2015

Pemetrexed, A Modulator of AMP-activated Kinase Signaling and an Inhibitor of Wild type and Mutant p53

Stuti Agarwal
Virginia Commonwealth University

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PEMETREXED, A MODULATOR OF AMP-ACTIVATED KINASE SIGNALING AND INHIBITOR OF TRANSACTIVATION OF WILD TYPE AND MUTANT p53

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

STUTI AGARWAL
Master of Science, Hamdard University, New Delhi, India, 2007

RICHARD G. MORAN, PH.D
Professor, Department of Pharmacology and Toxicology and the Massey Cancer Center

Virginia Commonwealth University
Richmond, Virginia,
April 2015
This dissertation is dedicated to my Grand mother Late. Mrs. Vimla Devi Agarwal, whom we lost in August 2014, in the battle against pancreatic cancer. I would also like to dedicate my work to my Grand father Late. Mr. Suresh Chand Agarwal and My Father Late. Mr. Shiv Kumar Agarwal.
ACKNOWLEDGEMENT

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My family have always been the driving force for my journey to set and achieve big goals in life and without their love, support and guidance I would have not accomplished a degree as prestigious as Ph.D. Knowing that they have and always be their for me in good and bad times, gives me strength to fight all the adversities come in the path of success and achievements. I want to thank my mother Mrs. Shikha Agarwal, sister Mrs. Smita Agarwal, brother Mr. Govind Agarwal, brother-in-laws Atul Kumar Agarwal and
Hitesh Kumar Agarwal and sister-in-law Sneha Agarwal, for all their support and love they have given me all these years and will continue to give. I also want to thank my In-Laws for their love and support.

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I love you and miss you Dad, especially when I fulfilled one of the dreams you always dreamt for me.
“Imagination is more important than knowledge. Knowledge is limited, imagination encircles the world.”

- Albert Einstein
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>4EBP1</td>
<td>eIF4E binding protein 1</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl CoA carboxylase</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole 4-carboxamide ribonucleoside</td>
</tr>
<tr>
<td>AICART/AICARFT</td>
<td>5-amino-4-imidazolecarboxamid ribonucleotide formyltransferase</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AK</td>
<td>adenosine kinase</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphocytic leukemia</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine-5'-monophosphate AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>Axl</td>
<td>Tyrosine kinase protein</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>=CH+</td>
<td>methenyl</td>
</tr>
<tr>
<td>=CH2</td>
<td>methylene</td>
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<tr>
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<td>methyl</td>
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</table>
CHO
formyl

DDATHF
5,10-dideaza-H4PteGlu, lometrexol

DeptoR
DEP-domain-containing mTOR-interacting protein

dFBS
dialyzed fetal bovine serum

DHFR
dihydrofolate reductase

DNA
deoxyribonucleic acid

dUMP
2'-deoxy-uridylate, uridylate

dUTP
2'-deoxyuridine-5-triphosphate

E1F4E
eukaryotic initiation factor 4E

eEF2
eukaryotic elongation factor 2

EGFR
Estrogen growth factor receptor

FBS
fetal bovine serum

FGAR
N-formylglycinamide ribonucleotide

fmol
femptomole

FPGS
folylpoly-γ-glutamate synthetase

FPLC
fast protein liquid chromatography

fZMP
5-formylaminoimidazole-4-carboxamide ribonucleotide

g
grams

GAR
Glycinamide ribonucleotide formyltransferase

GART/GARFT
glycinamide ribonucleotide formyltransferase

GOF
gain of function

H2PteGlu
dihydrofolate

H4PteGlu
5,6,7,8-tetrahydrofolate

HGPRT
hypoxanthine-guanine phosphoribosyltransferase

hTERT
human telomerase reverse transcriptase

HSP70
heat shock protein 70

Hx
hypoxanthine

IMP
inosine monophosphate
IMPCH  inosine monophosphate cyclohydrolase
IRS  insulin receptor substrate
kB  kilobase
KD  knockdown
kDa  kilodalton
M  molar
m  meters
MDR1  multiple drug resistance gene
MEF  mouse embryonic fibroblast
MFT  mitochondrial folate transporter
mg  milligram
ml  milliliter
mLST8  mammalian lethal with Sec12 protein 8
mM  millimolar
mmol  millimole
MPM  malignant pleural mesothelioma
MTHFR  5,10-methylene-H4PteGlun reductase
mTOR  mammalian target of rapamycin
MTX  methotrexate, 2-amino-10-methyl-folic acid
Mutp53  mutant p53
NFκB2  Nuclear factor kappa B 2
nM  nanomolar
nm  nanometers
nmol  nanomole
NSCLC  non-small cell lung cancer
PABA  para-aminobenzoic acid
PCNA  proliferating cell nuclear antigen
P53  tumor suppressor protein p53
<table>
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<tr>
<th>Acronym</th>
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<tbody>
<tr>
<td>P21</td>
<td>p53 transcriptional target of 21 kD</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCFT</td>
<td>proton-coupled folate transporter</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-OH kinase</td>
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<tr>
<td>Raptor</td>
<td>regulatory associated protein of mTOR</td>
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<td>RFC</td>
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<td>Rheb</td>
<td>Ras homolog enriched in brain</td>
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<td>Rictor</td>
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<td>receptor tyrosine kinase</td>
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<td>RTX</td>
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<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>SHMT</td>
<td>serine hydroxymethyltransferase</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>TdR</td>
<td>thymidine</td>
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<tr>
<td>TMP</td>
<td>thymidylate, thymidine-5-monophosphate</td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis complex</td>
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<tr>
<td>TSC1</td>
<td>hamartin</td>
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<td>Abbreviation</td>
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<tr>
<td>TSC2</td>
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<td>TTP</td>
<td>thymidine-5-triphosphate</td>
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<td>μg</td>
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<tr>
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New drug discoveries and new approaches towards diagnosis and treatment have improved cancer therapeutics remarkably. One of the most influential and effective discoveries in the field of cancer therapeutics was antimetabolites, such as the antifolates. The interest in antifolates increased as some of the antifolates showed responses in cancers, such as mesothelioma, leukemia, and breast cancers. When pemetrexed (PTX) was discovered, our laboratory had established that the primary mechanism of action of pemetrexed is to inhibit thymidylate
synthase (TS) (E. Taylor et al., 1992). Preclinical studies have shown that PTX has a broad range of antitumor activity in human and murine models of cancer (Adjei, 2000; Adjei, 2004; S. Chattopadhyay, Moran, & Goldman, 2007; Miller et al., 2000). Accordingly, in February 2004, the FDA issued first-line treatment approval for pemetrexed in malignant pleural mesothelioma and in 2008 for first line treatment for locally advanced or metastatic NSCLC (reviewed in (Rollins & Lindley, 2005). As an antifolate this level of therapeutic activity of PTX against lung cancers was surprising and atypical (Hazarika, White, Johnson, & Pazdur, 2004). This led us to the question whether the effects of pemetrexed on other folate-dependent targets could explain the clinical activity of the drug. Our lab showed that, in addition to inhibiting thymidylate synthase, PTX also inhibits aminoimidazolecarboxamide ribonucleotide formyltransferase (AICART), the second folate-dependent enzyme of de novo purine synthesis. Inhibition of AICART leads to massive accumulation of its substrate 5-amino-4-imidazolecarboxamide ribonucleotide (ZMP), causing activation of AMP-dependent kinase (AMPK), which ultimately leads to suppression of mTORC1 signaling, a central regulator of cell growth and proliferation. This secondary mechanism could explain the unusual activity of PTX against mesothelioma and lung cancers.

The large proportion of lung cancers are either null or mutant for p53 function. Therefore, this thesis focused on defining what the role of p53 is in the PTX-mediated AMPK activation and mTORC1 inhibition and how the loss of p53 affects mTORC1 signaling. These two questions proved to be interlinked. Chapter 2 investigates this relationship in detail. We found that, upon loss of p53, mTORC1 signaling is enhanced to a significant degree in colon carcinoma and lung cancer cell lines. Clearly, this observation required explanation. We found that the major factors responsible for these differences in mTORC1 activity upon loss of p53
were lower levels of two p53 target genes Tuberin (TSC2) and sestrin2. Immunoprecipitation studies of mTORC1 complexes from p53 wt and p53 null cells revealed quite interesting differences in the components of the mTORC1 complex. Immunoprecipitates from p53 null cells had higher levels of mTOR and lower levels of TSC2 and PRAS40 bound to raptor. This suggested that, in comparison to p53 competent cells, p53 null cells have more mTORC1 complex with enhanced activity due to decreased interaction of TSC2 and PRAS40, both of which are inhibitors of mTORC1. These observations explained the higher mTORC1 in p53 null cells and laid the foundation for determining the role of p53 in PTX-activated AMPK and mTORC1 inhibition.

In the experiments described in Chapter 3, we found that PTX-mediated AMPK activation inhibited mTORC1 regardless of the p53 status in colon carcinoma cells. This suggested that mTORC1 inhibition by PTX was either independent of p53 mediated negative regulation of mTORC1 or was somewhere bypassing it. Therefore, we compared the effects of PTX with the classic AMPK activator aminoimidazolecarboxamide ribonucleoside (AICAR). In spite of a common mechanism of AMPK activation, namely, expansion of cellular ZMP levels, signaling from AMPK activated by PTX or AICAR were quite different. PTX-activated AMPK phosphorylated the mTORC1 component Raptor but not tuberin (TSC2), whereas AICAR-activated AMPK phosphorylated both the targets. This differential behavior of two AMPK activators was due to differential behavior of p53 under these two treatments. Both, AICAR and PTX treatment led to increase in p53 levels but the p53 that accumulated after AICAR treatment was transcriptionally active while the p53 that accumulated after PTX treatment was not. Transcription of p53 targets, including TSC2 and sestrin2, was activated in AICAR- but not in PTX-treated cells. In the absence of p53 function, TSC2 was deficient and mTORC1 activity
enhanced, but Raptor phosphorylation by AMPK following PTX was robust and independent of both p53 and TSC2. Therefore we concluded that p53 deficiency suppresses TSC2 and upregulates mTORC1, but AMPK-phosphorylation of Raptor after pemetrexed treatment was sufficient to suppress mTORC1, even in TSC2 deficiency. This suggested pemetrexed as a drug for treatment of Tuberous Sclerosis, a genetic disease caused by functional inactivity of TSC1 or TSC2 due to point mutations in these genes.

Mutation of p53 is one of the most common genetic alterations in human cancers and tumors. Cancers that express mutant p53 tend to be more aggressive, resistant to chemotherapy and show worse prognosis then p53-null tumors (Elledge et al., 1993; Olivier et al., 2006). This tumor-promoting activity of mutant p53 has been correlated with acquired and novel transcriptional activities of mutant p53. It has been shown that mutp53 can activate the transcription of cell growth promoting genes, such as, NFκB2, PCNA, MDR1, Axl, EGFR, hTERT, and HSP70, which are not usually transcriptional targets of wt p53. Interestingly, we found that whereas DNA damaging drugs enhance the acquired oncogenic transcriptional activities of mutp53, PTX interferes with this transcription activation. We also found in Chapter 4 that PTX can limit or block the DNA damaging drug-mediated increment of transcriptional activation of mutp53. This suggests that blockade of transcriptional activation of mutp53 by pemetrexed may provide an additional therapeutic benefit in mutp53 bearing cancers.

As discussed in Chapter Three, although pemetrexed (with TdR) increases the levels of p53 and its binding to the promoter of its target gene, p21, this p53 is transcriptionally inactive. In order to understand the mechanism of the pemetrexed-mediated transcriptional defect of wt p53, we studied the PTX-mediated signaling towards ATM and ATR and their effects on their substrates Chk2 and Chk1, respectively. These studies suggested that the difference between
signaling under AICAR treatment and PTX treatment was that, unlike PTX, AICAR treatment was leading to DNA damage, followed by Chk2 phosphorylation at Thr68.

We found there were three major differences between AICAR and pemetrexed (+ TdR) mediated signaling: AICAR caused DNA damage, followed by ATM mediated phosphorylation of Chk2 at Thr68 and phosphorylation of p53 at Ser15 all of which lead to activation of p53 transcriptional activity, events which do not take place under PTX treatment. Studies aimed at understanding the effects of PTX on wt and mutp53 transcriptional activities are discussed in detail in Chapters Three and Four of this dissertation.

Overall, we concluded that PTX interferes with the transcription activity of wild type as well as gain-of-function mutant p53. The blockade of DNA damaging agent-mediated enhancement of mutp53 transcription activity by PTX, suggests the clinical relevance of PTX in carcinomas with mutp53. We suggest that this could be one of the contributing factors in the effects of PTX against human lung cancers.
Chapter 1

1 Overview and Introduction

1.1 STRUCTURAL, ABSORBATIVE AND TRANSPORT CHARACTERISTICS OF NATURALLY OCCURRING FOLATES

Folic acid (also known as folate, vitamin M, vitamin B9, vitamin Bc (or folacin), and pteroyl-L-glutamic acid) is a water soluble vitamin. Folates were first discovered in the early 1940's from yeast and liver extracts as a responsible factor for reversing macrocytic anemia in pregnant women (Hoffbrand & Weir, 2001). Folic acid was isolated and crystallized from spinach and its structure was determined (C. Baugh & Krumdieck, 1971). Folic acid is the most oxidized and stable form of the folates that can be utilized for cellular metabolism. Our current understanding of folates, their absorption at the cellular levels, metabolism and utilization at the molecular level is a result of a combined effort of basic and clinical research. Antifolates have been very useful tools in understanding these pathways. Three distinct moieties of folic acid are; (A) 2-amino-4-hydroxy-pteridine ring which is conjugated through a methylene group to (B) para-aminobenzoic acid (PABA), which forms a peptide linkage to (C) glutamic acid (Fig1-1). Tissue folates are commonly reduced forms of folic acid, which are formed due to its reduction to tetrahydro forms at the 5,6, 7,8 positions of pteridine ring.
The N5 or N10 nitrogen atoms of 5,6,7,8-tetrahydrofolate (H₄PteGlu) can be linked to methyl (CH₃), formyl (CHO), methenyl (=CH+), or methylene (=CH₂) groups. Additional glutamate residues are processively added to the γ-carboxyl tail by the enzyme folylpoly-γ-glutamate synthetase (FPGS), forming H₄PteGluₙ (Fig 1-1) (C. Baugh & Krumdieck, 1971; Tomsho, Moran, & Coward, 2008).

Humans obtain folates from dietary sources, including vegetables (particularly dark green leafy vegetables), fruits, fruit juices, nuts, beans, peas, dairy products, poultry, meat, eggs, seafood, grains and supplemental sources. Spinach, liver, yeast, asparagus, and brussels sprouts are among the foods with the highest levels of folates. Although the human body can synthesize all the components of a folate molecule individually, but it cannot produce two enzymes associated with folate de novo synthesis, present in microorganism. Microorganisms have dihydropteroate synthase, which conjugates the pteridine and a PABA ring to make dihydropteroic acid, and dihydrofolate synthetase, an activity of bacterial FPGS. A lack of dietary folates can lead to folate deficiency. A normal individual can store up to 500-20,000µg of folate; therefore, even with a complete lack of folates in the diet, it can take months before the signs of folate deficiency appear. This deficiency can result in many health problems, such as megaloblastic anemia and neural birth defect in developing embryos. Other folate deficiency related health problems include pregnancy complications, mental confusion, forgetfulness or other cognitive deficits, mental depression, sore or swollen tongue, peptic or mouth ulcers, headaches, heart palpitations, irritability, and behavioral disorders. The human body needs folate to synthesize DNA, repair DNA, and methylate DNA (C. Baugh & Krumdieck, 1971). It is especially important in aiding rapid cell division and growth such as in infancy and pregnancy, to produce healthy red blood and prevent anemia (Tomsho et al., 2008). In the US, grains, such as wheat flour, all purpose
flour, bread, juice, breakfast cereals etc., are fortified with folic acid to complete the daily requirement of the folic acid in human body; the concept of fortifying food with folic acid is now being accepted all around the world, including developing countries like China and India.

1.1.1 Intracellular uptake and compartmentalization of folate

The most abundant natural forms of folate found in the diet are polyglutamated derivatives of 5-CH3-H4PteGlu and 10-CHO-H4PteGlu (Fig1-1). As polyglutamation limits the transport of folates across membranes; nature has provided a group of enzymes called γ-glutamyl carboxypeptidases, which are located in the brush-border of the proximal jejunum. These enzymes hydrolyze 5-CH3-H4PteGlu polyglutamates to monoglutamates, that are substrate for transport (Halsted, 0321). Due to the hydrophilic nature of the charged folate molecule, its passive diffusion across cell membrane is minimal. The efficient transport of folates across membranes, its intestinal absorption and transport to systemic tissue involves four classes of transporters:

- The Reduced folate carrier (RFC)
- Folate receptors FRα and FRβ
- The proton-coupled folate transporter (PCFT)
- Folate transport mediated by ATP-binding cassette transport proteins and members of the SLC21 and SLC22 families of solute carrier

Reduced folate carrier (RFC)-The RFC (SLC19A1) was the first folate transporter studied at the kinetic, thermodynamic and molecular levels. The human RFC (hRFC) encoding gene is located on chromosome 21q22.3 (I. D. Goldman, Lichtenstein, & Oliverio, 1968; I. Goldman & Matherly, 1985; Matherly & Goldman, 2003; Sirotnak & Tolner, 1999). At neutral pH, the
reduced folate carrier (RFC) facilitates the transport of systemic 5-CH3-H4PteGlu, the principal folate found in mammalian serum, into cells. (G. I. & Oliverio, 1122; I. D. Goldman et al., 1968; Matherly, Seither, & Goldman, 1987; Matherly et al., 2007; Matherly, Hou, & Deng, 2007) (Fig1-2). Folates are negatively charged due to the two carboxyl groups in the glutamate side chain that are fully ionized at physiological pH. In order to facilitate transport via the RFC, there must be a substantial electrochemical potential difference for folates across the cell membranes (G. I. FAU et al., 1122). RFC mediated transport is highly sensitive to the transmembrane anion gradient, especially the organic phosphate gradient. This provides a driving force for RFC-mediated uphill transport to folates into cell (I. D. Goldman, 1971; Henderson & Zevely, 1983) (Fig. 1-3). The low affinity of the RFC for folic acid and its neutral pH optimum clearly distinguishes it from the PCFT. However, both RFC (Liu et al., 2005) and PCFT (Qiu et al., 2007) expression are markedly increased in the small intestine of mice when they are fed a folate deficient diet. The regulatory mechanism lying under this response is not well understood.

**Folate receptors, FRα, β and γ**- FRs, are encoded by three distinct genes designated α, β, and γ, all located on chromosome 11, are very high affinity folate binding proteins (Lu & Low, 2002; Salazar & Ratnam, 2007). These three FRs are homologous proteins (68–79% identical amino acid sequences) but show differential expression in different tissues. FRα is expressed in epithelial cells of the kidney, choroid plexus, retina, uterus, and placenta (Parker et al., 2005; Salazar & Ratnam, 2007). FRβ is expressed during normal myelopoiesis and is present in placenta, spleen, thymus, and in CD34+ monocytes (Ratnam, Marquardt, Duhring, & Freisheim, 1998; Reddy et al., 1999; Ross et al., 1989; H. Wang, Zheng, Behm, & Ratnam, 2000). The FRs have high affinity for folic acid (Kd 1–10 nM). FR-mediated folate internalization involves receptor mediated endocytosis (B. G. FAU, FAU, FAU, & Moran, 0207; Kamen, Wang,
Streckfuss, Peryea, & Anderson, 1998; Lu & Low, 1217; Ross et al., 1989).

(Adapted from PhD. Dissertation of Scott Rothbart, 2010)

Figure 1-1 Chemical structure of Folic acid and Tetrahydrofolate.

(Adapted from PhD. Dissertation of Scott Rothbart, 2010)
Figure 1-2. Schema of folate metabolism and folate-dependent reactions that occur in the cytosolic subcellular compartment of cells.

Enzyme abbreviations are AICART – aminooimidazole carboxamide ribonucleotide formyltransferase; cSHMT – cytosolic serine hydroxymethyltransferase isoform; DHFR – dihydrofolate reductase; GART – glycaminde ribonucleotide formyltransferase; TS – thymidylate synthase. Single arrows representing the direction of the reaction show irreversible reactions and reversible reactions are represented by double arrow.
When folate molecules bind to the FR on the cell surface, it causes invagination of the plasma membrane surface at that site, leading to formation of a vesicle (endosome). This endosome migrates to the cytoplasm, where it is acidified to a pH of ~ 6.5, resulting in dissociation of the folate from the FR complex (Yang, Chen, Vlahov, Cheng, & Low, 2007). The folate ligand is exported into the cytoplasm (Kamen, Peryea & Anderson, 1998; Rothberg, Ying, Kolhouse, Kamen, & Anderson, 1990) (Fig. 1-3). While FRs α and β can transport folate into cells, this is inefficient compared to transporters such as RFC (Sierra, Brigle, Spinella, & Goldman, 1995; Spinella, Brigle, Sierra, & Goldman, 1995).

ii) Proton-coupled folate transporter (PCFT)- The proton-coupled folate transporter (PCFT) is used to transport folate monoglutamates (including folic acid) across the apical surface of the proximal jejunum. PCFT symports folates optimally at pH 5.5 against its concentration gradient with protons along their concentration gradient into the enterocytes, explaining why the acidic microenvironment of the small intestine is favorable for PCFT-mediated folate transport (Qiu et al., 2006; Zhao & Goldman, 2007) (Fig. 1-3).

Folate transport mediated by ATP-binding cassette transport proteins and members of the SLC21 and SLC22 families of solute carrier- It has been suggested that there are other folate transport routes other than these highly specific transporters. The multidrug resistance-associated proteins MRP1-5 (ABCC1-ABCC5) and the breast cancer resistant proteins BCRP, (ABCG2) are relevant ATP-binding cassette exporters (Assaraf, 2006; Kruh & Belinsky, 2003; Wielinga et al., 2005)(Fig. 1-3). These are low affinity, high capacity transporters (Kms ~0.2 – 2 mM for folates/antifolates). Members of this family are widely expressed in mammalian cells and suppress the level of free folates or antifolates that accumulate in most cells grown in vitro (Fry, Yalowich, & Goldman, 1982).
Abbreviation of various transporters are as follows; PCFT= Proton-coupled folate transporters, RFC= Reduced folate carrier, FR= Folate receptors, MRP's and BCRP= multidrug resistance proteins and breast cancer resistant
Some of the shorter chain-length polyglutamate folates may be weak substrates for MRPs (Fry et al., 1982). The physiological importance of MRP2 in folate export is demonstrated by impaired biliary secretion of MTX in MRP2 (-/-) mice (Masuda et al., 1997).

In summary, it is during intestinal uptake that folic acid from fortified foods is reduced to 5-CH₃-H₄PteGlu. 5-CH₃-H₄PteGlu, which is absorbed in small intestine enters the portal circulatory system and is first delivered to the liver, where it can be passed through to the systemic circulation, can be secreted into the bile for reabsorption, or can be polyglutamated for storage. Systemic 5-CH₃-H₄PteGlu can be transported into cells by folate receptor-mediated endocytosis, a high affinity and low capacity process. The detailed discussion of this process is as follows.

### 1.1.2 Folate absorption in intestine

During intestinal absorption, polyglutamated folates are converted into monoglutamates and are transported across the apical brush border membrane of the proximal jejunum mediated by the PCFT. As PCFT transport is very efficient, concentrative and driven by a transmembrane proton gradient, the amount of folate transported to enterocytes is high enough to facilitate folate efflux across the basolateral membrane into the periserosal space and then enter the vascular system. The mechanism of folate export from enterocyte is unclear as neither PCFT not RFC are expressed at the basolateral membrane. However, MRPs, particularly MRP3 (Kruh & Belinsky, 2003) are expressed at this site and may be involved in the export of folates through this route.

After intestinal absorption, folates enter the hepatic portal system and are delivered to hepatic sinusoids. Once folates are in the liver, they have three potential destinations. i) Folate can be converted to polyglutamate storage forms mediated by FPGS; ii) they can be secreted in the bile at the hepatic canalicuar membrane, by MRP2 mediated process (Masuda et al., 1997), return to
the deudenum and jejunum for reabsorption and thus completing the cycle of enterohepatic circulation or iii) they can stay as monoglutamates or get converted into monoglutamates from stored polyglutamates in hepatocytes, and delivered directly from the hepatic portal vein, ultimately reaching the systemic circulation where they accumulate and participate in the processes of the one-carbon requirement of peripheral tissue.

1.1.3 Transport into systemic tissues

At neutral pH 7.4, RFC mediate the membrane transport of folate into systemic tissue. Although PCFT is co-expressed with the RFC in many tissues at the plasma membrane, due to the neutral pH at these sites, the function of the PCFT is minimal at these sites.

1.1.4 Polyglutamation and storage of folate

Polyglutamation of folates is attributed to the enzyme folylpoly-γ-glutamate synthetase (FPGS) (Moran, Werkheiser, & Zakrzewski, 1976). Polyglutamation is not only essential to increase the stability and retention of folates within the cell, but also to increase the affinity of folates for their target enzymes. Humans have two isoform of FPGS, cytosolic and mitochondrial, coded from same gene. These two isoforms help in maintaining the equal distribution of folate polyglutamate between the cytosol and the mitochondria (Cook & Blair, 1979; Freemantle, Taylor, Krystal, & Moran, 1995; S. M. Taylor, Freemantle, & Moran, 1995). Folates are transported into the mitochondria by the mitochondrial folate transporter (MFT), a family member of the inner mitochondrial membrane transport carriers that was cloned and characterized by our lab (McCarthy, Titus, Taylor, Jackson-Cook, & Moran, 2004; Perchiniak et al., 2007; Titus & Moran, 2000). Folate monoglutamates are the substrates for mitochondrial transport, suggesting substrate competition between cytosolic FPGS and the MFT (Freemantle et
al., 1995; Perchiniak et al., 2007). Chinese hamster ovary (CHO) cells that lack functional FPGS, known as AuxB1 cells, are unable to accumulate any cellular folates and therefore require an exogenous supply of purines, thymidine and glycine to the growth culture medium (T. R. FAU & Hanna, 0812; McBurney & Whitmore, 1974). These studies suggested that the purpose of mammalian folate metabolism is to produce purines, thymidine and glycine.

1.1.5 Intracellular folate metabolism

Folate metabolism takes place in both cytosolic and mitochondrial compartments and allow the recycling of folate molecules. Although, in the mammalian cells all the enzymes required to synthesize purines, thymidine, methionine and glycine are present in the cytosol. Cytosolic folate metabolism contributes to and is limited to purine, thymidine, and methionine synthesis. A study showed that the cytosolic isoform of FPGS when transfected into AUXB1 cells, relived the exogenous requirement of the purine and thymidine but not of glycine (Garrow et al., 1993; Lin, Huang, & Shane, 1993). It was found that mitochondrial folate metabolism fulfills the requirement of glycine supply to the cells (McCarthy et al., 2004) and cytosolic folate metabolism is essential for purine and pyrimidine synthesis. Synthesis of purines, thymidine and methionine is done in the cytosol using folate molecules as cofactors (Fig. 1-3). First, 5-methyl-tetrahydrofolate and homocysteine are converted into tetrahydrofolate and methionine. The tetrahydrofolate then can be converted to either 10-formyl-tetrahydrofolate or 5,10-methylene-tetrahydrofolate. The conversion of tetrahydrofolate (H₄PteGluₙ) to 10-formyl tetrahydrofolate consumes a formate molecule. Conversion of tetrahydrofolate to 5,10-methylene-tetrahydrofolate is done by the cytosolic serine hydroxymethyltransferase isoform (cSHMT). The two folate-dependent enzymes of purine synthesis, glycaminamide ribonucleotide formyltransferase (GART) and aminoimidazole carboxamide ribonucleotide formyltransferase (AICARFT), use the formyl
carbon of 10-formyl-tetrahydrofolate. 5,10-methylene-tetrahydrofolate can be converted either to 5-methyl-tetrahydrofolate or to dihydrofolate \((H_2PteGlu)\). Thymidylate synthase (TS) uses 5,10-methylene-tetrahydrofolate and deoxyuridylate to produce thymidylate and dihydrofolate. The dihydrofolate produced during thymidylate synthesis is then converted to tetrahydrofolate by dihydrofolate reductase (DHFR) (Fig. 1-3).

Mitochondrial folate metabolism is also important for glycine synthesis (Fig. 1-4). Cells keep separate pools for cytosolic and mitochondrial folate reactions (Appling, 1991). All the molecules required for folate metabolism such as glycine, serine and formate appears to get exchanged between the mitochondrial and cytosolic compartments (Pasternack, Laude, & Appling, 1994). In mitochondria, folates are utilized as cofactors to metabolize glycine. Conversion of serine and tetrahydrofolate into glycine and 5,10-methylene-tetrahydrofolate is mainly done by mitochondrial isoform of serine hydroxymethyltransferase. CHO-derived GlyA cells, that lack activity of the mSHMT isoform, but have an active cytosolic isoform, are auxotrophic for glycine (Kao, Chasin, & Puck, 1969). Later it was shown that GlyA cells have increased amounts of intracellular serine and decreased amounts of glycine when compared to wild-type CHO cells (Narkewicz, Sauls, Tjoa, Teng, & Fennessey, 1996). For a better regulation of glycine metabolism, mitochondria also contain a unique system known as glycine cleavage system (GCS). The GCS catabolizes glycine into formate, ammonia, and 5,10-methylene-tetrahydrofolate and NADH+ by using tetrahydrofolate and NAD (Kikuchi, Motokawa, Yoshida, & Hiraga, 1114).
Figure 1-4. Schematic of folate metabolism and folate-dependent reactions that occur in the mitochondrial subcellular compartment.

Enzyme abbreviations are GCS – glycine cleavage system; mSHMT – mitochondrial serine hydroxymethyltransferase isoform. Single arrows represent the direction of irreversible reactions and double arrows represent reversible reactions. The two reversible reactions catalyzed by mSHMT and GCS, converting tetrahydrofolate to 5,10-methylene-tetrahydrofolate, do not occur simultaneously; they are separate reactions that use the same molecules in different reactions with different enzymes.
In an in-silico study, mathematical models were generated to stimulate hepatic folate metabolism and suggested that both the mSHMT and GCS reactions are reversible, but are predicted to run in the direction of tetrahydrofolate to 5,10-methylene-tetrahydrofolate which ultimately is to generate 10-formyl-tetrahydrofolate (Nijhout et al., 2006). Although, both the reactions, by mSHMT and GCS, generate formate, they oppose each other in glycine regulation. While catabolizing glycine, the GCS directly generates formate. The 5,10-methylene-tetrahydrofolate converts into 10-formyl-tetrahydrofolate in two steps, and 10-formyl-tetrahydrofolate can be metabolized into formate and tetrahydrofolate. Later, it was shown that the mitochondrial folate metabolism is very important in yeast, especially in term of formate generation, as ~25% of the carbon units used in purine synthesis were from mitochondrially-derived formate (Pasternack et al., 1994). *In-silico* studies done by Pastenack et al. showed that in the absence of mitochondrial folate metabolism in *in-silico* mathematical models, thymidine and purine synthesis were reduced by ~40% and 60% respectively, and that other cytosolic folate-dependent processes were relatively unaffected (Pasternack et al., 1994). Thus, the role of mitochondrial folate metabolism is to produce glycine that can be metabolized into formate for the use in cytosolic synthesis of purines and thymidine.

### 1.1.6 Development of Cancer chemotherapy

The origin of effective cancer chemotherapy goes back to World War I. It began in the 1940's with the use of mustard gas by the German army as the first introduction of chemical warfare. Soldiers exposed to mustard gas showed dramatic symptoms like atrophy of lymphoid and testicular tissue and died within 2-3 weeks due to hypoplasia of the bone marrow (Krumbhaar & Krumbhaar, 1919). Later, Berendulum et. al showed the anticancer properties of mustard gas (BERENBLUM & SCHOENTAL, 1947). Studies on the effects of mustard gas on biological
systems led to an understanding of its selective toxicity towards proliferating cells (Gilman & Philips, 1946). On animal models, the most striking benefit of mustard gas was noted in chronic myelogenous leukemia, although quite significant responses was observed in Hodgkin's disease (cross ref. (GILMAN, 1963; Papac, 2001). Studies done by Gilman and other groups using mustard gas suggested that systemic administration of cytotoxic drugs could be a possible therapeutics for cancer.

1.1.6.1 Discovery and development of antifolates as cancer therapeutics

The origin of effective cancer chemotherapy goes back to World War I. It began in the 1940's with the use of mustard gas by the German army as the first introduction of chemical warfare. Soldiers exposed to mustard gas showed dramatic symptoms like atrophy of lymphoid and testicular tissue and died within 2-3 weeks due to hypoplasia of the bone marrow (Krumbhaar & Krumbhaar, 1919). Later, Berendulum et. al showed the anticancer properties of mustard gas (BERENBLUM & SCHOENTAL, 1947). Studies on the effects of mustard gas on biological systems led to an understanding of its selective toxicity towards proliferating cells (Gilman & Philips, 1946). On animal models, the most striking benefit of mustard gas was noted in chronic myelogenous leukemia, although quite significant responses was observed in Hodgkin's disease (cross ref. (GILMAN, 1963; Papac, 2001). Studies done by Gilman and other groups using mustard gas suggested that systemic administration of cytotoxic drugs could be a possible therapeutics for cancer.

1.1.6.2 Discovery and development of antifolates as cancer therapeutics

The discovery of antifolates led to a great advancement in the cancer chemotherapy. Syndey Farber showed that administration of folic acid to children suffering from acute lymphocytic
leukemia (ALL) stimulated the proliferation of cancer cells and exacerbated the disease (FARBER & DIAMOND, 1948). Further, Farber and colleagues demonstrated that the antifolate aminopterin (Fig.1-5) could induce remission of pediatric acute leukemia (FARBER & DIAMOND, 1948). Initially, it was thought that the mechanism of action of antifolate specifically targeted the fast dividing cells and thus supported the concept of targeting therapy to inhibit cell division. These effects of antifolates on cancer led to the modern era of antimetabolite cancer drugs starting in the late 1940's.

Antimetabolites such as aminopterin, methotrexate, pemetrexed, hydroxyurea, or N-(phosphonacetyl)-L-aspartic acid (PALA) interfer with DNA and RNA production and therefore cell division and the growth of tumors. Thus, antimetabolite therapy is very effective for some cancer cells. Aminopterin, an antifolate (Fig. 1-4), was first synthesized by Dr. Yellapragada Subbarow et al, and was subsequently was first used by Sidney Farber in 1947 to induce remission among children (FARBER & DIAMOND, 1948). Aminopterin was used in the United States from 1953 to 1964 for the indication of pediatric leukemia. Later, aminopterin was replaced by methotrexate due to manufacturing difficulties and to a better therapeutic index of methotrexate in a rodent tumor model (GOLDIN et al., 1955). In a study in 2005, Cole et. al suggested that aminopterin has greater cellular accumulation, metabolism and more reliable bioavailability than methotrexate. They concluded that considering the tolerable toxicity at the recommended dose and schedule, aminopterin deserves further study as an alternative to methotrexate. (Cole et al., 2005). In the 1950’s, several more antimetabolites were discovered such as the purine analog 6-mercaptopurine (Hitchings and Elion, 1954), which was shown to have anti-cancer activity in mice with acute leukemia (Hitchings and Elion, 1954; Skipper, Thomson, Elion and Hitchings,1954).
5-Florouracil (5-FU), a pyrimidine analog, is a suicide inhibitor (mechanism based irreversible inhibitor) for thymidylate synthase. It is one of the oldest chemotherapeutic drugs which is still being used in the treatment of several human cancers including colorectal, breast, stomach and pancreatic cancers, suggesting that “cytotoxic” chemotherapy is still one of the most useful and prevalent method of cancer treatment.

Due to dramatic results against various cancers, the efforts toward making new and better antifolates continued. Several of these agents have been rationally designed to target specific folate dependent enzymes or circumvent known mechanisms of resistance to classical antifolates. One of the most extensively studied and widely used antifolate is methotrexate (Fig. 1-5). Shortly after the discovery of methotrexate and its analogs, the primary folate-dependent therapeutic target of this drug was identified to be DHFR (Werkheiser, 1963) (Fig. 1-3, 1-6). Methotrexate is a tight-binding inhibitor of DHFR (Ki ~ 0.004 nM) (C. Shih, Habeck, Mendelsohn, Chen, & Schultz, 1998). DHFR inhibition by methotrexate prevents DNA and RNA synthesis by preventing the reduction of $H_2PteGlu_n$ to $H_4PteGlu_n$, the vital precursor for thymidylate and purine biosynthesis cofactors (Fig. 1-3 and 1-7). Additional $H_4PteGlu_n$-dependent reactions, such as serine-glycine interconversions and methionine synthesis, are also hindered. It is now known that, like natural folates, methotrexate is polyglutamated in cells (C. M. Baugh, Krumdieck, & Nair, 1973). This is important, as polyglutamation of methotrexate enhances intracellular retention of the drug and permits methotrexate to inhibit DHFR for longer periods of time, expanding the $H_2PteGlu_n$ pool (Rosenblatt et al., 1978). Polyglutamation also broadens the spectrum of target inhibition. Significant to cancer therapy, polyglutamation adds a layer of selectivity to methotrexate, as metabolites accumulate in tumor cells to a much greater extent than in bone marrow and intestinal tissues (Poser, Sirotnak, & Chello, 1981). Various
analogs of methotrexate were synthesized and tested as cancer therapeutics with the goal of maintaining the potency against DHFR while enhancing the substrate specificity of transport and polyglutamation, and limiting the uptake in normal tissues. Most attempts failed, as methotrexate was indeed a superior drug in this sense. However, the analog pralatrexate (10-propargyl-10-deazaaminopterin, Folotyn®) has been shown to meet these criteria and showed remarkable responses in some T-cell lymphomas. That study (Thompson, 2009) ultimately led to the U.S. Food and Drug Administration (FDA) approval of pralatrexate for relapsed peripheral T-cell lymphoma, a rare form of non-Hodgkins lymphoma, in 2009 (Thompson, 2009). Other rigorously studied antifolate that has proven itself to be promising, is Pemetrexed. The most sensitive cellular target of pemetrexed is thymidylate synthase.
Figure 1-5. Chemical structures of; A) aminopterin, the first antifolate. B) methotrexate
1.1.6.3 Second-generation antifolates targeting thymidylate synthase and purine synthesis pathway

In 1980s and 1990s, the efforts towards antifolate drug discovery has been shifted away from targeting DHFR, focusing on pharmacophores targeting the folate-dependent enzymes of thymidylate and purine biosynthesis. The first potent antifolate thymidylate synthase inhibitor (Ki ~ 3 nM) to come from this effort was CB3717 (Jones et al., 1981). CB3717 (Fig. 1-8) had antitumor activity in breast, ovarian, and liver cancers, but was ultimately withdrawn from the clinic due to life-threatening renal toxicity caused by poor solubility at the low pH of urine in the collecting ducts (Calvert et al., 1986; Jackman et al., 1991). Keeping this same 5,8-dideazafolate pharmacophore, analogs of CB3717 were synthesized in an attempt to increase solubility while maintaining specificity for thymdylate synthase. Ultimately, Raltitrexed (Tomudex®, ZD1694) was identified from this effort. Raltitrexed is a 2-desamino-2-methyl-N10-substituted-5,8-dideazafolate analog with a thiophene substitution for the PABA ring (Fig. 1-8) (Jackman et al., 1991). Compared to CB3717, these characteristics not only increased solubility, they made raltitrexed a superior substrate for both RFC-mediated transport and polyglutamation by FPGS (Jackman et al., 1991).

In addition to intracellular trapping of raltitrexed metabolites, polyglutamation increases the potency of raltitrexed as a TS inhibitor by more than 100-fold. Raltitrexed showed significant clinical response rates in colorectal and breast cancer patients. It is currently in widespread use outside the United States for the treatment of colorectal cancer, but never gained U.S. FDA approval, as it was not determined to be superior to the current standard of care for colorectal cancer, 5-fluorouracil (5-FU, also a TS inhibitor) with leucovorin, a reduced folic acid (Maughan et al., 2002; Popov et al., 2008).
The discovery of the first antifolate inhibitor of *de novo* purine biosynthesis, 5,10- dideaza-H4PteGlu (DDATHF, lometrexol) was serendipitous (Figure 1-8). The structure was originally proposed by G. Peter Beardsley as a potential TS inhibitor, but cell culture end-product reversal experiments performed in our lab showed it was targeting purine synthesis (Moran, Baldwin, Taylor, & Shih, 1989). Subsequently, enzyme kinetic studies demonstrated that DDATHF was a potent inhibitor of GART, the first folate-dependent enzyme of *de novo* purine synthesis (Baldwin et al., 1991; Moran et al., 1989; Sanghani & Moran, 1997) (Fig. 1-3 and 1-7). DDATHF is transported into cells via the RFC as well as the PCFT (Beardsley et al., 1989). DDATHF showed potent antitumor activity against a broad spectrum of carcinomas, but the development of DDATHF was halted in Phase I clinical testing due to induction of severe thrombocytopenia (Ray et al., 1993). Oral supplementation with folic acid ablated the unfavorable toxicity of lometrexol (DDATHF) (Alati et al., 1996; Roberts et al., 2000). These findings set a precedent for future clinical regimens to include folic acid and vitamin B12, although the protective mechanism is still not fully understood. Lometrexol posed a problem; it was made by a very complex 23 step process, and the product was mixture of diastereomers about carbon 6. The diastereomers needed to be separated before clinical trails, to meet FDA administration requirements. The fractional crystallization approach was not efficient and proved to have low yield. This led to the evaluation of alternative approaches to avoiding the preparation of diastereomeric mixtures. One strategy was to replace the 5-deazapteridine ring of lometrexol with the pyrrolopyrimidine ring, which removed the chiral center of lometrexol and resulted in the compound LY231514, which became known as pemetrexed (E. Taylor et al., 1992).
Figure 1-6. Human DHFR with bound dihydrofolate and NADPH.

