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Mechanism of Pentose Phosphate Pathway Regulation by Akt in *de Novo* Purine Synthesis During Essential Amino Acid Starvation

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Deron Trent Amador

Committee in charge:

    Professor Gerard Boss, Chair
    Professor Eliña Zuniga, Co-Chair
    Professor Elvira Tour

2012

The Thesis of Deron Trent Amador is approved and it is acceptable in quality and
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Chair

University of California, San Diego

2012

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ABSTRACT OF THE THESIS

Mechanism of Pentose Phosphate Pathway Regulation by Akt in *de Novo* Purine Synthesis During Essential Amino Acid Starvation

by

Deron Trent Amador

Master of Science in Biology

University of California, San Diego, 2012

Professor Gerard Boss, Chair
Professor Elina Zuñiga, Co-Chair

This study investigates the regulation of *de Novo* purine nucleotide synthesis by two pentose phosphate enzymes mediated by Akt, a kinase which may be affected by amino acid availability. HeLa cells starved of lysine for three hours show up to a 66% decrease in *de Novo* purine synthesis rates compared to control levels. A 40% reduction in PRPP availability after lysine starvation, the product of non-oxidative PPP and precursor substrate to the first committed step in *de Novo* synthesis, most likely contributes to this overall reduction in purine synthesis. Akt activation, as well as lysine return for one hour, almost completely restores *de Novo* synthesis rates to

control levels. TKT and TAL, enzymes unique to the PPP, show enzyme activity levels 57% and 49% of control levels during lysine starvation respectively, but can be restored to control levels upon Akt activation or lysine return. Cells expressing vectors of kinase-dead, dominant negative Akt fail to restore enzyme activity levels to control levels after lysine return. This strongly suggests that lysine starvation-induced reduction in Akt activity mediates TKT and TAL activity levels, thereby regulating *de Novo* purine nucleotide synthesis.

INTRODUCTION

This study explores the regulatory relationship between the role single essential amino acid starvation plays on the phosphatidylinositol 3-kinase/Akt (PI3K/AKT) signal transduction pathway and its subsequent regulation of pentose phosphate pathway (PPP) enzymes transketolase (TKT) and transaldolase (TAL), a process responsible for creating necessary precursor substrates for *de novo* purine nucleotide synthesis. Due to the potential complexity of the subject, the introduction has been structured in the following way: firstly, a brief description of each pathway and the major enzymes of interest within these pathways are discussed, followed by a discussion of the project itself.

THE PHOSPHATIDYLINOSITOL 3-KINASE/AKT SIGNALING PATHWAY - The PI3K/Akt signaling pathway begins with the binding of insulin or other growth factors to a transmembrane tyrosine receptor kinase, resulting in its auto-phosphorylation and the activation of the protein phosphatidylinositol 3-Kinase (PI3K). This activation leads to the recruitment of several proteins, ultimately resulting to the phosphorylation and activation of Akt (also known as protein kinase B). A detailed depiction of the signaling process is illustrated in Figure 1.

Akt is a serine-threonine kinase, activated by phosphorylation at Threonine-308 and Serine-437 [1] with the capability to phosphorylate proteins containing the amino acid sequence RXRXXS/T-B (X representing any amino acid and B representing those containing bulky hydrophobic residues) [2]. Three isoforms of Akt exist (Akt1, Akt2 and Akt3). Akt is often considered a "master regulator" due to its role in several vital cellular processes and its almost ubiquitous expression throughout all tissue types [3].

