of cell size, or critical size checkpoints, are in place throughout the evolutionary tree within the constraints of nutrients availability. This has been somewhat controversial in mammalian cells (Marshall et al., 2012). Separating cell growth and proliferation as independent processes appears impossible given the mounting evidence from the past two decades indicating that growth and proliferation are coupled. A large amount of evidence supports a pivotal role for the phosphatidylinositol 3-OH kinase (PI3K)/Akt/mTORC-1 axis in the establishment of this possible crosstalk. Studies showed that mutating several components of this signaling pathway influenced both cell size and number, hence organ size (Böhni et al., 1996; Leevers et al., 1996; Goberdhan et al., 1999). Likewise, mutations which inactivate genes which lead to deregulated mTORC-1 activity and failed metabolic checkpoints cause syndromes which are characterized by multiple tumor-like outgrowths in humans, such as Cowden syndrome (Liaw et al., 1997) and tuberous sclerosis (Brook-Carter et al., 1994). Conversely, experimental knockout of a number of genes heavily involved in PI3K activation and its downstream effectors causes organ hypoplasia and reduced body size in mice (Liu et al., 1993; Dummier et al., 2006).

Among the gene knockouts (KO) affecting organ and body size, one of the least characterized is APP. Mice carrying a homozygous deletion of APP are about 20% smaller than their wild type or heterozygous littermates (Zheng et al., 1995). This
phenotype has been attributed to a reduced food and liquid intake in APP KO mice, a possible result of a functional brain defect in hunger control. Nonetheless, the APP KO mouse has a phenotype that closely resembles what has been observed in knockout mice for proteins inherently linked to the coordination of growth and proliferation. Traditionally, most of the mammalian cell size checkpoints seemed to operate during G2/M. Recently, studies have indicated that cell size checkpoints are operating throughout the cell cycle (reviewed in Yasutis and Kozminski, 2013). Since depletion of APP leads to an increase in cell size, we cannot exclude that APP participates in an early cell size checkpoint.

APP AS A THERAPEUTIC TARGET IN ANTI-CANCER TREATMENT

Often contributing to malignant transformation, signaling molecules that regulate both cell cycle progression and cell growth are being pursued in cancer research (Sulić et al., 2005). Our findings showed the role of APP depletion in mediating necrotic death of cancer cells in hypoxia. Consequently, they provide a rationale to target APP signaling pathway in therapies against hypoxic NSCLC.

Hypoxia supports cancer stem cell (CSC) maintenance and we showed that APP targets hypoxic NSCLC cells. Previous studies implicated a role of APP in neural stem cell biology (Kwak et al., 2006). Numerous studies have demonstrated an intrinsic link between hypoxia and the establishment of CSC niche (reviewed in Keith and Simon, 2007). Thus, it would be worthy to see if APP may function as a cell growth regulator in cancer stem cells and if its depletion could
have any effect on cancer stem cell survival. Alternatively, the eradication of hypoxic tumor tissue could deprive NSCLC CSC of their natural niche. The importance of novel ways to target CSC in anti-cancer therapy, would significantly improve current treatments and potentially prevent tumor recurrence, cannot be underestimated.

Furthermore, increased expression of translation initiation factors and genetic predisposition resulting in the deregulation of ribosomal components of translation machinery are associated with malignant transformation (Ruggero, 2013). Therefore, anti-cancer therapies targeting the protein synthesis apparatus are being extensively investigated (Grzmil and Hemmings, 2012). Most of the drugs targeting deregulated protein synthesis are mTOR inhibitors (Wander et al., 2011). Some of them were found to be effective as anti-cancer agents and were approved for use in the clinic and some were additionally found to sensitize cancer cells to common chemotherapeutics like cisplatin (Huang et al., 2013). However, under hypoxic, stressful conditions, cells are under a dominant checkpoint and so mTORC-1 should already be inhibited. Since we have shown that APP regulates the rate of protein synthesis (independently of mTORC-1) in hypoxic environment, we propose that APP can be considered as a potentially attractive therapeutic target in hypoxia-rich tumors.
STRATEGIES TO TARGET APP/AICD SIGNALING

The phenomena observed upon APP depletion were reversed by overexpression of AICD. Unbound AICD is unstable and the factor Fe65 is thought to stabilize it (Kimberly et al., 2001). Nonetheless, Fe65 is only one of the many binding partners of AICD participating in AICD signaling and functions (Beckett, 2012). The identification of the AICD binding partner mediating all the effects presented here is beyond the scope of this dissertation. This AICD binding partner, once identified, could be targeted using a small molecule to disrupt its interaction with AICD. Using this strategy, we could reproduce the effects of APP genetic ablation in hypoxic tumor tissue. Small molecules could be more effective than large inhibitors, like GSI, in reaching hypoxic areas of tumors, which are, by definition, distant from the vasculature.

Cleavage of APP and AICD generation is reminiscent of Notch receptor cleavage and production of the NICD. The therapeutic efficacy of humanized monoclonal antibody mediated inhibition of Notch signaling is still under investigation. These antibodies were shown to prevent Notch cleavage and diminish Notch mediated downstream signaling (Falk et al., 2012). Similar antibodies could be utilized to prevent cleavage of APP and AICD dependent signaling. Still, antibodies are large molecules and their delivery to distant, hypoxic tumor tissue could be problematic.

Alternatively, novel signaling molecules downstream from APP and identified in this project, could be used as therapeutic targets, alone or in combination
with APP targeting. One of the most promising candidates is STYX, because it generates a phenotype that can be potentially detrimental to hypoxic NSCLC cells upon its inhibition.

In conclusion, we have demonstrated a novel role for APP in moderating cell growth during G₀/G₁ transition. Disruption of APP signaling leads to necrotic cell death of hypoxic NSCLC cells. These findings have far-reaching implications in our understanding of hypoxic cancer cell biology and provide a novel therapeutic target for the treatment of NSCLC.
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VITA

Anna Sobol was born in Łomża, Poland to Agnieszka and Michal Zaniewscy. She is married to Lucjan Sobol and is blessed with a 1-year-old son Matthew.

While in Poland, Anna attended the University of Bialystok to pursue master’s degree in biology. Each year she was awarded the scholarship for her academic achievements. After three and a half years she moved to Chicago, where she continued her education. She received Bachelor of Science degree in Biology with Molecular Biology emphasis from Loyola University, Chicago. She graduated with the highest honors. During her undergraduate work she was a recipient of the NSF founded Research for Undergraduate Fellowship. She was investigating the Microsatellites in evolutionary studies of Pickerels under the guidance of Dr. Howard M. Laten. Passionate about research she decided to pursue a doctoral degree.

In August 2010 she joined the Molecular Biology Program at Loyola. She is completing her doctoral work under the mentorship of Dr. Maurizio Bocchetta. Her dissertation focuses on the role of Amyloid Precursor Protein in the regulation of growth and cell cycle progression of Non-small cell lung cancer cells. For the last two years she was also working on the role of deubiquitination
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Anna received the first place award at the oral presentation competition during the Department of Pathology Graduation (June, 2013) and first place award at the poster competition during the Molecular Biology retreat (September, 2014).

Upon completion of her graduate studies, she intends to pursue a research preferentially in the area of oncology.