Adapted from http://en.wikipedia.org/wiki/dihydrofolate_reductase
In the beginning of the concept of the antimetabolite for cancer therapy started with targeting DHFR and later it shifted towards TS. Multitargeted antifolate Pemtrexed also targets AICART. Adapted from Muhsin M et al., 2004. Nat. Rev. Drug Disc. 3(10):825-826 { Muhsin,M. et al, 2004; }
1.1.6.4 Pemetrexed: a multi-targeted antifolate

Pemetrexed (L-glutamic acid, N-(4-(2-(2amino-4,7- dihydro-4-oxo-1H-pyrrolo(2,3-d) pyrimidin-5-yl)ethyl)benzoyl)) is arguably the most interesting of the new generation antifolates (Fig.1-8, 1-9). Although an analogue of folic acid, structurally and chemically it can be differentiated from MTX and DDATHF, as it has a 6-5 fused pyrrolo (2,3-d) pyrimidine nucleus (E. Taylor et al., 1992). Pemetrexed (LY231514, Alimta) was discovered from synthetic approaches aimed at eliminating the chirality of carbon 6 of DDATHF, in which a pyrrolopyrimidine ring replaced the 5-deazapteridine (E. Taylor et al., 1992) Surprisingly, this modification also changed the target profile. Pemetrexed polyglutamates were potent inhibitors of thymidylate synthase both in vitro (Ki ~ 1.3 nM) and in cell culture {; 234 Taylor 1228;}. Pemetrexed was reported to have effects on multiple enzymes involved in the folate metabolism, but the importance of some of these steps is questionable. End-product cell culture reversal experiments suggested that higher doses of pemetrexed had a significant secondary target, reversible with the addition of preformed purine (C. Shih et al., 1997; E. Taylor et al., 1992) This suggested that, like its predecessor DDATHF, pemetrexed was also targeting de novo purine synthesis. As structural analogues of folic acid, antimetabolites also use the same transporters (Westerhof et al., 1995). Pemetrexed enters the cells using the reduced folate carrier (RFC), a bidirectional transporter and major cellular transport system for folates (Zhao, Babani, Gao, Liu, & Goldman, 2000). Pemetrexed also uses folate receptor- α, a cellular membrane receptor. A low pH transporter, the PCFT, is also involved in pemetrexed internalization (S. Chattopadhyay, Wang, Zhao, & Goldman, 2004; Sierra & Goldman, 1998; Y. Wang et al., 2004; Y. Wang, Zhao, & Goldman, 2004; Zhao et al., 2000). As the microenvironment surrounding carcinoma cells is acidic due to secretion of lactic acid as a byproduct of anaerobic respiration, it has been suggested that the PCFT is, in fact, the
primary transporter of pemetrexed in solid tumors. Pemetrexed is one of the most efficient substrate of FPGS ever tested. Pemetrexed is polyglutamated 90 to 195 times more efficiently than methotrexate and 6 to 13 times more efficiently than lometrexol (Habeck et al., 1995). As pharmacological activity is achieved by polyglutamation, higher levels of glutamation not only increase retention of the pemetrexed inside the cell but also increase its specificity for its targets enzymes. Pentaglutamated pemetrexed has a Ki of 1.3 nm for its primary target TS in comparison to monoglutamated pemetrexed, which has Ki of 109 nM (I. D. Goldman & Zhao, 2002; Schultz, Patel, Worzalla, & Shih, 1999; C. Shih et al., 1997). Pentaglutamated pemetrexed is a potent inhibitor of TS, which catalyzes the transformation of deoxyuridine monophosphate to deoxothyimidine monophosphate (dTMP). Inhibition of TS decreases formation of dTMP, a progenitor of the nucleoside deoxothyimidine triphosphate (dTTP) needed for the DNA synthesis. The enzyme deoxycytidine deaminase is negatively regulated by dTTP. (I. D. Goldman & Zhao, 2002; Rustum et al., 1997; Schultz et al., 1999; Westerhof et al., 1995). As polyglutamated pemetrexed is also an inhibitor of GART and AICART, inhibition of these enzymes leads to inhibition of de novo purine synthesis. The DHFR-binding of pemetrexed is 1000 times less avid than methotrexate, and inhibition of DHFR by pemetrexed is reportedly minimal (I. D. Goldman & Zhao, 2002; C. Shih et al., 1997; Zhao & Goldman, 2007; Zhao et al., 2008)

1.1.6.4.1 De novo purine synthesis

Actively dividing cells require continuous availability of purine nucleotides due to active replication and transcription. This demand is mainly fulfilled by synthesis of purines in a de novo process known as de novo purine synthesis. The two parent nucleotides of purines in nucleic acids are adenosine monophosphate (AMP) and guanosine monophosphate (GMP). The de novo purine synthesis is an energy-consuming process consisting of 10 sequential enzymatic reactions
The whole purpose of this biosynthetic pathway is to build an end product, inosine monophosphate (IMP) from a 5-carbon molecule PRPP. Cells can also make IMP from the breakdown of nucleic acid using salvage pathways. Addition of preformed purines like hypoxanthine or inosine, can rescue cells from de novo purine synthesis inhibition or deficiency.

Hypoxanthine can be converted into IMP by hypoxanthine-guanine phosphoribosyl transferase (HGPRT). De novo purine synthesis is subjected to feedback inhibition by the end products of the pathway. The first and committed step of de novo purine synthesis is catalyzed by phosphoribosylpyrophosphate amidotransferase (PPAT). In this step, an amino group is donated by glutamine is attached at C-1 of PRPP, resulting into 5-phosphoribosylamine, a highly unstable intermediate with a half-life of 30 sec at pH 7.5. The activity of PPAT is inhibited by purine nucleotides IMP, AMP, GMP, and ATP (WYNGAARDEN & ASHTON, 1959). Out of ten reactions, two reactions of this pathway are dependent on the folate cofactor 10-CHO-H₄PteGlu (HARTMAN & BUCHANAN, 1959). The third step of de novo purine synthesis and the first folate dependent step of this pathway, is catalyzed by Glycinamide ribonucleotide Pemetrexed has been tested in and showed dramatic effects against various carcinomas including bladder, breast, cervix, colon, gastrointestinal tract, and pancreas (Adjei, 2000; Adjei, 2004; Britten et al., 1999). In the past, typical antifolates, especially TS inhibitors, have been ineffective against NSCLC but pemetrexed has been proven to have strong therapeutic effects against these diseases.
Figure 1-8. Chemical structure of inhibitors representing various classes of inhibitors of folate metabolism
Figure 1-9. Mechanism of action of pemetrexed.

THF= tetrahydrofolate; DHF= dihydrofolate; DHFR= DHF reductase; PRPP= phosphoribosyl pyrpp phosphate; GAR= glycaminide ribonucleotide; fGAR= N-formylglycinamide ribonucleotide; AICAR = 5-aminoimidazole-4-carboxamidine ribonucleotide; fAICAR= 5-formylaminomidazole-4-carboxamide ribonucleotide; IMP = inosine monophosphate
Figure 1-10. Folate dependent steps of de novo purine synthesis pathway.

De novo purine biosynthesis consists of ten sequential enzymatic reactions in which phosphoribosyl-1-pyrophosphate (PRPP) is converted to inosine monophosphate (IMP). IMP is converted to AMP and GMP thorough additional enzymatic steps (not shown). The two folate- dependent formyl transfer reactions of this pathway are catalyzed by GART and AICAR. AICAR, after entering mammalian cells, gets converted into ZMP, leading reaction in forward direction thus feeds into the reaction. PTX inhibits AICART, causing its substrate ZMP to accumulate. Additionally, IMP can be produced by salvage of the preformed purine, hypoxanthine, by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and the cofactor PRPP. Adapted from the Racanelli AC. et al., Cancer Res. 2009 Jul 1;69(13):5467-74.
Also pemetrexed is very effective against malignant pleural mesothelioma (MPM), a highly aggressive malignancy of the pleural cavities lining the lungs and chest usually associated with the exposure of asbestos fibers (Craighead et al., 1982). The median survival following diagnosis of MPM is approximately 11 months. This disease is resistant to most of the chemotherapeutic regimens including methotrexate and 5-FU with leucovorin (Harvey, Slevin, Ponder, Blackshaw, & Wrigley, 1984; Hazarika et al., 2004; Price, 1997; Solheim, Saeter, Finnanger, Finnanger, & Stenwig, 1992). A rigorous clinical study conducted in an area of northern Germany where exposure of asbestos was abnormally high, hence enriched in MPM patients, showed very effective results of pemetrexed on MPM (Hughes et al., 2002; Thodtmann et al., 1999). Also, cell-based combination studies of pemetrexed with cisplatin on MPM cells showed synergism and its extension to clinical study showed a survival benefit in this disease (Britten et al., 1999; Vogelzang et al., 2003). This led to the approval of pemetrexed and cisplatin as a first line treatment for MPM (Hazarika et al., 2004). Due to high retention of pemetrexed in the target tissue following plasma clearance, a dosage of 500 mg /m2 pemetrexed infused every 21 days was chosen as a very effective treatment modality for various cancers (S. Chattopadhyay et al., 2007). Folic acid and B12 supplementation with pemetrexed help in limiting toxicity (Hazarika et al., 2004).

Pemetrexed was also approved as a second line, single-agent treatment of locally advanced and metastatic NSCLC in 2004. The majority (90-95%) of lung cancers are of non-small cell origin and over a million of people are diagnosed with lung cancer each year worldwide (Parkin, Bray, Ferlay, & Pisani, 2001; Parkin, Bray, Ferlay, & Pisani, 2005). NSCLC is a highly progressive disease with a survival of 6 months following diagnosis if left untreated (Chemotherapy in addition to supportive care improves survival in advanced non-small-cell lung cancer: A
systematic review and meta-analysis of individual patient data from 16 randomized controlled trials (2008). This unusual success of pemetrexed against MPM suggested that it might be helpful in treatment of NSCLC. The effect of pemetrexed on NSCLC was initially tested in comparison to docetaxel (Taxotere), which was the standard treatment of the care for NSCLC at that time (Hanna et al., 2004). Although the median survival time of patients treated with pemetrexed and docetaxel were not significantly different (8.3 vs. 7.9 months median survival respectively), pemetrexed was significantly less toxic. This study played a favorable role in convincing FDA to approve pemetrexed for second-line treatment of NSCLC.

Various kind of tumors which have grown resistance against other antifolates have been shown to preserve sensitivity for pemetrexed (Jackman et al., 1995; Schultz et al., 1996; Zhao, Chattopadhyay, Hanscom, & Goldman, 2004; Zhao, Hanscom, Chattopadhyay, & Goldman, 2004). Pemetrexed retained activity in MCF-7 breast carcinoma and H630 colon carcinoma cells resistant to 5-FU and other TS inhibitors due to TS amplification (Schultz et al., 1996). The retention of pemetrexed antitumor activity in these cell lines was attributed to GART inhibition and other secondary targets of metabolites. Methotrexate resistance in patients with acute leukemia has been associated with low RFC expression (Gorlick et al., 1997) and these patient preserve sensitivity for pemetrexed. This presumably was because of the uptake of pemetrexed by the PCFT (Zhao et al., 2004).

Various studies were done with pemetrexed in combination with cisplatin chemotherapy in early stage NSCLC. Most of the evidence supporting this combination therapy, are based on the results of the trial on refinement of early stage lung cancer adjuvant therapy (TREAT), which included 132 patients with completely resected stage pIB-T3N1 NSCLC (Kreuter et al., 2013). The study showed a statistically significant improved feasibility rate of 95.5% for the pemetrexed- cisplatin
compared with 75.4% for the vinorelbine cisplatin treatment. Overall, the incidence of Grade 3/4 hematologic toxicities was significantly higher in vinorelbine cisplatin treatment.

In 2009, due to its favorable toxicity profile, results and convenient route of drug administration, FDA approved pemetrexed for maintenance therapy of NSCLC (Ciuleanu et al., 2009). This treatment strategy entails administering pemetrexed prior to disease progression following a platinum-based treatment cycle. Although this limits the treatment-free period following therapy, the low overt toxicity of pemetrexed combined with best supportive care has shown significance in survival benefit (Ciuleanu et al., 2009).

1.1.6.5 AICAR

AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide), enters the cells and is converted into ZMP, an analog of AMP that is capable of stimulating AMPK-dependent protein kinase activity (AMPK).

AICAR is being clinically used to treat and protect against cardiac ischemic injuries which, if left untreated, may lead to a myocardial infarction. Cardiac Ischemia is caused by insufficient blood flow to the myocardium (J. Corton, Gillespie, Hawley, & Hardie, 1999). The drug was first used in the 1980s as a method to preserve blood flow to the heart during surgery (Galinanes, Bullough D FAU, Mullane, K.M., FAU, & Hearse, 0903). Currently, the drug has also been shown as a potential treatment for diabetes by increasing the metabolic activity of tissues by changing the physical composition of muscle [Zarembo, Alan. An article in the Los Angeles Times claimed AICAR to be an “Exercise pill' could take the work out of workouts." Los Angeles Times]. Activation of AMPK by AICAR can inhibit basal or insulin stimulated glucose uptake, lipogenesis, glucose oxidation and lactate production on rat adipocytes (Gaidhu, Fediuc, & Ceddia, 2006).
1.1.6.5.1 **Mechanism of action of AICAR**

AICAR (commonly under the name Acadesine) is an analog of adenosine that enters cardiac cells to inhibit adenosine kinase and adenosine deaminase. In cardiac myocytes, AICA-riboside is phosphorylated to AICA-ribotide (ZMP) to activate AMPK without changing the levels of the nucleotides (Zhang, Frederick, He, & Balschi, 2006). Corton et al. showed similar results, where incubation of rat hepatocytes with AICAR, results in accumulation of the monophosphorylated derivative, ZMP, within the cell (J. M. Corton, Gillespie, Hawley, & Hardie, 1995). ZMP, an AMP mimetic, mimics both activating effects of AMP on AMPK, i.e. direct allosteric activation and promotion of phosphorylation by AMPK kinase. Due to activation of AMPK, AICAR affects several catabolic pathways (glycolysis, fatty acid oxidation), anabolic pathways (lipogenesis, glycogen synthesis, gluconeogenesis) and cell growth and survival pathways (mTORC1 and autophagy). In this dissertation, AICAR is used as an AMPK activator and its effects on the mTORC1 pathway were compared to those of PTX which were also shown to activate AMPK and inhibit mTORC1 (Racanelli, Rothbart, Heyer, & Moran, 2009; Rothbart, Racanelli, & Moran, 2010).

1.1.6.5.2 **Clinical evaluation of AICAR against cardiac ischemic injuries**

A brief period of coronary arterial occlusion followed by reperfusion prior to prolonged ischemia is known as preconditioning and has been shown to be protective of the effects of the prolonged ischemic period. Preconditioning preceding myocardial infarction may delay cell death and allow for greater salvage of myocardium through reperfusion therapy (Murry, Jennings, & Reimer, 1986). AICAR has been shown to precondition the heart shortly before or during ischemia (Burckhartt B, 1995). AICAR triggers a preconditioning anti-inflammatory state by increasing NO production via endothelial nitric oxide synthase. When AICAR is given 24 hours
prior to reperfusion, it prevents post ischemic leukocyte-endothelial cell adhesive interactions with increased NO production (Gaskin et al., 2009). AICAR also increases AMPK-dependent glucose uptake through translocation of GLUT-4, which is beneficial for the heart during post-ischemic reperfusion (3rd, Bergeron R, Shulman, G.I. & Young, 1999). The increase in glucose levels during AICAR preconditioning lengthens the period for preconditioning up to 2 hours in rabbits and 40 minutes in humans undergoing coronary ligation (Burckhartt et al., 1995; Murry et al., 1986). As a result, AICAR reduces the frequency and size of myocardial infarcts up to 25% in humans allowing improved blood flow to the heart. As well, the treatment has been shown to decrease the risk of an early death and improve recovery after surgery from an ischemic injury (Murry et al., 1986). This clearly suggests the importance of AICAR as a pharmacological agent in clinical therapeutics of heart diseases and cardiac surgeries. However, due to activation of AMPK, AICAR has been shown to act by inhibit mTORC1, a central pathway for cell growth and proliferation. Therefore, AICAR has attracted the attention of researchers for its anti-cancer properties.

1.1.6.5.3 Clinical Evaluation of AICAR as a cancer therapeutic agent

With the gradual inclination of chemotherapeutics towards targeted therapy, emerging evidence suggests that targeting cancer cell metabolism can be a promising and future therapeutic approach against human cancers. AMP-activated protein kinase (AMPK) is a known cellular metabolic sensor and plays an important role in the control of energy homeostasis in response to external stresses (Carling, 2005; Hardie, 2008; Kuhajda, 2008; Witczak, Sharoff, & Goodyear, 2008). The activation of AMPK is a signal of energy stress and the cell responds to AMPK activation by inhibiting or slowing down cell growth process and biosynthetic process and activating catabolic processes (Fogarty & Hardie, 2010). Therefore, the AMPK activators
AICAR and metformin has been intensively studied to determine their potential as cancer therapeutic agents (Fogarty & Hardie, 2010).

It has been shown that activation of AMP-activated protein kinase (AMPK) by AICAR, Metformin or the direct AMPK activator A23187 hampers cervical cancer cell growth through blocking the Wnt/β-catenin signaling activity (Kwan et al., 2013). This same group later reported that activated AMPK (p-AMPK) also inhibits cervical cancer cell growth by counteracting Forkhead box M1 (FOXM1) function (Yung, Chan, Liu, Yao, & Ngan, 2004). FOXM1 regulates a number of key cell cycle regulators that control the G1 to S and the G2 to M transitions (I. C. Wang et al., 2005). Some studies have shown that over-expression of FOXM1 might stem from the constitutively active ERK which confers metastatic activity to ovarian cancer cells and inhibition of ERK/FOXM1 has also been shown to repress the growth of ovarian cancer (Chan et al., 2012; Lok et al., 2011). Therefore activation of AMPK by AICAR followed by inhibition of ERK/FOXM1 and/or Wnt/β-catenin signaling has been suggested to be an effective therapeutic approach against ovarian and cervical cancers. Despite significant progress and success in the treatment of ALL, a significant number of children continue to relapse and for them, overall outcome remains poor. Some studies have studied the response of AICAR against ALL cells in culture. AICAR-mediated AMPK activation was found to be an antiproliferative agent in ALL. The mechanism of its anti-proliferative and apoptotic effects appear to be mediated via activation of p38-MAPK pathway, increased expression of cell cycle inhibitory proteins p27 and p53, and downstream effects on the mTOR pathway. Therefore, AICAR exhibits therapeutic potential as a targeted drug for the treatment of childhood ALL (Sengupta et al., 2007). The proliferation of various cancer cell lines was significantly inhibited by AICAR due to arrest in S-phase (Rattan, Giri, Singh, & Singh, 2005). Signaling pathway analysis suggested that this S-phase arrest was
accompanied by increased levels of p21, p27 and p53 proteins and attenuation of PI3K/AKT pathway. This inhibition of in vitro cell growth was also mimicked in vivo with a similar pattern of modulation of signaling pathways. AICAR mediated inhibition of cell proliferation due to S-phase blockade, occurs to a similar extent in both LKB1 wild-type and LKB1 knockout mouse embryonic fibroblasts. This suggests that AICAR mediated activation of AMPK can be LKB1 independent, a surprising outcome. (Rattan et al., 2005).

A considerable level of research has been performed with AICAR to understand the AMPK mediated mTORC1 inhibition. AICAR activated AMPK phosphorylates the Raptor subunit of the mTORC1 complex and inhibits mTORC1 signaling, causing cell cycle arrest at G1/S phase (Gwinn et al., 2008). As mTOR is a central regulator of cell growth and proliferation, its inhibition blocks cancer cells proliferation and cancer progression.

1.2 FOCUS OF THIS DISSERTATION

Whereas the evolution of cancer chemotherapy is continuing, the understanding of molecular and genetic mechanisms involved in the development and progression of cancer has been undergoing revolutionary growth, continually changing the landscape for chemotherapy. This also encouraged the molecular approach for cancer diagnosis and treatment. FDA approvals of the antifolate pemetrexed for NSCLC and MPM boosted the enthusiasm for antifolates as cancer therapeutics. As other TS inhibiting antifolates (5-FU/leucovorin and raltitrexed) have shown minimal response against MPM and NSCLC, the approval of pemetrexed as a first line therapy drug for these diseases suggested that the mechanism of action which is effective in NSCLC, is other then inhibition of TS. (Cunningham et al., 1996; Porta et al., 2005). Our lab recently showed that this TS-independent mechanism of action is due to inhibition of the enzyme AICART in the de novo purine synthesis pathway, which causes accumulation of the substrate of
this enzyme ZMP, leading to activation of the AMP-dependent protein kinase and to subsequent inhibition of mTORC1 (Racanelli et al., 2009; Rothbart et al., 2010). The studies of this dissertation are focusing on understanding the role of p53 in mTORC1 regulation, which is discussed in detail in Chapter 2. Understanding of the p53 mediated regulation on mTORC1 allowed us to address the question of how PTX effects are modulated by the p53 regulation on this pathway. In order to address this question, the differences in the downstream signaling of two AMPK activators, AICAR and pemetrexed were studied and directly compared during this dissertation. Although both of these agents cause activation of AMPK by increasing the amount of ZMP, the signaling downstream from AMPK was significantly different. Chapter 3 of this thesis is focused on understanding the causes for these differences, which seems to be due to differential behavior of p53 under these two drug treatments. Chapter 4 is mainly focused on determining the effects of pemetrexed on transcriptional activity of gain-of-function mutant p53.

The overall finding of this thesis work indicates that the atypical response of pemetrexed against several cancers including lung cancers may involve its effects on the transcriptional activity of wild type and mutant p53.

1.3 MATERIALS AND METHODS

1.3.1 Chemicals and reagents

Pemetrexed (LY231514, PTX) was obtained from Eli Lilly and Company (Indianapolis, IN). In some experiments, pemetrexed was purchased from LC Laboratories (#P-7177 Woburn, MA). AICAR (#A611700) was purchased from Toronto Research Biochemicals. PTX and AICAR were dissolved in PBS. Etoposide was purchased from Sigma Aldrich (#E1383) and was dissolved in DMSO. TRIzol Reagent (#15596026), DNase 1 (Invitrogen, # AM2222) SuperScript III First-strand Synthesis System (#18080), primers were from Invitrogen (Carlsbad,
CA) or Eurofin. Bradford Reagent (#5000006) was from Bio-Rad Laboratories. All other reagents were from Fisher Scientific (Waltham, MA) or Sigma Aldrich (St. Louis, MO) and were of the highest available purity.

Stock solutions of thymidine (560 µM) were routinely made by dissolving 0.00678 g of thymidine powder in 48 ml of 1x PBS. The concentration was determined by measuring absorbance at 267 nm from a 1:10 dilution of the stock solution in 1x PBS using an extinction coefficient of 9.7 mM⁻¹ cm⁻¹. The volume was adjusted accordingly with 1x PBS for a final concentration of 560 µM. This solution was filter-sterilized and was stored in single-use aliquots at -20°C. Thymidine was typically used at a final concentration of 5.6 µM in rescue experiments. DharmaFECT transfection reagent no. 2, siGENOME SMARTpool siRNAs targeting human p53 and scrambled siRNA pool no. 1 were purchased from Dharmacon (GE Healthcare, Lafayette, CO, USA). Four TSC2 siRNAs were purchased from Qiagen (Catalog # SI0001718, SI03027339, SI00011697, SI00011711) and mixed in equal amounts to make a pool. Complete EDTA-free Protease Inhibitor Cocktail tablets (#11873580001) were from Roche Applied Science (Indianapolis, IN). Thirty % Acrylamide/BIS solution 37.5:1 (#1610158), Laemmli Sample Buffer (#1610737), and Dual Color Precision Plus Protein Standard (#1610374) were from Bio-Rad Laboratories. Immobilon polyvinylidene fluoride (PVDF) membrane (#IPVH00010) was from Millipore (Billerica, MA). StartingBlock Blocking Buffer (#37542), Goat anti-rabbit IgG (#31462), and Goat anti-mouse IgG (#31348) secondary antibodies were from Thermo Scientific. Blotting Grade Blocker Non-fat Dry Milk (#1706404) was from Bio-Rad Laboratories. A list of antibodies and their sources can be found in Table 1.

1.3.2 Cell culture and reagents

HCT116 cell lines were a gift from Dr Bert Vogelstein. H460, A549, H441 and H661 cell lines
were purchased from ATCC (Manassas, VA, USA.) and grown in RPMI 1640 with 10% dialyzed fetal bovine serum (dFBS). H1299 cells expressing a ponasterone A-inducible p53 gene were made in Jennifer A. Pietenpol’s laboratory and were given to us by Dr. Sumitra Deb. Mutant p53 containing H1437 and H1048 NSCLC cells were generous gift form Dr. sumitra Deb. Mutant p53 containing H661 and H441 NSCLC cells were purchased from ATCC. All these cell lines were grown and maintained in RPMI 1640 with 10% dFBS and at 37°C with 5% CO2 with fresh media replacement after every 2-3 days. Immortalized p53-/-TSC2-/- MEFs and Sestrin 2 -/- MEFs were generous gifts from Dr. Andrei Budanov and were grown in DMEM (#11995 Gibco/Invitrogen Carlsbad, CA) supplemented with 10% dFBS and maintained at 37°C with 10% CO2 and given fresh media every 2-3 days. All cell lines were passaged by seeding in T-75 flasks at a density of 10^6 cells/ flask every 2-3 days. Passaging of adherent cell lines included washing with 1x phosphate buffered saline (#10010 Gibco/Invitrogen) and trypsinizing for 5 minutes at 37°C with 1x trypsin-EDTA (#15400 Gibco/Invitrogen).

1.3.3 Generating p53 mutant stable cell lines

HCT116 p53-/- or H1299 cells were plated in 6 well plates at a density of 2x10^5 cells per well and transfection was performed 24 hrs later with 2 µg of various mutant p53 plasmids containing different mutant form of p53. Forty-eight hrs later transfection media was replaced with RPMI + 10% dFBS. Cells were allowed to grow for 24hrs. 24 hrs later cells were trypsinized and replated in 10 cm dishes. These plasmids have selection marker for Zeocin. Thus, the cells which receive the plasmid and express it will be resistant to the Zeocin. Therefore, in parallel, cell survival assays were performed using the range of zeocin concentration from 20 µg/ml to 200 µg/ml on untransfected HCT116 and H1299 cells. Cells were plated in 24 well plate and 24 hrs later each well was supplied with a certain concentration of Zeocin containing media. Cellswere left in the
zeocin containing media for 2 weeks with replacing zeocin containing media at the interval of 2-3 days. The lowest concentrations of Zeocin which were able to kill 100% of cells were chosen for each cell line to use it for selection of cells expressing mut p53. The lowest concentration with 100% cell kill was found to be at 100 µg/ml for HCT116 and 40 µg/ml for H1299 cells. Therefore to select the transfected cells, cells were exposed to 100 µg/ml (HCT116) or 40 µg/ml (H1299) zeocin for 2 weeks, with selection media change after every 2-3 days. Cells were trypsinized and replated in 10cm dishes with Zeocin containing media. After two generations cells were plated to perform a western blot analysis to check the expression of mutant p53. As we found that the each plasmid was being expressed to the similar levels, we froze a batch of the cells in FBS with 10% DMSO. One batch was carried on for further experimental analysis.

1.3.4 Immunoblotting

Total Protein Isolation: Protein was typically harvested from 2-5 x 10^6 cells grown on 100 mm dishes. Prior to total protein harvest, one protease inhibitor cocktail tablet (manufacturer, catalog #) was dissolved in 50 ml 1x PBS and placed on ice. Tissue culture plates were kept cold throughout the harvesting procedure. Cells were washed once with cold 10 ml PBS (containing protease inhibitor), scraped, and pelleted at 1,000 rpm for 5 minutes at 4°C. Cells were lysed in cold buffer containing 62.5 mM Tris-HCl pH 6.5, 2% SDS, 5% glycerol, 5% 2-mercaptoethanol, 50 mM NaF, and a 1x concentration of Complete EDTA-free Protease Inhibitor Cocktail (manufacturer). Lysates were sheared through a 21-gauge needle for 30 times before being centrifuged at 14,000 rpm for 2 minutes. This shearing procedure was repeated, and lysates were spun at 14,000 rpm for 5 minutes. The protein concentration was determined using the Bradford Reagent according to the manufacturers protocol with BSA as a standard. Typical protein concentrations in lysates were 2-5 µg/µl. Protein was placed in single-use aliquots and stored at -
80°C.

**SDS-PAGE and Protein Transfer:** Gel electrophoresis and wet membrane transfers were performed using the Mini PROTEAN-3 Cell system (#1653301) from Bio-Rad Laboratories. Total protein was mixed with an equal volume of Laemml Sample Buffer, boiled for 5 minutes, and 20 µg of protein was loaded onto 1.5 mm SDS-polyacrylamide gels, poured according to the recipe provided with 30% Acrylamide/Bis 37:5:1 (#1610158) from Bio-Rad Laboratories. An aliquot (5-10 µl) of Dual Color Precision Plus Protein Standard was also loaded onto every gel for mass determination. Typically, protein was resolved on 7.5% or 12% gels in running buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS) at 50 volts for 30 minutes (or until protein migrated out of the stacking gel) followed by 120 volts for 1-1.5 hours to optimally separate the protein of interest.

Polyvinylidene fluoride (PVDF) membrane that had been pre-soaked in methanol was rinsed in water along with the gels and PVDF, gels, and sponges for the transfer were equilibrated for at least 20 minutes in cold transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 10% methanol). Six pieces of Whatman paper per gel were cut to the approximate size of an electrophoresis plate, and the transfer sandwich was assembled as follows: Layering on the black face of a transfer cassette, 3 pieces of Whatman paper (dipped in transfer buffer) were placed on top of 1 sponge. Air bubbles were removed by rolling with a 5 ml plastic pipette. The gel was centered on the Whatman paper, and a PVDF membrane was placed on top of the gel. Again, it was important to remove all air bubbles from the transfer sandwich by rolling. It also helped to keep the sandwich as wet as possible with transfer buffer during assembly. Whatman papers (dipped in transfer buffer) were placed on top of the membrane, followed by another sponge. The transfer cassette was closed, placed in its holder in the gel box along with a plastic ice block, and
the gel box was then filled with cold transfer buffer. Transfers were either ran on ice or in the cold room (4°C) at 100 volts for 45 minutes (one gel) or 50 minutes (2 gels). Following the transfer, membranes were dipped in methanol and dried on the lab bench for 15 minutes before being immunoblotted.

**Antibody Detection:** A general antibody detection procedure is described in this section. For detailed conditions for each antibody, refer to Table 1-1. Dried membranes were soaked briefly in methanol and non-specific proteins were blocked for 1 hour in either StartingBlock Buffer or 5% Blotting Grade Blocker Non-fat Dry Milk/0.1% TBS-T (0.5 M Tris-HCl pH 7.5, 0.14 M NaCl, 2.7 mM KCl, 0.1% Tween 20). Membranes were washed three times for 5 minutes in 0.1% TBS-T. Primary antibodies diluted in either StartingBlock Buffer or 5% BSA (#A4503) from Sigma were incubated on the membranes overnight at 4°C with rotation in sealed plastic bags to minimize antibody consumption. Membranes were washed 3 times with 0.1% TBS-T for 5 minutes and incubated for 1 hour in horseradish peroxidase-conjugated secondary antibody. Membranes were then washed 3 times with 0.1% TBS-T for 10 minutes. It was found that washing more stringently during this step greatly diminished non-specific background during exposure. Membranes were incubated with West Pico or West Dura SuperSignal chemiluminescence substrate (Pierce) for 5 minutes. Blots were exposed to autoradiography film and processed on an automated film developer. If the chemiluminescent conditions were not known, West Pico was applied first. If no signal was apparent, the blot was rinsed with 0.1% TBS-T, and West Dura (diluted by 40% with PBS) was applied. Signal was usually observed with one of these conditions, and rarely was a more stringent detection reagent applied. Blots presented in this dissertation are representative of findings from at least two biological replicates.
1.3.5 RNA interference

Cells were plated at 2 x 10^5 cells/well of a 6-well plate late in the day. Cells were transfected 24 hrs after seeding. siGENOME SMARTpool siRNAs (Dharmacon) (50 nM) were transfected with 0.1% DharmaFECT reagent according to the manufacturer’s instructions. For p53 knockdown the transfection media remained on the cells for 24 hours, at which time cells were washed with PBS and fresh media was replaced. Longer incubations resulted in visually apparent toxicity under a microscope. All experiments were controlled with a non-targeting siRNA SMARTpool (scrambled). Protein was harvested after 72 hours. Levels of proteins, targeted to knockdown, was analyzed by western blotting and knockdown was apparent by 48 hours and persisted at 72 hours post-transfection. For TSC2 knockdown, cells were transfected with transfection media for 24 hrs, followed by replacement with normal media. Cells were treated with the indicated drugs 36 hrs after transfection. Twenty-four hrs later, protein was harvested and western blot analysis was used to analyze protein levels.

1.3.6 Over-expression of WT and Mutant Raptor.

p53/-/ TSC2/- MEFS were plated in 6 well plates at a density of 2 x10^7 cells per well. Transfection was performed 24 hrs later with 3ug DNA using polyjet (SignaGen Laboratories) maintaining a ratio of 1:3 of DNA: polyjet. After 24 hrs, cultures were split into two 6 well plates. After 12 hrs, cells were treated with TdR or PTX + TdR. Twenty-four hr after treatment, cells were lysed for western blot analysis. Vectors used for transfection, pBABE Hygro-empty vector (ID-1765), pBABE-myc Raptor (ID- 18116) and pBABE myc-Raptor S722A/S792A (ID-18117) were originally made in the laboratory of Dr Reuben Shaw (Gwinn et al., 2008) and were purchased from Addgene.
1.3.7 Cellular growth assay

Adherent cells were seeded at a density of 20,000 cells/well of a 12-well plate and allowed to adhere overnight. Conditions were usually plated at least in duplicate and experiments were performed at least twice. Fresh media containing drugs were added the next day. Experiments typically lasted 72 hours after drug treatment. Following the incubation period, cells were washed 1x with PBS, trypsinized in 1.5 ml 1x trypsin-EDTA, and 1 ml of a single-cell suspension was counted electronically using a Z1 Coulter Particle Counter (Beckman Coulter Brea, CA). Data is presented as percent cell growth of experimental samples relative to controls grown in the absence of drug.

1.3.8 Total RNA Isolation

Total RNA was extracted from HCT116 cells grown to 75% confluency on a 100 mm tissue-culture dish using TRIzol Reagent according to the manufacturers protocol. All materials and reagents used were sterile and RNAse-free. Diethylpyrocarbonate (DEPC) H2O (0.01% v/v) was prepared by incubating at room temperature overnight and autoclaving before use. Pipettes were cleaned with RNAse ZAP (Invitrogen), and crosslinked with a UV Stratalinker 2400 (#400075 Stratagene La Jolla, CA). Cells were placed on ice, washed 1x with cold PBS, and lysed directly in 2 ml cold TRIzol Reagent. Cells were scraped, the slurry was transferred to a 14 ml round bottom Falcon tube, and the sample was incubated at room temperature for 5 minutes. An aliquot (400 µl) of chloroform was added to the sample in the fume hood. The sample was shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. The sample was spun at 7,000 rpm for 15 minutes at 4°C. The mixture separated into 3 phases. RNA remained exclusively in the aqueous upper phase. This aqueous phase was transferred to a fresh 14 ml tube and 1 ml of isopropanol was added to precipitate the RNA. This sample was
incubated at room temperature for 10 minutes before being centrifuged at 7,000 rpm for 10 minutes at 4°C. The supernatant was removed, and the RNA pellet was washed 1x with 75% ethanol in DEPC H2O. The sample was mixed and spun at 7,000 rpm for 5 minutes at 4°C. The ethanol was removed and spun again to remove excess ethanol. The pellet was re-suspended in 200 µl DEPC-treated H2O. For storage purposes, 75 µl dissolved RNA was suspended in 225 µl 100% ethanol and stored at -80°C. This sample could then be re-precipitated and solubilized if necessary. RNA concentration and purity was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE). Pure RNA has an OD260/280 of 2.0. The OD260/230 ratio was also used as a secondary indicator of RNA purity, and values below the range of 1.8-2.2 indicated the presence of copurified contaminants. RNA integrity was also determined by resolving RNA on a 1% TAE agarose gel at 100 volts for 30 minutes and then staining with ethidium bromide. A distinct banding pattern and intensity difference between 28s and 18s rRNA indicated that the RNA was intact.

**cDNA Synthesis:** cDNA was reverse-transcribed from 3 µg of total RNA using the SuperScript III First-strand Synthesis System from Invitrogen. SuperScript III Reverse Transcriptase is similar to the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), but has been engineered to diminish RNase H activity and enhance thermal stability. RNA was mixed with a final concentration of 5 µM oligo(dT)20 primer and 1 mM dNTP mix in a volume of 10 µl adjusted with DEPC-H2O. The mixture was incubated at 65°C for 5 minutes, then placed on ice for at least 1 minute. The cDNA synthesis mix was prepared in a separate tube by combining 2 µl of 10x RT buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 4 µl 25 of mM MgCl2, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT (40 U), and 1 µl of SuperScript III Reverse Transcriptase (200 U) per reaction. Ten µl of the cDNA synthesis mix was added to the RNA/primer mixture, the tube
was gently mixed, and cDNA was reverse-transcribed by incubating at 50°C for 50 minutes. The reaction was terminated by incubating at 85°C for 5 minutes before being held on ice. One µl of RNase H (2 U) was added to each tube and the tube was incubated for 20 minutes at 37°C. cDNA was stored at -20°C until use.

**Primer design:**

Primers to amplify the p21, Puma, HDM2, Bax, Pgp3, TSC2, Sestrin2, 4EBP1, NFκB2, PCNA, HSP70, hTERT, EGFR, MDM2, Axl and β-actin were designed with a 50-60% GC content, melting temperature between 55-75°C, and were 18-30 nucleotides in length. Six random nucleotides were added upstream of the restriction site on the 5’ end to allow for recognition and digestion by restriction enzymes. Strings of 3 or more G’s, C’s, or T’s were avoided in the 3’ end, but 1 G or C was placed at the 3’ end to help tack down the primer during annealing. Lyophilized primers were dissolved in HPLC-grade H2O to stock concentrations of 100 µM and were further diluted in HPLC-grade H20 to a working stock concentration of 15 µM before being added to Polymerase Chain Reactions (PCR). Primers were routinely stored as 100 µM stocks at -20°C as well as 15 µM working stocks in single-use aliquots. Sequences of primers are listed in table 1-2.

### 1.3.9 Chromatin Immunoprecipitation (ChIP)

Formaldehyde cross-linking and chromatin shearing The ChIP assay was adapted from Bronder et al (Bronder & Moran, 2003). Approximately 1 x 10^7 cells per condition were crosslinked at room temperature with 1% formaldehyde for 10 minutes, the reaction was then quenched for 5 minutes by the addition of 0.125 mM glycine. Cells were washed twice with PBS, scraped,
washed in buffer I containing 10 mM HEPES pH 7.5, 0.5 mM EGTA pH 7.5, 10 mM EDTA pH 8.0, 0.25% Triton X-100, then in buffer II containing 10 mM HEPES pH 7.5, 0.5 mM EGTA pH 7.5, 1 mM EDTA pH 8.0, 200 mM NaCl. All ChIP buffers contained 1 μg/μl aprotinin, 1 μg/μl leupeptin, 1 μg/μl peptatin, 1 mM PMSF, 0.2 mM Na3VO4 and 10 mM NaF. Cells were lysed in lysis buffer containing 25 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na Deoxycholate. Aliquots of 2 x 10^6 cells in 600 μl lysis buffer were sonicated with a Diagenode bath bioruptor for a total of 20 minutes with consecutive duty cycles of 30 seconds on, 30 seconds off at 4°C (5 minute cycles repeated a total of 4 times). These conditions were found to yield DNA fragments less than 1000 bp in size.

1.3.9.1 Immunoprecipitation

Lysates corresponding to 2 x 10^6 cells were precleared for 1 hour at 4°C with a 50% slurry of protein G-Sepharose (Amersham Biosciences) beads previously blocked with 8 μg of BSA and either 5 μg of sonicated lambda DNA or 6 μg of sonicated salmon sperm DNA, then incubated with 2 μg p53 Ab-6 (Calbiochem) antibody or 2 μg IgG (Millipore) antibody overnight. Antibody-protein-DNA complexes were captured by the addition of 30 μl of 50% blocked protein G-Sepharose bead slurry for 1 hour at 4°C. Beads were pelleted at 4,500 x g for 5 minutes at 4°C and the supernatant from the IgG immunoprecipitation was saved and the DNA contained within was referred to as input DNA. Beads were washed extensively twice with RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% SDS, 0.5% Na Deoxycholate, 1% NP-40), once with High Salt Buffer (500 mM NaCl, 50 mM Tris pH 8.0, 0.1% SDS, 1% Na Deoxycholate), once with LiCl Buffer (250 mM LiCl, 50 mM Tris pH 8.0, 0.5% Na Deoxycholate, 1% NP-40), and twice with TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) for 10
minutes each at 4oC rotating end-over-end. Protein-DNA complexes were eluted with elution buffer (2% SDS, 10 mM DTT, 0.1 M NaHCO3) while rotating end over end at 250C for 15 minutes and the cross-links were reversed by the addition of 0.2 M NaCl and incubation at 65oC overnight. DNA was treated with 10 μγ of RNase for 30 minutes at 37oC and with 20 μγ Proteinase K in 10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 0.25% SDS for 1 hour at 42oC, phenol-chloroform extracted, ethanol-precipitated and dissolved in 100 mL TE.

1.3.9.2 Quantitative PCR (Q-PCR)

Quantitative PCR was performed with 1μL of input DNA or ChIP DNA for each 25 μL reaction containing 12.5 μL Quantitect SYBR Green PCR Master Mix (Qiagen) and 0.3 μM of each primer. The amplification conditions were 95°C for 15 minutes, 40 cycles of 95°C for 45 seconds, for the noted Tm of each primer, for 45 seconds, 72°C for 45 seconds with a plate read, and concluding with a 5 minute extension at 72°C and a melting curve from 45°C to 100°C. Absolute quantities were calculated using a standard curve ranging from 100 ng to 0.8 ng of input DNA. The sequences of the primers flanking the p53 binding sites of the p21 promoter are; p21 3’RE- Fwd -5’-GAGGTCAGCTGCGTTAGAGG, Rev-5’-TGCAGAGGATGGATTGTCA-3’ and Tm used was 58°C.

1.3.10 m7GTP-CAP pulldown

To determine whether pemetrexed treatment enhanced residence of 4EBP1 at mRNA with a m7-guanosine CAP, cell lysates were incubated with m7GTP-sepharose beads, which have been previously shown to bind both eIF4E and 4E-BP1 (Holz, Ballif, Gygi, & Blenis, 2005). Following the indicated drug treatments, cells were lysed on ice for 30 min in IP buffer (25 mM HEPES pH 7.5, 1% NP40, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM
PMSF, 0.1% 2- mercaptoethanol, and 1x Roche Complete Protease Inhibitor Tablet). Cleared lysate (500 µg) was incubated with 40 µl of a 50% slurry of m7GTP-Sepharose (GE Lifesciences) for 2 hr at 4°C with rotation. Cap complexes were washed with IP buffer four times, resuspended in Laemmli Sample Buffer, and boiled for 5 min before being resolved on a 12.5% SDS-PAGE gel and immunoblotted as described above.
**Table 1-1 Antibody sources and conditions**

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<td>62</td>
<td>Starting block (SB)</td>
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Table 1-2 Primer sequences used for RT-qPCR

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<td>p21</td>
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<td>PIG3</td>
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<td>4</td>
<td>BAX</td>
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1.3.11 Immunoprecipitation and Kinase Assays

HCT116 cells were plated at 2x10^6 cells/ 15 mm dish and allowed to adhere for 48 hrs. Cell were harvested, lysed and immunoprecipitation was performed with 1mg protein under lower salt conditions (100mM) as suggested previously using anti-Raptor and anti-Rictor antibodies (37). Immunoprecipitation of mTORC1 or mTORC2, and subsequent in vitro kinase assays were carried out essentially as following; the cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.3% CHAPS, and 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM Na3VO4) supplemented with 1× Complete Protease Inhibitor Mixture from Roche Applied Sciences. The supernatant from the centrifugation at 15,000 × g for 20 min at 4 °C was immunoprecipitated using indicated antibodies and protein G- or protein A-Sepharose 4FF beads (Amersham Biosciences). The immunoprecipitates were washed three times with the lysis buffer. For in vitro kinase assay, the immunoprecipitates were further washed with wash buffer B (20 mM Tris-HCl, pH 7.5, 10 mM MgCl2). The immunoprecipitates were then mixed with 0.5 µg of recombinant 4EBP1(cat# SRP0253 from Sigma Aldrich) and 0.5 µg of recombinant Rheb (cat# SRP0225 from Sigma Aldrich) for mTORC1 in kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.2 mM ATP) and incubated for 20 min at 37 °C. The kinase reaction was stopped by the addition of 1× SDS sample buffer (3% SDS, 5% glycerol, 62 mM Tris-HCl, pH 6.7) and subsequent incubation at 95 °C for 5 min. The proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. All proteins were detected by Western blotting. GTPγS was purchased from Millipore. All antibodies used for western blot of IPs were from Cell Signaling, Inc.

1.3.12 Competative peptide block for antibody specificity

Same set of protein lysates or immunoprecipitaes are run on SDS-PAGE in duplicate and
proteins are transferred onto the PVDF membrane. Then, membrane is cut vertically in two parts separating the two sets of samples. One membrane is incubated overnight at 4°C with 1:500 dilution of α-Rheb antibody and other membrane is incubated with 1:500 dilution of α-Rheb Abs + cometenive Rheb-peptide solution (Rheb antibodies are preincubated with 500ng or 1µg of Rheb-peptide for 30 min at RT). Secondary antibody incubation and exposure to the Licore was done as suggested above in the immunoblotting section.

1.3.13 Overexpression of HA-TSC2 and Flag-Sestrin2

HCT116 p53 null cells were transfected with 3 µg DNA using Polyjet (DNA:Polyjet at 1:3). Cells were lysed and immunoblotted 48 hr after transfection. For m7GTP pulldowns, cells were plated in 10 cm dishes and transfected using 3 µg DNA; 48 hr later, cells were lysed in m7GTP buffer. 4EBP1-eIF4E complexes were captured on m7GTP beads, as described (Holz et al., 2005). Samples were resolved on 12.5% SDS-PAGE gels. Flag-Sestrin2 plasmid was obtained from Dr. Andrei Budanov and HA-TSC2 was purchased from Addgene (Plasmid 24939, deposited by Dr Kunliang Guan (Inoki, Li, Xu, & Guan, 2003)).
Chapter 2

2 Deletion and hot-spot mutations of p53 enhances mTORC1 activity by decreasing TSC2 expression and lysosomal localization

2.1 INTRODUCTION

AKT, AMPK and mTORC1 are three main nodes of the central control system for responses against metabolic changes and/or metabolic stress in the cells, which branch out into a number of signaling forward and feedback loops. An equally important tumor suppressor protein, p53, is thought to also play direct and indirect roles in influencing these pathways, although the understanding of the mechanism of this control is limited. This chapter will investigate the molecular mechanism of regulation of p53 on mTORC1 signaling and the importance of its functional status in control of mTORC1 in human colon and lung carcinoma cells.

2.1.1 Discovery and structure of the tumor suppressor p53

p53 was first identified in 1979 as a phosphoprotein that co-immunoprecipitated along with SV40 large and small t antigen, with sera from mice or hamster bearing SV40- induced tumors. This co-immunoprecipitated protein traveled at 53000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) (Linzer & Levine, 1979). Linzer et al. Later, a temperature sensitive mutant of the SV40 large T-antigen gene was found which caused the
accumulation of p53 after a temperature shift. This observation suggested that p53 was involved in SV40-mediated transformation and thus p53 was hypothesized to have oncogenic properties (Linzer & Levine, 1979). Subsequent rigorous studies on p53 and SV40 showed that this accumulation of p53 was actually the result of inactivation of p53 due to binding with large T-antigen rather than being involved in induction of the tumors directly. In 1984 when p53 was reconstituted in a p53 null Ab-MuLV-transformed cell line, the expression of p53 was found to be essential for tumor cells to exhibit a fully transformed phenotype, manifested in lethal tumors in syngeneic mice (Rotter, Wolf, & Nicolson, 1984; D. F. Wolf, Harris, & Rotter, 0926). In 1983, Oren et al. cloned the p53 gene and did genetic manipulation of its sequence; they studied the consequences of such manipulations on cell growth and survival (Oren & Levine, 1983). Later they studied the mechanism of varying levels of p53 in transformed and non-transformed cells (Reich, Oren, & Levine, 1983) and suggested that levels of p53 are regulated at the level of mRNA as well as the protein stability level. Until then the mechanistic role of p53 in cancer progression was not clear. However, in 1989, Levine and group published a landmark paper which demonstrated p53 as a tumor suppressor gene. This led to change in understanding of p53 function and its role in preventing tumor progression. Later studies by Levine's group supported the idea of p53 being a tumor suppressor and suggested that the p53 proto-oncogene can act negatively to block transformation (Finlay, Hinds, & Levine, 1989).

The p53 gene is located on the short arm of human chromosome 17 (17p13) and consists of 11 exons spanning approximately 20 kb of DNA (Benchimol et al., 1985; Lamb & Crawford, 1986). The p53 protein consists of 393 amino acids organized into four functional domains (Fig 2-1). The amino terminus of p53 contains two acidic transactivation domains, amino acids 1-42 and 43-60 (Fields & Jang, 1990) that play an important role in the transactivation mechanism of its
target genes; various transcription factors, e.g., TATA binding proteins (TBP) and TBP associated factors (TAFs) bind to these regions (Seto et al., 1992; Thut, Chen, Klemm, & Tjian, 1995). The p53 transactivation domain has one nuclear export sequence located at amino acids 12-27 containing two serine residues (serine 15 and serine 20), which are known to be phosphorylated after DNA damage, resulting in nuclear retention of p53 (Y. Zhang & Xiong, 2001) and both of these phosphorylation sites play an important role in deciding the functional fate and stability of p53. Downstream from the transactivation domain there is a proline rich domain (amino acids 64-92). This domain consist of five repeats of the src homology 3 (SH3) binding motif PXXP where P represents proline and X any other amino acid (Walker & Levine, 1996). This region has been linked to p53-mediated apoptosis but suggested to be dispensable for transactivation and cell growth arrest (Sakamuro, Sabbatini, White, & Prendergast, 1997). The proline rich sequence is followed by a DNA binding domain that stretches from amino acids 102 to 292 and enables p53 to specifically bind to a specific consensus binding sequence (p53 response element, p53RE) within gene promoters (Kern et al., 1991). Typically these sequences contain two copies of the 10 bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 10-13 bps where Pu is a purine base and Py is a pyrimidine base (el-Deiry, Kern, Pietenpol, Kinzler, & Vogelstein, 1992). The DNA binding domain contains the majority of the mutational hotspots in human cancer; the most frequently occurring mutations are in the peptides that make the closest contacts with DNA, explaining why many p53 mutants are unable to bind DNA (Cho, Gorina, Jeffrey, & Pavletich, 1994) or binds to complete different and newly recognized targets (also discussed in chapter 4).

The C-terminal region of p53 consist of a flexible linker and a tetramerization domain. The linker region connects the central core domain (N terminal domain) to the tetramerization
domain, a 32 amino acid peptide required for the formation of tetramers. p53 binds to DNA in tetramer form (Jeffrey, Gorina, & Pavletich, 1995). The tetramer is formed from 4 identical monomers. Each monomer contains a turn, a β-strand, a second turn and an α-helix. Two monomers form a dimer in which the α-helix is anti-parallel to the β-strand in the corresponding monomer. Tetramers are formed by two dimers interacting through their α-helices and therefore the p53 tetramer is known as a dimer of dimers (Jeffrey, Gorina S, and Pavletich, N.P., 1994). This tetramerization is required for efficient p53 transactivation in vivo and subsequent growth suppression (Pietenpol et al., 1994). A nuclear export sequence (NES) is located at amino acids 340-351 within the oligomerization domain and tetramerization masks the NES resulting in nuclear retention of p53 (Stommel et al., 1999). The extreme C-terminal basic domain, amino acids 363-393, was initially thought to be a negative regulator of sequence specific binding (Hupp & Lane, 1995). However it is widely accepted that p53 binds nonspecifically to DNA via the C-terminal domain (CTD) and then slides along the DNA searching for p53 consensus sequences and that, therefore, the CTD promotes DNA binding (McKinney, Mattia, Gottifredi, & Prives, 2004). There are three known nuclear localization signals within p53, one located in the flexible linker sequence and the other two within the CTD (Addison et al., 1990; Shaulsky et al., 1990).

2.1.2 Functional importance of p53

The p53 pathway can rapidly respond to cellular stimuli, especially to stresses that endanger genomic instability. p53 is known as “the guardian of genome” (Deppert, 2007; Efeyan & Serrano, 2007) or “cellular gatekeeper” (Levine, 1997) because of its central role in coordinating the cellular responses to the broad range of cellular stress factors. p53 functions as a mediator and often decision maker for deciding whether the cell should respond to stress with cell cycle
arrest, senescence, DNA repair, cell metabolism changes or apoptosis. All these decisions are mediated through the transcription activation or repression of p53 target genes (B. Vogelstein, Lane, & Levine, 2000; Vousden & Lane, 2007). As a transcription factor, p53 upregulates and down-regulates a broad spectrum of genes, thus regulation of p53 demands very tight and finely tuned controls (Menendez, Inga, & Resnick, 2010).

The conventional models for the regulation of p53 function are focused on three mechanisms: p53 stabilization induced by ATM/ATR mediated phosphorylation after DNA damage, sequence-specific DNA binding, and target gene activation by docking of p53 on the gene promoter or interaction with transcriptional machinery. Some recent studies suggest that the regulation of p53 cannot be categorized solely by these three levels of regulation (Iwakuma & Lozano, 2000; Marine et al., 2006; Menendez et al., 2010). This chapter will focus not on the mechanisms of control of p53, but rather on the mechanism of the p53 dependent regulation of mTORC1 in cancer cells.

2.1.3 AKT-AMPK-mTORC1 Pathway

As a evolutionary mechanism, cells respond to changes in the intracellular and extracellular environment by altering either gene expression or by the even faster and often transient set of effects collectively known as signal transduction. The expression of a protein is regulated firstly at the transcriptional level with the synthesis of RNA from DNA and secondly at the translational level in which protein-encoding information in mRNA is read. As a very integral and efficient process, translation demands substantial amounts of energy and cellular material (Calkhoven, Muller, & Leutz, 2002). Before initiating the process of growth and proliferation, cells must ensure the availability of resources of energy and required raw material like amino acids, nucleic acids, etc. It is necessary for cells to keep an account of the energy status of the
cells before committing to growth and proliferation.

A key pathway that senses and responds to change in environment is the AKT-AMPK-mTORC1 cascade. AKT and AMPK are antagonistic in this pathway and have opposing regulatory effects on the signaling through mTORC1, which in turn controls initiation of cap-dependent translation (Fig. 2-2). AKT, also known as Protien Kinase B, is a downstream effector for PI3K, which directly phosphorylates TSC2 (Tuberous sclerosis complex 2) on the number of residues including Ser 939, Ser 981 and Thr 1462. (Cai et al., 2006; K. Inoki, Li, Zhu, Wu, & Guan, 2011; Manning, Tee, Logsdon, Blenis, & Cantley, 2002; Potter, Pedraza, & Xu, 2002). Although TSC2 is an important target of AKT but it is not the sole target for this kinase. Recent studies have shown that AKT can positively regulate mTORC1 by phosphorylating PRAS40 (Proline-rich AKT substrate 40), a recently discovered binding partner and inhibitor of mTORC1 (Y. Sancak et al., 2007; Vander Haar, FAU, Bandhakavi S FAU - Griffin, Timothy,J., FAU, & Kim, 0424). In the presence of growth signal, AKT is phosphorylated at Ser-473 (by mTORC2) and Thr-318 (by PI3K) and activated. Activated AKT phosphorylates TSC2 and/or PRAS40. This phosphorylation of PRAS40 leads to conformational changes of PRAS40 causing dissociation from the mTORC1 complex. Dissociation of PRAS40 from the mTORC1 complex causes activation of mTORC1 signaling to its downstream targets, suggesting PRAS40 to be an inhibitor of mTORC1. Prior studies proposed this phenomenon to be mediated through 14-3-3 binding of the phosphorylated PRAS40 (Oshiro et al., 2007; Vander Haar et al., 0424; L. Wang, Harris, Roth, & Lawrence, 2007). Inhibition of mTORC1 upon dissociation of PRAS40 was nicely demonstrated by in vitro kinase assays done by Sancak Y et al. using immunoprecipitated mTORC1 complex and recombinant S6K1 as a substrate (Y. Sancak et al., 2007).

Thus, AKT can regulate and activate mTORC1, independent of TSC2 by phosphorylating and
dissociating an mTORC1 inhibitor PRAS40. Interestingly, PRAS40 also contains a TOR signaling motif that has been proposed to negatively regulate the mTORC1 activity by competing with the binding of 4EBP1 and S6K to Raptor (L. Wang et al., 2007). Therefore, PRAS40 is a direct inhibitor of mTORC1 that antagonizes the activation of mTORC1 by Rheb.GTP (Y. Sancak et al., 2007). Because the TSC1-TSC2 complex is absent in some lower eukaryotes, higher eukaryotes might have evolved a TSC1/2 complex-Rheb.GTP regulation module to fine tune the regulation of mTORC1 for more complex and suddenly changing environmental stimuli. Due to its critical role, mTORC1 is tightly regulated by forward and feedback mechanisms. Some recent studies have proposed that PRAS40 is not only an inhibitor of mTORC1 but also a substrate. mTORC1 mediated phosphorylation of PRAS40 inhibits its inhibitory activity and augments the removal of inhibition on signaling downstream from mTORC1 (Fonseca, Smith, Lee, MacKintosh, & Proud, 2007; Oshiro et al., 2007; L. Wang et al., 2007). This was proposed to be a positive feedback mechanism for AKT induced mTORC1 signaling. As TSC2 is an inhibitor of mTORC1, TSC2 null mouse embryonic fibroblasts have constitutive mTORC1 signaling, and show insensitivity towards insulin-mediated activation of AKT. AKT signaling is largely inhibited due to a negative feedback mechanism, suggesting that hyperactive Rheb can overcome PRAS40-mediated inhibition of mTORC1 (Y. Sancak et al., 2007).

When the TSC1/2-Rheb-GTP-mTORC1 module of signaling is regulated by AKT, activated mTORC1 also participates in regulation of the activity of insulin-PI3K-AKT signaling by at least one feedback mechanism. Some early studies suggested that activated mTORC1 suppresses AKT signaling by inhibition of insulin receptor substrate 1 (IRS1) (Haruta et al., 2000; Takano et al., 2001). Recent studies suggested that S6K, the downstream target of mTORC1, when
phosphorylated, further phosphorylates and represses IRS1 (Harrington et al., 2004; Um et al., 2004). It is becoming increasingly clear and accepted that this feedback regulation is not only important for maintaining cell homeostasis but also plays an important role in insulin resistant diabetes and in cancer therapeutics by rapamycin analogs (Easton, Kurmasheva, & Houghton, 2006; Um, D'Alessio, & Thomas, 2006). Whereas insulin-PI3K-AKT signaling senses the availability of growth hormones and promotes growth and anabolic processes, the cell has an antagonist effector molecule for this mechanism to maintain the cell's energy homeostasis called AMP-activated protein kinase (AMPK).

2.1.4 AMP-activated protein Kinase (AMPK)

AMPK is a central metabolic switch found in all eukaryotes that regulates glucose and lipid metabolism in response to alteration in nutrients and intracellular energy status. Activation of AMPK in response to diminished energy (ATP) in cells promotes catabolic processes to generate ATP, while inhibiting anabolic processes that consume it (Hardie, 2007). On a daily basis, mammalian cells are exposed to factors that can lead to genetic abnormalities in the PI3K-AKT and MAPK signaling pathways that lead to constitutive activation of cell proliferation and growth pathways through mTORC1 (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011; Hay & Sonenberg, 2004; Shaw & Cantley, 2006). AMPK mediates an endogenous regulatory mechanism and negatively controls cell growth and proliferation and thus represents a effective and new approach for cancer therapeutics (W. Wang & Guan, 2009).

2.1.4.1 AMPK sensitivity for AMP: ATP levels

Today we know that AMPK recognizes the ratio of AMP:ATP and is very sensitive to this ratio in the cell (Hardie & Hawley, 2001). Initially it was thought that AMPK is allosterically
modulated by adenosine-5'-monophosphate (AMP) (Yeh, Lee, & Kim, 1980). The AMPK is sensitive to the AMP:ATP and, to a lesser extent, the ADP:ATP ratio. In cells, the ratio of ADP:ATP is maintained by an enzyme adenylate kinase which maintains the reaction of ADP to AMP and ATP in equilibrium ($2\text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$). Healthy cells keep the ATP:ADP ratio at approximately 10:1 by ATP synthase ($\text{ADP} + \text{Pi} \rightarrow \text{ATP}$). The driving force for ATP synthase comes from the downhill flow of protons across the inner mitochondrial membrane, a gradient maintained by oxidative phosphorylation pumping protons against this gradient (Yoshida, Muneyuki, & Hisabori, 2001).

2.1.4.2 Interplay between ATP synthase and adenylate kinase

ATP synthase disrupts the equilibrium imposed by adenylate kinase in that it drives the above equation towards increasing levels of ATP. Therefore, to maintain equilibrium, the adenylate kinase reaction is driven from the right to left, generating ADP from AMP. The adenylate kinase reaction together with ATP synthase reaction maintains the ATP:AMP ratio at approximately 100:1 under the conditions of ATP homeostasis. When cells are under stress, the consumption of ATP increases and in order to maintain equilibrium, the adenylate kinase reaction is driven from left to right giving rise to an increased level of AMP in the process. AMPK is a heterotrimeric kinase complex composed of a catalytic ($\alpha$) subunit and two regulatory ($\beta$ and $\gamma$) subunits. Under energy starvation conditions, when glucose or the ATP/AMP ratio decreases below a threshold, AMP binds to the $\gamma$ subunit leading to the phosphorylation of the catalytic ($\alpha$) subunit at Thr 172 (Hawley et al., 2010).
Figure 2-1. Structure of p53 protein.

Map of the functional domains of the p53 protein, the amino-acid residues spanning each domain are indicated below the domain name. p53 consists of two amino terminal acidic transactivation domains (TAD), a proline rich (PRD), a central DNA binding domain (DBD), a flexible linker (L), a tetramerization domain (4DE) followed by the C-terminal regulatory domain (CTRD). The nuclear export sequence (NES) and nuclear localization signals (NLS) are also noted.
Figure 2-2. Schematic diagram of mTORC1 upstream and downstream targets. Where does p53 fit in this pathway?
Phosphorylated and activated AMPK phosphorylates TSC2 at Ser1387 (D. M. Gwinn et al., 2008) and turns on its GAP (GTPase activating protein) activity towards Rheb. Biochemical studies confirmed that Rheb-GTP is a substrate of TSC2. Increase in GTPase activity of TSC2 leads to conversion of Rheb-GTP into Rheb-GDP (Garami et al., 2003; K. Inoki, Li, Xu, & Guan, 2003; T. Sato, Nakashima, Guo, & Tamanoi, 2009; Tee, Manning, Roux, Cantley, & Blenis, 2003b; H. Zhang et al., 2003). Rheb-GTP is an essential factor for mTORC1 kinase activity even in vitro (T. Sato et al., 2009). In the absence of TSC2 function, Rheb GTP levels increase leading to hyperactivity of mTORC1 (Roccio, Bos, & Zwartkruis, 2000). Recently, it was shown that phosphorylation of Raptor by AMPK is required for the inhibition of mTORC1 activity (D. M. Gwinn et al., 2008). This study discovered that AMPK negatively regulates mTORC1 pathway by phosphorylating two proteins upstream of mTORC1 leading to the concept that activation of AMPK can be used for mTORC1 inhibition.

2.1.5 mTOR

mTOR (mammalian target of rapamycin), a 290 KDa Ser/Thr kinase of the phsophatidylinositol 3-kinase related protein kinase (PIKK) family, is a highly conserved, nutrient-responsive regulator of cell growth and proliferation. mTOR is present as two biochemically and functionally distinct complexes: a complex with Raptor known as mTORC1 or a complex with Rictor known as mTORC2 (Wullschleger, Loewith, & Hall, 2006; Y. Zhang, Billington, Pan, & Neufeld, 2006). A major source of information and knowledge about mTOR came after the discovery of the antifungal and immunosupressent drug rapamycin, a macrocyclic lactone, which is a potent and specific inhibitor of mTOR. Rapamycin forms an intracellular complex with the peptidyl-prolyl cis–trans isomerase FKBPI2 (FK506-binding protein). This drug/receptor complex binds to the FRB domain of mTOR located N-terminal to the kinase domain (Fingar &
Blenis, 2004). Although the mechanism of mTORC1 inhibition by rapamycin is still poorly understood but some studies suggest that it could be due to weakening of Raptor-mTOR interactions (Kim et al., 2002; Murakami et al., 2004). Prior studies focused on understanding the influence of TOR on overall metabolism, used transcriptional profiling of rapamycin treated yeast, drosophila and mammalian cells; the results suggested that the inhibition of mTORC1 by rapamycin affects translation of approximately 5% of all genes in the genome (Hardwick, Kuruvilla, Tong, Shamji, & Schreiber, 1999). Guertin et al. reported that treatment with rapamycin mimics the signals of glucose and amino acid starvation, which clearly suggested the critical role of TOR complexes in energy sensing pathways (Guertin, Guntur, Bell, Thoreen, & Sabatini, 2006; Hardwick et al., 1999; Peng, Golub, & Sabatini, 2002)

Rapamycin inhibits mTORC1 signaling. Interestingly, effects of rapamycin on the two targets of mTORC1 are different. Whereas rapamycin strongly inhibits phosphorylation of S6K1, it’s effects on 4EBP1 phosphorylation are minimal after short intervals of exposure. 4EBP1 is an essential protein for inhibition of mTORC1 mediated cap-dependent translation initiation (Fig. 2-3). Also, it has been shown that tumors develop resistance towards Rapamycin or Rapalogs and re-occurrence of cancer is prevalent with this drug treatment (Vignot, Faivre, Aguirre, & Raymond, 2005). mTORC2 is insensitive to Rapamycin upon short exposure but mTORC2 complex diminishes upon longer exposure of Rapamycin. mTORC1 is composed of at least four subunits: mTOR, mLST8/Gbl, PRAS40, and Raptor (Reiling & Sabatini, 2006). The 36 kDa protein mLST8/GL interacts with the TOR kinase domain but has no intrinsic catalytic activity. It consists almost entirely of seven WD40 repeats, a motif known to facilitate protein–protein interaction and signal transduction (Kim et al., 2003; T. F. Smith, Gaitatzes, Saxena, & Neer, 1999). Seven WD40 repeats can also be found in Raptor in addition to a novel RNC domain and
three HEAT repeats, also known to facilitate protein-protein interaction (Hara, Maruki, Long, Yoshino, Oshiro, Hidayat, Tokunaga, Avruch, & Yonezawa, 0820a; Kim et al., 2002). Raptor acts as a scaffold for the recruitment of the 4EBP1 and P70S6K1 substrates to mTORC1 (Nojima et al., 2003; Schalm, Fingar, Sabatini, & Blenis, 2003). mTORC2 plays a very important role in the feedback regulation of the mTORC1. Increased levels of P-389 S6K1 downstream of mTORC1 leads to the single site phosphorylation of Rictor (T1135) that in turn exerts a negative regulatory effect on the mTORC2 dependent phosphorylation and activation of PI3K/AKT. This way mTORC1 can directly regulate mTORC2 and vice-versa (Dibble, Asara, & Manning, 2009). mTORC2 is believed to be rapamycin insensitive. However, recent findings show that prolonged (>24h) rapamycin treatment can disrupt mTORC2 assembly and function by sequestering newly synthesized mTOR molecules (Sarbassov, Ali, Kim, Guertin, Latek, Erdjument-Bromage, Tempst, & Sabatini, 2004a; Sarbassov et al., 2006). Rictor shares conserved domains among eukaryotes, but their functions have so far not been elucidated (Jacinto et al., 1206; Sarbassov, Ali, Kim, Guertin, Latek, Erdjument-Bromage, Tempst, & Sabatini, 2004b). The domain structure of the TOR complex is quite complex and sophisticated suggesting the probability of many other proteins are likely to interact, perhaps transiently, an indication that there may be a much more complex mechanism of mTORCs regulation than currently understood. mTORC1 is active when the energy status of the cell is high. However, AMPK is activated when the cell is under energy stress, limiting mTORC1 activity. This antagonistic regulation by mTORC1 and AMPK critically decides cell fate and whether to allow cell growth is allowed or whether cell growth is suppressed based on insufficient energy charge.
Figure 2-3. Regulation of Cap dependent translation by mTORC1 target, 4EBP1 and S6K1.
2.1.6 AMPK activators

Recently, AMPK activators have been studied as potential chemotherapeutic drugs due to the role of AMPK in cell growth control and its signaling crosstalk between critical metabolic and oncogenic pathways. Treatment of MEFs with an AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) inhibits mTORC1 activity and cell growth (Jones et al., 2005). AICAR mediated activation of AMPK was also found cytotoxic in Acute Lymphoblastic Leukemia (ALL) cells via inhibition of mTORC1 pathway and increased expression of cell cycle inhibitors and apoptotic proteins, p53 and p27 (Sengupta et al., 2007). Use of Metformin, a LKB1-dependent AMPK activator (D. Gwinn et al., 2008) is associated with a statistically significant lower incidence of cancer in those diabetic patients using this drug versus patients with similar disease on other therapeutic agents (Libby et al., 2009). There was a detailed discussion on AICAR in chapter 1 of this thesis. Intraperitoneal injections of metformin in mouse models of lung tumorigenesis activated AMPK and inhibited mTORC1 in liver tissue, but in lung tissues mTORC1 was inhibited due to inhibition of insulin-lime growth factor-I receptor (IGF-1R/IR) mediated signaling (Memmott et al., 2010). Metformin inhibits mTORC1 activity in MCF-7 cells in an AMPK dependent manner (Zakikhani, Dowling, Fantus, Sonenberg, & Pollak, 2006).

Studies from this laboratory recently showed (Racanelli, Rothbart, Heyer, & Moran, 2009; Rothbart, Racanelli, & Moran, 2010) that pemetrexed, an antifolate, also activates AMPK and leads to inhibition of the mTORC1 pathway. In this thesis we demonstrate for the first time that this effect of PTX, although affected by loss or mutation of p53, is still p53-independent and mTORC1 inhibition is easily observed regardless of p53 status.

We recently showed that pemetrexed, an AMPK activator has a strong effect on both
downstream targets of mTORC1, S6K1 and 4EBP1 (Racanelli et al., 2009; Rothbart et al., 2010). Treatment with an AMPK activator not only blocks the mTORC1 signaling but also modifies lipid synthesis, apoptosis and many major pathways involved in cell survival and growth (Hardie, 2007; Mihaylova & Shaw, 2011). Due to involvement of AMPK in several other catabolic and metabolic processes, the activation of AMPK leads to a multifaceted attack on tumor cells. Thus, AMPK activators can be used as chemotherapeutic agents.

2.1.7 TSC1/TSC2 complex

Tuberous sclerosis (TSC) is an autosomal dominant genetic disease with an estimated prevalence of about 1 in 6000 newborns, which is caused by the loss of function of either TSC1 or TSC2 tumor suppressor genes. Loss of function of these genes leads to the formation of benign but progressively growing tumors in several vital organs including kidney, brain, heart and skin. The tumors that form in the brain can lead to mental retardation and then seizures, causing poor quality of life and ultimately, death. The tumor-like growths formed in this disease are named hamartomas in the brain, skin, kidneys, heart and other organs. Common clinical features are facial angiofibromas, renal angiomyolipomas, hypopigmented macules, cardiac rhabdomyomas, and cortical tubers and subependymal glial nodules in the brain. The greatest source of morbidity is the brain tumors, which cause seizures in 80-90% of affected individuals, mental retardation in about half of affected individuals, and behavioral abnormalities (mostly autism) in over half of affected individuals. (Cheadle, Reeve, Sampson, & Kwiatkowski, 2000; Gomez MR, Sampson JR, Whittemore VH. 1999; Young & Povey, 1998) Two genes causative of TSC have been identified, TSC1 and TSC2. TSC1 is located on chromosome 9q34 (van Slegtenhorst et al., 1997) and encodes a 130 kDa protein named hamartin while TSC2 (Identification and characterization of the tuberous sclerosis gene on chromosome 161993)The European
Chromosome 16 Tuberous Sclerosis Consortium, 1993) is located on chromosome 16p13.3 and encodes a 200 kDa protein named tuberin. A variety of mutations have been reported in these genes, which include point mutations, large deletions/ rearrangement/ insertion for TCS2 and insertion, deletion, nonsense and splicing mutation for both the genes. (Reviewed in (Cheadle et al., 2000; Young & Povey, 1998). TSC1 contains two coiled-coil domains, which have been shown to mediate binding to tuberin (Hodges et al., 2001) the TSC1/TSC2 heterodimer has been shown to function as a tumor suppressor (Plank, Yeung, & Henske, 1998; van Slegtenhorst et al., 1998). Histological studies of tumor lesions of TSC patients show some diversity, however, the tumors that arise as a result of loss of function of either TSC1 or 2 shares common features suggesting that TSC1 and TSC2 are involved in the regulation of the same pathway which feeds into the cell cycle, cell growth control, and vesicular trafficking and adhesion (van Slegtenhorst et al., 1998). Some recent studies showed that the TSC1/TSC2 heterodimer is involved in the regulation of cell growth and proliferation downstream of PI3K- AKT in a signaling pathway feeding into mTORC1 in both D. melanogaster and mammalian cells, suggesting that TSC1/TSC2 are a regulator of the mTORC1 pathway (K. Inoki, & Guan 2003; Manning & Cantley, 2003). Within the TSC1- TSC2 complex, TSC1 stabilizes TSC2 (Benvenuto et al., 2000; Chong-Kopera et al., 2006) while TSC2 acts as GTPase activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain) (Garami et al., 2003; K. Inoki, Li, Xu, & Guan, 2003; T. Sato, Nakashima, Guo, & Tamanoi, 0721; Saucedo et al., 2003; Stocker et al., 2003; Tee & Blenis, 0123; Y. Zhang, Saucedo, Ru, Edgar, & Pan, 2003). GTP bound Rheb potently activates mTORC1 (Y. Sancak et al., 2007). When active TSC1-TSC2 complex converts Rheb-GTP to Rheb-GDP and inhibits mTORC1, thus, loss of TSC2 promotes cell growth and tumorigenesis. TSC2 has multiple sites for AKT, MAPK, RSK, and extracellular
signal-regulated kinase (ERK) phosphorylation (Ballif et al., 2005; Dan et al., 2002; Liu, Cai, Espejo, Bedford, & Walker, 2002; Manning et al., 2002; Roux, Ballif, Anjum, Gygi, & Blenis, 2004; Tee, Manning, Roux, Cantley, & Blenis, 2003a), all of which appear to be inhibitory to its function. It was clear that in the presence of growth factor or insulin stimulation AKT blocks the TSC2 mediated inhibition of mTORC1 by its phosphorylation at several sites (K. Inoki, Li, Zhu, Wu, & Guan, 2002; Manning et al., 2002), but the mechanism by which that AKT mediated phosphorylation inhibits TSC2 tumor suppressor function is still unknown. Also, there are several contradictory studies which suggest that TSC2 can be located in the cytoplasm (Nellist et al., 1999), the membrane portion of cell (Wienecke, Konig, & DeClue, 1995) or even in nucleus (Lou, Griffith, & Noonan, 2001). This clearly suggests that the possible mechanism central to control of TSC2 could be the translocation of the TSC1-TSC2 complex between cellular compartments. To address this question Cai et al. (Cai et al., 2006) fractionated nuclear, cytoplasmic and membrane fractions and showed that tuberin subcellular translocation is dependent on AKT mediated Ser/Thr phosphorylation of TSC2 and thus dependent on the status of the growth factors available to cells. They also showed that upon growth factor stimulation 14-3-3 proteins mediate the translocation of TSC2 into the cytosol; however, TSC1 enhances TSC2 retention at the membrane. The most interesting finding of this study was that upon growth factor stimulation, Rheb and TSC2 co-localization are disrupted, suggesting that the regulation of TSC2-Rheb-GTP on mTORC1 is mechanistically regulated by the translocation of TSC2 between cytosol and membrane bound Rheb-GTP. They suggested that when TSC2 and Rheb are co-localized, TSC2 GAP activity converts Rheb GTP into Rheb GDP and in the absence of Rheb- GTP, mTORC1 signaling is not activated (Cai et al., 2006). A follow-up study (J. Zhang et al., 2013) from the same group was done to understand the mechanism of interaction of
TSC1/TSC2-Rheb-mTORC1. They showed that the tuberous sclerosis complex localizes to peroxisomes, including TSC1, TSC2, and also Rheb. This signaling node regulates mTORC1 in peroxisomes in response to reactive oxygen species (ROS). This study also suggested that peroxisome-localized TSC2 functioned as a GTPase for Rheb-GTP and converted it to Rheb-GDP in response to ROS leading to mTORC1 inhibition; this mTORC1 inhibition was diminished in cells expressing peroxisome-localization deficient mutants of TSC2 (J. Zhang et al., 2013). Menon et al. in their very recent study addressed the similar question of how TSC2-Rheb regulates mTORC1 in the presence of growth factor and amino acid availability conditions. They showed that upon insulin stimulation there is no change either in the GAP activity of TSC2 or in the stability of the TSC complex (Menon et al., 2014). It has been known before that both low levels of growth factors or amino acids can inhibit mTORC1 activity (Y. Sancak et al., 2010b). Study of Menon et. al (Menon et al., 2014) added further understanding of how these two factors (insulin and amino acids) are quite independent of each other in regulating the localization of the TSC complex and mTORC1 to the lysosome. They also showed that activity of the PI3K-AKT pathway induces the dissociation of the TSC complex from the lysosome in an AKT dependent manner. This sophisticated study unified the mechanism by which independent pathways affect the recruitment of mTORC1 and the TSC complex to Rheb at the lysosomai surface and raises the concept that lysosomal localization serves to integrate growth signals and the decision between survival or proliferation (Menon et al., 2014). This suggest that the presence or absence of TSC2 function plays an important role in deciding the control of PI3K/AKT vs mTORC1 on S6K1 (Jaeschke et al., 2002).

2.1.8 Rheb

Rheb is a small GTPase initially isolated as a Ras homolog enriched in brain (Yamagata et al.,
Rheb expression was also induced in quiescent fibroblast cells after the addition of serum (Yamagata et al., 1994). In addition, Rheb expression was induced in PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla after the addition of EGF or FGF (Yamagata et al., 1994). Rheb has been found to be highly conserved during evolution and was found to play critical roles in cell growth, cell cycle, autophagy and amino acid uptake (Aspuria & Tamanoi, 2004; Patel et al., 2003). Rheb is a monomeric 21 KDa protein which belongs to the Ras superfamily of GTPases (Wennerberg, Rossman, & Der, 2005). For a long time, Ras superfamily G-proteins were classified into one of five subfamilies, Ras, Rho, Rab, ARF, and Ran (depending on what for the classification?); members of each subfamily share high sequence homology in their effector domain and they also have similar functions (Bourne, Sanders, & McCormick, 1991; Mackay & Hall, 1998; Moore, 1998; Moss & Vaughan, 1998; Schimmoller, Simon, & Pfeffer, 1998; Vojtek & Der, 1998). Rheb proteins are highly conserved during evolution and are found from yeast to humans but are absent in plants. Mammalian cells have two different Rheb genes: RHEB1 and RHEB2 (also called RHEBL1) but lower eukaryotes such as yeast or Drosophila have only one gene. The translation products of these two genes share 54% identity and 74% similarity and it is thought that they perform similar functions. However, their tissue expression profiles differ, with Rheb1 ubiquitously expressed while Rheb2 expression is more limited (Saito, Araki, Kontani, Nishina, & Katada, 2005). For the purposes of this dissertation study, only Rheb1 will be discussed further. Recently, Rheb has received significant attention, partly because Rheb proteins play roles in regulating growth and cell cycle, an effect thought due to its role in the insulin/mTOR/S6K signaling pathway. Rheb acts downstream of AKT and activates S6K phosphorylation dependent on TOR. Rheb is known to be an activator of mTORC1 (Aspuria & Tamanoi, 2004; T. Sato, Nakashima, Guo, & Tamanoi,
Like other Ras superfamily proteins, Rheb also activates downstream effectors only when bound with GTP. All Rheb proteins contain short stretches of sequences involved in the recognition of the guanine ring and phosphates of guanine nucleotides, known as G1 – G5 boxes, (Aspuria & Tamanoi, 2004). Tamanoi’s group have made a number of contributions to the understanding of Rheb proteins. They showed that Rheb family members share some very critical features (Urano, Tabancay, Yang, & Tamanoi, 2000) first, an arginine residue corresponding to the glycine at the 12th codon of Ras is conserved in all the Rheb homologues. Second, they have very similar effector domain sequences in which 10 of the 17 residues are identical, and 4 of the remaining residues are similar. Third, they all terminate in the CAAX motif (C is cysteine, A is an aliphatic amino acid, and X is the C-terminal amino acid that is usually methionine, alanine, serine, glutamine, or cysteine) that is required for farnesylation (Clark et al., 1997; Urano et al., 2000; Yang, Urano, & Tamanoi, 2000) a posttranslational modification, important for membrane localization and function of Rheb.

2.1.8.1 Rheb Expression and cancer

Rheb is frequently overexpressed in human carcinoma and has been shown to markedly sensitize the epidermis of transgenic mice overexpressing Rheb in basal epidermal keratinocytes; these mice show squamous carcinoma induction following a single dose of Ras-activating carcinogen 7,12-dimethylbenz(a)anthracene (Lu et al., 2010). A meta-analysis of previously published cancer cytogenetic and transcriptional databases suggested that the chromosome 7q36.1–q36.3 region containing the RHEB gene is frequently amplified in some human cancer histologies: an increased RHEB expression was observed in liver, lung and bladder cancers. A direct correlation between Rheb mRNA levels and breast cancer prognosis and progression has been
shown by microarray database mining (Jiang & Vogt, 2008). Studies done to determine the significance of Rheb in the oncogenic transformation of chicken embryonic fibroblast suggested that Rheb played an essential role in oncogenic transformation (Jiang & Vogt, 2008). The expression of Rheb Q64L and N153T in these fibroblasts induced morphological changes, including increased size and vacuolization, and conferred upon the cells the ability for anchorage independent growth.

2.1.8.2 Subcellular localization of Rheb

The initial studies on Rheb were mainly done using ectopicly overexpressed Rheb; some of these studies indicated that Rheb localizes on perinuclear and vesicular structures. (Buerger, DeVries, & Stambolic, 2006; Saito et al., 2005; Takahashi, Nakagawa, Young, & Yamanaka, 2005). There have been reports of Rheb localizing to several endosomal membrane fractions: lysosomes, peroxisomes, and mitochondria. A literature consensus on the location of Rheb in the cell has not yet been reached. Studies done by Sancak et al using overexpression of Rheb showed that exogenously expressed Rheb is localized in lysosomal membranes, based on colocalization with the lysosomal marker LAMP2 (Y. Sancak et al., 2010a). Later this observation was confirmed using antibodies to detect endogenous Rheb (Menon et al., 2014). Subcellular membrane localization of Rheb is dependent on its farnesylation and postprenylation modification events including Rce1 cleavage and Icmt-mediated carboxyl methylation (Hanker et al., 2010; Takahashi et al., 2005).

2.1.8.3 Interconversion of Rheb-GTP and Rheb-GDP

As with other Ras superfamily members, Rheb binds to both guanine nucleotides GTP and GDP as reported in rat, S. pombe and M. drosophila (Yamagata et al., 1994; Yan et al., 2006; Zheng et
al., 2011). Extensive biochemical studies with *S. pombe* suggested that Rheb binds to GDP (Zheng et al., 2011). This binding was specific to guanine nucleotides as excess GDP or GTP competed with bound GTP but this was not seen with CTP, UTP or ATP. It is also well known that Rheb has an intrinsic GTPase activity (Zheng et al., 2011). Some studies showed that the rate of GTPase activity of Rheb was much slower than seen with Ras but some studies reported these rates to be comparable (Yamagata et al., 1994). TSC2, upon activation, works as a GTPase activating protein and enhances the intrinsic GTPase activity of Rheb. In the presence of excess GTP, GDP dissociates form Rheb suggesting that Rheb shuttles between GDP-bound and GTP-bound forms (Zheng et al., 2011).

### 2.1.9 Sestrin1/2

The GADD (Growth arrest and DNA damage) genes that are induced in response to genotoxic treatments encode several functionally distinct proteins. The proteins encoded by these genes are centrally involved in adaptive or detrimental responses to cellular stresses (Fornace, 1992; Fornace, Jackman, Hollander, Hoffman-Liebermann, & Liebermann, 1992; M. L. Smith & Fornace, 1996). One very recently added gene in the list of GADD genes is Sestrin2 also known as Hi95. Budanov et al., while studying the genes affected by long term oxidation stress, reported the Hi95 gene to be involved in regulation of cell viability in response to genetic and oxidative stress conditions (A. V. Budanov et al., 2002). Oxygen homeostasis is tightly regulated and controlled by an oxygen sensing mechanism and by oxidative stress responsive genes, which are induced to compensate for oxygen deficiency (hypoxia). In order to identify novel genes induced under prolonged hypoxic conditions, Budanov et al. compared gene expression profiles in human glioblastoma A172 cells maintained under normoxic conditions and following
prolonged hypoxia, using microarrays (A. V. Budanov et al., 2002). Hi95 was one of the genes to be induced under this hypoxia; this was of interest because of the strong homology to the p53 responsive gene PA26, a GADD gene (Velasco-Miguel et al., 1999). Budanov et al further demonstrated that this induction of Hi95 was p53-independent after hypoxia but was p53-dependent after DNA damage (A. V. Budanov et al., 2002). Hi95 induction in MCF7 cells suppresses growth, sensitizing them to DNA damaging drug and serum deprivation but protecting them from H$_2$O$_2$ treatment and ischemia. Hi95 is now called sestrin 2. Sestrin1 and Sestrin2 have been recently described as p53 target genes (A. V. Budanov et al., 2002; Peeters et al., 2003; Velasco-Miguel et al., 1999) involved in the regulation of mTORC1. DNA damage and oxidative stress induce Sestrin1 and Sestrin2 and show cytoprotective function (A. V. Budanov, Sablina, Feinstein, Koonin, & Chumakov, 2004). Recent studies by Budanov and Karin showed that sestrin1/2 also play important roles in the inhibition of mTORC1. Exogenous expression of Sestrin1/2 activates AMPK and TSC2 phosphorylation and lead to mTORC1 inhibition. They showed that induction of p53 by genotoxic stress induces Sestrin2 which further leads to activation of AMPK and TSC2 leading to inhibition of mTORC1 and they concluded that sestrin1/2 work as the connecting link between AMPK and TSC2 (A. V. Budanov & Karin, 2008b). This chapter also involves the role of Sestrin 2 on p53 and its regulation on mTORC1.

### 2.1.10 Raptor

Raptor, the regulatory associated protein of mTOR not only interacts with mTORC1 but also with mTORC1 substrates S6K1 and 4EBP1. These two proteins interact with Raptor via a domain known as a TOR signaling motif and become phosphorylated by the mTORC1 kinase domain. Therefore Raptor works as a scaffold protein that facilitates the recruitment of the
substrates of mTORC1. This recruitment allows phosphorylation by mTORC1 of S6K1 at a single site, Thr389, and of 4EBP1 at 4 different sites (Ser65, Ser70, Ser37/46) (Fingar et al., 2004;, Maruki, Long, Yoshino, Oshiro, Hidayat, Tokunaga, Avruch, & Yonezawa, 2002; Loewith et al., 2002; Ma & Blenis, 2009; Nojima et al., 2003; Schalm & Blenis, 2002; Schalm & Blenis, 2002; Schalm et al., 2003). mTORC1 mediated phosphorylation of S6K1 is essential for the assembly of the eIF3 translation initiation complex Fig. 2-2 (M. K. Holz, Ballif, Gygi, & Blenis, 2005b). On the other hand, 4EBP1 is a strong translational repressor which binds to the complex of capped mRNA with initiation factor eIF4E, blocking further enucleation of the translational initiation complex by other factors. Only after phosphorylation of 4EBP1 by mTORC1 will 4EBP1 release eIF4E and allows its interaction with other factors, prompting the initiation of cap dependent translation (Ma & Blenis, 2009).

In a very important paper, Gwinn et al showed that activation of AMPK leads to phosphorylation of TSC2 at Ser1387 and of Raptor at Ser792 leading to inhibition of mTORC1 kinase activity (D. M. Gwinn et al., 2008). Although Ser792 phosphorylation is important for the regulation of mTORC1, it is not the only modification of Raptor. Using tandem mass spectrometry, Raptor was found to be is phosphorylated at six different phosphorylation sites mainly in two clusters (cluster1, Ser696/Thr706 and cluster 2 Ser855/Ser859/Ser 863/Ser877). This observation was confirmed with site-specific antibodies for these sites. This raises the question of whether AMPK activators, which can strongly phosphorylate Raptor at Ser792, may inhibit mTORC1. The 3rd chapter of this dissertation will be discussing whether, PTX, which activates AMPK and robustly phosphorylate Raptor at Ser 792 leads to inhibition of mTORC1.

2.1.11 p53 mutations and cancer

p53 is mutated in approximately 50% of human cancers, making it the most commonly mutated
gene in human malignancies (B. Vogelstein, 1990). As described above, p53 is capable of triggering apoptosis or growth arrest, both of which aid in maintaining genome stability. Due to the involvement of p53 in these pathways which protect the cell from transformation, extensive research has been done to understand the impact of the presence of mutant p53 in the genome. The majority of p53 mutations in human cancers disrupt the ability of p53 to bind to DNA (Kato et al., 2003). In a study including 280 tumors containing p53 with somatic base substitution mutations, 98% of the mutations fell within a 600 base pair region of p53 encompassing exons 5 through 8 (amino acids 110-307). Out of the 280 analyzed, 227 were from solid tumors. Colon, esophagus, breast and non-small cell lung cancer contained the highest number of mutations (Hollstein et al., 1991). In heterozygous cells, a mutant p53 allele can alter the function of the wild-type p53 allele, either by having a dominant negative effect on wild-type p53, or by resulting in gaining new oncogenic properties, independent of the wild-type p53 allele (Baker et al., 1989). Analysis of tumors containing allelic deletions in the short arm of chromosome 17, the region encoding p53, showed that the remaining allele contained point mutations indicative of loss of heterozygosity (Baker et al., 1989). Upon co-translation of mutant p53 and wild-type p53, mutant p53 was found to oligomerize with wild-type p53, driving it to a mutant phenotype characteristic of a dominant negative effect (Milner & Medcalf, 1991). Transfection of mutant p53 into p53 null tumors resulted in lethal tumors, demonstrating that mutant p53 may gain oncogenic properties (D. Wolf, Harris, & Rotter, 1984).

2.1.12 Interplay between p53-TSC2-Sestrin2 and mTORC1

Feng et al. showed that upon DNA damage induced by etoposide, p53 competent cells show activation of AMPK followed by inhibition of mTORC1. They suggested that, under genotoxic stress, p53 negatively regulates mTORC1 via p53 dependent activation of AMPK (Feng, Zhang,
Levine, & Jin, 2005). It was not known, before this thesis, whether un-stimulated endogenous levels of p53 have any effects on mTORC1 regulation. Later it was reported that activation of p53 by DNA damaging agent leads to increase in TSC2 (Feng et al., 2007) and sestrin2 (A. V. Budanov et al., 2002) mRNA and protein levels in p53 competent cells. Budanov et al showed that upon overexpression of exogenous Sestrin2, AMPK mediated phosphorylation of TSC2 increases, leading to decrease in mTORC1 activity. These investigators suggested that Sestrin1/2 was an essential participant in the interaction of TSC2 and AMPK for TSC2 phosphorylation and activation (A. V. Budanov & Karin, 2008a).

All these studies cumulatively suggest that the involvement of p53 in mTORC1 pathway could be multimodal and dependent on the type of stress stimulant. Most of these studies are done either by activation of p53 using DNA damaging drugs or by introducing ectopic TSC2, sestrin1/2 or AMPK.

2.2 FOCUS OF THIS CHAPTER

In this chapter, we focus on understanding the differences in control of mTORC1 activity in the presence and absence of p53 and followed those observations onto the effect of mutant p53 on control of mTORC1.
2.3 RESULTS

2.3.1 mTORC1 activity is suppressed by basal p53 function in an AMPK independent manner.

Feng et al (Feng et al., 2005) established a connection between p53 and mTORC1 when they showed that stabilization of p53 by the DNA damaging agent VP16 (etoposide) suppressed the phosphorylation of the mTORC1 target S6K1 in MEFs and that this effect does not occur in MEFs lacking p53. We observed the same phenomena for HCT116 colon carcinoma cells isogenic for p53 deletion, ie, S6K1 becomes hypophosphorylated in wt HCT116 cells treated with VP16 but not in the HCT116 cell line devoid of p53 (p53−/−) treated with VP16 (Fig. 2-4A). However, a second very significant effect was evident: untreated p53 (−/−) HCT116 cells displayed a robust hyperphosphorylation of S6K1 at T389 over that in HCT116 with p53 function (Fig. 2-4A). The effect of DNA damage on mTORC1 activity was shown by Feng et al. (Feng et al., 2005) to be mediated by AMPK activation; however, the stimulation of mTORC1 with loss of p53 in untreated cells was not due to differences in the activation of AMPK, as the phosphorylation of AMPK at S172 was independent of p53 (Fig. 2-4B).

There was a substantial decrease in the 4EBP1 detected in 7mGTP pulldowns in HCT116 p53−/− cells (Fig. 2-5A), an index of unphosphorylated 4EBP1 capable of binding to capped mRNA; the level of eIF4E on these beads was identical in p53 +/+ and −/− cells. Others have shown that 4EBP1 is phosphorylated at as many as four residues by mTORC1 and that electrophoresis in high percentage acrylamide gels can resolve several 4EBP1 species as indicated in the lysate section of Fig. 2-5A (Gingras, Raught, & Sonenberg, 2001; Herbert, Tee, & Proud, 2002; M. K. Holz, Ballif, Gygi, & Blenis, 2005a; Ma & Blenis, 2009). The lowest band in western blot from lysates (Fig. 2-5A) represents unphosphorylated 4EBP1 and the higher bands (α, β, and γ)
represent species with progressively higher phosphorylation states; the phosphorylated forms are shunted to the proteasome and degraded (Ma & Blenis, 2009).

The diminution of the unphosphorylated form of 4EBP1 was also observed as lower levels of the fastest migrating band detected in immunoblots of lysates from p53 -/- HCT 116 compared with wt p53 HCT116 (Fig. 2-5A). These data suggest that the basal level of p53 in HCT116 cells has a substantial suppressive effect on mTORC1 activity, and that deletion of p53 releases mTORC1 from this control.

In order to understand whether this difference in total levels of 4EBP1 in p53 +/+ and -/- cells is completely due to increased phosphorylation and decreased stability of the protein or its is partially due to transcriptional differences as, p53 is a transcription factor, we analyzed mRNA levels of 4EBP1. p53 +/+ and p53 -/- HCT116 cells were grown in RPMI media + 10% dFBS and were harvested 24 hrs later cells in Trizol; mRNA was extracted followed by cDNA synthesis. Real Time qPCR analysis showed that 4EBP1 transcript levels were 3-4 fold lower in the absence of p53 suggesting that p53 was directly or indirectly involved in the transcriptional regulation of 4EBP1 (Fig. 2-5B). Therefore, we hypothesized that p53 regulates 4EBP1 transcription and would be binding to the 4EBP1 promoter. In order to test our hypothesis, we did in-silico analysis to find probable p53 binding site on 4EBP1 promoter. We found that almost 1000 bp upstream of promoter there is a probable p53 binding half site. Thus we performed chromatin immunoprecipitation (ChIP) using anti-p53 antibody followed by DNA precipitation and Q-PCR, however, we did not find any increase in the binding of p53 at the 4EBP1 promoter compared to IgG control for nonspecific binding (data not shown). This suggested that p53 indirectly but positively regulates 4EBP1 mRNA transcription without binding to 4EBP1 promoter.
Figure 2-4. mTORC1 activity is suppressed by p53 in an AMPK independent manner.

A) HCT116 cells isogenic for p53 were treated with DMSO or VP16. 24 hrs later whole cell lysates were analyzed for P-T389 S6K1, as an indicator of mTORC1 activity using immunobloting. Levels of p53 and actin were checked as well. B) Levels of activated AMPK measured by P-ser172 AMPK, are unaffected by the status of p53.
Figure 2-5. Difference in 4EBP1 transcript and protein levels in p53 WT and null HCT116 cells.

A) Translation repressor species of 4EBP1 is diminished upon loss of p53; HCT116 p53 wt and null cells were grown in RPMI for 24 hrs followed by 7m-GTP pull down. B) 4EBP1 transcript levels are 3 fold higher in the presence of p53; HCT116 WT and null cells were grown as above followed by RNA extraction, cDNA synthesis and real time qPCR. Shown in B is combined data from three individual experiments.
2.3.2 **Negative regulation of mTORC1 by p53 is also seen in other carcinoma cell lines.**

To determine whether this effect of endogenous p53 on mTORC1 was peculiar to HCT116 or was more general, we tested other carcinoma cell lines. H1299 lung carcinoma cells with ponesteron-A inducible wt p53, when induced with ponesteron for 24 hrs, showed remarkable decrease in phosphorylation of S6K1 (Fig. 2-6A). The hypophosphorylated species of 4EBP1 was also increased in cells induced with ponesteron-A, in comparison to uninduced cells, seen as the shift of the band towards down and increase in the intensity of lowest band (Fig. 2-6). We examined the activity of mTORC1 in other carcinoma cell lines with wt p53 after transfection of siRNA pools directed against p53. In the NSCLC lines H460 and A549 and in HCT116 (Fig. 2-6B), the endogenous wt p53 levels were largely eliminated by siRNA treatment and, concomitantly, p-T389 S6K1 was enhanced and the level of unphosphorylated 4EBP1 was reduced, indicating a higher mTORC1 kinase activity upon loss of p53. We concluded that the endogenous level of p53 in several carcinoma cell lines was exerting a marked controlling effect on mTORC1.
Figure 2-6. Negative regulation of mTORC1 by p53 is more of a generality amongst various cancer cells.

A) Lung cancer cells H1299, consists of ponasteron inducible p53 vector, were induced with ponetsron for 24 hrs followed by immunoblotting to analyze P-T389 S6K1 and 4EBP1 levels as an indicator of mTORC1 activity. B) p53 was knocked out using siRNA inference in HCT116, H460 and A549 cells. Cells were harvest and lysed 48 hours after p53-siRNA transfection. As an indicator of mTORC1 activity P-T389 S6K1 and 4EBP1 migration was analyzed by using specific antibodies by immunobloting.
2.3.3 Mutation of p53 also causes hyperactivity of mTORC1.

Human tumors lose p53 function most commonly due to mutation in one allele followed by loss of the other from the genome. Hence, we tested whether hyperactivity of mTORC1 was observed in carcinoma cells with a single allele of mutant p53 in the same genetic background. For this, p53-/− HCT116 colon carcinoma cells were stably transfected with three mutant p53 species commonly seen in clinical samples, namely R175H, R248W, and R273H (Fig. 2-7). These same mutant p53 species and also V143A were stably transfected into H1299 small cell lung carcinoma cells and were compared with H1299 cells bearing a ponasterone-A-inducible wt p53 gene (Fig. 2-9). In HCT116 (Fig 2-7A), mTORC1 targets S6K1 and 4EBP1 were as hyperphosphorylated in cells bearing any of these p53 mutant forms as they were in p53 null cells. Likewise, the binding of 4EBP1 to mGTP beads in lysates of the mutant p53-bearing carcinoma cells was minimal and comparably decreased from that seen in p53 wt cells as that in p53-null cells (Fig. 2-7B). The effect of loss or mutation of p53 on 4EBP1 levels was also observed at the transcriptional level as the level of 4EBP1 mRNA was interestingly quite low in p53-/− and mutant p53 expressing cells in comparison to wt p53-bearing isogenic HCT116 (Fig. 2-8).

Ponasterone-A induced wt p53 in H1299 reduces mTORC1 activity as seen by the decreased phosphorylation of S6K1 and 4EBP1, and increased levels of unphosphorylated 4EBP1 (Fig. 2-9A). Like p53 null H1299, mutant p53 bearing H1299 also showed minimal binding of 4EBP1 to mGTP beads in lysates in compassion to isogenic cells expressing inducible wt p53 (Fig. 2-9B). Like HCT116, loss or mutation of p53 lead to decreased levels of 4EBP1 mRNA levels in H1299 cells (Fig. 2-10).
We concluded that several mutant p53 could not reinstate the control of mTORC1. Despite the high levels of p53 expression typical of p53 mutant tumors, hyperactivity of mTORC1 was equivalent to that in p53 null cells. Hence, one of the functions of wild-type p53 is to exert a control on mTORC1 that is missing in the mutants.
Figure 2-7. Mutations in the DNA binding domain of p53 enhance mTORC1 activity equivalently to loss of p53.

Expression and phosphorylation of S6K1 and 4EBP1 were determined by immunoblotting of HCT116 p53-/- cells stably transfected with hot spot mutants of p53 (A). The binding of 4EBP1 to m7GTP bound beads is severely depressed in HCT116 cells stably transfected with p53 mutants in comparison to HCT116 with WT p53 (B).
Figure 2-8. Mutations in the DNA binding domain of p53 decreases 4EBP1 mRNA expression to the level comparable to loss of p53.

HCT116 cells were grown in the same conditions (RPMI + 10%dFBS) for 24 hours to allow the 60% confluency followed by RNA extraction, cDNA synthesis and RT-qPCR analysis to determine the levels of 4EBP1. The expression of 4EBP1 in cells stably transfected with mutant p53 was compared with p53+/+ and p53-/− cells. mRNA levels are normalized to actin. (plotted as ±sd, n=3). Data suggested that mutant p53 overexpression does not compensate for loss of wt p53 function and the mRNA levels of 4EBP1 in mutant p53 is as low as in p53-/− cells.
Figure 2-9. Mutations in the DNA binding domain of p53 enhance mTORC1 activity equivalently to loss of p53.

The expression and phosphorylation of S6K1 and 4EBP1 were determined by immunoblotting of H1299 cells stably transfected with hot spot mutants of p53 and compared H1299 transfected with an inducible p53 construct (A). The binding of 4EBP1 to m7GTP bound beads is severely depressed in H1299 cells stably transfected with p53 mutants in comparison to H1299 expressing Ponasteron-A inducible WT p53 (B).
Figure 2-10. Mutations in the DNA binding domain of p53 in H1299, decreases 4EBP1 mRNA expression, comparable to loss of p53.

H1299 cells were grown in the same conditions (RPMI + 10% dFBS) for 24 hours to allow the 60% confluency followed by RNA extraction, cDNA synthesis and RT-qPCR analysis to determine the levels of 4EBP1. The expression of 4EBP1 in H1299 stably transfected with mutant p53 was compared with H1299 expressing ponasteron-A inducible WT p53 and parental H1299. mRNA levels are normalized to actin. (plotted as ±sd, n=3). Induction of WT p53 increases the levels of 4EBP1 mRNA in comparison to mutant p53 expressing cells as well as p53 null cells.
2.3.4 In-vitro kinase assay of mTORC1 immunoprecipitated from p53 null cells showed higher mTORC1 kinase activity.

When we saw the higher cellular mTORC1 activity in p53 null cells than that in p53 competent cells, we tested if this difference could be seen in-vitro. In order to address this question we performed in vitro kinase assays. We immunoprecipitated mTORC1 from p53 +/- and +/- HCT116 cells under low salt condition (Y. Sancak et al., 2007) using Raptor antibodies. Here, low salt condition refers to 100mM NaCl in wash buffer used for washing the bead-antibody-mTORC1 complex. This condition was used in order remove nonspecific protein sticking to beads, yet preserving the weak and transient but specific mTORC1 interacting proteins in the complex. The kinase assays were performed using Rheb-GTPγS as an essential cofactor and recombinant 4EBP1 as substrate (described in methods and materials) (Fig. 2-11A). The levels of P-T37/46 4EBP1 were analysed by immunoblotting using phosphospecific antibodies for this modified residue of 4EBP1. We found that mTORC1 immunoprecipitated from p53 +/- cells was catalytically more active and able to phosphorylate 4EBP1 to significantly higher levels than was mTORC1 from p53+/+ cells (Fig. 2-11A). Three independent experiments were performed and immunoblots were quantified using Licore imaging and quantitation. Data complied from all three experiments is represented as a bar graph with standard deviations representing the variation among replicate biological repeats (Fig. 2-11B).

In vitro kinase assays on mTORC1 complexes immunoprecipitated with antibody against Raptor indicated that the mTORC1 isolated from p53 +/- HCT116 cells was more catalytically active than that from p53 +/- cells (Fig 2-11A,B). Hence, the differences responsible for the enhanced kinase activity of mTORC1 from p53 +/- cells in either the components of this complex or the post-translational modifications of these components were at least partially stable to
immunoprecipitation. The composition of the isolated IPs was investigated. The level of Raptor and of the AMPK-dependent phosphorylation of Raptor at S792 were equivalent in Raptor IPs from p53 wt and null cells (Fig 2-12A, B left panels). Although the level of mTOR seen in lysates was unchanged (Fig 2-12A, right panel), the amount of total mTOR associated with Raptor was enhanced in p53 deficient cells (Fig 2-12A,B left panels). The Raptor-bound mTOR had higher p-S2448, a modification catalyzed by S6K1, but this appears to reflect the higher levels of mTOR bound to Raptor (Fig 2-12A, left panel). Although the levels of PRAS40 and of p-T246 PRAS40 were unchanged in lysates of p53 null cells, the binding of total and p-T246 PRAS40 to anti- raptor IPs was lower in p53 null cells (Fig. 2-12A, left panels and B right panel). Contrary to the levels of the other components, the levels of TSC2 were substantially decreased in lysates of p53-null cells (Fig. 2-9A, right panel), as they also were in lysates of all of the mutant p53s studied (Fig. 2-7A). Apparently as a result of these lower expression levels of TSC2, the level of TSC2 in the IPs from p53 null cells was decreased compared to that in p53 wt cells (Fig. 2-12A, left panels, B right panel). Overall, there were higher levels of mTOR in mTORC1 complexes in p53 null HCT116 cells and a lower content of PRAS40 and TSC2 bound to mTORC1, factors that would each promote higher mTORC1 kinase activity.

In order to confirm that these differences are mTORC1 specific, immunoprecipitation under same conditions and buffer was performed using anti-Rictor antibodies and the component of mTORC2 complex were observed using immunoblotting (Fig. 2-13). The levels of Rictor in lysates as well as in IPs were the same in both p53+/+ and +/- cells (Fig 2-13 left and right panels). The levels of mTOR and S-2448 mTOR were also equivalent in both lysates and IPs from both cell lines (Fig 2-13 left and right panel). Interestingly, we found more phosphorylation of AKT at Ser473 and T308 in the lysates of p53 competent cells (Fig. 2-13, right panel).
Figure 2-11. In vitro kinase assay shows higher kinase activity in mTORC1, immunoprecipitated from p53 null cells.

A) The mTORC1 complex from p53-/- cells has enhanced kinase activity in vitro. mTORC1 was immunoprecipitated with an anti-Raptor antibody under low salt (100 mM) conditions and in vitro kinase assays were performed using 4EBP1 as a substrate; p-T37/46 4EBP1 generated was determined by immunoblot. (B) Licor densitometry data from three independent in vitro kinase assays (mean ±sd).
Figure 2-12. The enhanced kinase activity of the mTORC1 complex is retained during immunoprecipitation in cells lacking p53.

(A) The components of the mTORC1 complex differ between p53+/+ and p53−/− cells. Anti-Raptor immunoprecipitates from isogenic HCT116 cells were probed with antibodies against the indicated proteins; lysates were probed in parallel. (D) The level of Raptor, PRAS40, and TSC2 in p53-null HCT116 cells was measured by densitometry from 3 independent experiments and expressed (±sd) relative to the level in p53 wt control cultures. **p=0.0045 for mTOR, *p=0.0117 for PRAS40, *p=0.040 for TSC2.
Figure 2-13. The differences in mTORC1 components are specific to it and not seen in mTORC2.

mTORC2 was immunoprecipitated in the same way as mTORC1 using anti-Rictor antibody. Immunoblotting was performed on immunoprecipitate and levels of Rictor, P-S2448 mTOR and mTOR were analyzed (left panel). Lysates from which IP was performed, were probed to analyze the levels of various components of mTORC2 (right panel).
2.3.5 Higher Rheb bound to mTORC1, immunoprecipitated from p53 null cells under low salt conditions

In our immunoprecipitation studies, we found higher Rheb bound to the mTORC1, immunoprecipitated from p53 null cells (see below). Others have not successfully detected endogenous Rheb in mTORC1 immunoprecipitates. Therefore, when we detected the band at the size corresponding to Rheb (21 KD) we set out to confirm whether the band detected in our studies is indeed Rheb or it is a non-specific band. We tested this by performing a competitive-peptide blocking experiment. The principle behind this experiment is that, in the presence of excess Ab-specific peptide (against which antibody has been raised), antibody will not be able to bind to the corresponding protein band on the immunoblot due to competitive binding of peptide with the antibody. Thus, the blot incubated with antibody (-competitive peptide) will show signal at the correct size, suggesting the presence of the corresponding protein, while the blot incubated with antibody (+ competitive peptide) will not. In this experiment we probed the lysates of p53 null and competent cells with either anti-Rheb antibody alone or Ab + peptide solution (i.e. anti-Rheb antibody pre-incubated for 30 minutes with 100ng or 1µg of Rheb-epitope peptide). The blot incubated with anti-Rheb antibody in the absence of peptide showed the presence of band at 21kD, however the blot incubated with anti-Rheb antibody, pre-incubated with Ab + 1µg peptide solution, showed the absence of this band (Fig. 2-14). This indicated that the peptide competed with the Rheb present in the lysates and that the 21 KDa band was indeed Rheb. We concluded that the antibody we used for the detection of Rheb is indeed specifically binding to Rheb.

mTORC1 complexes, immunoprecipitated from p53 +/- and +/- cells were equally divided and loaded in duplicate on SDS-PAGE gels followed by transfer onto the PVDF
membranes. One blot (representing 1 set of protein samples) was incubated overnight with Rheb-antibody alone and other blot (representing a second identical set of the protein samples) was incubated with Rheb-antibody with 1µg of competitive peptide (Ab+ peptide solution). After incubation with the secondary antibody followed by detection on a Licore, we found that the blot incubated with anti-Rheb antibody in the absence of competitive peptide, showed the Rheb band at 21 Kd, in immunoprecipitate from p53-/- cells, however this band was not present on the blot incubated with Ab + peptide (Fig. 2-15). This indicated that higher Rheb is bound to the immunoprecipitate from p53 null cells than to that from p53 competent cells.

In our studies, the mTORC1 immunoprecipitation was done using wash buffer with 100 mM NaCl concentration. We hypothesized that the probable cause of unsuccessful attempts of detecting Rheb in mTORC1 immunoprecipitates by others, was the use of wash buffers with salt concentrations too high to allow survival of these complexes. We also hypothesized that Rheb interacts with mTORC1 transiently so that with the use of high salt concentrations (150 mM to 500mM) as previously used by others would be immunoprecipitation conditions too stringent to catch loosely bound interactions. Therefore, we performed mTORC1 immunoprecipitation from lysates of p53 -/- and +/- cells, using increasing concentration of NaCl (from 50 to 500 mM) in the wash buffer. Rheb was detectable in the mTORC1 complex, immunoprecipitated at 50mM and 100mM salt-wash buffer, but, with the increase of the salt concentration in the wash buffer, binding of Rheb with mTORC1 complex was decreased and completely vanished at 500mM salt. To confirm the mTORC1 specific binding of Rheb, nonspecific mouse IgGs (same species as of α-Raptor antibody) were used for immunoprecipitation as a negative control. We saw that Rheb was immunoprecipitated only under low salt conditions and only in IPs done with anti-Raptor antibody (Fig. 2-16). This suggests that Rheb loosely interacts with the mTORC1 complex and
this interaction is higher in p53-/- cells. This could be due to higher Rheb levels in lysosomal membranes of p53 null cells, as suggested by Catherine Bell’s experiments (data not shown). The mRNA and protein levels of Rheb are equivalent in p53 null and competent cells (Fig. 2-17, 2-14 respectively). As expected, under 500 mM salt condition, PRAS40 binding was remarkably decreased as previously shown by Sancak et al. (Y. Sancak et al., 2007) (Fig 2-17).
Figure 2-14. Rheb peptide block shows the specificity of the α-Rheb antibody.

p53 null and wt HCT116 cells were seeded and harvested after 24 hrs. 25 µg of cell lysates from p53 +/+ and -/- cells were loaded in triplicate. Proteins were transferred on the PVDF membrane. Membrane was blocked using start block and cut into three strips, A, B, C. A) Incubated with α-Rheb antibody; B) α-Rheb antibody + 100 ng peptide; C) α-Rheb antibody + 1 µg peptide. All three membranes were simultaneously incubated with secondary antibody and signal was detected by Licor.
Figure 2-15. Higher Rheb is bound to mTORC1 complex immunoprecipitated from p53 null cells.

Immunoprecipitation was performed using lysates from p53 +/- and -/- HCT116 cells. Immunoprecipitates were divided equally and ran on SDS-PAGE gels in duplicate followed by transfer on PVDF membranes A) Left blot was incubated with α-Rheb antibody + 1μg peptide solution, overnigh at 4 degree. B) This was incubated only with α-Rheb antibody (-peptide) overnight at 4 degree. Secondary antibody incubation and detection by licore was done simultaneously. The red arrow indicates the Rheb band at the size of 21 kD.
Figure 2-16. At 100mM or low salt condition of wash buffer, Rheb is detectable and higher in the immunoprecipitates of p53 null cells.

p53 null and wt HCT116 cells were seeded and harvested after 24 hrs. Immunoprecipitation was performed using α-Raptor antibody and wash buffers with salt concentration as indicated. Immunoblotting was performed to analyze Raptor, p-S792 Raptor, mTOR, p-S2448 mTOR, PRAS40, p-T246 PRAS40 and Rheb using corresponding antibodies and signal was detected by Licor.
Figure 2-17. Steady state mRNA levels in p53 null and competent cells.
HCT116 p53−/− and +/+ cells were grown in triplicates in the same conditions (RPMI + 10%dFBS) for 24 hours to allow the 60% confluency followed by RNA extraction, cDNA synthesis and RT-qPCR analysis to determine the levels of Rheb. Data is plotted after normalization to actin (plotted as ±sd, n=3).
The transcript and protein levels of TSC2 and Sestrin 2 are lower in p53 null HCT116 cells.

We found that the levels of TSC2 bound to the mTORC1 complex are lower in p53 null cells than to that in p53 competent cells. It was not known whether the uninduced, basal level of p53 can regulate these two genes. Therefore, we asked if the total levels of TSC2 and sestrin2 would be decreased upon loss or mutation of p53. Steady state mRNA levels of TSC2 and Sestrin2 were measured by RT-qPCR. mRNA was extracted from HCT116 p53 competent or null cells followed by cDNA synthesis. Real time q-PCR analysis showed that levels of TSC2 are approximately 2 fold lower in p53 -/- cells than in p53 +/+ cells (Fig. 2-18, left panel). This apparent transcriptional effect was even larger in the case of Sestrin2 where p53+/+ cells express almost 5 fold higher levels of Sestrin2 mRNA than p53-/- cells (Fig 2-18, right panel). Previous reports have also indicated an effect of p53 on levels of TSC2 mRNA (Feng et al., 2007) and DNA-damaging drugs were reported to increase Sestrin2 mRNA (A. V. Budanov et al., 2002). We further investigated if this difference was translated into protein levels. Indeed, we saw significantly lower levels of TSC2 and Sestrin2 protein levels in p53 -/- HCT116 cells (Fig. 2-19).
Figure 2-18. Steady state mRNA levels of TSC2 and Sestrin2 are lower in the absence of p53.

p53 +/+ and p53 -/- HCT116 cells were grown in the same conditions (RPMI + 10% dFBS) for 24 hours to allow the 60% confluency followed by RNA extraction, cDNA synthesis and RT-qPCR analysis to determine the levels of TSC2 (left panel) and sestrin2 (right panel). mRNA levels are normalized to actin.
Figure 2-19. Protein levels of TSC2 and Setsrin2 are reduced to great extent upon loss of p53.

p53 +/+ and p53 +/- HCT116 cells were grown in the same conditions (RPMI + 10% dFBS) for 24 hours to allow the 60% confluency. Cells were harvested and lysed. Immunoblotting was performed in order to analyze the levels of TSC2, Sestrin 2 and p53. Actin is shown as a control for equal protein loading across the samples. Data represents three individual biological repeats, run on the SDS page and immunoblotted at the same time.
2.3.7 The levels of TSC2 mRNA and protein increased upon activation of p53 by DNA damaging drug etoposide suggesting p53 transcriptional regulation of these two genes.

In order to confirm if the levels of TSC2 were regulated by p53, we treated HCT116 p53+/+ cells with etoposide, a conventional method of inducing DNA damage leading to activation of p53 and its transcription activity. HCT116 p53+/+ and p53-/- cells were treated with DMSO or etoposide (VP16, 20µM) for 24 hrs. Cells were harvested and processed further for immunoblotting or RNA extraction. Upon RT-qPCR analysis we found that the levels of TSC2 mRNA were increased by almost 2 fold in cells treated with VP16 in p53 wt cells but not in cells treated with VP16 genetically null for p53 (Fig. 2-20A). The protein levels of TSC2 were also increased after VP16 treatment only in p53 +/- cells but not in p53-/-cells (Fig. 2-20B). Hence, we show here that activation of AMPK can lead to increase in the levels of AMPK dependent phosphorylation of TSC2 at S1387 (Fig. 2-20B). We conclude that upon activation of p53 after VP16 treatment, the increased levels of TSC2 and Sestrin2 allow the interaction of activated AMPK and TSC2 and the phosphorylation of TSC2 at Ser1387.

2.3.8 Carcinoma cells that are p53 null or that express only mutant p53 have decreased levels of TSC2 and Sestrin2

HCT116 and H1299 cells expressing mutant p53 were studied to compare their TSC2 and Sestrin2 mRNA and protein levels with their isogenic p53 null and wt p53 cells. The steady-state levels of TSC2 and Sestrin2 mRNA were determined by RT-qPCR. Both mRNA populations for TSC2 and for sestrin2 were decreased with loss or mutation of p53 (Fig. 2-21). The protein levels of TSC2 were also diminished in both HCT116 and H1299 cells without p53, or with mutant p53 (Fig. 2-22).
Figure 2-20. Levels of TSC2 mRNA and protein increased upon activation of p53 after etoposide treatment.

HCT116 p53+/+ and -/+ cells were treated with either DMSO or VP16 (20µM) for 24 hours. (A) Cells were harvested to extract RNA followed by RT-qPCR to determine steady state levels of TSC2 mRNA. mRNA levels are normalized to actin mRNA levels. (B) Cells were harvested and lysed followed by immunoblotting to analyze the levels of P-T1387 TSC2, TSC2 and p53. Actin is used a marker for equal protein loading.
Figure 2-21. Carcinoma cells that are p53 null or that express only mutant p53 have decreased levels of TSC2 and Sestrin2 mRNA.

Cells were grown in the same conditions (RPMI + 10% dFBS) for 24 hours to allow the 60% confluency followed by RNA extraction, cDNA synthesis and RT-qPCR analysis to determine the levels of TSC2 (left panel) and sestrin2 (right panel). mRNA levels are normalized to actin (plotted as ±sd, n=3). Data suggests that either loss or mutation of p53 leads to decreased levels of these two mRNA species in both colon carcinoma cells HCT116 cells as well as in lung carcinoma cells H1299.
Figure 2-22. Protein levels of TSC2 and Setsrin2 are reduced to great extent upon mutation in p53 and is comparable to loss of p53.

Cells were grown in the same conditions (RPMI + 10%dFBS) for 24 hours to allow the 60% confluency. Cells were harvested and lysed. Immunoblotting was performed in order to analyze the levels of TSC2. Actin is shown as a control for equal protein loading across the samples. Cells expressing mutant p53 or p53-/- cells have remarkable decrease in TSC2 level (A) HCT116 cells, (B) H1299 cells.
2.3.9  Complementation of the control of the mTORC1 pathway in p53 null cells by TSC2 and Sestrin2.

Because of the centrality of TSC2 to the control of mTORC1 kinase activity and to the role of Sestrins in the activation of TSC2 by AMPK (A. V. Budanov & Karin, 2008a), we questioned whether the hyperactivity of mTORC1 seen in p53 -/- HCT116 cells was due to the lower levels of TSC2 and Sestrin2. When FLAG-tagged Sestrin 2 or HA-TSC2 were individually transfected into p53 -/- HCT116, the phosphorylation of S6K1 was reduced almost to the levels seen in wt HCT116; when both constructs were co-transfected, the level of p-T389 S6K1 was identical to that in wt HCT116 (Fig. 2-23A). Likewise, transfection of TSC2 or Sestrin2 or of both constructs into p53 -/- HCT116 returned the level of 4EBP1 seen in 7mGTP pulldowns back to that seen in p53 +/- HCT116 (Fig 2-23B). We concluded that the enhanced mTORC1 activity seen in p53 null cells was due to diminished function of TSC2 due to lower levels of TSC2 itself and of lower levels of Sestrin2.
Figure 2-23. Complementation of the control of the mTORC1 pathway in p53 null cells by TSC2 and Sestrin2.

Increasing the levels of TSC2 and Sestrin2 in p53 null cells reverses the elevated mTORC1 activity in p53 null cells. (A) FLAG-tagged Sestrin2, HA-TSC2, or an empty vector were transfected into p53−/− HCT116 cells and p-T389 S6K1 was determined by immunoblotting. (B) Exogenous TSC2 and Sestrin2 restores 7mGTP cap binding in p53 null cells. 7mGTP pulldown assays were performed on p53−/− HCT116 cells following transfection as in (A); bound 4EBP1 and eIF4E were detected by immunoblot.
Figure 2-24. Proposed model depicting the mechanism of p53 mediated regulation of mTORC1.

A) p53 wt cells have more TSC2, more PRAS40, less Rheb and less mTORC1 complex present at lysosomal membrane. Due to higher TSC2 levels Rheb mediated activation of mTORC1 will decline, causing lower mTORC1 activity in p53 wt cells. B) In the absence of p53, TSC2 levels are low, PRAS40 levels bound to mTORC1 are low and levels of Rheb on the lysosomal membrane is high, leading to higher mTORC1 activity.
2.3.10 Discussion

The single most frequent genetic change in human tumors is mutation of the p53 gene at positions that alter the transcriptional activity of the protein. Typically, mutation in one allele is followed by a loss of the other allele, with major changes in levels of transcriptional targets of the wt protein. As a less frequent event, some tumors have lost both alleles of this gene. We show here that either mutations of p53 that modify its transcriptional function or complete loss of the gene upregulates mTORC1 activity. The intermediate step that mediates this upregulation appears to be lower expression of TSC2, a previously suggested transcriptional target of p53 (Feng et al., 2007) and Fig. 20, 21, 22). The fact that replacement of TSC2 levels by transfection reverses the effect of p53 loss on mTORC1 activity argues for this causal relationship. These observations are supported by the studies done by my colleague Catherine Bell. In her studies of subcellular fractionation and confocal microscopy, she showed that p53 null cells have lower levels of TSC2 and increased levels of Rheb localized in the lysosomal membrane, the site of mTORC1 activity (data not shown). This observation suggested that the decreased levels of TSC2 in p53 null cells resulted in a lower distribution of TSC2 to lysosomal membranes and that this effect is, surprisingly, associated with an enhanced level of Rheb in fractions containing both mTOR and Raptor, presumably representing the active mTORC1 complex (Fig. 2-24). These differences in lysosomal localization of TSC2 and Rheb are more obvious in cells completely lacking TSC2 expression, i.e., TSC2-null MEFs (Experiments done by Ms. Bell and data not shown). The current literature suggests that redistribution of TSC2 to lysosomal membranes plays an intrinsic role in control of mTORC1 activity. Interestingly, by causing a primary decrease in TSC2 levels at the lysosomal membrane, we also observed the redistribution of Rheb (experiment of Catherine Bell, data not shown). Two recent papers suggest the involvement of
another p53 target, the Sestrins, in mTORC1 localization at lysosomal membranes (Parmigiani et al., 2014). We observed the reinstatement of control of mTORC1 following exogenous expression of Sestrin2 in p53 null cells, apparently a reflection of the enhanced efficiency of the residual levels of TSC2 in p53-null cells in the presence of higher Sestrin2. This interpretation reflects the current view of a dynamic equilibrium of the binding of TSC2 at the lysosomal membrane, affecting mTORC1 activity.

We have used immunoprecipitation (this thesis), membrane fractionation (Catherine Bell) and confocal microscopy (Catherine Bell) to study effects of p53 on the distribution of components of mTORC1. All three approaches indicated that the decrease in TSC2 levels in p53 null cells is followed by a decreased occupancy of TSC2 at the position of active mTORC1. The effect of p53 loss on enhanced Rheb colocalization with mTORC1 was indicated by membrane fractionation and confocal microscopy. This phenomenon was also captured in IPs done at low (100mM) NaCl concentration in wash buffer, but not at high salt-wash buffer, as would be expected for a transient interaction. Decreases in PRAS40 were detectable in IPs, when these immunocomplexes were washed with lower levels of salt (Y. Sancak et al., 2007), as were higher distribution of mTOR into Raptor complexes; these redistributions were not obvious from membrane distribution studies or confocal microscopy, but reflected the changes in mTORC1 activity in p53 null cells.

The enhancement of mTORC1 we observed in p53 null cells was also seen in carcinoma cells expressing mutant p53s at the very high levels of this protein usually found for such mutations, a fact that emphasizes the centrality of the p53-driven transcriptional program in control of mTORC1 activity. There have been prior indications that AMPK-mediated TSC2 control of mTORC1 depends on p53 function and involves the p53 target Sestrin2. Feng et al
showed that stabilization of p53 by DNA damaging agents caused phosphorylation and activation of AMPK, leading to mTORC1 inhibition; these effects were TSC2 dependent (Feng et al., 2005). A recent study (H. Wang et al., 2008) demonstrated that this DNA damage-initiated effect on AMPK involved ATM effects on p53 and p63, as well as DNA-PK; these effects of DNA damage are clearly distinct from those reported here. The effect isolated by our studies is clearly not one which involves activation of p53 by a DNA damage effect, seen by Feng et al., but rather an activity mediated by the low steady state levels of wt p53. These effects, which are downstream of and not mediated by AMPK, represent distinctly different level of involvement of p53 in control of mTORC1 activity. Therefore, we concluded that loss or mutation of p53 enhances mTORC1 activity by decreasing the TSC2 expression and its localization at lysosomal membrane as depicted in Fig. 2-24.
Chapter 3

3 Involvement of p53 in pemetrexed-activated AMPK mediated inhibition of mTORC1

3.1 INTRODUCTION

In the previous chapter we determined the effects of p53 on mTORC1 regulation and concluded that p53 negatively regulates mTORC1 activity via regulating the expression of TSC2 and Sestrin2. We also showed that there were differences in the components of the mTORC1 complex in p53 +/+ and +/- cells, including more mTOR and less TSC2 and PRAS40 in p53-/- cell in comparison to p53 +/+ cells, which would contribute to the higher mTORC1 activity in p53 compromised cells. A very interesting finding by my colleague Catherine Bell, suggested that p53 competent cells have decreased Rheb and increased levels of TSC2 at the lysosomal membranes, apparently leading to decreased mTORC1 activity in these cells. It is clear from these data that, upon loss of p53, negative regulation of mTORC1 is relieved leading to an increase in cell growth and proliferation. This correlation of p53 and mTORC1 is evident in most cancers where function of WT p53 is diminished or lost and mTORC1 activity is high. As most cancers show hyperactive mTORC1, mTORC1 inhibition offers a very attractive and promising approach for targeted chemotherapy.
This chapter is focused to understand the molecular mechanism of an AMPK activator pemetrexed (PTX), which inhibits mTORC1. Our lab has shown that PTX inhibits AICARFT, a second folate dependent enzyme in de novo purine synthesis which when inhibited, causes robust buildup of its substrate ZMP (Racanelli, Rothbart, Heyer, & Moran, 2009; Rothbart, Racanelli, & Moran, 2010). ZMP is an AMP mimetic and thus can activate the central energy sensor of the cell, AMPK. Our lab also showed that this activation of AMPK by PTX inhibits mTORC1 in an LKB1 independent manner (Racanelli et al., 2009; Rothbart et al., 2010). Whereas the majority of lung cancers have a p53 null or p53 mutant phenotype, our studies in chapter two suggests that mTORC1 activity increases to a significant level upon loss or mutation of p53. Because PTX is active against lung cancers and also can inhibit mTORC1, we wanted to understand if PTX mediated inhibition of mTORC1 is modulated by p53 status. Therefore the focus of this chapter is to understand the role of p53 in PTX-mediated activation of AMPK and inhibition of mTORC1. In the pursuit of an understanding of how activation of AMPK by pemetrexed PTX would behave in the presence and absence of p53 function, we compared it with another AMPK activator, AICAR. This chapter also shines light upon the mechanism whereby two AMPK activators that initially activate AMPK via ZMP accumulation, can lead to two different downstream effects based on their ability to activate p53. Therefore, this chapter builds a bridge between our findings from chapter 2 and a long-standing question of how PTX is so effective against lung cancers. This chapter also lays a platform for understanding the effects of PTX on gain-of-function mutant p53 in chapter 4.

3.1.1 Accumulation and Stabilization of p53 by Post Translational Modifications (PTMs)

Normally, p53 is maintained at very low concentration due to its relative short half-life of approximately 20 minutes, much less than that of mut p53 (Strano et al., 2007). p53 is rapidly
turned over in unstressed cells through its interaction with MDM2, a RING family type E3 ligase, which promotes poly-ubiquitination and proteasomal degradation (Honda, Tanaka, & Yasuda, 1997). Therefore, after a genotoxic stress or a variety of other cellular insults, levels of p53 immediately increase by stabilization of the p53 protein. Kastan et al. showed that, after the genotoxic stress of IR-induced DNA damage, protein levels of p53 increased, apparently due to by a post-translational mechanism (Kastan, Onyekwere, Sidransky, Vogelstein, & Craig, 1991). Human p53 harbors an array of serine (S)/ threonine (T) phosphorylation sites that span the entire protein, but these sites are highly concentrated in the N terminal transactivation and C-terminal regulatory domains (Fig. 3-1). It has been shown that the phosphorylation of p53 at Ser15 and possibly at ser20 causes p53 accumulation by blocking the interaction between MDM2 and p53 thereby inhibiting its degradation (Shieh, Ikeda, Taya, & Prives, 1997; Siliciano et al., 1997; Toledo & Wahl, 2006). PTMs of p53 have been extensively investigated; modifications can include phosphorylation, acetylation, methylation, ubiquitination, adenylation and/or sumoylation (Reviewed in (Toledo & Wahl, 2006). After different cellular stresses, several of these PTMs are thought to act as a barcode dictating the multiple cellular functions of p53. p53 phosphorylation at the N terminus has been reported to have significant redundancy; a single residue can be phosphorylated by several kinases and one kinase can phosphorylate several residues (J. P. Kruse & Gu, 2009). This section will address the literature on those phosphorylation sites that are investigated in this chapter. Fig. 3-1 summarizes the known PTMs of p53. This chapter also compares the effects of two AMPK activators and a DNA damaging agent on the phosphorylation levels of several key p53 residues and what is known about the consequences of these phosphorylation events on p53 stability and transactivation is summarized below.
Figure 3-1. Post-translational modifications of p53.

A map of the post translational modifications of p53 where each residue known to be modified is shown. Phosphorylation (P) sites are indicated in yellow, acetylations (A) in pink, ubiquitination (U) in grey, methylation (M) in as blue and neddylation (N) in green. Red circles indicate the phosphorylation sites and green circles indicate acetylation sites, analyzed in the studies of this chapter.
Figure 3-2 Schema of mTORC1 pathway.

This is a schematic representation of AKT-mTORC1-AMPK pathway. Black arrows show activating phosphorylation events and red arrows show inhibitory events.
3.1.1.1 Serine 15 and serine 20

The most studied post-translational modifications of p53 are serine 15 and serine 20 phosphorylation. These phosphorylation sites have had the attention of the p53 community not only because the biology of these phosphorylation sites is complex but also because they are believed to be crucial in manipulating the stability and trans-activation of p53 (Siliciano et al., 1997; Toledo & Wahl, 2006). The E3 ligase, MDM2, interacts with amino acids 18-23 of p53, and phosphorylation at serine 15 is thought to block this interaction and promotes the accumulation of p53 (Shieh et al., 1997). However, there is a diverse and contradictory literature about the importance of ser15 phosphorylation in the transactivation of p53. Fuchs et al suggested that phosphorylation of serine 15 is not required for p53 accumulation or transcriptional activation but rather that S15 phosphorylation enhances subsequent phosphorylations on neighboring residues (Fuchs, O'Connor, Fallis, Scheidtmann, & Lu, 1995). Serine 15 lies within a nuclear export signal and phosphorylation of S15 results in nuclear retention of p53 (Zhang & Xiong, 2001). Lambert et al. showed that after IR, phosphorylation of serine 15 increases which in turn increases the ability of p53 to recruit and associate with the transcriptional co-activator proteins CBP and p300 (Lambert, Kashanchi, Radonovich, Shiekhattar, & Brady, 1998). The acetyltransferase activity of p300 recognizes the C-terminal domain of p53 as a substrate resulting in acetylation at lysines 373 and 382 enhancing the sequence-specificity of p53 binding to DNA (Gu & Roeder, 1997a). Several kinases have been linked to the phosphorylation of serine 15, including ATM, ATR, AMPK, DNA-PK, ERK, p38 and CDK9 (Reviewed in (Toledo & Wahl, 2006). Phosphorylation of serine 20 also aids in p53 accumulation and nuclear retention in a similar fashion as was described for serine 15.
phosphorylation, as both residues lies within the nuclear export sequence region. However, it has been reported that S20 phosphorylation also is not required for p53 stabilization and activation (Chehab, Malikzay, Stavridi, & Halazonetis, 1999). Chk1, among several other kinases, have been identified to be capable of phosphorylating serine 20 and, rather surprisingly, other than facilitating p53 stabilization, no further functions of this modification have been elucidated (Shieh, Ahn, Tamai, Taya, & Prives, 2000a). Studies with mice containing serine to alanine mutations in p53 at ser15 or ser 20 or both showed the redundancy in the functional requirement of the phosphorylation of these two residues. Individual mutations in gene knock-in experimental mice only showed a marginal difference in the stability and transactivation activity of p53. However, mice bearing both ser15/20 mutations of showed a more severe phenotype, including tissue specific reduced apoptotic capacity, compromised replicative senescence and a latent development of a spectrum of tumors (Chao, Herr, Chun, & Xu, 2006; Toledo & Wahl, 2006).

3.1.1.2 Serine 37

Dohoney et al showed that, following DNA damage by UV- or γ-irradiation, p53 is stabilized and phosphorylated at a number of residues, including serines 15 and 37. Phosphorylation at serine 37 appears to be important in p53-dependent transcription after UV and IR treatment. Mutating this serine residue to an alanine significantly impairs the transactivating ability of p53 in a MDM2-luciferase reporter assay (Dohoney et al., 2004). A previous study in which the N-terminal domain of p53 was fused to the DNA-binding domain of the yeast GAL4 protein, showed that phosphorylation of S15, but not S37, is critical for p53-dependent transactivation (Dumaz & Meek, 1999)

Sequence-specific DNA binding of p53 is also enhanced when serine 37 is phosphorylated. Serine 37 phosphorylation has been reported to be important for the transactivation and pro-
apoptotic activities of p53 (Li et al., 2006). The phosphorylation of serine 37 was shown to stimulate the interaction between p53 and the transcriptional coactivators p300 and PCAF which acetylate p53 at lysine 382 and lysine 320, respectively, activating the sequence-specific DNA-binding of p53 (K. Sakaguchi et al., 1998).

3.1.1.3 Serine 46

Phosphorylation of serine 46 has been implicated in p53-dependent apoptosis. A study in which knock-in mice expressing the human p53 gene with a S46A mutation were compared with mice expressing the wild-type human p53 gene. These mice were generated by homologous recombination and LoxP/Cre-mediated deletion to introduce a Ser46 to Ala missense mutation into the human p53 knock-in allele in mice (p53hki(S46A)). This study suggested a modest reduction in p53 mediated transcription activation of some pro-apoptotic targets and compromised apoptosis in MEFs and embryonic stem cells from these mice (L. Feng, Hollstein, & Xu, 2006). When p53 S46 is mutated to an alanine, mouse embryonic fibroblasts (MEFs) and mouse embryonic stem cells had a reduced ability to induce apoptosis after UV treatment when compared to cells containing wild-type p53. Mayo et al. showed that during mild VP16-induced DNA damage, serine 46 is not phosphorylated and p53 thereby activates the transcription of MDM2 inducing the autoregulatory feedback loop. After extensive VP16-induced DNA damage, serine 46 is phosphorylated and the transcriptional activation by p53 becomes more extensive, for instance, PTEN transcription is activated and increased apoptosis was observed. These results were confirmed by genetic manipulation studies. A nonphosphorylatable serine 46 to alanine p53 mutant (S46A) targeted the MDM2 promoter in preference to that for PTEN. A serine 46 to aspartate mutant (S46D, a phosphorylation mimic) targeted PTEN in preference to MDM2. Thus this study concluded that phosphorylation of S46 is essential and sufficient for
choosing PTEN as a target gene over MDM2. This must be in order to overcome the induction of MDM2 and the formation of the autoregulatory feedback loop of MDM2-p53 (Mayo et al., 2005).

### 3.1.1.4 Serine 392

Studies done by Sakguchi et al. suggested that phosphorylation of serine 392 significantly enhanced tetramer stability, possibly through hydrogen bonds between the phosphorylated residue and the N-terminal region of p53 (K. Sakaguchi et al., 1997). DNA binding and transcription activation of p53 is also enhanced after phosphorylation of serine 392 during UV treatment (Keller, Zeng, Wang, Zhang et al., 2001a). Phosphorylation of the C-terminal S392 following UV-radiation activates specific DNA binding through stabilizion of the p53 tetramer (Matsumoto, Furihata, & Ohtsuki, 2006). For a long time it was believed that S392 phosphorylation is a response to DNA damage induced by UV radiation but a group recently showed that phosphorylation of Ser392 can also occur during the induction of p53 by a range of stimuli including treatment of cells with the MDM2 inhibitor, Nutlin 3a. (Cox & Meek, 2010). Phosphorylation of serine 392 appears to be important for the anti-proliferative activity of p53. When SV3T3 mouse cells were transfected with p53 containing an alanine at this residue, this mutant p53 was unable to suppress colony formation as compared to cells transfected with wild-type p53 (Milne, Palmer, & Meek, 1992).

### 3.1.1.5 Acetylation

Lysine acetylation is a powerful mechanism for activating function. It has been said to work at three levels in the case of p53: 1) it promotes p53 stabilization by blocking ubiquitination; 2) it inhibits the formation of the HDM2/HDMX repressive complex at the promoter of target genes
and thus alleviates promoter inhibition; 3) it assists in the recruitment of transcription cofactors at the promoter for p53 transcriptional activity. Of the nine acetylation sites have been identified for p53, six are lysine residues (K370, K372, K373, K381, K382 and K386) in the C-terminal regulatory domain. These sites are shown to be acetylated by CBP/p300 and ubiquitinated by HDM2 (J. Kruse & Gu, 2008; Nakamura, Roth, & Mukhopadhyay, 2000). Histone acetyl transferases which are responsible for these modifications include the structurally related p300, CBP, p300/CBP associated factors (PACF) and the MYST (named for the members MOZ, Ybf2/Sas3, Sas2 and Tip60) family of HATs, Tat-interactive Protein of 60 kDa (TIP60) and human Males absent On the First (hMOF) (J. Kruse & Gu, 2008; Sykes et al., 2006; Sykes, Stanek, Frank, Murphy, & McMahon, 2009). It has been shown that p53 can be modified by acetylation both in vivo and in vitro and the site of p53 that is acetylated by its co-activator, p300, resides in a C-terminal domain known to be critical for the regulation of p53 DNA binding. Therefore, the acetylation of p53 at this region can dramatically stimulate its sequence-specific DNA-binding activity, possibly due to an acetylation-induced conformational change (Gu & Roeder, 1997a; Luo et al., 2004).

A seventh lysine residue, K320, in the tetramerization domain is acetylated by PACF (J. Kruse & Gu, 2008). Acetylation of this residue promotes p53-mediated activation of Cyclin-Dependent Kinase inhibitor 1A (CDKN1A), commonly known as p21, results in cell cycle arrest and thus favors cell survival (Knights et al., 2006). More recent studies have discovered two new additional sites of acetylation, K120 and K164 which are in the DNA binding domain and are acetylated by TIP60/hMOF (Sykes et al., 2006) and CBP/p300 (Tang, Zhao, Chen, Zhao, & Gu, 2008) respectively. Nevertheless, despite all the studies suggested that acetylation of the C-terminal domain plays a critical role in p53 mediated processes, mice expressing acetylation-
deficient p53 generally did not exhibit any major depression in cell cycle control, apoptosis or tumor suppression (L. Feng, Lin, Uranishi, Gu, & Xu, 2005). This observation is in accordance with the fact that only rarely do human cancers have mutations in the C-terminal domain of p53 (http://p53/free.fr/).

3.1.2 Transcriptional Regulation by p53

As described in the previous section, p53 contains a DNA binding domain which specifically binds the p53 response element (p53RE) within promoter regions of its genes, and a C-terminal domain which has been shown to non-specifically bind DNA and then coordinate the linear search of DNA for p53REs (el-Deiry, Kern, Pietenpol, Kinzler, & Vogelstein, 1992; McKinney, Mattia, Gottifredi, & Prives, 2004). This section will briefly describe the mechanisms whereby p53 regulates gene transcription.

3.1.2.1 p53 Response Element

The p53RE contains two copies of the 10 bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 10-13 bps where Pu is a purine base and Py is a pyrimidine base (el-Deiry et al., 1992; Miner & Kulesz-Martin, 1997). It has been shown that tetrameric p53 is able to recognize, bind and transactivate genes from noncanonical consensus sequences containing only half of the consensus sequence (Jordan et al., 2008). Overall, the literature suggests that different p53RE have different binding affinities for p53. A study using a red-white p53 reporter system in Saccharomyces cerevisiae measured the ability of p53 to transactivate a reporter gene from various p53REs. It was shown that there is as much as a 1000-fold difference between the transactivation from weaker p53RE to stronger p53RE. The p21 promoter has two p53Res located 1.2 and 2.4 kb upstream of the transcriptional start site. The sequence of the upstream
p21 p53RE has the highest affinity for p53, while the p53RE within the BAX promoter has the weakest affinity (Inga, Storici, Darden, & Resnick, 2002). The promoters of three other p53-regulated cell cycle genes, cyclin G, 14-3-3 and GADD45 also have quite high binding affinities, compared with that of the proapoptotic genes PUMA, BAX and p53AIP (Weinberg, Veprintsev, Bycroft, & Fersht, 2005). Posttranslational modifications of p53 and p53 binding partners also contribute to the diversity of the transcriptional response. For instance, the phosphorylation of serine 46 discussed above has been shown to selectively induce p53 to transactivate the proapoptotic gene p53-regulated Apoptosis Inducing Protein1 (p53AIP). In the latter study, the authors selected the p53 binding sequences from the human genome using in-silico analysis and cloned and found a novel mitochondrial protein designated as p53AIP. They showed that the expression of p53AIP can be induced by wt p53 after DNA damage in MCF-7 cells. The induction of p53AIP led to enhanced apoptosis and this phenomenon was dependent on the phosphorylation of p53 at Ser46 (Oda et al., 2000).

3.1.2.2 Transcriptional Initiation

For successful transcriptional initiation, the chromatin around a promoter must be modified to form an open chromatin structure (euchromatin) that allows the general transcriptional initiation machinery to bind to the DNA. The role of p53 in this process is critical. After p53 has bound to the p53RE, histone acetyltransferases (HATs) are recruited to acetylate the histones surrounding p53 (Lill, Grossman, Ginsberg, DeCaprio, & Livingston, 1997). P300/CBP are recruited to the promoter following the interaction of CBP with the transactivation domain of p53. p300/CBP acetylates the histones bound to DNA in the vicinity of p53, as well as the C terminal domain of p53, increasing the activation of gene transcription (Gu & Roeder, 1997b). After the opening of regional chromatin, the general transcription machinery can be recruited to form the preinitiation
complex (PIC). p53 is known to act with several components of the PIC to initiate transcription. The assembly of a complex containing p53, TBP, the TBP associating factors (TAF), TAFII60, TAFII40 and TAFII250 appears sufficient to activate transcription.

The composition of the PIC required for transcriptional activation differs between genes as well as between stress stimuli promoting transcription of a particular gene. Two examples of this are the p21 and FAS promoters. Espinosa et. al showed that prior to any cellular stress, p53 is poised at the p21 promoter along with components of the PIC, TBP and paused RNA polymerase (Espinosa, Verdun, & Emerson, 2003). After UV stress, levels of p53 bound to the promoter increase, leading to completion of the transactivation process by p53-dependent recruitment of TAFII250. This stimulates the phosphorylation of serine 2 in the C terminal domain (CTD) of the paused RNA polymerase, converting it to the elongation form. In contrast to p21, the FAS promoter does not contain a TATA box. Prior to any stress, p53 is also poised on the FAS promoter, but the levels of paused RNA polymerase are significantly lower than found at the p21 promoter (Espinosa et al., 2003). After UV treatment, p53 levels increase, recruiting HATs and TAFII250 to the promoter. There is no net loss of paused RNA polymerase at the promoter after the appearance of the elongation form, suggesting that the initiation and elongation rates were similar. The difference between these two promoters demonstrate a strategic placement of p53 on the gene promoters depending on their role in cellular mechanisms. Cell cycle genes, such as p21, are poised for rapid transcriptional activation after cellular stress while the transcriptional activation of the proapoptotic gene Fas is slower due to the lower levels of RNA polymerase poised at the promoter (Espinosa et al., 2003).

3.1.2.3 Transcriptional Elongation

After RNA polymerase is cleared from the promoter, other elongation factors are recruited to the
promoter vicinity, some of which interact with p53. The phosphorylation of serine 392 of p53 is often implicated in complexes with elongation factors and, as described above, this phosphorylation promotes oligomerization of p53 and subsequent DNA binding (Hupp, Meek, Midgley, & Lane, 1992; K. Sakaguchi et al., 1997). During the discovery that casein kinase 2 (CK2) is able to phosphorylate serine 392 of p53 after UV treatment, p53 was found to be in a complex with subunits from the FACT (facilitated chromatin transcription) complex, a known elongation factor (Keller, Zeng, Wang, Zhang et al., 2001b). It is also shown that the human p-TEF (positive transcription elongation factor) is responsible for converting the paused RNA polymerase to elongating polymerase through phosphorylation at serine 2 in the carboxy terminal domain (Marshall, Peng, Xie, & Price, 1996). The Cdk9 kinase is the component of p-TEF responsible for phosphorylating serine 5 of the CTD of RNA polymerase (Ramanathan et al., 2001; Zhu et al., 1997). Cdk9 is known to interact with p53 and phosphorylate it at serine 392; the stabilized p53 then promotes transcription of Cdk9, thereby promoting transcriptional elongation in a feed-forward system (Claudio et al., 2006).

3.1.2.4 Transcriptional Repression

A less studied role of p53 is transcriptional repression. After binding to some promoters, p53 interacts with mSin3a recruiting HDAC1 (histone deacetylase), which removes the acetyl moieties from the chromatin causing the chromatin to close around the promoter and repressing transcription (Murphy et al., 1999). The physical binding of p53 to promoters may restrict the binding of other transcription factors required for transcriptional activation. This is observed during hypoxic stress: under these conditions p53 binds to the alpha-fetoprotein promoter, inhibiting the binding of the transcriptional activator HNF3 and, hence, repressing transcription (Y. I. Lee et al., 2000). Although the mechanism of transcriptional repression is not understood
yet, the binding of p53 to the Rad51 promoter, repressing transcription of this gene, is critical to homologous recombination during DNA damage (Arias-Lopez et al., 2006).

### 3.1.3 p53 Dependent Apoptosis

Yonish et al. demonstrated the importance of p53 in the mechanism of apoptosis when they showed that tumor cells null for p53 underwent spontaneous cell death after the introduction of a wild-type p53 by transfection, suggesting that p53 was involved in the apoptosis pathway (Yonish-Rouach et al., 1991). Bax was found to be a direct transcriptional target of p53 and the first example of a proapoptotic gene directly regulated by p53 (Miyashita & Reed, 1995). The restoration of p53 in murine leukemia cells caused an increase of Bax mRNA and protein levels (Selvakumaran et al., 1994). Since then several other pro-apoptotic genes have been identified that are regulated by p53, including p53AIP1, APAF1, Caspases 1, 6 and 10, FAS, PUMA, DR4 and DR5. Upon binding to the promoters of these genes, p53 activates transcription, thereby promoting increased levels of the downstream components of the apoptosis cascade (Reviewed in (Riley, Sontag, Chen, & Levine, 2008). Evidence for a role of p53 in the regulation of apoptosis that was independent of transcription was first hypothesized after transcriptionally inactive mutants of p53 were found to induce cell death (Haupt, Rowan, Shaulian, Vousden, & Oren, 1995).

### 3.1.4 p53 senses cellular stresses induced by chemotherapeutic agents

Understanding of p53 as a cellular stress response protein, it was of interest to know how p53 responds to stresses induced by chemotherapeutic agents. Most cellular responses by p53 have been studied after IR or UV exposure. Ionizing radiation, camptothecin and bleomycin directly
and rapidly induce DNA strand breaks, promoting the accumulation of significant levels of p53 within one hour of treatment. Cells electroporated with DNaseI or the restriction enzyme AluI also accumulated p53, leading to the conclusion that DNA strand breaks initiate p53-dependent signaling pathways (Nelson & Kastan, 1994). Further analyses showed that as little as one DNA strand break per cell was sufficient to induce a p53 dependent growth arrest (Huang, Clarkin, & Wahl, 1996).

Much less is known about the p53 response to nucleotide pool deprivation. Linke et al, for the first time proposed the concept that p53 could sense nucleotide levels and then would become stabilized (Linke, Clarkin, Di, Tsou, & Wahl, 1996). In their experiments, WS1 embryonic skin cells containing wild-type p53, treated with antimetabolites inhibiting different parts of de novo pyrimidine and purine synthesis resulted in a G1 cell cycle arrest. However, p53 null cells slipped into early S-phase and then arrested. Interestingly, dNTP biosynthesis inhibitors caused arrest in early S-phase regardless of p53 status. In the studies of Linke and colleagues, the G1 arrest was reversed upon the addition of the limiting nucleotides and no apparent DNA damage was detected by metaphase chromosome analysis, therefore, the p53 dependent nucleotide deficiency arrest was not caused by DNA damage (Linke et al., 1996). Feng et. al have shown that DNA damage induced activation of p53 leads to phosphorylation and activation of AMPK in a p53 dependent manner (Z. Feng, Zhang, Levine, & Jin, 2005). Whereas finding of Jones et. al have suggested that upon metabolic stress, i.e upon glucose deprivation, AMPK activation leads to phosphorylation of p53 at ser15. This ser15 phosphorylation of p53 is required to induce AMPK mediated metabolic checkpoint and cell growth arrest (R. G. Jones et al., 2005a). These two studies suggest that connection of p53 and AMPK but with different theories. Former suggests that p53 mediated effects on mTORC1 are AMPK activation
dependent and later suggest that AMPK mediated effects on cell growth are p53 and p53 phosphorylation at ser15, dependent. Clearly these differences in the observation by two groups could be due to different type of cell stresses used in the studies. A section of this dissertation is dedicated to understand the response of p53 under the treatment of DNA damaging agent VP16 and two AMPK activators, PTX and AICAR.

### 3.1.5 ATM/ATR regulation of p53

To protect the genome from genetic assault caused by environmental factors like radiation, reactive oxygen species, ultraviolet light, and other environmental mutagens, cells have evolved complex mechanisms, collectively referred to the DNA damage response. This pathway acts to rectify DNA damage to allow the minimization of genetic infidelity. The cellular response to DNA damage involves both repair mechanisms and checkpoint responses. Checkpoint responses are thought to have evolved to delay the cell cycle progression in order to prevent error prone DNA replication. Many of the published studies on the involvement of p53 in the DNA damage response were done using radiation or DNA damaging agent such as etoposide (VP16). Etoposide is a topoisomerase inhibitor, which forms a ternary complex with DNA and the topoisomerase II. Topoisomerase II is a helicase, which unwinds the DNA during replication. Thus binding of VP16 with topoisomerase stabilizes DNA and topoisomerase II complex, prevents re-ligation of the DNA strands, and causes DNA double strand break.

ATM-Chk2 and ATR-Chk1 pathways play key roles in the DNA damage response (Sancar, Lindsey-Boltz, Unsal-Kacmaz, & Linn, 2004). ATM and ATR are large kinases with sequence similarity to the phosphatidylinositol-3-kinase (PI3K) family. Each of them phosphorylates multiple protein substrates (Abraham, 2001). The two key, well-studied substrates are Chk1 and Chk2 which are selectively phosphorylated by ATR and ATM, respectively. Chk1 and Chk2 are
serine-threonine checkpoint effectors, that is, they are themselves phosphorylated early in the checkpoint response, but then have activities as kinases, essential to the imposition of the G1/S, or G2/M phase blocks in cell-cycle traverse (Bartek & Lukas, 2003) dependent on the intensity and type of DNA stress.

The ATM–Chk2 and ATR–Chk1 pathways respond to different types of damaged DNA structures (Fig.3-3). ATM is recruited to and activated primarily at DNA double-strand breaks (DSBs) in conjunction with the MRE11:RAD50: NBS1 (MRN) sensor complex (J. H. Lee & Paull, 2005; Paull & Lee, 2005; Suzuki, Kodama, & Watanabe, 2005) whereas ATR is activated via recruitment to single-stranded DNA (ssDNA) upon single strand break in association with its partner protein, ATRIP (Dart, Adams, Akerman, & Lakin, 2004; Lupardus, Byun, Yee, Hekmat-Nejad, & Cimprich, 2002; Zou & Elledge, 2003). ATM and Chk2 are activated potently by radiation and genotoxins that induce DSBs, but only weakly, if at all, by agents that block DNA replication without inducing damage (Matsuoka et al., 2007). This piece of information is important for the understanding and explanation of the later section of this chapter. In response to DSBs, inactive subunits of ATM homodimerize, then autophosphorylate each other and dissociate to form partially active monomers (Bakkenist & Kastan, 2003; Kastan & Bartek, 2004). ATM autophosphorylated at S1981 is often taken as an indicator of its activation. Modification of this residue has been shown to be involved or at least linked to ATM activation under most circumstances (Bakkenist & Kastan, 2003; Kastan & Bartek, 2004). However, this residue is not essential for ATM function, at least in mice (Pellegrini et al., 2006). ATM monomers are then recruited to the DSBs site with the MRN sensor complex where they can locally act on multiple substrates. Of the several substrates for ATM, the two most important, or at least the most studied, are H2AX (Fernandez-Capetillo, Lee, Nussenzweig, & Nussenzweig,
2004) and ChK2 (Lukas, Falck, Bartkova, Bartek, & Lukas, 2003).

Figure 3-3. Schematic representation of activation of ATM-Chk2 and ATR-Chk1 pathway following genotoxic stress.

The ATM-Chk2 and ATR-Chk1 pathways are activated selectively by DSBs and by tracts of ssDNA, respectively. Here, for clarity and simplicity multiple proteins involved in the DNA repair mechanism are represented as DRPC = DNA repair protein complex. Studies in later part of this chapter suggest that AICAR or VP16 treatments lead to DNA damage activating p53 and its transcriptional activity, however, PTX might cause replication halt leaving p53 transcriptionally inactive. This concept is presented here in the form of question marks in red color.
ATM phosphorylates Chk2 on T68, a residue located within an N-terminal serine/threonine (SQ/TQ) rich motif (Ahn, Li, Davis, & Canman, 2002). Once the SQ/TQ motif of one Chk2 molecule is phosphorylated, it interacts with the phosphopeptide-binding Fork-head associated (FHA) domain of another, homodimerize transiently; autophosphorylation ensues and ATM achieves a fully activated state (Ahn et al., 2002; Cai, Chehab, & Pavletich, 2009; Oliver et al., 2006). It has been suggested that these activated Chk2 monomers dissociate from the site of damage and disperse throughout the nucleus to act on several substrates involved in the response to DNA damage, which includes proteins involved in cell cycle progression, gene transcription, and apoptosis (Lukas et al., 2003). One of the most studied substrates of Chk2 kinase is the p53 protein, which was shown to be phosphorylated at ser15 by Chk2 (Chehab, Malikzay, Appel, & Halazonetis, 2000; Oliver et al., 2006; Shieh, Ahn, Tamai, Taya, & Prives, 2000b). ATM has been thought to activate p53 by directly phosphorylating it upon DNA damage. ATM also phosphorylates MDM2 and MDMX, which regulate the stability of p53, and thus is thought of as responsible for the stabilization of p53 upon DNA damage (Chen, Gilkes, Pan, Lane, & Chen, 2005; Lavin & Kozlov, 2007). There is also increasing evidence that supports the concept that ATM may have substrates and function in the cytoplasm (Lavin, 2008).

ATR–Chk1 signaling is also thought to be activated upon blockage of DNA replication. Replication fork stalling generates ssDNA directly; however, this structure can also arise through the action of nucleotide excision repair (NER) or at dysfunctional telomeres. As a result of nucleotide depletion or DNA damage lesions caused by ultraviolet light, blockage of DNA replication can occur, which causes the uncoupling of DNA polymerase from the replication helicase, generating ssDNA tracts (Byun, Pacek, Yee, Walter, & Cimprich, 2005). These ssDNA
tracts are rapidly coated by trimeric ssDNA binding protein complex and replication protein A (RPA) and Chk1 are recruited at these tracts with ATR-interacting proteins (ATRIP) (Zou & Elledge, 0626). However, it is important to remember that the ATR-Chk1 pathway is also activated in response to DSBs after which nucleolytic strand resection exposes ssDNA. Serine 15 of p53 is phosphorylated by ATR after IR, UV or HU treatment. Kinase inactive mutants of ATR interfere with phosphorylation of p53 in late S-phase during IR treatment and completely suppress p53 phosphorylation after UV treatment, suggesting an important role of ATR in the S-phase checkpoint (Tibbetts et al., 1999). DNA breaks induced by camptothecin induce ATR which in turn phosphorylates H2AX, an event required to recruit DNA repair proteins Mre11, Rad50 and NSB1 (MRN) (Furuta et al., 2003). As the DSB response can be generated by replication of damaged DNA, when leading strand DNA polymerase encounters single strand nicks, it leads to activation of both ATM-Chk2 and ATR-Chk1 pathway. Thus, the effects of ATM and of ATR are not mutually exclusive, especially when cells are exposed to genotoxic stress, inducing ionizing radiation and most of the cytotoxic chemotherapy agents.

DNA damage induces cell cycle delays or arrest at the G1/S and G2/M transitions (the G1 and G2 checkpoints), and also causes a transient decrease in the rate of DNA synthesis (the intra-S checkpoint). All these cell cycle delays are dependent on and carried out by different proteins including p53, although it has been suggested that ATM and ATR have overlapping targets.

3.2 FOCUS OF THIS CHAPTER

In this chapter, I investigate the differential dependence of two AMPK activators on p53 in the mechanisms whereby they inhibit mTORC1. Surprisingly, while comparing these AMPK activators, AICAR and PTX, we found that even after the similar initiation mechanism of AMPK
activation (ZMP accumulation), some of the downstream signaling differs between the two compounds, while some remains the same. We showed that whereas PTX-activated AMPK is a p53 and thus TSC2-independent inhibitor of mTORC1, AICAR-activated AMPK leads to the activation of TSC2 and thus suggests the involvement of TSC2 in the AICAR mediated inhibition of mTORC1. It is also shown that this difference is due to the differences in p53 transcriptional activity following treatment with these drugs. AICAR causes an increase in the levels of transcriptionally active p53, thus, allowing the p53 dependent increase of TSC2 and sestrin2 levels, followed by AMPK-mediated (Sestrin2 is required for AMPK mediated phosphorylation of TSC2 at Ser1387 (Gwinn et al., 2008; R. J. Shaw et al., 2004) activation and phosphorylation of TSC2. On the other hand, p53 accumulating after PTX is transcriptionally compromised. Even under the conditions of limited TSC2 function and hyperactive mTORC1, PTX is effective and sufficient for inhibition of mTORC1 activity. This is due to the p53-independent, AMPK-mediated phosphorylation of S792 Raptor by PTX, which is shown to be necessary and sufficient for mTORC1 inhibition.

In order to understand the underlying mechanism of differential behavior of p53 under these two AMPK activators, we compared these effects of these AMPK activators with those coming on after treatment with the control DNA damaging agent, etoposide (VP16). We found very interesting differences and similarities in the posttranslational modifications of p53 under these treatments. We also observed the differences of the ATM/ATR mediated Chk2/ Chk1 activation which may explain the differences in the p53 transcriptional activity under these drug treatments.
3.3 RESULTS

3.3.1 Overactive mTORC1 in p53 null cells is suppressed by PTX.

HCT116 p53 +/- and p53-/- cells were treated with PTX in the presence of TdR in order to circumvent the effect of PTX on thymidylate synthase and restrict its effects to AMPK activation and downstream mTORC1 signaling. Phosphorylation of the downstream targets of mTORC1 was suppressed in both HCT116 cells and their isogenic p53-null derivative after PTX treatment, in response to activation of AMPK (Fig. 3-4). PTX activates AMPK to the same extent in both p53+/+ and p53-/- HCT116 cells as determined by assessing phosphorylation using p-Thr172AMPK antibodies (Fig 3-6). The phosphorylation of T389 on S6K1 was diminished by PTX treatment in both HCT116 p53 (+/+) and (-/-) cell lines (Fig. 3-4). An immunoblot with phosphospecific antibody against 4EBP1 phosphoT70 indicates equivalent levels of suppression of mTORC1 activity following PTX treatment of HCT 1116 cells with and without p53 function (Fig 3-4). 4EBP1 detected with a pan-antibody showed multiple and much lighter bands in untreated p53 null cells in comparison to wt p53 cells (Chapter 2-Fig. 2-5, Fig. 3-4) and (Holz, Ballif, Gygi, & Blenis, 2005) and these bands shifted towards lower phosphorylation state after PTX treatment in both p53 -/- and p53 +/- cells. Using RT-qPCR, it was also noted that the level of 4-EBP1 mRNA was significantly lower in cells null for p53 than in wild-type cells (Fig. 2-5B). Overall, it appears that PTX can inhibit mTORC1 activity independent of p53 status at the level of both of its substrates, S6K1 and 4EBP1(Fig. 3-4). The effect of PTX on 4EBP1 binding to capped mRNAs was assessed using the 7mGTP bead pull down assay; the results of these experiments suggested that the levels of 4EBP1 bound to 7mGTP beads are increased substantially after PTX treatment, independent of p53 status (Fig. 3-5), but that the amount of 4-EBP1 accumulating in p53 null cells on capped RNA is decreased when p53 is lost.
Nevertheless, treatment with PTX inhibits mTORC1 in a p53-independent manner, preserving levels of hypophosphorylated 4EBP1 and prolonging the integrity of 4EBP1-eIF4E complexes. As hypophosphorylated species are the active inhibitor of cap-dependent translation initiation and are not targeted by the proteasome, the binding of this species of 4EBP1 increased on 7mGTP beads, which mimic the 7mGTP cap of mRNA (Fig 3-5). Once again, the levels of 4EBP1 bound to beads in lysates from TdR-treated p53 WT cells is quite a bit higher than that from TdR-treated p53 null cells, as shown previously in untreated p53 isogenic HCT 116 (Chapter 2-Fig. 2-5A). Overall, the effects of PTX on S6K1 phosphorylation are strong but the effects on 4EBP1 seemed to be more extensive and are probably a greater contributing factor for inhibition of mTORC1 signaling to cap-dependent protein synthesis initiation, in agreement with experiments previously performed by Scott Rothbart in this laboratory (Rothbart et al., 2010).
Figure 3-4 Overactive mTORC1 in p53 null cells is suppressed by PTX. PTX activates AMPK in p53 null cells and can reduce mTORC1 activity in these cells.

p53+/+ and p53/- HCT116 cells were treated with 1 µM PTX + TdR for 24 hrs. AMPK activation and phosphorylation of S6K1 and 4EBP1 were assessed by immunoblotting.
Figure 3-2. PTX mediated increase in the 4EBP1 binding to 7mGTP beads is p53 status independent.

HCT116 wt and p53 null cells were treated with 1 µM PTX + TdR for 24 hrs. m7GTP pull down was performed using sepharose beads bound to m7GTP mimicking mRNA. Pulldowns were immunoblotted to analyze the levels of bound 4EBP1 to the beads. Lysates were probed to analyze the phosphorylation of S6K1 and 4EBP1.
3.3.2 Unlike AICAR, PTX treatment does not lead to activation of TSC2 by phosphorylation at Ser1387

AMPK is thought to negatively affect mTORC1 by two signals: activation of TSC2 GAP activity following phosphorylation of TSC2 S1387 (Garami et al., 2003; Sato, Nakashima, Guo, & Tamanoi, 2009; Tee, Manning, Roux, Cantley, & Blenis, 2003) and direct inhibition of mTORC1 kinase by phosphorylation of Raptor at S792 (Gwinn et al., 2008). These events were monitored after PTX- or AICAR treatment of HCT116 cells. The phosphorylation of AMPK at T172 in either p53 WT or null HCT116 cells was equivalent after either PTX or AICAR and was not p53-dependent (Fig 3-6). The AMPK-dependent phosphorylation of TSC2 at S1387 was seen in HCT116 cells with WT p53 after AICAR treatment (Fig. 3-7A), but not after PTX plus thymidine (Fig. 3-7B). In fact, the phosphorylation of S1387 of TSC2 was diminished in PTX-treated wt HCT116 cells, and the p-S1387 immunoblot had to be exposed longer to detect any signal. The level of p-S1387 TSC2 in treated cells was clearly dependent on p53 function: neither TSC2 nor p-S1387 TSC2 was easily detected in AICAR- or PTX-treated p53 -/- HCT116 (Fig. 3-7A,B). There was an increased expression of TSC2 in AICAR-treated p53-wt cells (Fig. 3-7A), which was not seen in p53 wt cells treated with PTX (Fig. 3-7B); this effect proved to be due to differences in p53 transactivation after these drugs (discussed below). We drew the conclusion that there was a defect in AMPK signaling to TSC2 in p53-null cells and PTX treated cells due to a deficiency of TSC2. In contrast, robust inhibitory phosphorylation of Raptor was observed after either AICAR or PTX and was unaffected by deletion of p53 in HCT116 cells (Fig. 3-7). We observed some interesting effects in these experiments, which raised some intriguing questions; 1) why does PTX-activated AMPK not phosphorylate TSC2, 2) why does
PTX not cause an increase in TSC2 protein levels when TSC2 is clearly a target of p53 (Fig. 2-18 to 22, and Fig. 3-7B), and 3) How is PTX-mediated inhibition of mTORC1 independent of TSC2 activation? Our later studies suggested that the answers to questions 1 and 2 are related, as discussed below.

3.3.3 p53 stabilized by PTX is transcriptionally compromised

As shown previously (Z. Feng et al., 2007) Chapter 2, Fig 2-18 to 22), TSC2 is a transcription target of p53. When we observed that the levels of TSC2 increased in wt p53 HCT116 cells after AICAR treatment but not after PTX treatment and that p53 null cells have lower levels of TSC2, the levels of p53 and its stabilization by AMPK activators were studied. In order to understand the difference of effects of two AMPK activators on p53, the accumulation of p53 was examined in HCT116 cells exposed to either PTX in the presence of thymidine or AICAR and these levels were compared with those in cells treated with VP16, a widely used topoisomerase II inhibitor known to damage DNA. PTX or AICAR caused the accumulation of p53 in HCT116 cells that was similar to that seen in VP16 (Fig 3-8), although the levels of p53 in PTX-treated cells were usually somewhat lower that in AICAR or VP16. Likewise, AICAR caused a robust enhancement of the level of several proteins whose genes were known to be p53 transcriptional targets (Budanov et al., 0923; Z. Feng et al., 2007; Riley et al., 2008), namely p21, sestrin 2, human mdm-2, and Bax (Fig. 3-8), and TSC2 (Fig. 3-7A), in a pattern identical to that seen after DNA damage. Very surprisingly, the level of these proteins did not change in cells treated with PTX (Figs 3-8, 3-7B respectively). RT-qPCR studies indicated that the steady-state levels of mRNA for these genes reflected the protein levels seen after each drug; i.e., VP16 and AICAR augmented the levels of p21, Sestrin 2, and TSC2. The steady state levels of human MDM2, PUMA, PIG3, BAX, well known transcriptional targets of p53 mRNAs also showed a modest to
substantial increase after VP16 and AICAR but there was no change in the mRNA for these p53 target genes after PTX (Fig 3-9). Thus, the transcriptional response of the p53 that accumulated in PTX-treated cells, was severely compromised. Budanov et. al have shown that Sestrin2 promotes the AMPK-mediated activation and phosphorylation of TSC2 at Ser1387. Because PTX-activated AMPK does not phosphorylate TSC2 at Ser1387, it seemed that levels of TSC2 and Sestrin2 were not sufficient for AMPK-mediated phosphorylation of TSC2.
PTX and AICAR mediated AMPK activation is p53 status independent.

PTX and AICAR both activate AMPK in cells with and without p53 to the same extent. p53+/+ and p53-/- HCT116 cells were treated with 1 µM PTX + TdR for 24 hrs or with 250 µM AICAR. AMPK activation was assessed by immunoblotting using antibody against P-ser172 AMPK, where ND= no drug treatment.
Figure 3-4. PTX and AICAR mediated AMPK leads to differential signaling downstream of AMPK.

PTX and AICAR both activate AMPK in cells with and without p53 to the same extent. p53+/+ and p53−/− HCT116 cells were treated with (A) 1 µM PTX + TdR (B) 250 µM AICAR for 24 hrs. AMPK mediated phosphorylation of TSC2 and Raptor was detected by using anti- P-ser1387 TSC2 and P-ser792 Raptor antibodies by immunoblotting. Total TSC2 and Raptor were also detected.
Figure 3-5. PTX causes accumulation of p53 but does not activate p53-dependent transcription.

Wild-type HCT116 cells were treated with VP16, DMSO, or TdR, or PTX + TdR, or AICAR (500 µM) for 24 hrs. Cell lysates were probed for p53 and its transcriptional targets p21, Sestrin2, HDM2, and BAX by immunoblot. PTX causes accumulation of p53 but does not activate p53-dependent transcription.
Figure 3-6. PTX stabilized p53 does not activate transcription of its target genes.

Wild-type HCT116 cells were treated with VP16, DMSO, or TdR, or PTX + TdR, or AICAR (500 µM) for 24 hrs. Total cell RNA was harvested followed by cDNA synthesis and q-RT PCR to analyze steady state levels of mRNAs of p53 transcription targets; p21, MDM2, PUMA, PIG3, BAX, TSC2 and Sestrin2. Three independent biological repeats combined and expressed (±sd) and P values <.0001.
3.3.4 PTX effects on mTORC1 are TSC2 and Sestrin2 independent.

TSC2 and Sestrin2 are two genes involved in the negative regulation of mTORC1 that are p53 transcription targets (Budanov et al., 0923; Z. Feng et al., 2007) chapter 2). As shown in Fig. 3-7B, after PTX treatment p53 transcriptional activity is compromised, an effect that mutes TSC2-mediated inhibition of mTORC1. We addressed if PTX effects were dependent on TSC2 and sestrin2 levels. First, p53-/- MEFs and p53-/-, TSC2 -/- MEFs were treated with PTX and mTORC1 signaling was observed at the level of pThr389 S6K in order to assess the effect of deletion of TSC2. mTORC1 activity was equally inhibited after PTX treatment in MEFs whether or not they expressed TSC2, in spite of the fact that TSC2-/- MEFs showed higher mTORC1 activity than TSC2 WT MEFs (Fig. 3-10A). In related experiments, the effects of PTX were compared in MEFs from WT or Sestrin 2 -/- mice; PTX mediated inhibition of mTORC1 was unaffected by the genetic loss of Sestrin2 (Fig. 3-10B), again, in spite of the fact that mTORC1 activity was higher in cells that had lost Sestrin 2 function. Because genetic deletion of TSC2 is a lethal event unless done in a background of p53-/- mice, the above experiments only allowed test of the effects of loss of TSC2 in a p53 null background. Hence, we depleted TSC2 in p53 wild type HCT116 cells with pools of siRNA to isolate the effect of TSC2 loss itself from the loss of p53. Untreated HCT116 cells in which TSC2 was depleted with siRNA had a substantial increment in mTORC1 activity as seen from the increased phosphorylation of S6K1 and 4EBP1 (Fig. 3-11). Nevertheless, the hyperactive mTORC1 in p53 wt HCT116 cells with siRNA-depleted TSC2 was suppressed by PTX (Fig. 3-11), as was also seen in p53-null HCT116 cells (Fig 3-4), TSC2 null MEFs in a p53 null background (Fig. 3-10A.), and Sestrin2-null MEFs (Fig. 3-10B). We concluded that enhanced activity of mTORC1 in p53 null cells is caused by the p53 dependence of transcription of TSC2 and Sestrin2 (Chapter
2), but that PTX controlled mTORC1 even in cells null for TSC2, Sestrin 2 or p53 function.

3.3.5 PTX effects on cell growth are not affected by p53 or TSC2 loss, AICAR effects are.

WT HCT116 cells and their p53-null derivative were treated with PTX in the presence of thymidine to determine whether p53 loss altered growth suppression by activated AMPK. These experiments were also done in the absence of thymidine to determine the effect of p53 on PTX activity against thymidylate synthase and activation of AMPK, conditions germane for clinical use of this drug. There was no difference in the response to PTX between the p53 wild type and +/- HCT116 cell lines under either condition (Fig. 3-12A), although a decrease in sensitivity in the presence of thymidine was observed, as expected from previous experiments in this laboratory (Rothbart et al., 2010). This would concur with the observation that PTX treatment in the presence (Fig 3-8) or absence of thymidine (see below) did not activate the transcriptional program of p53. In contrast, HCT116 cells wt for p53 function were more sensitive to AICAR than HCT116 null for p53 (Fig. 3-12B), in accord with the fact that p53 increased levels of TSC2 in AICAR-treated cells, while PTX did not (Fig. 3-7). Nearly identical results were found using MEFs with and without p53 and TSC2 function (Fig. 3-12 C,D). These observations suggested that PTX mediated inhibition of mTORC1 was independent of p53 and TSC2 function, and this fact was evident even at the level of cell growth. We concluded that suppression of growth by AICAR in carcinoma cells would be muted by loss of p53, but that the loss of p53 or TCS2 would be without effect on therapeutics with PTX.
Figure 3-7. Overactive mTORC1 in p53 null cells is suppressed by PTX, independent of TSC2 and Sestrin2.

PTX decreases mTORC1 signaling in cells lacking p53 and TSC2, as well as in cells lacking Sestrin2. TSC2+/+ and -/- MEFs, and Sestrin2+/+ and -/- MEFs were treated with PTX + TdR for 24 hrs and p-T389 S6K1 evaluated by immunoblot.
Figure 3-8. PTX inhibits enhanced mTORC1 activity after TSC2 transient knock down.

PTX controls mTORC1 activity in cells after TSC2 silencing. WT HCT116 cells were transfected with siRNA against TSC2 for 36 hrs, then were treated with PTX + TdR for 24 hrs; p-T389 S6K1 and p-T70 4EBP1 were determined by immunoblot.
Figure 3-9. Growth inhibition by PTX was not affected by loss of p53 or TSC2. (A) The presence of p53 does not influence cell proliferation after PTX treatment. Wt HCT116 (filled symbols) and p53−/− (open symbols) cells were treated with PTX alone (circles) or PTX + TdR (inverted triangles) for 72 hrs before cells were harvested and counted using a Coulter counter. (B) HCT116 cells with wt p53 show higher sensitivity to AICAR than HCT116 cells null for p53. HCT116 p53+/+ and p53−/− cells were treated with AICAR for 72 hrs and final cell numbers were determined as in (A). In (A) and (B), each symbol is the mean ± sd of three experiments, each of which was performed in duplicate. (C) PTX attenuates the growth of wt MEFs and MEFs lacking p53 and/or TSC2 equally. The growth of MEFs null for p53 (open circles) or null for both p53 and TSC2 (filled inverted triangles) were compared with that of wild type MEFs (filled circles) after 72 hrs exposure to increasing concentrations of PTX with thymidine. (D) MEFs lacking TSC2 and/or p53 are less sensitive to AICAR. MEFS were treated with increasing concentrations AICAR for 72 hr as in (C). The studies on growth inhibition in HCT116 cells were performed by Richard Moran.
3.3.6 Phosphorylation of Raptor is necessary and sufficient for suppression of mTORC1 in PTX-treated HCT116 cells.

Shaw and his colleagues have previously demonstrated that AMPK can regulate mTORC1 by the phosphorylation of Raptor as well as the control by phosphorylation of TSC2 that was discovered earlier (R. J. Shaw & Cantley, 2006; R. J. Shaw et al., 2004). Our data suggest that PTX activated AMPK mediated inhibition of mTORC1 is TSC2 independent, which suggested that phosphorylation of Raptor at Ser 792 by AMPK might be the affecting factor. To test whether the phosphorylation of Raptor by AMPK was involved in and sufficient to suppress mTORC1 kinase activity in the absence of TSC2 function, p53-/- TSC2 -/- MEFs were transfected with a vector encoding wild type human Raptor or a construct for Raptor in which the two serines phosphorylated by AMPK were mutated to alanines (AA Raptor, (R. J. Shaw et al., 2004). The vectors carrying WT or AA Raptor resulted in expression of recombinant Raptor in substantial excess over endogenous Raptor (Fig. 3-13). Raptor was phosphorylated at S792 in vector transfected cells after PTX and even more so in PTX-treated cells transfected with WT Raptor, but cells transfected with AA Raptor and treated with PTX showed a strong suppression of pS792 Raptor. We drew the conclusion that the Raptor made from transfected Raptor vectors was competing with endogenous Raptor for binding to mTORC1 complexes. Treatment of MEFs transfected with empty vector or WT Raptor with PTX resulted in suppression of mTORC1 kinase activity as seen by lower levels of phosphorylation of S6K1 at S389 and of 4EBP1 by phosphorylation at T70. However, neither of these phosphorylation events was suppressed in AA Raptor-transfected cells after treatment with PTX (Fig 3-13). Similar dominance of Raptor phosphorylation was observed when p53 competent and p53 null HCT116 cells were transiently transfected with either empty vector, or vector containing WT or AA raptor
(Fig 3-14). Both cell types when transfected with AA raptor followed by PTX treatment showed no decrease in P-Thr398 S6K1 suggesting no inhibition of mTORC1 in the absence of raptor phosphorylation (Fig. 3-14). Hence, we concluded that, as in MEFs lacking TSC2, in HCT116 cells the suppression of mTORC1 following PTX was mediated by phosphorylation of Raptor by AMPK and that, in cells in which endogenous Raptor in mTORC1 complexes was replaced by a mutant Raptor incapable of AMPK mediated phosphorylation, mTORC1 suppression after PTX treatment did not occur.
Figure 3-13. Phosphorylation of Raptor is necessary and sufficient for suppression of mTORC1 in PTX treated cells.

TSC2-/- p53-/- MEFs were transfected for 36 hrs with pBABE-Hygro containing WT or AA Raptor, then treated with PTX + TdR for 24 hrs. Phosphorylation of S6K1 and 4EBP1 were used as indices of mTORC1 activity.
Figure 3-14. Phosphorylation of Raptor is necessary and sufficient for suppression of mTORC1 in PTX treated HCT116 cells.

WT and p53 null HCT116 cells were transfected for 36 hrs with pBABE-Hygro containing WT or AA Raptor, then treated with PTX + TdR for 24 hrs. Phosphorylation of S6K1 and 4EBP1 were used as indices of mTORC1 activity.
3.3.7 **AICAR mediated inhibition of mTORC1 is TSC2 dependent**

AMPK activated by both AICAR and PTX leads to the phosphorylation of Raptor. As shown in Fig. 3-7A, phosphorylation of Raptor is necessary and sufficient for inhibition of mTORC1 in HCT116 cells and in MEFs treated with PTX. Hence, one would think that in TSC2 null cells, AICAR mediated phosphorylation of raptor might also be sufficient for the inhibition of cell growth. Contrary to this idea, AICAR mediated inhibition of cell growth is decreased by the loss of p53 or of TSC2 and p53 (Fig. 3-12B,D). Thus, we compared PTX- and AICAR-activated AMPK mediated phosphorylation of Raptor and TSC2. WT HCT116 cells were treated with PTX in the presence of TdR or with AICAR (Fig. 3-15). The levels of p-Thr172 AMPK were equivalent under both the treatments. However, PTX-activated AMPK mediated phosphorylation of Raptor to a greater extent than did AICAR treatment at the doses used in these experiments (Fig. 3-15). As previously seen (Fig 3-7A), the level of phosphorylated TSC2 as well as total TSC2 increased under 250 µM AICAR treatment. This suggest that when TSC2 GAP activity is deficient in p53 null or TSC2 null cells, AICAR is less growth inhibitory due to a weaker phosphorylation of Raptor and its dependence on TSC2 mediated inhibition of mTORC1 (Fig. 3-12B,D) and equivalent growth inhibition may require a higher dose of AICAR in absence of p53. In cells competent for p53 function, AICAR causes both Raptor phosphorylation and TSC2 phosphorylation, and growth inhibition was more intense. On the other hand, PTX-activated AMPK only phosphorylates Raptor due to the deficit of TSC2 subsequent to the inactivity of p53 transcription caused by PTX, so that there is no difference in potency in p53 WT or null cells (Fig. 3-12A,C). In order to probe the degree to which inhibition of mTORC1 depends on activation of TSC2, the effect of deletion of TSC2 on the inhibition of mTORC1 by AICAR was studied in p53-/- TSC2-/- MEFs. p53-/- TSC2+/+ MEFs showed
almost equivalent inhibition of mTORC1 by PTX as did p53-/- TSC2-/- MEFs, while AICAR was substantially less effective in p53-/- TSC2-/- MEFs (Fig. 3-16). This supported the concept that AICAR-activated AMPK activates TSC2 GAP activity as well as directly inhibiting Raptor function to inhibit mTORC1 but PTX relies only on phosphorylation of Raptor.

Hence, while one would predict that AICAR would be more active against p53 WT tumors, it would substantially lose activity against p53 null tumors, and probably also tumors bearing DNA binding domain p53 mutations (Chapter 2) and would be substantially less active against tumors that had lost TSC2 function, such as tuberous sclerosis syndrome. On the other hand, PTX would not be affected by loss of p53, nor by loss of TSC2 and should retain activity against tumors with these genotypes.

3.3.8 Schema of differential signaling to mTORC1 by PTX- and AICAR-activated AMPK

AICAR activates AMPK via ZMP while also causing the accumulation of a transcriptionally active p53, promoting the transcription of both Sestrin2 and TSC2. Consequently, AMPK-catalyzed phosphorylation of Raptor and TSC2 occurs and mTORC1 is inhibited (Fig. 3-17, right). PTX, likewise, causes activation of AMPK via ZMP, but subsequent p53-dependent transcription is defective. As a result, Sestrin2 and TSC2 levels are diminished and AMPK cannot activate TSC2. Nevertheless, PTX-activated AMPK causes a robust phosphorylation of Raptor sufficient to inhibit mTORC1 kinase (Fig. 3-17, left).
p53+/+ HCT116 cells were treated with 1 μM PTX + TdR or 250 dRIs for 24 hrs. AMPK activation assessed by immunoblotting using antibody against P-ser172 AMPK. Phosphorylation of Raptor and TSC2 was assessed by immunoblotting using antibodies against P-ser792 Raptor and P-Ser1387 TSC2 respectively.

Figure 3-15. Differential effects of PTX and AICAR, downstream to activated AMPK

p53+/+ HCT116 cells were treated with 1 μM PTX + TdR or 250 dRIs for 24 hrs. AMPK activation assessed by immunoblotting using antibody against P-ser172 AMPK. Phosphorylation of Raptor and TSC2 was assessed by immunoblotting using antibodies against P-ser792 Raptor and P-Ser1387 TSC2 respectively.
p53/- TSC2+/+ and p53/- TSC2-/- cells were treated with 1 C2-pter AI or 250 ere trea for 24 hrs. S6K phosphorylation was assessed by immunoblotting using antibody against P-T389 S6K1 as an indicator of mTORC1 activation.

Figure 3-16 AICAR mediated inhibition of mTORC1 is TSC2 dependent
(right) AICAR activates AMPK via ZMP while also causing the accumulation of a transcriptionally active p53, promoting the transcription of both Sestrin2 and TSC2. Consequently, AMPK-catalyzed phosphorylation of Raptor and TSC2 occurs and mTORC1 is inhibited. (left) PTX, likewise, causes activation of AMPK via ZMP, but subsequent p53-dependent transcription is defective. As a result, Sestrin2 and TSC2 levels are diminished and AMPK cannot activate TSC2. Nevertheless, PTX-activated AMPK causes a robust phosphorylation of Raptor sufficient to inhibit mTORC1 kinase.
3.3.9 Post-translational modifications of p53 following AMPK activation

Earlier sections of this thesis have shown that PTX stabilized p53 is transcriptionally inactive. In order to reveal the cause of this phenomenon, we studied the post translational modifications of the p53 under these treatments. The post-translational modifications of the p53 accumulating in PTX-treated HCT116 cells were compared to those after AICAR or VP16. To assure that the PTM-specific blots in these experiments reflected the stoichiometry of modification rather than the level of p53, we first estimated the relative levels of p53 in control cells and cells after each drug treatment, then loaded different volumes of lysates onto a gel to achieve equivalent loading of p53. There were striking differences in the post-translational modification profiles of p53 after treatment with VP16 or either of the two AMPK-activating drugs (Fig. 3-18A): after VP16 treatment, p53 was modified at a number of residues that reflect the molecular events of the DNA-damage response (Appella & Anderson, 2001; Siliciano et al., 1997), including N-terminal acetylation at K373 and K382, and C-terminal phosphorylation at S20, S37, and S46. None of these post-translational modifications was observed with either activator of AMPK, PTX or AICAR. Like VP16, both AICAR and PTX induced phosphorylation of p53 at S392. The sole modification observed that distinguished the transcriptionally inactive p53 in PTX-treated cells from the transcriptionally active p53 in AICAR (and VP16) was phospho-S15, a modification previously found essential for transcriptional response to p53 (Fig. 3-18A) (R. G. Jones et al., 2005b; Siliciano et al., 1997).

3.3.10 Differential activation of Chk1 and Chk2 by AMPK activators

In order to understand the cause of the differences in the posttranslational modifications of p53 observed after, VP16, AICAR or PTX treatment, we studied the activation of the central effector kinases ATM/ATR by studying the phosphorylation of their downstream targets Chk2 and Chk1,
respectively. The phosphorylation of Chk1 did not occur after either AMPK activators, AICAR or PTX, but was robust after DNA damage induced by VP16 (Fig. 3-18B). This difference in phosphorylation of Chk1 might be the underlying explanation of the differences in the posttranslational modifications of p53 between AICAR and VP16 such as Ser S20, S37, and S46 and acetylation at K373 and K382. However, phosphorylation of Chk2 was observed in both treatments associated with p53 transcriptional activation, DNA damage by VP16 and AMPK activation by AICAR, but not following PTX (with thymidine)(Fig 3-18B). Hence, it would appear that the p53-dependent transcription following activation of AMPK by AICAR is associated with phosphorylation of Chk2 and subsequent phosphorylation of p53 serine 15, probably by Chk2 (Chehab et al., 2000; Oliver et al., 2006; Shieh, Ahn, Tamai, Taya, & Prives, 2000b) while PTX neither activates phosphorylation of Chk2 and p53 serine 15 nor p53-dependent transcription. This in turn would suggest that ATM is activated by AICAR treatment of HCT116 cells, as well as treatment with VP16.

3.3.11 Stabilization and phosphorylation of p53 at ser15 are ATM mediated.

p53 wt HCT116 cells, when pretreated with a specific inhibitor of ATM, Ku-60019, for 2 hrs followed by treatment with either VP16, PTX + TdR, PTX, or AICAR showed decreases in the p53 levels stabilized by these drugs; the levels of ser-15 phosphorylation of p53 was remarkably decreased (Fig. 3-19), strongly implying that ATM was essential for S15 phosphorylation of p53. Decrease in the levels of P-T68 Chk2 was also seen, an indicator of ATM inhibition by Ku-60019. This suggested that although there are several perplexing differences in the effects of these drugs on p53 and p53 activating pathway, they all directly or indirectly activate ATM and leading to ATM mediated stabilization of p53. As PTX in the presence of thymidine stabilizes p53 but does not show increases in the levels of p53 pS15, this suggested that although PTX
stabilizes p53 in an ATM dependent manner, the cause of activation of ATM might be different and thus leading to a different signaling than seen with VP16 or AICAR, as noted by the absence of pS15 p53, psr345 Chk2, pT68 Chk2 and pS139 H2A.X. This suggests that in the absence of DNA damage, p53 is transcriptionally inactive.

However, PTX in the absence of thymidine, causes DNA damage, leads to ATM dependent increase in the levels of p53, pSer15 p53, psr345 Chk1 but it does not phosphorylate Chk2 at Thr68. This indicates that although PTX causes DNA damage and activates ATM, but presumably due to some mechanistic differences in activation of ATM is leading to a differential signaling downstream from ATM to p53 transcriptional activation and leaving p53 transcriptionally compromised. We propose that this mechanistic difference in activation of ATM could be due to difference in type and extent of genotoxic stress or DNA damage like DSBs vs SSBs or replication halt.
**Figure 3-18. Differential p53 post translation modification and Chk1 and Chk2 phosphorylation following AMPK activation.**

P53 +/+ HCT116 cells were left untreated or treated with 20 µg VP16, or 1 µg PTX + TdR or 500 µg AICAR for 24 hrs. (A) Cells were harvested and lysates were probed to assess the levels of p53 under each treatment followed by densitometry to find p53 levels to calculate the amount of protein need to be loaded to get equal levels of p53 loading. After loading protein so that levels of p53 are equal, blots were probed for different posttranslational modifications commonly known and correlated with p53 activation. (B) Equal amount of protein loaded to assess the levels of phosphorylation of Chk1 and Chk2 by using antibodies against P-ser345 Chk1 and P-T68 Chk2.
Figure 3-19. Pretreatment with ATM inhibitor Ku-60019 decreases levels of p53, P-serp53 and P-Thr68 Chk2 in all treatments.

p53 competent HCT116 cells were treated with Ku-60019 for 2hrs or left untreated followed by treatment either with VP16, AICAR or PTX with or without TdR. Lysates were probed for the assessment of p53, P-ser15 p53, PThr68 Chk2 and Chk2 using corresponding antibodies.
3.3.12 Binding of p53 and presence of Acetyl Histone 4 at p21 promoter is equivalent after PTX or AICAR treatment

As p53 accumulated after PTX treatment was not activating the transcription of its target genes, we decided to analyze whether this p53 was binding to the promoter of p21. We performed Chromatin Immunoprecipitation (ChIP) studies using α-p53 antibody to measure the relative occupancy of p53 at p21 promoter. We found that the levels of p53 bound to p21 promoter after VP16 treatment increased several fold. Although the levels of p53 bound to the p21 promoter after PTX treatment were lower than after VP16, they were equivalent to that after AICAR treatment, which were sufficient to transcriptionally activate p21 (Fig. 3-20, blue bars). The presence of p53 at the p21 promoter without transactivating transcription. It led us to question the next step required for promoter accessibility by the transcriptional machinary, namely the recruitment of histone acetylases (HATs) to histones in the vicinity of the promoter, creating a euchromatin structure. We performed ChIP using acetyl-histone H4 to analyze the presence of acetylated hostones at the promoter region of the p21 gene, an indicator of euchromatin form of gene. The levels of acetylated histone 4 occupancy at p21 were increased after VP16, PTX and AICAR treatment (Fig. 3-20, green bars). As expected this increase was higher under VP16 treatment in comparision to that after AICAR and PTX treatment, which were equivalent (Fig. 3-20, green bars). This suggested that binding of p53 or recruitment of HATs at p21 promoter were not the limiting factors for p53 transcriptional activity under PTX treatment (Fig. 3-20) and that, hence, the deficit in PTX was further downstream in the transcriptional response.
3.3.13 AICAR leads to a delayed DNA damage but PTX (with TdR) does not

Because it seemed that AICAR and VP16 both activated ATM, but PTX did not, it was of interest to determine the generic question of whether the activation of AMPK resulted in DNA damage. The initiation of DNA damage after AMPK activation was followed by observation of phosphorylation of histone H2A.X and comparison with the time course of DNA damage with VP16. H2A.X phosphorylation was robustly induced by as little as 3 hours exposure to VP16 and was maintained for at least 24 hr (Fig 3-21A). This time course coincided with the occurrence of robust Chk2 phosphorylation and equally robust and sustained phosphorylation of p53 ser15, while Chk1 phosphorylation was more delayed in onset as was the accumulation of p21 after treatment with VP16. Unlike the rapid time course of p53 S15 phosphorylation in VP16-treated cells, the phosphorylation of p53 S15 was a delayed event in AICAR-treated cells. Interestingly, and quite surprisingly, H2A.X phosphorylation was detected after AICAR treatment on a time scale coincident with Chk2 and p53 ser15 phosphorylation, and p21 transcription (Fig 3-21A). Notably, the phosphorylation of H2A.X, of Ser15 and of Chk2 were less pronounced after AICAR than after VP16, but they were easily measurable and appeared to explain the transcription of p21 in AICAR. The time course of these events in cells treated with PTX and thymidine indicated no H2A.X phosphorylation, no Chk1/ Chk2 activation, no p53 ser15 phosphorylation and no p53-dependent transcription of the p21 gene at any time point studied (Fig. 3-21B). We concluded that the difference between AICAR and PTX activation of AMPK reflected a relatively weak and late-occurring but quite noticeable DNA damage response coincident with Chk2 phosphorylation induced by AICAR, whereas this did not occur with PTX.
3.3.14 PTX-activation of AMPK does not result in the DNA damage response, but PTX inhibition of thymidylate synthase does.

The transcriptional response of the p53 target gene p21 coincided in time with increased levels of pH2A.X, a common marker of DNA damage and/or replication halt, in AICAR-treated cells, while PTX (in the presence of TdR) mediated activation of AMPK did not lead to DNA damage. The primary target of PTX is thymidylate synthase, but the biochemical consequences of TS inhibition on growth are prevented by thymidine (Taylor et al., 1992). Thus in the absence of TdR, we can study the effect of PTX mediated thymidylate synthase inhibition as well as effects on AMPK activation. We fully expected that the effects of PTX on thymidylate synthase would induce DNA damage leading to stabilization of a transcriptionally competent p53 and set up experiments to test whether this was the case. Under these conditions, a robust phosphorylation of H2A.X was seen in HCT116 cells, indicating severe genotoxic stress (Fig 3-22). Interestingly, the DNA damage or replication arrest induced by PTX led to phosphorylation of Chk1 but Chk2 was not phosphorylated and, remarkably, p21 transcription still did not occur. This was completely unexpected, but suggested that phosphorylation of Chk2 is necessary for p53-mediated transcriptional activation of p21. Interestingly, an appreciable level of phosphorylation of p53 S15 was seen, but this did not translate to transcriptional activation of p21.
Promoter of p21 seems to recruit HATs and Presence of RNA Pol II through out the gene length suggesting normal initiation of transcription.

DMSO       VP16
TdR
PTX+TdR
ND      AICAR

Figure 3-20 Chromatin immunoprecipitation suggest equivalent binding of p53 and presence of acetylated histone 4 at p21 promoter after AICAR and PTX treatment.

p53 +/- HCT116 cells were left untreated or treated with 20µM VP16, or 1 µM PTX + TdR or 500 µM AICAR for 24 hrs. Cells were harvested and lysed followed by chromatin immunoprecipitation using α-p53 or α-Acetyl-histone4 antibody. Precipitated DNA was used for realtime qPCR analysis to see the binding of p53 and acetylated-histone4, respectively.
Figure 3-21. AICAR leads to a delayed DNA damage but PTX (with TdR) does not.

WT HCT116 cells were treated with VP16 (20 μM) AICAR (500AR or PTX (1 μm + Tdr (5.6 μm) for given time durations. Cells were harvested and lysates (A) from VP16 and AICAR (B) VP16 and PTX + Tdr, treated cells were probed for P-Ser345 Chk1, Chk1, P-T68 Chk2, Chk2, P-S139 H2A.X, H2A.X, P-ser15p53 and p21 using corresponding antibodies.
Fig. 3-22. PTX-activation of AMPK does not result in the DNA damage response, but PTX inhibition of thymidylate synthase does.

Cells were either treated with VP16, PTX +/- TdR, AICAR or in combination of VP16 and PTX
+- TdR or VP16 and PTX. Cells were harvested and lysates were probed for the assessment of p53, P-ser15 p53, p21, P-Ser139 H2A.X, H2A.X, P-Ser345 Chk1, P-T68 Chk2 using antibodies against corresponding residues.

3.3.15 Blocking VP16 effects by pre-treatment with PTX.

The initial phases of phosphorylation of H2A.X were seen after six hr exposure to VP16 and this exposure resulted in enhanced p21 transcription (Fig 3-23B). When HCT116 cells were pretreated for 20 hours with PTX plus thymidine prior to exposure to VP16 (Experimental scheme Fig. 3-23A), remarkably, the DNA damage caused by VP16 was blocked, as was the phosphorylation of Chk1 and the transcription of p21 (Fig. 3-23B). Chk2 phosphorylation was not blocked by pretreatment with PTX and thymidine, conditions that activate AMPK but do not inhibit thymidylate synthase. Hence, activation of AMPK even with phosphorylation of Chk2 and of S15 p53, was not sufficient for p53-dependent transactivation of p21 without DNA damage.

When thymidine was not added to PTX in the medium, so that the genotoxic stress subsequent to inhibition of thymidylate synthase occurred as well as activation of AMPK, extensive H2A.X phosphorylation was seen, Chk1 phosphorylation was observed, and Chk2 was not, but p21 transcription was not stimulated (Fig. 3-23B) as also seen before (Fig. 3-22). When VP16 was added to cells pretreated for 20hrs with PTX alone, Chk1, Chk2 and ser15 p53 phosphorylation was seen and but there was still no p21 transcription was observed. Interestingly, pretreatment with PTX (+ Tdr) blocks VP16 mediated DNA damage, suggesting that PTX mediated activation of AMPK blocks cells from undergoing the events needed for the development of DNA strand breaks after stabilization of Topoisomerase cleavable complexes.
with VP-16 (see Discussion). As PTX in the absence of thymidine, itself causes phosphorylation of H2A.X, it was not clear from this data that whether PTX blocks VP16 mediated DNA damage. Steady state levels of mRNA of p53 transcription target genes P21, MDM2, PUMA, PIG3, BAX showed no increase upon VP16 treatment in cells pre treated with PTX (+/- TdR) for 20 hrs (Fig. 3-23C). However, cells treated with VP16 alone for 6hr or AICAR alone for 24 hrs showed remarkable increase in the steady state levels of these above mentioned mRNA (Fig. 3-23C).

These data suggested that DNA damage and Chk2 phosphorylation were required for p21 transcriptional activation, that AMPK activation by PTX in the presence of thymidine was insufficient for p53 transcriptional activation, that DNA damage by itself could be segregated from p53-dependent transcriptional activation by whether or not Chk2 was activated and p53 S15 was phosphorylated. Overall, the effects of PTX (+/- TdR) interfered with those of VP16, blocking the transcription activity of p53 induced by VP16.

3.3.16 PTX can block DNA damage effects even after prolonged exposure of VP16

PTX (+ TdR) can block DNA damage induced by 6 hr exposure of VP16, concomitant with the block of p21 transcription induction. We asked whether PTX would be able to hold its blocking effects under the prolonged exposure of VP16. P53 competent HCT116 cells were treated with PTX (+ TdR) for 20 hrs followed by addition of VP16 at 6, 12 and 24 hrs before harvestation. As expected, VP16 caused stabilization of p53 and induces the DNA damage by 6 hrs with an increase by 12 and 24 hrs. This DNA damage was harmonized with the increase in p21 protein levels (Fig. 3-24). Interestingly, we found that the PTX (+ TdR) can block the DNA damage induced by VP16 for 12 hrs, however, by 24 hrs the effects of VP16 seems to start dominating over PTX + TdR, indicated by some induction of p-S139 H2A.X at 24 hr VP16 treatment in cells.
pretreated with PTX+ TdR. This induction of p-S139 H2A.X was concur with increase in p21 levels suggesting that DNA damage is essential for VP16 mediated activation of p21 transcription by p53 (Fig. 3-24).

3.3.17 Blocking AICAR effects by PTX.

In order to understand if PTX, an AMPK activator can block the effects of another AMPK activator, AICAR, that appeared to initiate a DNA damage response, we co-treated cells with AICAR and PTX with both drugs added at the same time (experimental scheme, Fig. 3-25A). As with VP16, PTX in the presence of thymidine blocked AICAR mediated DNA damage indicated by phosphorylation of H2A.X, phosphorylation of ser15 p53 and Chk2, p21 transcription. In the absence of thymidine, PTX itself causes DNA damage thus it cannot be interpreted from this data whether PTX blocks AICAR mediated DNA damage. Phosphorylation of p53 S15 was observed with both PTX alone and PTX with AICAR treatment (fig. 3-25B). AICAR mediated phosphorylation of Chk2 is blocked by PTX concomitant with block in transcription of p21. Steady state levels of mRNA of p53 transcription target genes; P21, MDM2, PUMA, PIG3, BAX shows no increase upon AICAR treatment when combined with PTX (+/- TdR) (Fig. 3-25C).

We conclude that in the presence of the thymidine, PTX-activated AMPK neither cause DNA damage nor allowed the DNA damage mediated by VP16 or AICAR, yet it blocks the p21 transcriptional response. Hence, in the absence of thymidine, PTX blocks thymidylate synthase as well as activates AMPK and causes DNA damage or replication stress but still does not activate transcriptional activity of p53. Surprisingly, the effects of PTX without thymidine (AMPK and inhibition of thymidylate synthase) also block the effects of VP16 and AICAR on p53 transcriptional activation. We concluded that the activation of p53 transcriptional activity
depends on the type of genotoxic stress and DNA damage as well as the phase of cell cycle arrest induced (see Discussion).
Fig. 3-23. PTX blocks VP16 mediated transcriptional activation of wt p53: p53 competent HCT116 cells were treated alone either with VP16, PTX, AICAR with or without TdR or VP16 was added to media in PTX (+/- TdR) after 20hrs as shown in (A). Cells were harvested 6 hrs later for (B) protein analysis by immunoblotting, (C) steady state mRNA analysis using RT-qPCR. Experiment is replicated twice showing similar patterns of changes in mRNA levels.
p53 wt HCT116 cells were treated with PTX + TdR for 24 hrs or left untreated followed by addition of VP16 (20µM) for indicated time points. Cells were harvested and lysed for immunoblotting to analyze p-S139 H2A.X, an indicator of DNA damage and p21.

Figure 3-24. PTX (+TdR) blocks DNA damage and p21 transcription even after prolonged exposure to VP16.
p53 competent HCT116 cells were treated alone either with VP16, AICAR or PTX with or without TdR or with combination of AICAR and PTX (+/- TdR) for 24 hrs as described in (A) followed by protein analysis by immunoblotting (B), steady state mRNA analysis by RT-qPCR(C).

Figure 3-25. PTX limits AICAR mediated transcriptional activation of wt p53
3.3.18 The effects of PTX plus TdR are caused by the PTX, not by thymidine.

PTX in the presence of thymidine blocks VP16 and AICAR mediated DNA damage, indicated by decreased levels of p-S139 H2A.X in cells pretreated with PTX + TdR followed by VP16 or AICAR treatment (Fig. 3-23,24,25). However, in the absence of thymidine, PTX itself causes genotoxic stress, shown by increased levels of p-S139 H2A.X (Fig. 3-23,24,25). Thus, we asked the question whether the DNA damage block seen by PTX + TdR, is due to PTX mediated effects or is an effect of TdR present in the media, originally added to circumvent the effects of PTX on thymidylate synthase inhibition. p53 competent HCT116 cells were pretreated with PTX (+ TdR) or TdR for 20 hrs followed by addition of VP16 6hr prior to cell harvest or were cotreated with AICAR and PTX (+ TdR) or TdR. As expected, VP16 and AICAR mediated induction of DNA damage and p21 protein levels were blocked only in cells pretreated with PTX (+TdR) and not with TdR only (Fig. 3-26). This suggested that PTX mediated activation of AMPK leads to some interesting yet unclear signaling events, which do not allow the DNA damage to take place, presumably due to cell cycle arrest at G1 phase.
p53 wt HCT116 cells were treated with PTX+TdT, TdT or AICAR alone. For combination treatment, cells were pretreated with PTX + TdT or TdT for 20 hr followed by VP16 treatment 6 hrs before harvesting the cells. For AICAR combination studies with PTX + TdT or TdT, indicated drugs were added simultaneously for 26 hrs. Cells were harvested and immunoblotting performed to analyze p53, p-H2A.X and p21. β-actin was used as loading control.

Figure 3-26. VP16 and AICAR induced DNA damage and p21 transcription is blocked by PTX, not TdT.
3.4 DISCUSSION

The studies in this chapter were started with a fairly simple but important question of how p53 status affected PTX mediated effects on mTORC1? Very early in the studies we found that PTX activated AMPK could still inhibit mTORC1 in cells which have lost p53 function, inspite of the fact that the mTORC1 is hyper-activated in these cells. This led us to question why PTX effects on mTORC1 are p53 independent. In order to understand that, PTX effects were compared with those of AICAR, a moderately well studied AMPK activator. We found that the effects of PTX and AICAR were the same on AMPK activation but different downstream to that. PTX-activated AMPK only phosphorylates Raptor and thereby blocks mTORC1 kinase activity, whereas AICAR-activated AMPK phosphorylates both TSC2 and Raptor. Thus, AICAR activated both controlling arms whereby AMPK inhibits mTORC1, whereas PTX could only activate one of these arms.

Involvement of p53. The difference in the effects of PTX and AICAR proved to be due to differential transcriptional activity of p53; p53 stabilized by AICAR was transcriptionally active and caused the transcription of TSC2 and sestrin2, two molecules involved in and required for AMPK mediated phosphorylation and activation of TSC2, whereas PTX stabilized p53 is transcriptionally inactive, unable to promote the transcription of the TSC2 and sestrin2 genes. In the presence of low levels of these proteins, AMPK does not phosphorylate TSC2 and thus TSC2 GAP activity is not enhanced. This answered the question why PTX effects on mTORC1 are p53 independent; it does not activate p53 regulated TSC2 arm of pathway. Thus p53 function it does not make a difference in the inhibition of mTORC1 by PTX. We further found that the effects
of PTX on mTORC1 are indeed TSC2 and sestrin2 independent.

**PTX-mediated AMPK signaling**  This raised the hypothesis that, if PTX does not exploit TSC2 GAP activity, then raptor phosphorylation has to be taking the whole burden of inhibition of mTORC1. In order to test this hypothesis we transfected TSC2/-p53/- MEFs with WT and double mutant Raptor (AA: S792A and S772A) that cannot be phosphorylated by AMPK. We found that upon PTX treatment, cells which have WT raptor, show inhibition of mTORC1 but not those with AA raptor. In the absence of TSC2, WT raptor when phosphorylated by AMPK led to inhibition of mTORC1 in these cells suggested that AMPK mediated phosphorylation of Raptor is dominant, necessary and sufficient for inhibition of mTORC1. This was reflected in the cell growth assays that PTX mediated inhibition of cell growth was p53 and TSC2 independent while loss of p53 or TSC2 was diminishing the growth inhibitory effects of AICAR. This is a critical and an important observation, as this property of PTX can be harnessed in the cancers that have lost p53 or TSC2 function and have enhanced activity of mTORC1.

**AICAR-mediated AMPK signaling.**  Given that the effects of PTX-activated AMPK on mTORC1 suppression was independent of TSC2 function, it was of interest to determine if AICAR mediated suppression of mTORC1 requires TSC2 function or whether the phosphorylation of Raptor by AICAR is sufficient. Treatment of MEFs null for TSC2 with AICAR showed that AICAR could inhibited mTORC1 only in TSC2 competent MEFs and not in MEFs null of TSC2. This suggested that at the dose of AICAR used in this experiment, is dependent on both TSC2 and Raptor phosphorylation. Presumably, at higher doses of AICAR, Raptor phosphorylation could also become sufficient to mTORC1 inhibition.
**P53 PTMs** PTX stabilized p53 was transcriptionally crippled and thus does not increase the levels of TSC2 and Sestrin2. We set off to understand the reason for this phenomenon. We used DNA damaging agent VP16 as a control, which is well studied and known to activate the transcriptional activity of p53. We saw very interesting differences and similarities between AICAR and VP16 or AICAR and PTX. Whereas AICAR and VP16 both stabilize and activate p53 transcriptional activity, they had very clear and surprising differences in the posttranslational modifications of p53. VP16 activated p53 was post transnationally modified at all the residues analyzed in this study, but AICAR activated p53 was only phosphorylated at Ser15 and Ser392. Interestingly, PTX stabilized p53 was only phosphorylated at Ser392, a residue thought to be responsible for tetramer formation of p53. Therefore the only difference at the level of posttranslational modification evident between AICAR and PTX, was p-S15. This suggested that absence of p-S15 of p53 might be the causative factor for transcriptional inactivity of p53 in PTX treatment. We also analyzed the binding of p53 and occupancy of acetylated histone 4 at p21 promoter, and found that occurrence of both the events were equivalent in AICAR and PTX (+TdR) treatments, eliminating the possibility of these events to be a limiting factor for p53 transcriptional inactivity under PTX treatment. Phosphorylation of p53 at ser15 and ser20 stabilizes p53 by inhibiting its interaction with MDM2, an E3-ubiquitin protein ligase.

**Signaling upstream of p53** As ATM and ATR are the kinases known to phosphorylate Chk2 and Chk1, respectively, upon DNA damage which then phosphorylate p53 at S15, we studied the activation of these proteins and their target protein H2AX, as an indicator of DNA damage. We found that Chk1 was phosphorylated only under VP16 treatment, known to cause DSBs, but not
under AICAR treatment, in spite of the fact that AICAR also caused H2A.X phosphorylation. While both AICAR and VP16 treated cells show p-S15 p53 and increased p21 levels, ATR-mediated Chk1 phosphorylation was absent in AICAR as it was in PTX-treated cells. This suggested that phosphorylation of Chk1 is not required for the transcriptional activation of p53. Interestingly, unlike PTX (+TdR), both VP16 and AICAR treated cells showed increased levels of ATM-mediated phosphorylation of Chk2. Therefore, the absence of phosphorylation of Chk2 and p53 at S15 were the only differences noted between AICAR and PTX. It was clear by the time course studies that phosphorylation of; p53 at S15 and Chk2 at T68 was coinciding with the induction of H2A.X, an indicator of DNA damage after VP16 or AICAR treatment. None of the events; p-S345Chk1, p-T68Chk2, p-S15 p53 were present in PTX (+ TdR) nor was the DNA damage indicator p-S139H2A.X. This suggested that in the absence of DNA damage, ATM does not get activated, which, in turn, does not phosphorylate and activate Chk2, leaving ser15 p53 un-phosphorylated and p53 transcriptionally inactive.

We decided to check if DNA damage is required for the transcriptional activation of p53. PTX in the absence of TdR inhibits thymidylate synthase and can cause genotoxic stress. We decided to test if PTX in the absence of the thymidine can activate DNA damage followed by transcriptional activation of p53. When cells were treated with PTX alone, it caused a remarkable increase in the levels of p53, p-S15 p53, p-S345Chk1 and p-S139 H2A.X with no increase of p21 protein levels. This suggests that even after genotoxic stress, p53 stabilized by PTX is not transcriptionally active. This showed that the genomic stress caused by PTX-mediated inhibition of thymidylate synthase is not sufficient to activate p53 transcriptional activity. Interestingly, the genomic stress caused by PTX could cause activation of ATR mediated activation of Chk1 without causing an increase in p21 levels. The only difference
between the signaling after treatments stabilizing transcriptionally active p53 (VP16 and AICAR) versus treatment stabilizing transcriptionally inactive p53 (PTX + TdR and PTX alone), was pT68 Chk2. This suggested that in the absence of Chk2 activation, and therefore activation of ATM, p53 is transcriptionally compromised.

**PTX interfered with the DNA damage response.** Interestingly, when we pretreated cells with PTX (+ TdR), followed by exposure to VP16, we saw that the DNA damage induced by VP16 was blocked, concomitant with a failure to promote transcription of p21. Similar observations were made with the co-treatment of PTX + thymidine and AICAR: PTX + TdR blocked the AICAR-mediated DNA damage and hence, p53 could not transcribe p21.

We know by the data generated by Scott Rothbard, a former graduate student of our lab, that PTX in the presence of thymidine blocks cells at the G1/S border and PTX without thymidine inhibits thymidylate synthase and causes S phase cell-cycle block. From this information, we inferred that the pretreatment of cells with PTX in the presence of thymidine blocks cells at G1 phase of cell cycle and does not allow the induction of DNA damage by VP16 or AICAR. This was a surprise, since topoisomerase poisons were thought to act at several parts of the cell cycle.

When cells are treated with PTX (-TdR), it causes a S phase cell cycle block and increases in p-S139 H2A.X but it does not transactivate p53. Cells pretreated with PTX followed by VP16, apparently had all signaling events caused by ATM activation (p-ser15 p53, p-ser345Chk1, p-T68Chk2, p-ser139 H2A.X) induced but there was still no increase in the p21 levels. A possible explanation could be that upon PTX treatment, due to thymidine insufficiency, DNA replication is halted but it does not allow the double stranded DNA damage caused by VP16 and thus is not capable of transactivation of p53. Similar effects were seen when cells were treated with PTX
and AICAR together. In addition we found that PTX decreased the level of p-T68 Chk2 activated by AICAR in the cells pretreated with PTX. Perhaps the transcriptional inactivation of p53 caused by PTX does not allow the production of a protein essential to the DNA damage response, e.g. 53BP1.

This chapter raises many important questions and unleashes some very interesting phenomenon taking place under unique pharmacological stimuli. This study clearly challenges the correlation of signaling events like p-S15p53, p-S345Chk1, p-T68Chk2 and p-S139H2A.X and p53 transcriptional activity.

3.5 CONCLUSIONS

Studies in this chapter lead to the conclusion that PTX effects on the mTORC1 pathway are independent of p53 and TSC2 function, which makes it a suitable drug for cancers with hyperactive mTORC1 due to loss or mutation of these genes. We also uncovered the mechanism of this independence by showing that p53 accumulated under PTX treatment, is transcriptionally compromised and thus does not allow the activation of TSC2 transcription and its GAP activity leaving the mTORC1 inhibition by PTX, TSC2 function independent. PTX mediated effects on mTORC1 inhibition are due to robust phosphorylation of Raptor by AMPK and this effect is necessary and sufficient to inhibit mTORC1, even in cells with enhanced mTORC1 activity.

Later we went on to address the reason of p53 transcriptional incompetence under PTX treatment and we show some very interesting effects of this drug on the ATM/Chk2-ATR/Chk1-P53 pathway. We believe that, in the presence of thymidine, the G1 cell cycle arrest blocked the DNA damaging effects of VP16 and AICAR and allow the accumulation of a transcriptionally inactive p53. On the other hand, in the absence of thymidine PTX inhibits thymidylate synthesis and arrest cells at S phase of cell cycle, which probably leads to a replication halt, which neither
activates p21 transcription itself nor allows VP16 or AICAR mediated activation of p21 transcription.

3.6 SURPRISES FROM THIS STUDY

These surprises are discussed in more detail in the future directions section of this thesis (Chapter 5).

- PTX (+ TdR) stabilizes p53 without causing phosphorylation of p53 at ser15.
- Chk1 activation has been reported to inhibit transcriptional activity of p53. In my studies, etoposide activates Chk1 and AICAR does not, but both activate p53 transcriptional activity.
- PTX (+Tdr) can block the DNA damage mediated by VP16 or AICAR and blocks p21 transcription. This provides strong evidence that DNA damage is required for VP16 or AICAR activation of p53.
- PTX-activated AMPK, does not cause DNA damage and does not activate p21 transcription. Hence, AMPK activation per se does not activate p53.
- We found that although both AICAR and VP16 lead to transcriptional activation of p53, AICAR treatment does not lead to increase in pS345Chk1 but levels of pS15p53 and pT68Chk2 are increased. On the contrary, VP16 treatment led to increase in the phosphorylation of all these three proteins; pS345Chk1, pS15p53 and pT68Chk2. This challenges the correlation between activation of Chk1, Chk2, phosphorylation of p53 at ser15 and trans-activation of p53.
Chapter 4

4 Interference of PTX with transcriptional activity of mutant p53 may help to explain its effects against lung cancers

4.1 ABSTRACT

The role of p53 as a tumor suppressor gene is very well studied and p53 has been established as one of the most frequently mutated genes in human cancers. Until a decade or two ago, it was believed that the mutation of p53 most often led to a nonfunctional protein. However, substantial evidence has accumulated that supports the concept that DNA binding domain mutations of p53, can also confer properties that intensify its oncogenic activity. Such mutant p53 proteins with neomorphic activities are referred to as Gain-of-Function (GOF) mutant p53s. Different missense mutations confer different and unique activities and the literature on this field is quite extensive. This chapter addresses a very unique phenomenon observed under PTX treatment that PTX not only prevents transcriptional activation by wild type p53, but also interferes with GOF mutant p53 transcription. Most human lung cancers have GOF mutp53s that often lead to transcriptional activation of growth and chemoresistance promoting genes not induced by wt p53. The previous chapter of this dissertation determined that PTX increases the levels of WT p53 but blocks its transcriptional activity. Therefore, we hypothesized that PTX
might also block the transcriptional activity of mutp53s. This proved to be the case.

4.2 DISCOVERY OF MUTANT P53 AND ITS ROLE IN CANCER

The p53 protein was first identified in a complex with the simian virus 40 large T-antigen (Lane & Crawford, 1979). It was demonstrated that many tumors expressed abundant levels of this protein, and that the levels of the p53 protein positively correlated with the progression of cancer (DeLeo et al., 1979); thus, p53 was initially though to be an oncogene. This concept was reinforced when ectopic expression of the newly cloned p53 cDNA was shown to cooperate with oncogenic Ras to transform primary cells in culture (Eliyahu, Raz, Gruss, Givol, & Oren, 1984). Later, when several groups compared the sequence of their cloned p53 cDNAs, the striking results was that each clone differed in sequence (Levine & Oren, 2009). It was later recognized that these early experiments demonstrating that p53 overexpression could transform cells and promote in vivo tumor growth were actually performed with mutant version of p53 that has been isolated from tumor cells (Hinds, Finlay, & Levine, 1989; Levine & Oren, 2009). Thus, the concept of gain-of-function of mutant p53 came to be recognized. Mutation in p53 gene can lead to three possible outcomes (Fig.4-1).

A) Loss of function

B) Dominant Negative behavior

C) Gain of function

4.2.1 Loss of function of p53

More than 85% of mutations found in the p53 gene are single point mutations that result in missense proteins. Mutations, which lead to complete loss of p53 tumor suppresser function,
would severely compromise the ability of the cells to respond to genotoxic stress. Mutations of p53 can occur by deletion, insertion, truncation or point mutation and tumors with a mutant p53 allele very frequently subsequently undergo loss of heterozygosity with loss of the wild type allele of p53 resulting in complete loss of p53 or p53 function. Some of these point mutations lead to formation of protein which is functionally inactive (Fig. 4-1) (Hollstein et al., 1994).

4.2.2 Dominant Negative Behavior

Point mutation in one allele results in a missense protein with loss of function of one allele. Some of these mutations, especially when expressed in higher amount, can exert a dominant negative effect on protein coded by the remaining wild-type allele. This results in an abrogation of the ability of wt p53 to inhibit cellular transformation (Fig. 4-1) (Brosh & Rotter, 2009; Oren & Rotter, 2010). This dominant negative behavior is either due to formation of mutant/wild type co-tetramers (Chan, Siu, Lau, & Poon, 2004) or the incorporation of wild type p53 into mutant p53 super-tetrameric aggregates (Xu et al., 2011). This is rare in human cancers as the wild type allele is usually lost by deletion or gene conversion.

4.2.3 Gain of function (GOF) mutants of p53

Unlike most of the tumor suppressor genes, which have one allele inactivated by mutations or truncations followed by silencing of the second wild type allele (Weinberg, 1997), the vast majority of cancer-associated mutations in p53 are missense mutation with substitution of one base pair, which lead to translation of a different amino acid in that position and can give rise to a protein with an unique functionality. The great majority of these mutations are clustered in the central-most region of the p53 known as the DNA binding domain (Fig. 4-2). Of the several mutations known and mentioned in the literature, some occur at higher frequency and are known
as “hot spot” mutations (Harris & Hollstein, 1994). The wild type p53 is a very short-lived protein expressed at low steady state levels in normal conditions, but is stabilizes during any kind of genotoxic or cellular stress. However, missense mutations lead to expression of altered full length proteins with a substantially increased half life (Fig. 4-3)(Strano et al., 2007). Each mutation leads to a very different and unique protein and protein function, and generalizations about p53 across mutations can be misleading. The selection of inactivating mutations in p53 during tumor progression might be preserving their activity as dominant negative inhibitors of wild type p53, but the extensive prior literature on this point provides enough evidence to say that some missense mutation give an additional survival advantage; these mutant p53s are known as Gain of Function mutants (GOF) (Fig.4-1, 2,3).
Figure 4-1 Schematic representing differences in the mutant forms of p53 at the level of their transcriptional specificity.
Figure 4-2 Structure of wt and Gain-of-function mutant p53.

Top) WT p53, Bottom) GOF p53, where read lines in the DNA binding domain represent the mutations present in the cells lines used in this study.
Figure 4-3. Schematic representation of difference in half-life and effects on downstream cell processes after DNA damage.

A. Wild type p53, B. Gain-of-function mutp53.
One of the clearest pieces of evidence for GOF effects derives from experiments in which the expression of tumor derived mutant p53s in non-transformed, otherwise p53 null cells are inoculated into mice; such constructed cell lines greatly increased their tumor forming ability in nude mice compared with cells transfected with wt p53. Wolf et al. showed that L12, an Ab-MuLV-transformed, p53 null cells, when transfected with a tumor derived p53 gene, gave rise to a L12 derived cell with mutant p53 which efficiently transcribed p53 mRNA and synthesized the p53 protein. When injected into syngeneic mice, L12 derived cells expressing this mutant p53 protein showed much more tumorogenicity than parental L12 cells. Tumors formed by parental L12 later regressed but tumors formed by L12 derived clones with mutp53 made lethal tumors, suggested an advantage of survival gained by mutant p53 over loss of p53 function (Wolf, Harris, & Rotter, 1984). Dittmer et al. reported that the expression of human or murine mutant p53 in p53 null cells exerted a new and additional phenotype to these cells. They showed that expression of mutant p53 in p53 null cells increases their tumorigenic potential in nude mice or enhances plating efficiency in agar cell culture. Introduction of mutant p53 in p53 null cells increased the expression of the gene regulated by the multidrug resistant enhancer promoter element suggesting that other then loss of function, mutations can also lead to “Gain of function”(Dittmer et al., 1993) which is oncogenic.

These pioneering studies were done either in cell culture or by injecting cells in nude mice. However, to understand and confirm this phenomenon, mouse models were required to demonstrate that mutation of p53 is not equivalent to loss of p53 function. Almost a decade after the p53 knock out mouse was generated, two “knock- in” mouse of Li-Fraumeni Syndrome (LFS) were generated (Lang et al., 2004; Olive et al., 2004). In an attempt to better recapitulate the human disease, these groups created tumor derived mutp53 expressing, genetically
engineered mice, by inserting a tumor derived p53 cDNA into the endogenous TP53 locus, using homologous recombination. Two hot spot mutations were used; structural mutant p53 R172H and the contact mutant p53R270H, which are murine equivalent of human p53 codon 175 and 273, respectively. Also, p53R172H/- and p53R270H/- mice developed biologically different tumors compared to p53-/- mice, including a variety of carcinomas and more frequent endothelial tumors. They showed that p53R172H/+ and p53R270H/+ mice developed allele-specific tumor behavior, which is quite distinct from that of p53+/- mice. This suggested that there was a difference in tumor behavior upon loss of one allele versus mutation in one allele. These results clearly suggested that mutation in p53 generated a genetically altered tumor along with a more metastatic capability, supporting the hypothesis of GOF mutant p53 (Lang et al., 2004; Olive et al., 2004). Similarly, in an analysis of a LFS patient, a germ line missense mutation in TP53 have been shown to be associated with an earlier age (approximately 9 yrs) onset of tumor when compared to germ line deletion in TP53, suggesting a gain of function effect of missense p53 mutants in human tumors (Bougeard et al., 2008). Also, several studies have shown that the presence and level of expression of mutant p53 has a strong co-relation with poor prognosis of several types of human tumors, including breast cancer (Elledge et al., 1993; Olivier et al., 2006).

4.3 **VARIOUS ASPECTS OF CELLULAR PROGRAMMING MODULATED BY MUTANT P53:**

In addition to knockout mice and human epidemiology data, cell based assays also have implicated that mutant p53 affects different aspects of tumorigenesis by targeting different modes of cellular programming. Some of them are discussed below (Fig.4-4):
4.3.1 RNA synthesis and proliferation

Bossi et al showed that inhibition of mutant p53 by RNA interference reduces cell proliferation in vitro, and in vivo it increases tumorigenicity and resistance to cancer drugs. Overall they demonstrated that knock down of mutant p53 weakens the aggressiveness of human cancer cells and provides evidence of the GOF hypothesis (Bossi et al., 2006). A mutant form of p53 protein interacted with the NF-Y transcription factor and, after DNA damage, this interaction causes a p53/NF-Y dependent increase in RNA synthesis. They showed that mutant p53 binds NF-Y target promoters and, upon DNA damage, recruits p300, leading to histone acetylation. The recruitment of mutant p53 to the CCAAT sites was severely impaired upon abrogation of NF-Y expression. Endogenous NF-Y, mutant p53, and p300 proteins formed a triplex complex upon DNA damage. This study suggested that aberrant transcriptional regulation might explain the ability of mutant p53 proteins to act as an oncogenic factor (Di Agostino et al., 2006).

4.3.2 Cell Survival

Various experiments done for the purpose of differentiating loss of function of p53 and from the effects of expression of a mutant p53 suggested that mutant p53 showed an advantage of survival over loss of p53. Stambolsky et al. showed that mutant p53 can be recruited to the vitamin D-receptor (VDR) regulated genes and modulates their expression. Mutant p53 can increase the accumulation of VDR in the nucleus. It can augment the transactivation of some genes and relieve the repression of others. Overall, mutant p53 can convert vitamin D into an anti-apoptotic agent that promotes cell growth and survival of tumor cells (Stambolsky et al., 2010).

4.3.3 Chemoresistance

Several studies have compared the properties of the cells with and without p53 mutations within
the same cell population. These experiments were usually done by introducing human tumor derived mutant p53 species into p53-null H1299 cells. Clonogenic survival assays performed on these cells suggested that cells overexpressing the His175 p53 mutant, but not the His273 mutant, recover preferentially from etoposide treatment. Moreover, etoposide-induced apoptosis were substantially reduced in the presence of p53His175 or p53His179, whereas p53His273 and p53Trp248 did not have much protective effect. In contrast, both p53His175 and p53His273 showed increased resistance against low concentrations of cisplatin, but resistance reduced with higher concentration and showed no protection at all against high concentrations. This suggested that particular p53 mutants might offer a selective survival advantage to tumor cells during chemotherapy. These findings define a new type of mutant p53 selective gain of function which may compromise the efficacy of cancer chemotherapy (Fig.4-3,4) (Blandino, Levine, & Oren, 2002).

4.3.4 Abnormal centrosome and spindle checkpoints

Fibroblasts from Li-Fraumeni syndrome heterozygotes expressing mutant p53s, when exposed to a spindle depolymerizing agent, generates polyploid cells more frequently than p53 null fibroblasts from LFS heterozygotes. This study also showed that, this class of dominant gain of function mutants (p53-RSC (relaxed spindle checkpoint allele)) does not need any new transcriptional activity of mutant p53 to exert this behavior. This suggested that one of the ways by which mutant gain of function p53 can help tumor survival and progression can be a direct promotion of genetic instability (Gualberto, Aldape, Kozakiewicz, & Tlsty, 1998).

4.3.5 Gene amplification

Albor et al. showed that mutant p53 proteins can interact and activate topoisomerase I, and that
this could be a mechanism for induction of genomic instability by mutant p53 proteins (Albor, Kaku, & Kulesz-Martin, 1988). Later, this group followed up with the study of the effects of exogenous mutant p53 protein expression in genomic stability in human p53-/- Saos-2 cells. They established the correlation between the presence of mutant p53 and an increased number of PALA (N-(phosphoacetyl)-L-aspartate)-resistant colonies, a phenotype caused by gene amplification of the aspartate transcarbamylase gene. They showed by immunoprecipitation that mutant p53 interacts with topoisomerase I and upon continuous expression of mutant p53 for several generations, the number of PALA resistant colonies increases after subsequent exposure of PALA. Furthermore, following exposure to camptothecin (which stabilizes topoisomerase I cleavage complexes and mediates non homologous recombination) the number of PALA resistant colonies increased. When combining the expression of mutant p53 with exposure to camptothecin, an additive increase in the number of PALA resistant colonies was evident. These studies suggested that mutant p53 mediated gene amplification processes independent of its capability to inactivate wild type p53 protein but dependent on interaction of mutant p53 with topoisomerase I (El-Hizawi, Lagowski, Kulesz-Martin, & Albor, 2002).

4.3.6 Somatic cell reprogramming and stem cell characteristics

A well-known hallmark of some of the most aggressive and deadly cancers is a poorly differentiated phenotype thought to result from the presence of stem-like cancer cells. (Ben-Porath et al., 2008). The increased expression of a set of embryonic stem cell (ESC) genes and a decreases expression of genes that are targets for polycomb identifies a poorly differentiated lung adenocarcinoma. This gene expression signature was the marker of poor prognosis and worse overall survival in lung adenocarcinomas but not all poorly differentiated non-small cell lung cancers exhibit such a gene expression profile (Hassan, Chen, Kalemkerian, Wicha, & Beer,
Human cancers are known to consist of a heterogeneous set of diseases which are from one another by pathologic presentation and molecular signature.

Breast cancer in one of the best fitting example of this phenomenon and each breast cancer subtype on the basis of pathological presentation is also heterogeneous when viewed on a molecular basis. Mizuno et al. studied if a p53 mutation could allow cells within a tumor to acquire a stem cell-like state by coordinating expression of stem cell identified genes. Using microarray and database studies, they demonstrated that breast and lung cancers with p53 mutations exhibit a stem cell-like transcriptional pattern. These data suggest the model that loss of p53 function enables acquisition of stem cell properties, which are positively selected during tumor progression (Mizuno, Spike, Wahl, & Levine, 2010).

4.3.7 Disruption of tissue architecture

Breast cancer is thought to arise from mammary epithelial cells found in acini, which collectively form terminal ductal lobular units. Each acinus consists of a single layer of polarized luminal epithelial cells surrounding a hollow lumen (Bissell, Radisky, Rizki, Weaver, & Petersen, 2002). Freed-Pastor et al. used 3D culture system to understand the involvement of mutant p53 in the distortion of tissue architecture. They showed that depletion of mutant p53 is sufficient to phenotypically revert breast cancer cells to more acinar-like morphology (Freed-Pastor et al., 2012)
Figure 4-4 Schematic representation of mechanism of mutp53 mediated induction of chemo-resistance in cancer cells
4.3.8 Angiogenesis

Angiogenesis is one of the requirements for tumor growth and progression because unlimited growth of cells demands high supply of nutrient and thus blood supply. There are some studies, which suggest that mutant p53 transcriptional programming includes some genes like ID4, which are directly involved in angiogenesis. ID4 (inhibitor of DNA binding 4) is a member of a family of proteins that function as dominant-negative regulators of basic helix-loop-helix transcription factors. The ID4 protein binds to and stabilizes mRNAs encoding pro-angiogenic factors IL8 and GRO-alpha. This results in the increase of the angiogenic potential of cancer cells expressing mutant p53 (Fontemaggi et al., 2009).

4.3.9 Migration, invasion and metastasis

One of the biggest challenges faced cancer therapeutics is migration, invasion and metastasis of cancer cells. There is some evidence that suggests that mutant p53 might play a role of an oncogene by helping and promoting cell migration, invasion, and metastasis. When immune deficient SCID mice are transplanted with human T cell acute lymphoblastic leukemia (T-ALL), only those cells which either possessed a mutant p53 gene or lacked the wild type allele infiltrated and induced the lethal hematopoietic disease. The mutant p53 genes exert a distinct pattern in vivo and in vitro: mutants which showed greatest cell proliferation of T-ALL lines in vitro and colony formation in methylcellulose cultures, also showed greatest tissue invasiveness of T-ALL cells in vivo (Hsiao et al., 1994). It was clear that mutant p53 gave an additional advantage for survival and invasion in vivo and in vitro over lack of wild type p53 function. Some specific hot spot mutants of p53 play a role in the generation of lymphohematopoietic metastatic potential and tissue invasiveness as assayed in SCID mice, whereas the expression of wild-type p53 is capable of keeping this metastatic potential in check (Hsiao et al., 1994).
4.4 MOLECULAR MECHANISM OF TUMOR PROMOTING ACTIVITY OF GOF MUTANT P53

All known mutants of p53 are either loss of function or gain of function proteins. A huge focus of researchers in the p53 field is to understand and elucidate the underlying molecular mechanism of the GOF behavior. A significant amount of research has been done to address this question and there are several molecular mechanisms that have been proposed to explain the gain of function of mutant p53. These are discussed below.

4.4.1 Interaction and Inhibition of the activity of other p53 family proteins

Mutant p53 has been suggested to interact, sequester and interfere with the activity of proteins involved in the antitumor protective mechanisms of cells. The p63 and p73 proteins, which are not targets of WT p53, have been shown to interact with gain-of-function mutant p53 (Fig.4-3) (Di Como, Gaiddon, & Prives, 1999; Marin et al., 2000). The p63 and p73 genes each encode several isoforms derived by combination of multiple transcription start sites and alternative splicing. Some of those isoforms share or mimic WT p53 function. Upon expression or accumulation of mutant p53, p63 and p73 bind to mutant p53 and thus cannot perform their transcriptional activity, leading to deregulation of p63 and p73 target genes. p63 and p73 target genes are mainly involved in apoptosis and cell proliferation regulation. Thus, the binding of mutant p53 to p63 and p73 severely diminishes their transcriptional activity and diminishes apoptosis (Irwin et al., 2003; Strano et al., 2000). The core domain of the mutant p53 is sufficient for the binding to p63 and p73 (Gaiddon, Lokshin, Ahn, Zhang, & Prives, 2001; Strano et al.,
Thus, this region of mut p53 might serve as a protein-protein interaction module which contributes to gain of function activity. Keeping the complexity of the proteins involved in the interaction network of the WT p53 in mind, it would not be unexpected to find additional new interacting partners of GOF mutant p53 in the future.

4.4.2 Mutant p53 as a bona-fide transcription factor

Almost a decade of research has investigated this aspect of gain of function p53. Levin's group reported that a p53 mutant with mutation at two critical residues at the N-terminus loses its gain of function activity (Lin, FAU, & Levine, 1995). A genome-wide approach has shown that mutant p53 modulates the levels of numerous transcripts. Most of these genes are found to be involved in cell cycle, cell growth and cell proliferation, cell migration, angiogenesis, and chemoresistance suggesting that regulation of these genes by GOF mutant p53 could be pivotal in its function as a pro-oncogenic protein (S. Deb, Jackson, Subler, & Martin, 1992; M. W. Frazier et al., 1998a; Iwanaga & Jeang, 2003; Y. I. Lee et al., 2000; Ludes-Meyers et al., 1996; Margulies & Sehgal, 1993; Mizuarai, Yamanaka, & Kotani, 2006; Scian et al., 2003; Subler, Martin, & Deb, 1994; Tsutsumi-Ishii, Tadokoro, Hanaoka, & Tsuchida, 1995). Consequently, gain-of-function mutant p53 might trigger other pathways by modulating the transcription of genes, which may represent the molecular basis of the broad-spectrum gain-of-function activity. Despite continuous efforts from the scientific community, the understanding of molecular details of the transcription activity of mutant p53 is still very scarce mainly due to different behavior of each mutant. However, some of the possible mechanisms for this regulation of transcription by mutant p53 are as follows;
4.4.3 Interaction of gain-of-function mutant p53 with transcription factors leading to differential regulation of WT p53 targets.

Wild type p53 has been shown to interact with a number of transcription factors (E2F1, SP1, NF-Y, YY1, TBP, TAFs), giving rise to protein-protein-DNA complexes. Mostly, these macromolecular complexes modulate the transcription of genes whose promoters lack wild type p53 binding sites. Often these promoters are inhibited by wt p53 in specific phases of the cell cycle after DNA damage (Farmer, Friedlander, Colgan, Manley, & Prives, 1996; Lu & Levine, 1995; Manni et al., 2001; mbriano et al., 2005; St Clair et al., 2004). Some studies have shown interaction of wt p53 with NF-Y on CCAAT box-containing promoters, and upon DNA damage this complex recruits histone deacetylases (HDACs) and releases histone acetyl transferase (HATs), causing repression of the key cell cycle control genes like cyclin A, cyclin B1, cdk1, cdc25c and cdk1-associated kinase activity (mbriano et al., 2005). Interestingly, Di Agostino recently reported that mutant p53 interacts with NF-Y upon DNA damage and leads to response opposite to that of wt p53. This interaction provoked the expression of cyclin A, cyclin B1, cdk1, cdc25c and cdk1-associated kinase activity leading to mutp53/NF-Y complex-dependent increase in DNA synthesis. Upon DNA damage, mutant p53 binds NF-Y target promoters and recruits p300, leading to histone acetylation and promoting the initiation of transcription of those genes. They further showed that even in normal conditions, NF-Y and mutant p53 are present on NF-Y regulated gene promoters along with HDACs, independent of DNA damage. This suggests that binding of mutant p53 with NF-Y is dependent on the presence of NF-Y and on the CCAAT box of the promoter; upon DNA damage this association increases. This leads to the recruitment of the p300 HATs in a mutant p53-dependent manner. The switch between p300 and HDACs is shifted towards increased acetylation and reduced methylation of neighboring
histones leading to the promotion of initiation of transcription from these promoters. This observation opened up several interesting questions. The foremost is: since both WT and mutant p53 can interact with both p300 or HDACs in complex containing NF-Y, then how are p300 or HDACs recruited in an opposed manner by Wt and mutant p53? Second, as mutant p53 is present in a complex with NF-Y/HDACs in unstressed condition, what is the role of this complex under these conditions? However, the overall conclusion of the study by Di Agostino et. al. was that mutant p53 enhances transcription of proliferative targets of NF-Y.

In fact, mutp53 interacts with many other sequence specific transcription factors and shows differential regulation from that by wt p53. One such factor is Sp-1 which regulates transcription by binding to Sp-1-response elements (Sp-1-REs) and interacts with both mut p53 as well as wt p53 (Chicas, Molina, & Bargonetti, 2000; Gualberto et al., 1998). Transcription regulation outcomes by p53:Sp-1 interaction depends on the type of p53 protein. While wt p53 leads to inhibition of Sp-1 dependent transcription activation, presumably by interfering with the DNA binding of Sp-1 (Bargonetti, Chicas, White, & Prives, 1997), mutp53 elicits co-operative effects on Sp-1 mediated transcription activation. Similarly, wt p53 interaction with the proto-oncogene ETS-1 is inhibitory to its activity (E. Kim et al., 2003; Pastorcic & Das, 2000), whereas mutp53:ETS-1 interaction promotes ETS-1 mediation transcription activation (Sampath et al., 2001). This suggest that the macromolecular complex assemblies containing WT or mut p53 are functionally distinct.

**Interaction of gain-of-function mutant p53 with DNA.**

The question of whether mutp53 transactivation may require its direct binding with DNA has been a discussion of controversy for a long time. This is mainly due to a lack of evidence suggesting that mutp53 can bind to DNA with specificity. This question has been approached
mainly by analysis of the ability of mutp53 proteins to bind at wt p53 recognition sequences, and the lack of binding was interpreted as an inability of mutp53 to bind DNA specifically. However, most of the promoters activated by mutp53 do not contain wt p53 binding consensus sequences nor show similarity to it, suggesting that mutp53 regulates these promoter by binding at response elements different than wt p53-Res (Dittmer et al., 1993; M. W. Frazier et al., 1998b; Gualberto et al., 1998; Tsutsumi-Ishii et al., 1995; Yang, Pater, & Tang, 1999). However, from studies performed on mut p53-regulated promoters and not based on wt p53-REs, it was clear that mutp53 binds with DNA (Bargonetti et al., 1997; Chicas et al., 2000; Y. I. Lee et al., 0907; Zalcenstein et al., 2003). Chromatin immunoprecipitation studies revealed that mutp53 proteins do physically bind with their responsive promoters in vivo, suggesting that mutp53 targets DNA in a specific manner but independent of Wtp53 canonical binding sequence (Zalcenstein et al., 2003). One of the biggest barriers in the determination of the putative binding site for mutp53 on DNA is that most of the promoters activated by mutp53 do not show any sequence homology of mut p53 binding site. However, Kim and Deppert's lab has proposed the idea that mutp53 binding with DNA is not sequence specific but it is largely determined by DNA structure. This idea was originated after the observation that mutp53, but not wt p53, interacts with Matrix Attachment Region (MAR) elements, which are regulatory DNA sequences shown to play a role in high order chromatin organization, and chromatin modification (E. Kim & Deppert, 2004). Weissker et al tested the interaction of the mutant p53 with DNA representing high complexity with regard to sequence and secondary structure. They showed a specific binding of the mutp53 with lambda DNA and suggested the possibility that p53 might be able to interact with nuclear MAR DNA sequences (Weissker, Muller, Homfeld, & Deppert, 1992). There are several studies which suggest that wt and mutp53 interact with promoters as macromolecular complexes.
that show distinct functionality. On the other hand, it is shown that regulation of the MDR1 promoter by wt p53 and mutp53 are from different promoter regions (Sampath et al., 2001). There have been several studies focusing on defining the new targets of mutp53 which are now categorized as neo-morphic genes.

4.5 POTENTIAL TRANSCRIPTION TARGET GENES OF MUTANT P53

The mechanism for oncogenicity of mutp53 which has been studied the most, is transcriptional regulation of mutp53. Mutp53 have been reported to modulate the transcription of various genes (Table 4.1) that are briefly discussed below. These genes are: MDR1, NfkB2, Axl, PCNA, hTERT,, hsp70 and EGFR, all of which have been functionally implicated in increased aggressive behavior of tumors. The characteristics of each of these neomorphic p53 targets are briefly summarized, below.

4.5.1 Multidrug resistant gene 1 (MDR1)

Each cancer has different response towards cytotoxic drugs; some are relatively sensitive and some are more refractory. Multidrug resistance (MDR) describes the phenomenon of an acquired simultaneous resistance to unrelated drugs. Multidrug resistance was first described in 1969 and than in 1970 when chinese hamster ovarian cancer cells were exposed to increasing concentration of actinomycin D (Biedler & Riehm, 1970; Simard & Cassingena, 1969). Though these cells were selected with one drug, they were later found to be resistant to a range of clinically important natural products, including anthracyclines (doxorubicin and duanomycin), the vinca alkaloids (vincristine, vinblastin and vindesine), etoposide and colchicine. Extensive studies with cell lines and transplantable tumors have shown that MDR can develop rapidly. The genes involved in MDR have been identified and their role in drug resistance has been confirmed
by gene transfer. Mechanisms identified to date include reduced drug accumulation, involved the P-glyco-protein (Pgp; mdr1 gene) (Childs & Ling, 1994) mainly, but also the MDR-associated protein (mrp) gene (Cole et al., 1992). MDR cells overexpressing MDR1 show resistance to a broad spectrum of drug structures and thus it has been thought that Pgp may be acting by altering the intracellular pH or modifying membrane potential. Classic studies demonstrated that cells exposed to increasing concentrations of any of the natural products (except bleomycin) underwent amplification of the mdr gene, with the appearance of homogeneously staining regions in CHO cells (Biedler & Riehm, 1970) and double minute chromosomes in mouse and human cells (Biedler & Riehm, 1970). Studies done with purified MDR1 and functionally reconstituting it into liposomes to investigate its properties show that Pgp alone is sufficient to transport many drugs.

4.5.1.1 MDR1 and mutant p53

The most frequently expressed drug resistance genes, MDR1 and MRP1, occur in human tumors with mutant p53 and their over expression is correlated with the presence and transcriptional activity of mutant p53 (de Kant, Heide, Thiede, Herrmann, & Rochlitz, 1996; Fukushima et al., 1999; Galimberti et al., 1998; Oka et al., 1997; Sampath et al., 2004). Sampath et al showed that mutant p53 did not activate either the MRP1 promoter or the endogenous gene but strongly up-regulated the MDR1 promoter and expression of the endogenous MDR1 gene. Transient transfection of mutant p53 caused increased expression of endogenous MDR1 in comparison to empty vector or transcriptionally inactive mutant p53 transfection. Oka et al studied some surgically removed colorectal tumors using immuno-histochemistry and found that p53 and MDR1 were co-expressed in a significant number of samples (P < 0.002). Although they did not find any relationship between MDR1 or p53 protein accumulation with histologic grade or stage.
but their study clearly demonstrated that MDR1 expression is closely associated with p53 protein accumulation in human colorectal cancers (Oka et al., 1997).

4.5.1.2 MDR1 and cancer

There are several studies, which suggest that levels of MDR1 are correlated with poor prognosis of cancer chemotherapy. The most significant observations were made in leukemia and pediatric cancers. For example, using an anti-MDR1 monoclonal antibody, Ma et al. were the first to demonstrate that MDR1 may be overexpressed in acute myelogenous leukemia (AML) (Ma et al., 1987). In two patients, leukemia cells were negative for MDR1 at diagnosis of AML, but one patient became positive at first relapse and the other on recovering from second induction chemotherapy. Subsequently, two large studies correlated mdr1 mRNA expression with response to treatment in acute leukemia and showed that the higher the expression of MDR1, the worse was the response of the disease to chemotherapeutics. (Pirker et al., 1991; SSato et al., 1990). Development of agents to overcome multidrug resistance (MDR) is important in cancer chemotherapy.

4.5.2 Nuclear factor Kappa B 2 (NFkB2)

The Rel/NFkappaB family of transcription factors is involved in multiple cellular processes, including inflammation, cell cycle regulation, apoptosis and oncogenesis. Constitutive activation of NFkappaB has been described in a great number of solid tumors and this activation appears to support cancer cell survival and to reduce the sensitivity against chemotherapeutic drugs (Scian et al., 2005). Additionally, some of these drugs induce this transcription factor and, through this mechanism, lower their cytotoxic potential (Bug & Dobbelstein, 2011). Nuclear factor-κB2/p100 has been reported to promote endometrial carcinoma (a common cancer in females) cell survival.
under hypoxia in a HIF-1α independent manner. Inhibition of NFκB has been shown to enhance the sensitivity to antineoplastic- or radiation-induced apoptosis in vitro and in vivo (Yeramian et al., 2011).

4.5.3 AXL

The Axl subfamily of mammalian receptor tyrosine kinases (RTKs), also known as the TAM family, consists of Axl, Tyro3 (or Sky), and Mer. Axl was first isolated during a transfection experiment using cDNA from a patient with myeloproliferative disorders (Crosier & Crosier, 1997; Neubauer et al., 1994). Axl is a 140 kDa protein has been found to be involved in the transformation of various cell types and thus believed to have oncogenic potential (Janssen et al., 1991; McCloskey, Pierce, Koski, Varnum, & Liu, 1994). Axl expression is observed predominantly in fibroblasts, myeloid progenitor cells, macrophages, neural tissue, ovarian follicles, cardiac and skeleton muscle but rarely present in normal epithelial cells (Crosier & Crosier, 1997).

TAM receptor tyrosine kinases are known to play a role in cell adhesion as well as in intracellular signaling. In fact, it was observed that Axl mediates adhesion of 32D myeloid cell line (McCloskey et al., 1997), whereas Lee et al. (1999), demonstrated that increased expression of the Axl receptor induces transformation of NIH 3T3 cells into highly tumorigenic cells in nude mice (W. P. Lee et al., 1999).

4.5.3.1 Axl and cancers

Axl was described in CML patients and its overexpression in fibroblasts suggested Axl’s transforming activity (Janssen et al., 1218; McCloskey et al., 1994; O'Bryan et al., 1991).
Following its original identification, upregulation of Axl has been reported in a variety of cancers including breast, gastric, prostate, ovarian and lung cancers (Berclaz et al., 2001; Jacob et al., 1992; Shieh et al., 2005; W. Sun, Fujimoto, & Tamaya, 2004; Wimmel, Glitz, Kraus, Roeder, & Schuermann, 2001b; Wu et al., 2002).

4.5.3.2 Metastatic potential of Axl

The expression of Axl was found to be higher in the metastatic prostate carcinoma cell line DU145 than in the less aggressive prostate carcinoma cell line PC-3 or in normal prostate cells (Jacob et al., 0805; Sainaghi et al., 2005). Moreover, Sainaghi et al. also showed that Axl/Gas6 interaction induces mitogenic activity in DU145 and PC-3 cell lines, which is not mediated by inhibition of apoptosis and is proportional to Axl expression (Sainaghi et al., 2005).

4.5.3.3 The role of Axl in non-small cell lung cancer (NSCLC): A key player in resistance against chemotherapy.

Overexpression of Axl has been related to increased resistance of cancer cells to chemotherapeutic drugs in numerous types of cancer. Axl has been shown to be overexpressed in NSCLC (Shieh et al., 0221; Wimmel, Glitz, Kraus, Roeder, & Schuermann, 2001a) and, recently, two independent studies reinforced the idea that Axl is important for NSCLC and demonstrated that Axl plays a key role in acquiring resistance to chemotherapy. One of these reports by Zhang et al. (2012) demonstrated increased activation of Axl and evidence for epithelial-to-mesenchymal transition (EMT) in the mutant epidermal growth factor receptor (EGFR) lung cancer models with acquired resistance to erlotinib (a tyrosine kinase inhibitor used in the treatment of lung cancer) in vitro and in vivo. Inhibition of Axl led to restoration of sensitivity to erlotinib, indicating that Axl may represent a promising therapeutic target to
prevent or overcome acquired resistance to drugs (Zhang et al., 2010).

4.5.3.4 The role of mutant p53 in Axl transcription regulation

The receptor protein tyrosine kinase Axl is upregulated at both RNA and protein levels in H1299 lung cancer cells expressing any of a few mutant p53s-R175H, -R273H, and -D281G. On the other hand, knockdown of endogenous mutant p53 levels in human lung cancer cells H1048 (p53-R273C) and H1437 (p53-R267P) resulted in reduction in the level of Axl as well. Using ChIP, they showed the direct nucleation of GOF p53 on the Axl promoter and facilitation of the acetylation of the Axl promoter, suggesting that mutp53 regulates transcriptional activation of the Axl gene. Furthermore, knockdown of Axl using siRNA led to decrease tumorogenicity and migration of cancer cells, suggesting that increase in Axl levels by mutp53, help in cancer progression and survival. (Vaughan et al., 2012).

4.5.4 Proliferating Cell Nuclear Antigen (PCNA)

PCNA is known to be involved in the DNA replication and thus molecular marker for cell proliferation. PCNA was first identified in the sera of group of patients with systemic lupus erythematosus, as an antigen to an autoimmune antibody (Mathews, Bernstein, Franz, B, Jr, Garrels, & Garrels, 1984). Later studies showed that this antigen was actively expressed in proliferating human cancer cells and showed a pattern of expression similar to a ‘cyclin’ protein with peak expression during the late G1 and S phase (Bravo R FAU - Fey„S.J. et al., 0222). Three identical PCNA molecules associate in a head-to-tail fashion to form a homotrimeric ring that encircles the DNA double helix as a sliding clamp and make a critical platform on replicating DNA for the coordinate recruitment and regulation of other proteins essential for DNA replication. PCNA-interacting proteins important for DNA metabolism
include polymerases, ligases, topoisomerases, and proteins controlling replication initiation, cell cycle control, chromatin and epigenetic regulation, gene transcription, cell survival, and metabolism (Maga & Hubscher, 2003; Moldovan, Pfander, & Jentsch, 2007; Naryzhny & Lee, 2010; Ulrich & Takahashi, 2013; Warbrick, 2000).

4.5.4.1 PCNA and Cancer

PCNA engenders special interest among cancer researchers because of its role in cell proliferation. Because cell growth is a requirement for cancer progression at both primary and metastatic sites, and PCNA is an essential factor for DNA replication, inhibition of PCNA has been exploited as an anticancer strategy. Due to its involvement in more than one cell survival mechanisms, circumvention of its inhibition by cells (developing resistance) is less likely than therapies targeting other signaling pathways (Stoimenov & Helleday, 2009). Small molecule inhibitors and peptides against PCNA have been used to target it and study its mechanism and function. PCNA has also been widely used as a tumor marker, but there are conflicting results regarding the correlation between PCNA expression in tumor tissues and patient prognosis (Grossi et al., 2003; Heimann, Ferguson, Recant, & Hellman, 1997; Wang et al., 2006).

4.5.4.2 The role of mutant p53 in transcription of PCNA

Shivkumar et al. have shown that low levels of wild-type p53 transcriptionally activate the human PCNA promoter but higher levels of wild-type p53 inhibit promoter activation in vivo. Interestingly, in contrast to what is seen with wt p53, expression of tumor derived mutant p53 at low levels failed to activate the PCNA promoter but at higher levels significantly increased the transcription of PCNA (Shivakumar, Brown, Deb, & Deb, 1995). This study indicated that mild DNA damage which moderately elevates wt p53 levels increases the expression of PCNA,
probably to assist DNA repair. As mutant p53 is always present at high levels in tumor cells, they constitutively induce the expression of PCNA to avail the cell proliferation property of PCNA. They also showed that where wild-type p53 binds to the human PCNA promoter in a sequence specific manner, a tumor-derived p53 mutant does not require the wild-type p53 response element for transactivation of the human PCNA promoter (Shivakumar et al., 1995).

4.5.5 Human Telomerase Reverse Transcriptase (HTERT)

The ribonucleoprotein complex telomerase is continuously expressed in stem and germ cells and repressed in most normal somatic cells. Almost 70–90% of cancer cells stably express this enzyme (Shay & Bacchetti, 1997), which is reactivated during malignant transformation. This reactivation grants unlimited proliferation capacity for the tumor cells by de novo synthesis of telomeric (TTAGGG)n sequences, circumventing the “end-replication problem” of DNA synthesis (Dahse, Fiedler, & Ernst, 1997; N. W. Kim et al., 1994; Mansfield et al., 2007; Smogorzewska & de Lange, 2004). Inactivation of mammalian telomerase leads to telomere attrition, eventually leading to uncapped telomeres, which elicit a DNA damage response and cell cycle arrest or death.

4.5.5.1 hTERT and cancer

It has been shown that telomerase activity is strongly correlated with the state of malignancy and metastatic potential of cancer cells. hTERT has been suggested to be a useful molecular marker in cancer prognosis. hTERT represents the catalytic subunit of telomerase with reverse transcriptase activity (Albanell et al., 1997; Bryan, Sperger, Chapman, & Cech, 1998; Smogorzewska & de Lange, 2004; Triginelli et al., 2006; Uen et al., 2007). Telomerase as an enzyme consist of two subunits, an RNA template molecule known as hTERC and enzyme unit
hTERT. To render its enzymatic activity hTERT needs hTERC (Albanell et al., 1997; Bryan et al., 1998; Dahse et al., 1997; N. W. Kim et al., 1994; Mansfield et al., 2007; Smogorzewska & de Lange, 2004; Triginelli et al., 2006; Uen et al., 2007) and various telomerase associated proteins (Smogorzewska & de Lange, 2004).

4.5.5.2 Transcription of hTERT by mutant p53 and other oncogenic factors.

Since transcription activity of mutant p53 has been correlated with transcription regulation of various genes, Scian et al. in 2004, investigated and, indeed, showed that hTERT is a transcriptional target of GOF mutp53 using microarray analysis, later confirmed by qPCR analysis (Scian et al., 2004). Transient transcriptional assays, testing the ability of tumor derived mutant p53 to mediate transactivation of hTERT promoter, suggested that mutp53 transactivates the hTERT promoter. ChIP followed by qPCR analysis demonstrated that mutp53 was present on the hTERT promoter suggesting its involvement in transactivation of the gene (Scian et al., 2004).

4.5.6 Heat Shock protein 70 (hsp70)

One class of the heat shock proteins (Hsp70s) are 70 kDa, conserved, ubiquitously expressed and consist of eight homologous chaperones. These proteins play a crucial role in protein folding and their translocation from one compartment to the other of the cell. Hsp70, a protein unfolding machine, binds and releases stretches of hydrophobic amino acids in an ATP dependent process (Morano, 2007; Tavaria, Gabriele, Kola, & Anderson, 1996). Hsp70 proteins have a substrate-recognizing domain, which interacts with sequences of hydrophobic amino acid residues in newly synthesized proteins as they emerge from the ribosomes. Hsp70 binds tightly
to partially synthesized protein sequences and prevents them from aggregating and being rendered nonfunctional. When the protein is synthesized, a nucleotide exchange factor stimulates the release of ADP and binding of fresh ATP, opening the binding pocket of Hsp70. Then the client protein is free to fold on its own, or to be transferred to other chaperones for further processing.

4.5.6.1  **Hsp70 and cancer and its transcriptional regulation by mutant p53**

Hsp70 has been implicated in four crucial steps of tumorigenesis: (Ciocca, Arrigo, & Calderwood, 2013) (1) stabilizing oncogenic proteins (Gray, Prince, Cheng, Stevenson, & Calderwood, 2008; Khaleque et al., 2005) (2) inhibiting programmed cell death (Garrido et al., 2012) and replicative senescence; (3) induction of tumor angiogenesis (J. Sun & Liao, 2004) (4) activation of invasion and metastasis (Durech, Vojtesek, & Muller, 2012; J. Sun & Liao, 2004).

Most of the client proteins for the hsp70-hsp90 complex participate in functions that promote cell growth, proliferation, and cell survival associated with carcinogenesis. The mechanisms of the induction of HSP70/90 in cancer are not fully clear. Tsutsumi-Ishii et. al showed that mutant p53 transactivates HSP70 and analysis using in vitro mutagenesis of the heat shock response element (HSE) suggests that a HSE with heat shock factor binding ability is required for transactivation of the heat shock protein70 promoter by mutated p53 genes. (Tsutsumi-Ishii, Tadokoro, Hanaoka, & Tsuchida, 0519)

4.5.7  **Epidermal growth factor receptor (EGFR)**

The ErbB transmembrane receptor family consist of various members including, EGFR (ErbB1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4). These receptors are structurally similar and consist of three domains: an extracellular domain, a trans-membrane domain, and an
intracellular domain. The extracellular domain has a ligand-binding region, and several ligands, including Epidermal Growth Factor (EGF) bind to it. The ligand binding leads to receptor homo- or hetero-dimerization between EGFR and other ErbB family members at the cell surface, causing internalization of the dimerized receptor. The receptor dimerization results in autophosphorylation of the intracellular EGFR tyrosine kinase domain. The phosphorylated tyrosine kinase stimulates an intracellular signal transduction cascade which leads to activation of several downstream pathways (including the Ras-Raf-MEK-ERK, PI3K- AKT-mTOR, and JAK-STAT3 pathways), leading to cell proliferation and growth (Scaltriti & Baselga, 2003; Toyooka et al., 2011).

4.5.7.1 EGFR and Cancer

EGFR tyrosine kinase mutations are one of the most frequent mutations in human cancers, which usually occur between exons 18 to 21 of the EGFR gene. These mutations leads to constitutively activated ligand-independent EGFR protein production, leading to carcinogenesis (Lynch et al., 2004; Paez et al., 2004). About 80-90% of EGFR mutations are either short in-frame deletions in exon 19 or point mutations resulting in substitution of arginine for lysine at codon 858 (L858R) in exon 21 (Mitsudomi & Yatabe, 2007). Cancer cells with constitutively active mutant EGFR are highly dependent on the ligand-independent activity of this protein or its overexpression, a phenomenon called “oncogene addiction” (Gazdar, Shigematsu, Herz, & Minna, 2004). This constitutive expression or activity allows cancer cells to circumvent the normal cell growth and proliferation regulation mechanism and, at the same time, the resulting oncogenic addiction leads to a greater sensitivity of these cancers towards small molecule inhibitors of these oncogenic proteins. In first line treatment, EGFR inhibitors show approximately 75% response rate in patients with typical EGFR mutations (Mok et al., 2009).
4.5.7.2 Transcription regulation of EGFR by mutp53

wt and tumor derived mutp5, can both transactivate the EGFR promoter (S. P. Deb, Munoz, Brown, Subler, & Deb, 1994). Transient transfection assays with a promoter-CAT construct containing the entire EGFR promoter (pER-1.CAT) were performed to compare the transactivation efficiency of wt and mut p53. This experiment suggested that, although wt p53 can transactivate the EGFR promoter, it is a very weak transactivation in comparison to that caused by several mutants of tumor-derived p53. Although each p53 mutant had a different efficiency toward transactivation of EGFR, p53-R281G was proven to be the strongest transactivator of this promoter (Ludes-Meyers et al., 1216).

4.6 POST TRANSLATIONAL MODIFICATION OF MUTP53

The posttranslational modification of wt p53 has been studied extensively. However, a knowledge base of PTMs on mutp53 is not well established, although it has been demonstrated that PTMs play some role in the activity of mutp53. One would think that, as PTMs are thought to play a major role in the activity and function specificity of wt p53, mutation on these residues would abolish the function of wt p53 and should be frequently observed in cancers. Interestingly enough, whereas mutations have been indicated in all coding exons of p53, more than 95% of the base substitution mutations are located in exons 4-9, which encode the DNA binding domain of the protein. About 30% of mutations locate within six “hotspot” residues that are frequently mutated in almost all cancer types (Hollstein et al., 1994) that is, Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282, none of which are known to be post-translationally modified. As indicated previously, most frequent mutations are on residues not subject to post translational modification. A probable explanation of this is that a mutation in a residue of p53 subject to a controlling PTM have a survival disadvantage or that, the PTM is important for mutp53 function.
so is not selected in vivo. Whereas most of the PTM sites described in the DNA binding domain (DBD), do not fall in a hotspot for tumor mutations, the codons for Lys132, Thr155, Ser215, Glu258, Asp 259, and Cys277 have been suggested to have over 90 cancer-associated mutations/codon. (Cross ref. (Nguyen, Menendez, Resnick, & Anderson, 2014))

4.6.1 Phosphorylation of Serines and Threonines in mut p53

Like wt p53, mutp53 can also be post-transnationally modified in response to various stress and DNA damage signals. In 1993, one of the pioneering studies by Ullrich et al showed that, in the absence of stress signals, the posttranslational modification pattern of mutp53 is different than that of WT p53 (Ullrich et al., 1993). Later, it was shown that phosphorylation and acetylation of p53 was substantially higher in tumor cells than in non-transformed cells. This pattern was true irrespective of tumor types or the presence of mutp53. Out of 10 sites analyzed, phosphorylation at residues 15, 81, and 392 were the most frequently observed modifications along with some acetylation sites. (Minamoto et al., 0802). Conservation of PTM residues in tumor cells suggests that mutp53 function may either needs these PTMs or atleast these PTMs do not affect mutp53 oncogenic activity negatively (M. Matsumoto, Furihata, & Ohtsuki, 1222; M. Matsumoto et al., 2004). Perhaps the most studied PTM of WT and mutant p53 is Ser15. One of the major limitations in the studies of mutp53 is that each mutant protein behaves very different in different conditions and cell types, which may make the conclusions from one study contradictory or not in accordance with the conclusions of the other studies. Indeed, different studies to understand the importance of Ser15 modification in mutp53 show contradictory results (Nagata et al., 1999; Ullrich et al., 1993). However, later studies that included many more cells lines and conditions suggested that there is no conclusive correlation between ser15 modification and stability, localization DNA binding, transcriptional activity of mutp53 under stressed and unstressed
Mutation of p53 is one of the most common genetic alterations in human cancers and tumors that express mutant p53 are usually more aggressive, resistant to chemotherapy, and show worse prognosis than p53-null tumors. This tumor promoting activity has been correlated with the transcription activity of mutant p53. One of the main foci of this dissertation was to understand why PTX effects on mTORC1 regulation are independent of wt p53 (chapters 2 and 3). This chapter is mainly focused to understand the effects of PTX on the transcription activity of tumor-derived mutp53. As we found that PTX blocks transcriptional activation of WT p53 towards its target genes, we hypothesized that PTX might block the transcription activation of GOF mutp53 as well. As discussed in the introduction of this chapter, novel transcription targets of this neomorphic mutp53 are chemoresistance, cell growth and proliferation promoting genes. Therefore, blockade of transcription of these genes by mutp53, under PTX treatment, might offer a therapeutic advantage for cancers with mutp53 like lung cancer and breast cancer.

4.8 RESULTS

4.8.1 Sensitivity of mutant p53 cells lines to PTX

PTX has been approved by the US FDA for first line treatment for non-small cell lung cancer. One of the most common genetic mutations in lung cancers is mutation of p53 and as PTX is the only antifolate with activity against lung cancer, we sought to understand the mechanism of the effects of PTX against lung cancer. We used H1437 NSCLC cells with a R267P mutation in p53 and H1048 NSCLC cells with R273P mutation in p53. These cells lines have been shown to have gain- of-function mutp53 activity (Vaughan et al., 2012).
We tested the growth suppression of these NSCLC cells lines by PTX treatment in the presence (Fig. 4-5A) or absence of thymidine (Fig. 4-5B). Cells were treated for 72 hrs followed by electronic counting using a coulter counter (Fig 4-5). We found that the growth of both lung cancer cell lines, H1437 (with a R267P mutation in p53) and H1048 (R273P) are suppressed by PTX. This suggested that both lung cancer cell lines, with mutant p53, are sensitive to PTX.

4.8.2 PTX blocks mTORC1 pathways in mutp53 containing lung cancer cells

We showed in the previous chapter that cell lines expressing mutant p53 have higher mTORC1 activity than that with wt p53 (Chapter 2) and that PTX treatment mediates activation and phosphorylation of AMPK at Thr172 leading to inhibition of mTORC1 (Chapter 3). This PTX-activated AMPK mediated inhibition of mTORC1 was p53 function independent. Therefore, we hypothesized that PTX will inhibit the enhanced mTORC1 activity in cells with mutp53. Lung cancer cell lines H1437 and H1048 were treated with either thymidine or PTX (1µM) with thymidine (5.6µM) in order to determine the AMPK-mediated effects on mTORC1 in these cells. Phosphorylation of AMPK at P-Thr172 was enhanced in both H1437 (Fig. 4-6A) and H1048 (Fig. 4-6B). This activation of AMPK leads to a decrease in the phosphorylation of pT70 4EBP1 suggesting that PTX inhibits mTORC1 via activating AMPK in cells with mutp53 (Fig. 4-6). It is shown previously in this thesis (Chapter 2) that mTORC1 activity is remarkably higher in cells with mutp53 have mTORC1 activity than in isogenic cells with WT p53. Now, this data suggests that PTX can decrease the mTORC1 kinase activity in the cells with gain of function mutp53.
Figure 4-3. Growth suppression curve of H1437 and H1048 cells after PTX (+/-TdR) treatment

Cells were treated for 72 hrs followed by counting by coulter counter. A. Cells were treated with PTX (+/- TdR), B. Cells were treated only with PTX
Figure 4-6 Effects of PTX on AMPK and mTORC1 pathway.

Cells were treated either with thymidine (Tdr) or PTX (1μM) + Tdr (5.6 μM) for 24 hrs followed by immunoblotting to analyze the P-Thr172 AMPK, pT704EBP1, 4EBP1 and p53 in A) H1437 cells B) H1048 cells.
4.8.3 PTX increases the levels of mutp53 without increasing the levels of its transcription targets

We have shown in chapter 3 that treatment of p53+/+ HCT116 cells with VP16, AICAR, or PTX leads to accumulation of WT p53. VP16 mediated activation of p53 leads to transactivation of its target genes such as p21, Sestrin2, Bax and HDM2, whereas the p53 accumulated in PTX treated cells is transcriptionally inactive. As a logical extension of that study we sought to understand how PTX affects gain of function mutant p53. Bug et al. (2011) showed that treatment with the anthracyclines, doxorubicin or duanorubicin induces the accumulation of mutant p53 and this may augment any new oncogenic functions of mutp53 (Bug & Dobbelstein, 2011). We decided to compare the effects of PTX and DNA damaging agents on mutp53.

We first studied the effects of PTX on mutp53 levels and on its transcription target Axl. Treatment of lung cancer cells, H1437 (Fig. 4-7A) and H1048 (Fig.4-7B) with PTX in the presence of thymidine increases the protein levels of mutp53. Surprisingly, PTX was not only inhibiting the increase in mutp53 transcription target Axl, it was causing a decrease in this protein. (Fig. 4-7). In chapter 3, we found that PTX accumulated p53 is transcriptionally inactive; hence, we asked whether PTX treatment would augment the oncogenic transcriptional activity of mutp53.

We treated H1048 (R273P) cells either with VP16 (20µM), Doxorubicin (2µM) or PTX (1µM) with TdR (5.6µM) for given time periods, followed by immunoblotting to check the levels of mutp53 and two of its transcription targets Axl and NFκB2 (Fig. 4-8A,B). Treatment with doxorubicin and PTX both leads to increase in mutp53 levels (Fig. 4-6,7,8). Considering that the basal levels of mutp53 in these cells are significantly higher than the basal levels of wt p53 in cancer cells, it is an interesting effect exerted by PTX. As expected, doxorubicin (Fig. 4-8A) and
VP16 (Fig. 4-8B) treatments activate mutp53 transcription activity towards its target genes Axl and NFκB2 but PTX does not (Fig.4-8). Axl and NFκB2 are known to promote tumor progression, providing a survival advantage to the cancer cells. This data suggests that whereas mutp53 cells, upon treatment with either VP16 or doxorubicin, will have the survival advantage provided by mutp53 transcription targets, PTX treated cells will not.
Figure 4-7 Effects of PTX on mutp53 and its transcription target Axl.
Cells were treated either with thymidine (Tdr) or PTX (1um) + Tdr (5.6 um) for 24 hrs followed by immunoblotting to analyze the levels of mutp53 and of Axl in A) H1437 cells B) H1048 cells.
Figure 4-8 Effects of VP16, doxorubicin and PTX on mutp53 levels and its transcription targets.

H1048 cells were treated either left untreated or treated with (A) doxorubicin (2μm) for 3, and 6 hrs or PTX (1μm) + Tdr (5.6 um) for 24 hrs (B) VP16 (20um) for 6,12,and 24 hrs or PTX (1μm) + Tdr (5.6 um) for indicated time durations followed by immunoblotting to analyze the levels of p53 and its transcription targets.
4.8.4 Unlike etoposide and doxorubicin, PTX does not lead to an increase in the transcription of mutp53 target genes

In these initial experiments, we saw that DNA damaging agents mediated an increase in the abundance of two mutp53 transcriptional targets, Axl and NFkB2, but that the time dependency of these increases could not be predicted and were different for the two transcriptional targets. Hence, we tested if that regulation was at the levels of transcription, as would be expected by analogy with the effects of wt p53. We first started with analyzing the levels of mutant p53 in four NSCLC cell lines; H1437, H1048, H661 and H441 (Fig. 4-9). As expected, the levels of p53 in H1048, H661 and H441 cells were very high in comparison to HCT116 with wt p53. Interestingly the level of mutant p53 was remarkably low in H1437 cells (Fig. 4-9). We treated four NSCLC cell with VP16 for 3, 6, 12 and 24 hrs, doxorubicin for 3, 6 and 12 hrs and PTX 6, 12 and 24 hrs followed by RNA extraction. In this analysis, we were looking for increases in the abundance of mut53 transcriptional targets, and we considered that mRNA levels might show a more consistent pattern. A cDNA synthesis reaction was performed from these RNA populations using random hexamer followed by RT-qPCR analysis to measure steady state levels of mRNAs. Drug-treated H1048 (R273P) lung cancer cells were analyzed for steady state mRNA levels of several mutp53 transcription targets: NFκB2, HSP70, Axl, PCNA, and hTERT. The mRNA levels of NFκB2, HSP70 and PCNA were increased after treatment with either etoposide or doxorubicin. The levels of Axl and hTERT mRNA levels were increased after etoposide but not after doxorubicin (Fig. 4-10). These increments were time course dependent as levels of mRNA of different genes are enhanced at different time points by the same drugs. None of these mRNAs were increased after PTX treatment (without thymidine) at any time point (Fig. 4-10).
Upon observing no increase in the mRNA levels of these genes after PTX treatment, we further tested if PTX in the presence of TdR would also have similar effects. Indeed, the effects of PTX with or without thymidine were almost identical (Fig. 4-11) as none of mutp53 targets showed any increase at the mRNA level after PTX or PTX + TdR treatment.

In order to understand if these effects of PTX on mut p53 were cell line dependent or more of general effects in different cell lines, we performed similar experiments in H1437, which bear a p53 gene with a mutation that confers a R to P change at codon 267. The levels of NFκB2, Axl, EGFR, MDR1, HSP70, and PCNA were increased by both VP16 and doxorubicin, however hTERT mRNA was increased only after etoposide treatment but not by doxorubicin (Fig 4-12). Once again the effects of the two DNA-damaging drugs were different for different genes. As expected, PTX did not increase the levels of mRNA of any of these neomorphic target genes. Interestingly, we saw an increase in p21, a wt p53 transcription target upon VP16 and doxorubicin but not after PTX treatment in this cell line that had no wt p53 allele (Fig. 4-12). This suggests that this p53 mutant itself still had some remaining wt p53 activity. The level of p53 in these cells also supports the similarity of behavior between the R267P mutant and wt p53, in that the levels of the R267P mutant p53 were maintained at lower levels than the levels expected for gain of function mutant p53s (Fig. 4-9). H1437 cells were also analyzed for differences in response after PTX and PTX + TdR. H1437 cells also showed almost identical results when treated with PTX in the presence or absence of thymidine (Fig. 4-13).

Two other lung cancer cells H441 (R158L) and H661 (R158L) were also tested to investigate the generality of the PTX mediated effects. H441 cells were treated in the same way as H1048 and H1437, and the data are shown in Fig 4-14. Levels of NFκB2, EGFR, HSP70, PCNA, and hTERT increased after VP16 or doxorubicin treatment in a time-dependent manner in H441.
The mRNA levels of hTERT were not affected by doxorubicin, there was an evident increase in the levels of hTERT mRNA after VP16 treatment. The levels of Axl mRNA were increased by VP16 but not by doxorubicin (Fig. 4-14). Again, none of the mRNAs levels for any of these mut p53 neomorphic targets was increased after PTX treatment (Fig. 4-14), suggesting that it was a generality that PTX does not increase mutp53-mediated GOF transcription of these oncogenic genes.

When H661 cells were studied, which have a mutation in p53 at codon 158 (R158L) NFκB2, and hTERT were increased after either VP16 or doxorubicin treatment. The mRNA levels of Axl, HSP70 and PCNA were increased only after doxorubicin treatment with no evident increase after VP16 treatment. As seen in the other three cells lines, none of these mRNAs were increased after PTX treatment (Fig. 4-15).

Overall this data suggested that, whereas both DNA damaging agents, VP16 and doxorubicin, lead to enhanced transcriptional activity of mutp53 in several lung cancer cell lines, PTX does not cause transcriptional activation of mutp53 transcription targets in any. These data is summarized in table 4-1. Hence, mutp53 containing lung cancers can exploit the survival advantage provided by the increased levels of oncogenic transcription targets of mutp53 followed by VP16 or doxorubicin, but not following PTX treatment.
Figure 4-9. Protein levels of p53 in different cell lines

All the cells were cultured in the same conditions and harvested. Equal levels of protein was loaded to compare the p53 levels in these cells lines. β-actin is used a loading control.
Figure 4-10. mRNA levels of various oncogenic genes in H1048 cells after DNA damaging drugs increased but not after PTX.

H1048 cells treated with etoposide (20um) for 6,12 and 24hrs, Doxorubicin (2um) for 3,6, and 12 hrs and PTX (1um) for 6,12, and 24 hrs. Steady state mRNA levels were analyzed using RT-qPCR. This experiment has been repeated twice.
mRNA levels in H1048 (R273P) cells

Figure 4-11. mRNA levels of various oncogenic genes in H1048 cells after DNA damaging drugs increased but not after PTX.

H1048 cells treated with etoposide (20um) for 6,12 and 24hrs, Doxorubicin (2um) for 3,6,and 12 hrs and PTX (1um) +/- TdR (5.6um ) for 24 hrs. Steady state mRNA levels were analyzed using RT-qPCR. This experiment ahs been repeated twice.
Figure 4-12. mRNA levels of various oncogenic genes in H1437 cells after DNA damaging drugs increased but not after PTX.

H1437 cells treated with etoposide (20μm) for 6, 12, and 24hrs, Doxorubicin (2μm) for 3, 6, and 12 hrs and PTX (1μm) for 6, 12, and 24 hrs. Steady state mRNA levels were analyzed using RT-qPCR. This experiment has been repeated twice.
Figure 4-13. mRNA levels of various oncogenic genes in H1437 cells after DNA damaging drugs increased but not after PTX.

H1437 cells treated with etoposide (20um) for 6,12 and 24hrs, Doxorubicin (2um) for 3,6,and 12 hrs and PTX (1um) +/- TdR (5.6um ) for 24 hrs. Steady state mRNA levels were analyzed using RT-qPCR. This experiment has been repeated twice.
mRNA levels in H441 (R158L) cells

Figure 4-14. mRNA levels of various oncogenic genes in H441 cells after DNA damaging drugs increased but not after PTX.

H441 treated with etoposide (20um) for 6,12 and 24hrs, Doxorubicin (2um) for 3,6,and 12 hrs , PTX (1um) for 6, 12 and 24 hrs. Steady state mRNA levels were analyzed using RT-qPCR. This experiment has been repeated twice.
Figure 4-15. mRNA levels of various oncogenic genes in H661 cells after DNA damaging drugs increased but not after PTX.

(A) H661 cells treated with etoposide (20μm) for 6, 12 and 24 hrs, Doxorubicin (2μm) for 3, 6, and 12 hrs and PTX (1μm) for 6, 12, and 24 hrs. Steady state mRNA levels were analyzed using RT-qPCR. This experiment has been done once.
Table 4-1 Summary: the highest increase in levels of mRNA in RT-qPCR analysis.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug</th>
<th>NFKB2</th>
<th>PCNA</th>
<th>hTERT</th>
<th>AXL</th>
<th>MDR1</th>
<th>EGFR</th>
<th>Hsp70</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1048 (R273C)</td>
<td>VP16</td>
<td>10</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>---</td>
<td>---</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>5</td>
<td>3</td>
<td>1.7</td>
<td>0.7</td>
<td>---</td>
<td>---</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>PTX (+/- TdR)</td>
<td>1</td>
<td>1</td>
<td>1.1</td>
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<tr>
<td>H1437 (R267C)</td>
<td>VP16</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>4.5</td>
<td>2.6</td>
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<td></td>
<td>Doxorubicin</td>
<td>7.3</td>
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<td>2.5</td>
<td>2.9</td>
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<td></td>
<td>PTX (+/- TdR)</td>
<td>0.8</td>
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<tr>
<td>H441 (R158L)</td>
<td>VP16</td>
<td>10</td>
<td>2</td>
<td>4</td>
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<td></td>
<td>Doxorubicin</td>
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<td>PTX (+/- TdR)</td>
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<td>1</td>
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<tr>
<td>H661 (R158L)</td>
<td>VP16</td>
<td>2</td>
<td>1.2</td>
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<td></td>
<td>Doxorubicin</td>
<td>2.2</td>
<td>2.7</td>
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<td></td>
<td>PTX (+/- TdR)</td>
<td>1</td>
<td>1.1</td>
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These numbers represent the highest fold increase in the mRNA levels upon indicated treatments. Numbers represent the ratios of mRNA levels found in the treated cells divided by that in untreated cells. Experiments done on H1437, H1048 and H441 cells were repeated twice and pattern of change in mRNA levels is reproducible. Experiment on H661 cells was done once.
4.8.5 PTX can block the VP16 mediated transcription activation of mutant p53

It has been previously reported that an increase in NFκB2, PCNA, MDR1, EGFR, Axl, Hsp70, and hTERT caused by transcriptional activation by mutp53 may lead to survival advantage to cancer cells and allow the development of chemo-resistance. We also had shown in Chapter 3 that PTX did not activate wt p53 and, in addition, could block the transcriptional activation of the p21 gene by wt p53. Because PTX does not cause activation of mutp53 transcription activity, we hypothesized that combining PTX with VP16 might block VP16 mediated the GOF transcriptional activation of mutp53 and inhibit the enhancement of oncogenic targets of mutp53. H1048 cells containing mutp53 (R273P) cells were either left untreated or treated with VP16 (20µM) for 6 hrs or PTX (1µM) in the presence or absence of TdR (5.6µM) for 24 hrs. For combination treatment, H1048 cells were treated with PTX for 20hrs followed by VP16 treatment for 6 hrs. Cells were harvested in Trizol and RNA was extracted. RT-qPCR analysis was performed to measure the steady state levels of mRNAs for the several genes discussed above. We found that, after 6 hrs treatment with VP16 alone, the levels of mRNA from genes of interest were increased significantly; NFκB2 increased 10-14 fold, PCNA increased 2.5-3 fold, AXL increased 3-3.5 fold, Hsp70 2-2.5 fold, and hTERT 5-8 fold (Fig 4-16). However, when cells were pretreated with PTX (+/- TdR) for 20 hrs followed by 6hr exposure to VP16, there was a 50-60% blockade of VP16-mediated increase in NFκB2 mRNA levels, a remarkable effect considering that NFκB2 is strongly transactivated by VP16 activated mutp53. More interestingly, we found a complete suppression of VP16 mediated increment in PCNA, AXL, Hsp70 and hTERT mRNA levels when cells were pretreated with PTX in the presence or absence of TdR (Fig. 4-16). This clearly suggests that pretreatment with PTX does not allow the VP16 mediated transcriptional activation of mutp53. This led us to hypothesize that if PTX can
block the transcription activation of mutp53 transcriptional targets induced by DNA-damaging drugs, then combination treatment of PTX will increase the cytotoxic effect of these drugs.

4.8.6 **Combination drug treatment shifts the growth curve towards left**

As we found that PTX can block the VP16 mediated enhancement of the transcriptional activity of mutp53, we hypothesized that combination treatment of these two drugs might lead to better cell growth suppression. H1048 and H1437 cells were either treated separately or co-treated with various concentrations of PTX and VP16 for 72 hrs followed by measurement of cell densities by coulter counter. We found that combination treatment of PTX and VP16 shows better growth suppression of H1437 and H1048 cells in comparison to either of the two drugs alone (Fig. 4-17), as evidenced by a shift of the dose-response curves for PTX to the left. It now remains to be proven whether blockade of transcriptional activity of mutp53 might be playing role in the cell growth suppression of the combination treatment.
Figure 4-16. PTX can block the VP16 mediated transcription activation of mutant p53.

H1048 cells were either left untreated or treated with VP16 (20µM) or PTX (1µM) in the presence or absence of TdR (5.6µM) for 24 hrs. For combination treatment H1048 cells were treated with PTX for 20hrs followed by VP16 treatment for 6 hrs. Steady state mRNA levels were analyzed by RT-qPCR. This experiment has been repeated twice and effects shown in this experiment are reproducible.
Figure 4-17. Combination of PTX and etoposide increases the sensitivity of GOF mutant p53 containing lung cancer cells.

(A,B) H1048 cells treated with increasing concentration of VP16 and PTX and cell densities were measured by couler counting after 72 hrs. C,D) H1437 cells treated with increasing concentration of VP16 and PTX and cell densities were measured by couler counter after 72 hrs. Data is plotted after normalizing with untreated cells, which is considered as 100% growth.
4.9 DISCUSSION

We asked a fairly simple but important question of how PTX will affect the cells with gain of function mutp53. We addressed this question using lung cancer cell lines with gain of function mutp53, because the most frequent gene mutated in lung cancer is p53.

As, in the third chapter of this thesis, we showed that cells with mutp53 have equivalent mTORC1 activity as isogenic p53 null cells, but much higher levels of p53. Hence, this study becomes very important, because it suggests that PTX can block the effects of loss of p53 as well as the effects of mutp53 on mTORC1.

We asked if PTX can inhibit transcriptional activity of mutp53 as it does the transcriptional activity of wt p53. Indeed, PTX blocks the transcriptional activation of GOF mutp53. This is a therapeutically very important phenomenon as it has been previously reported that treatment with DNA damaging drugs leads to enrichment in chemoresistant cells in cell culture due to increase levels of mutp53 (Blandino et al., 2002). It has been suggested that DNA damaging drug leads to increase in mutp53 levels, increasing the expression of proteins responsible for or factors in the development of chemo-resistance in these cancers and that, hence, provide growth advantage in the faced of drug treatement by supporting tumor progression ((MDR1) (Y. Matsumoto, Takano, & Fojo, 1997; Y. Matsumoto, Takano, Kunishio, Nagao, & Fojo, 2001; Y. Matsumoto, Tamiya, & Nagao, 2005) (NFKb2) (Harte et al., 2014; Ludes-Meyers et al., 1216; Scian et al., 1203; Scian et al., 2004; Shivakumar et al., 1995; Vaughan et al., 2012). This is the first time that it has been shown that a chemotherapeutic drug can inhibit the activation of GOF transcription from mutp53 and suggests that this effect may by involved in the therapy with PTX. As PTX can block the transactivation of transcriptional target of mutp53 gene, it suggests that
combination treatment of PTX with other drugs that usually stimulate mut p53 GOF transcription can be therapeutically more effective than these drugs alone.

Interestingly we found H441 and H661 cells, both of which have the same mutation in p53 (R158L) show different transcriptional activation profiles after doxorubicin or etoposide treatment. This suggested that mutation of other genes, perhaps KRAS in H441 and p63 in H661, was affecting the mut p53 selectivity towards its transcriptional targets as well as the intensity of transactivation. It is clear that each mutant p53 behaves differently and responds differently towards different DNA damaging drugs, with an overall of similarity but quite different details of the response. This complexity is rather daunting to explain. There are several questions left unanswered by my studies that beg experimental explanation. Some of those are discussed below;

1. Why do PTX and DNA damaging drug treatments lead to increased protein levels of mutp53? Our current understanding of mutp53 indicate that the untreated levels of mutp53 are much higher than untreated levels of wt p53 due to an increased half life, most likely due to a lower binding of mdm2 to the mutant forms of p53. Out of the several theories to explain this, he most likely one is that mutp53 is constantly phosphorylated at ser15 and thus not allow the binding of MDM2, avoiding degradation; the well studied mechanism of wt p53 stabilization after genotoxic stress. This raises a question that if mutp53 is already phosphorylated at ser15 than by what mechanism do these treatments increase mutp53 levels? However, we know by our studies in chapter 3 that PTX (with thymidine) does not mediate phosphorylation of ser15 of WT p53, but we don’t know what happens in the case of mutp53.
2. Why is the transcription activity of mutp53 compromised after PTX treatment: Studies in the chapter 3 done on PTX mediated effects on transcriptional activity of wt p53 suggests that PTX stabilizes a transcriptionally compromised p53 and we propose that this p53 is transcriptionally inactive due to the inability of PTX to induce a DNA damage signal. As we see that PTX leads to an increased levels of mutp53 without enhancing its transcriptional activity, it would be interesting to analyze the status of H2A.X in cells treated with VP16 or PTX to see if PTX effects on DNA damage are the same in cells with mutp53.

3. What are the post-translational modifications of mutp53 under PTX vs VP16 and doxorubicin. In Chapter 3, we examined the posttranslational modifications after PTX treatment and after VP16 treatment, and found that the post-transcriptional modifications of p53 were a reflection of the type of genotoxic stress induced by the drugs. Therefore, it will be interesting to know the posttranslational profile of mutp53 after different treatment to predict the underlying mechanism of action of these drugs.

4. What are the mechanism(s) of the PTX-mediated effects on transcriptional activation by wt p53 and mut p53. Our current understanding suggests that transcriptional activity of mutp53 is regulated very differently than that of wt p53. As PTX affects are similar towards transactivation inhibition of both wt and mut p53, this suggests a common mechanism by PTX is at play which interferes with transcription from both wt and mutp53. Thus a better understanding of the mechanism by which PTX blocks transcriptional activity of wt and mut p53 might improve the fundamental understanding of this field.
Chapter 5

5 Overview and perspective

It has been shown previously that some of the very frequent mutations in lung cancers, like EGFR mutations and amplifications, K-Ras mutations, and PI3K mutations act upstream of mTORC1 and feed into hyperactivity of mTORC1 in these cancers. However, the most frequent mutations found in lung cancer is p53, but it is not known how loss or mutation of p53 affects mTORC1 kinase activity.

My PhD. thesis work started by addressing the fairly simple but very important question of how p53 regulates mTORC1 activity. Later, after understanding the affects of deletion or hot-spot mutation of p53 on mTORC1 activity, we explored how this regulation may affect the therapeutics of PTX on cancer. This dissertation has also discovered a very important mechanism of the regulation of the mTORC1 pathway by mutp53 and how PTX affects the oncogenic affects of GOF mutp53 in lung cancer cells.

The loss or hotspot mutation of p53 enhance mTORC1 activity.

Our very initial studies in this quest led to the discovery that p53 null cells have higher mTORC1 activity because p53 null cells had lower levels of TSC2 and sestrin2. The mTORC1 complexes
in cells null for p53 had lower levels of TSC2 and PRAS40 bound with a increased levels of mTOR suggesting that upon loss of p53 more mTOR is present as mTORC1 complex and something fundamentally different was happening with the recruitment of components of the complex. These finding were confirmed and strengthened by experiments by my colleague Catherine M. Bell who performed subcellular fractionation and confocal microscopy studies to analyze the distribution and co-localization of mTOR, TSC2 and Rheb in cells with or without p53. The site of function of mTORC1 is currently thought to be the lysosomal membrane and TSC2 has been reported to be localized at this site under serum starved condition. Accordingly Ms Bell found that the levels of TSC2 are decreased in the lysosomal membrane of p53 null cells, and the decreased levels of TSC2 were concomitant with an increased level of Rheb at lysosomal membrane of p53 null cells. We also showed that cells with gain of function mutant p53 also have enhanced mTORC1 activity and this is also due to decrease in TSC2 and Sestrin2 levels in absence of WT p53 and its transcription activity. This is first time anyone had showed these effects. We concluded that deletion or hotspot mutation of p53 enhances mTORC1 activity by decreasing TSC2 levels, its localization at lysosomal membrane and at least partially as result of increased levels of Rheb at lysosomal membrane.

Future perspective
We still don’t understand the reasons for the differences seen in the components of mTORC1 complexes from wt and null p53 cells.

1. More mTOR present as mTORC1 complex: mTOR can be present in the form of two complexes, mTORC1 and mTORC2. Using an anti-raptor antibody for immunoprecipitations of mTORC1, more mTOR was pulled down in p53 -/- cells. As the levels of mTOR and Raptor are the same in the lysates from both cell lines this
suggested that, in p53 null cells, more Raptor-bound mTOR is present i.e, more mTORC1 complex is present. It seems as if the dynamic distribution of mTOR between mTORC1 and mTORC2 complex might be of importance for the mechanism of regulation of mTORC1 activity.

2. **Lower levels of PRAS40 bound to the complex:** PRAS40 is an inhibitor of mTORC1 kinase activity unless phosphorylated by AKT at T-246. In our immunoprecipitation studies, we saw that the levels of PRAS40 bound to mTORC1 complex is decreased in p53 null cells. We think that possibly PRAS40 interacts with mTORC1 complex via TSC2 and that the lower levels of TSC2 in p53/-/- cells led to decreased levels of PRAS40 binding to mTORC1 in these cells, contributing to the higher mTORC1 activity. However, there is no direct experimental evidence for this hypothesis, and studies to confirm or negate it will be very useful.

3. **Higher levels of Rheb in lysosomal membranes of p53 null cells:** Subcellular fractionation and confocal microscopy studies done by Ms. Bell, indicated that the levels of Rheb are increased at the lysosomal membrane of p53 null cells. Although we don’t understand the mechanism of this difference yet as there was no difference in the total Rheb protein levels and steady state mRNA levels in the cells. We think that possibly Rheb-GTP has higher affinity for lysosomal membrane than Rheb-GDP and this could explain the increased localization of Rheb in p53 null cells. Immunoprecipitations using an anti-raptor antibody also suggested more Rheb present in the mTORC1 complex from p53 null cells, an extremely controversial observation.

4. **Colocalization of TSC2 and Rheb with mTOR in mutp53 cells:** We showed that the levels of TSC2 are decreased in cells with mutp53 in comparison to p53 competent
cells and this phenomenon was consistent with the increased levels of mTORC1 activity. It would be of interest to see if the localization of TSC2 and Rheb in mutp53 cells are similar to that seen in p53 null cells.

**Because p53 negatively regulates mTORC1, it was expected that loss of p53 would diminish the therapeutic effects of PTX; it did not.**

My initial experiments of this study showed that PTX can effectively down-regulate the enhanced mTORC1 activity in p53 null cells. This result was more interesting than surprising. We continued on this study and sought to understand the mechanism of the p53-independent mechanism of PTX mediated inhibition of mTORC1. We found that, whereas AICAR-activated AMPK phosphorylates both TSC2 and Raptor, PTX-activated AMPK only phosphorylates Raptor but not TSC2. We discovered that the levels of TSC2 are not increased in p53 competent cells after PTX treatment because of a failure to activate p53 transcription. Therefore, we concluded that PTX-stabilized p53 does not increase levels of TSC2 and Setsrin2, so that, as in p53 null cells, PTX treated cells cannot phosphorylate and activate TSC2. We proved this postulate and showed that Raptor phosphorylation was sufficient for mTORC1 inhibition. We tried to understand the transcriptional incompetence of the p53 stabilized by PTX. It was not due to the binding of p53 at the promoter of p21, nor the recruitment of HATS to regional histones. We shifted our attention to the clues left by the pattern of PTMs in p53 after PTX.

**We conludeded that PTX (±TdR) does not cause transcriptional activation because it does not cause DNA damage of the nature caused by drugs such as VP16 or AICAR, which do**
activate transcription by p53.

Studies done to test this hypothesis brought several surprises to us. As expected, cells treated with PTX in the absence of TdR led to increased levels of H2A.X pS139, indicating genotoxic stress but we still did not observe any increase in the levels of p21.

Future perspectives

1. How does AMPK activation by PTX stabilize p53 ?

PTX in the presence of thymidine stabilizes p53 without causing phosphorylation of p53 at S15, a PTM thought to be required for the stabilization of p53 after DNA damage as it inhibit the interaction of p53 with it E3 ubiquitin ligase and protects p53 from proteolysis. How p53 can be stabilized without phosphorylation of S15 is as yet unknown.

2. Why does PTX in the absence of TdR not induce p53 transcription ? PTX without thymidine allows the effects of inhibition of thymidylate synthase to be expressed, including the incorporation of dUMP into DNA and subsequent damage to DNA following patch excision. It is of great interest that how inhibition of thymidylate synthase is not sufficient to activate p53 transcriptional activity. It seems that phosphorylation of p53 at S15 works as an enhancer of p53 stabilization but it is not a mandatory event for p53 stabilization, as we see higher levels of p53 in treatments that induce phosphorylation at S15 (VP16, AICAR and PTX (-TdR)), than in PTX+ TdR.

3. Chk1 activation has been reported to inhibit transcriptional activity of p53. Studies done by Beckerman and Prives suggested that HU-stabilized p53 is transcriptionally inactive due
to activation of Chk1. Following inhibition of Chk1 activation by an ATR inhibitor or by knocking down Chk1 using siRNA, p53 transcriptional activity can be regained. Interestingly, in my studies VP16 activates Chk1 and AICAR does not, however they both stabilize transcriptionally active p53, suggesting that the presence or absence of Chk1 activation is not mandatory for p53 transactivation. This is of great interest to dissect the phenomenon of how the activation of Chk1 can play as an inhibitor of p53 transactivation under one stimuli (HU), an activator in other (VP16) or just remain a non-affecting factor in another (AICAR, PTX (+TdR)).

4. **PTX (+TdR) can block the DNA damage by VP16 or AICAR and blocks p21 transcription.**

We found that cells pretreated with PTX (+TdR) blocked the VP16 or AICAR mediated DNA damage signal and increase of p21 levels. This is strong evidence that DNA damage is required for VP16 or AICAR mediated transcriptional activation of p53. A former PhD. student of our lab, Dr. Scott Rothbart showed that cells treated with PTX with thymidine are arrested at G1 stage of cell cycle, while cells treated with only PTX (- TdR) are arrested in S phase. This suggested that blockade of cells at the G1/S border or in S-phase does not allow the DNA damage to occur even after VP16 treatment, thus blocking DNA damage signaling, leaving p53 transcriptionally inactive. However, PTX in the absence of TdR blocks cells at S phase, probably due to a replication halt caused by thymidylate synthase inhibition. This replication halt allows the increased levels of pS139 H2AX but does not induce DNA double strand break DNA damage signal and thus does not allow p53-mediated induction of p21 levels. This also explains the PTX (+/-TdR) mediated block of VP16 and AICAR effects on p53 as cells are already arrested at G1 and S phase under PTX (+TdR) or PTX, respectively, without double strand
break. Therefore addition of VP16 or AICAR is incapable to induce DNA damage and in the absence of a certain type DNA damage (DSBs and SSBs), p53 is transcriptionally inactive.

5. **PTX-activated AMPK does not cause DNA damage and does not activate p21 transcription.** Whereas AICAR-activated AMPK causes DNA damage and leads to induction of p21 mRNA and protein levels, PTX-activated AMPK neither causes DNA damage nor induction of p21 levels. This suggests that AMPK activation per se does not induce DNA damage and AICAR induced DNA damage proceeds via an unknown mechanism.

6. **The relationship between activation of Chk1 and Chk2, phosphorylation of p53 at S15, and p53 transactivation.**

A great deal of research has been done in order to understand how DNA damage leads to stabilization, post-translational modification and transactivation of p53. A general understanding is that DNA damage leads to stabilization of p53 by multiple and specific posttranslational modifications by the activity of several kinases: ATM/ATR/ Chk2/Chk1, leading to transcriptional activation of p53. My studies are suggesting that the correlation of S15 phosphorylation of p53 with p53 stabilization does not necessarily apply to conditions other than DNA damage. Also, multiple phosphorylations at specific residues of p53 have been reported to be necessary for p53 transactivation, which does not seem to be the case, at least under the condition of AICAR treatment. In addition, Chk1/Chk2 have been shown to be involved in the S15 phosphorylation of p53, which is also not in accordance with our studies as AICAR shows pS15 but no p-S345 Chk1 and PTX (-TdR) shows p-S15 but no pChk2. Therefore, dissection of
the interdependence of these events would be a great contribution in the field of p53 signaling pathways, especially after treatment with AMPK activators.

Transactivation of unique genes by GOF mutant p53 and PTX

We found that PTX does not activate transcriptional activity of wt p53 and it blocks VP16 mediated transactivation of mutp53. In four lung cancer cell lines with different hot-spot mutations I analyzed the transcriptional activity of mutp53 at 7 different genes after treatment with the DNA damaging agents, VP16 and Doxorubicin or PTX (+/- TdR). All of these genes have been described in prior literature as being transcriptional targets of GOF mutp53. The overall pattern suggested that, whereas treatment with VP16 and doxorubicin leads to transactivation of mutp53 and increases the levels of mRNA from these several genes (albeit to different levels in different cell lines), PTX (+/-TdR) does not allow the transactivation of these genes in any cell line. More interestingly, PTX can block the VP16 mediated transactivation of GOF mutp53. This finding is very important as this suggests that where cancer cells with mutp53 can have a survival advantage upon treatment with VP16 or doxorubicin due to increased expression of the proliferative and cell survival genes, treatment with PTX will block this effect.

Future perspectives

1. Mechanism of transactivation of mutp53: Not much is known about the transactivation of mutp53. The fact that PTX can block the transactivation of both wt and GOF mutp53 suggests the use of some common mechanism of transactivation. Therefore, analysis of
DNA damage signaling under PTX treatment in mutp53-carrying cells might be of interest and may reveal the mechanism of these neomorphic properties.

2. Why do PTX or DNA damaging drugs lead to increased protein levels of mutp53: As little is known about the stabilization of mutp53, these drugs can be used as a tool to manipulate the pathway and understand the possible mechanism of mutp53 accumulation in cancer cells.

3. Importance of post translational modification of mutp53 in its oncogenic activity: Understanding the levels of various posttranslational modification of mutp53 after different drug treatment or genotoxic stresses might help in understanding the mechanism of transactivation of this protein.

4. Understanding the protein-protein interaction: mut p53 has been reported to interact with p53 family proteins (p73 and p63) and inhibit their p53-like activity as one of its several mechanisms by which mutp53 provides survival advantage to cancer cells. Therefore, it will be interesting to understand if these different drugs lead to different protein-protein interactions with p73 or p63, providing or avoiding a survival advantage.


    Acadesine extends the window of protection afforded by ischaemic preconditioning in

    uncoupling of MCM helicase and DNA polymerase activities activates the ATR-
    dependent checkpoint. Genes Dev., 1;19(9):1040-52

    Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane


    phase I evaluation of the quinazoline antifolate thymidylate synthase inhibitor, N10-

    87(1):87-91.

    Targeting grb7/erk/foxm1 signaling pathway impairs aggressiveness of ovarian cancer


69. FARBER, S., & DIAMOND, L. K. (1948). Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid [*FOLIC ACID/antagonist; *LEUKEMIA EDAT- 1948/06/03 00:00 MHDA- 2008/10/24 09:00 CRDT- 1948/06/03 00:00 AID - 10.1056/NEJM194806032382301 [doi] PST - ppublish]. N Engl J Med. 3;238(23):787-93.


296


94. GILMAN, A. (1963). The initial clinical trial of nitrogen mustard [*MECHLORETHAMINE EDAT- 1963/05/01 MHDA- 1963/05/01 00:01 CRDT- 1963/05/01 00:00 PST - ppublish], Am J Surgery, 105:574-8


251. Nguyen, T. A., Menendez, D., Resnick, M. A., & Anderson, C. W. Mutant TP53 posttranslational modifications: Challenges and opportunities [TP53; acetylation; methylation; p53; phosphorylation; transcription; ubiquitylation EDAT-2014/01/08 06:00 MHDA-2014/01/08 06:00 CRDT-2014/01/08 06:00 PMCR-2015/06/01 00:00 PHST-2013/09/06 [received] PHST-2014/01/02 [accepted] PHST-2014/02/11 [aheadofprint] AID-10.1002/humu.22506 [doi] PST-ppublish]. Hum Mutat, 35(6):738-55.


ribofuranoside (AICAR) on childhood acute lymphoblastic leukemia (ALL) cells: Implication for targeted therapy. Mol Cancer, 10;6:46.


[*NEOPLASMS/experimental; *PURINES/effects EDAT- 1954/05/01 MHDA-1954/05/01 00:01 CRDT- 1954/05/01 00:00 PST - ppublish]. Cancer Res., 14(4):294-8.


progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. Mol Cell Biol., 25(24):10875-94.


409. WERKHEISER, W. C. (1963). The biochemical, cellular, and pharmacological action and effects of the folic acid antagonists [*BIOCHEMISTRY; *ENZYME INHIBITORS; *FOLIC ACID ANTAGONISTS; *NEOPLASM THERAPY; *OXIDOREDUCTASES; *PHARMACOLOGY; *REVIEW EDAT- 1963/09/01 MHDA- 1963/09/01 00:01 CRDT- 1963/09/01 00:00 PST - ppublish]. Cancer Res., 23:1277-85.


Vita

Stuti Agarwal was born on July 6th, 1982, in Lucknow, India. She completed her Bachelor’s degree of Science from Lucknow University in 2004 and attended Hamdard University in the following fall for Master’s in Science, Department of Biotechnology. She qualified the National Eligibility Test, India for lecturereship and worked for two years as part time lecturer in New Delhi, India. She started her graduate studies at Virginia Commonwealth University in the department of Pharmacology and Toxicology in the fall of 2009, where she later joined the laboratory of Richard G. Moran in the spring of 2010. She was inducted in Phi Kappa Phi honor Society in 2010, VCU. In 2011 she received “Clayton Award” in the recognition of academic excellence to graduate student of school of medicine, VCU. In 2012 she recived the “Nomination- Phi Kappa Phi ScholarshipAward. In 2012, she joinied Tiranga- Indian National at VCU as general secretary and in 2013-14 she served as the President of Tiranga- Indian National at VCU. Tiranga awarded her “Excellent Leadership” award for her leadership skills. She received “University Service Award” in two consecutive years; 2012 and 2013, in the recognition of the outstanding commitment and service activities to various professional organizations and Greater Richmond community. In At Virginia Commonwealth University she presented a poster on her dissertation work at the AACR 105th Annual Meeting in 2014. Stuti joined Virginia Academy of Science (VAS) in 2013, as a secretery of Medical Science section. Stuti aided in the organization, execution of the Medical Sciences section of the 73rd annual meeting. She was one of the judges in VAS, 73rd annual meeting for poster and oral presentations by graduate student of Virgina. She served twice as the one of the judges in the local school science fairs in 2013 and 2014.