These cellular processes include cell survival, proliferation and growth [4], as well as angiogenesis [5], glucose uptake and metabolism [6]. In order to accomplish this, Akt can have several downstream targets for each physiological response, which is further complicated by the fact that these targets themselves may mediate more than one cellular process. For instance, Akt can inhibit several pro-apoptotic proteins resulting in the enhancement of cell survival: Akt blocks FOXO mediated transcription of pro-apoptotic proteins [7] and phosphorylates BAD, resulting in its inhibition of apoptotic function [8,9]. FOXO, however, plays a regulatory role in other cellular processes such as cellular metabolism and proliferation [10]. Proliferation can be regulated by several Akt-mediated targets involved in cell-cycle progression. One such process implicates the phosphorylation of p27, inhibiting localization of the cyclin-dependent kinase inhibitor to the nucleus therefore rendering it unable to inhibit cell cycle progression. Proliferation is enhanced not due to the inactivation of p27 function but by its sequestration to the cytosol [10,11]. Another prime example is the phosphorylation of glycogen synthase kinase (GSK3). Inhibition of GSK3 upon phosphorylation prevents kinase activity on its substrate glycogen synthase, promoting glycogen synthesis [11]. Additionally, inhibition of GSK3 prevents subsequent phosphorylation and targeting for degradation of cyclins D and E, transcription factors *c-jun* and *c-myc*, which all regulate G1-to-S-phase cell cycle progression [12]. GSK3 has also been shown to directly inhibit pro-survival factors such as Bcl-2 family members, its inactivation thereby increasing cell survival and proliferation [11]. In short, Akt-mediated phosphorylation of GSK3 enhances glycogen synthesis, cell cycle progression, survival and proliferation.
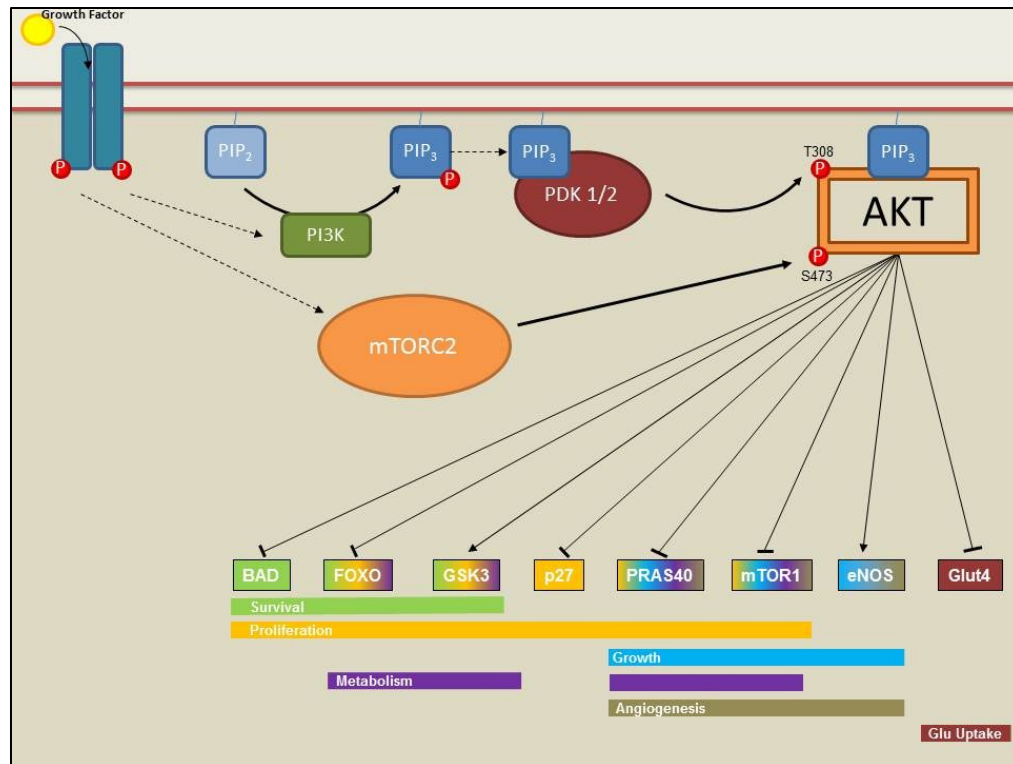
FIGURE 1. **General schematic diagram of the phosphatidylinositol 3-Kinase/Akt signal transduction pathway with targets of Akt and resulting cellular processes.** Transmembrane receptor tyrosine kinases are bound by insulin or other activating growth factors which in turn activate phosphatidylinositol 3-kinase (PI3K) via direct binding or phosphorylation of scaffolding adaptors (not pictured). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$). The production of PIP$_3$ stimulates localization and binding of phosphoinositide dependent protein kinase 1 (PDK1) and Akt to PIP$_3$ at the cell membrane. Activated PDK1 phosphorylates Akt at T308. Akt can also be phosphorylated at S473 by the receptor tyrosine kinase mediated activation of mammalian target of rapamycin complex 2 (mTORC2). Akt mediated phosphorylation can then result in the activation or inhibition of target substrates controlling several cellular processes. Multiple substrates are illustrated to demonstrate the versatility of Akt and overlap of target function. Akt phosphorylates BCL2 antagonist of cell death (BAD) and Forkhead Box Protein O (FOXO), sequestering them from their apoptosis-inducing target genes and therefore promoting cell survival. Cell cycle progression is promoted by the phosphorylation and inhibition (via cytosolic sequestration) of p27^{Kip1}, a cyclin dependent kinase inhibitor. Cell proliferation is promoted by glycogen synthase kinase 3 (GSK3) phosphorylation. Association with Akt promotes localization of glucose transporter 4 (Glut4) to the cell membrane to increase glucose uptake. Endothelial nitric oxide synthase (eNOS) stimulates angiogenesis by releasing nitric oxide after activation via Akt phosphorylation. Akt mediated activation of mTORC1 regulates several cellular process within a complex relationship [10].

A great clinical significance exists between the regulatory role of the PI3K/Akt signaling pathway, its downstream targets and disease. Of greatest concern is the part it plays in cell transformation, malignancy and tumor progression. With few possible exceptions, PI3K signaling is studied more commonly in human cancer than any other pathway. It is not unrealistic when you examine the mechanism behind its oncogenic activity: PI3K function is activated in human cancer (whereas other tumor suppressor pathways are inhibited), making it a prime candidate for therapeutic targeting; it is generally much easier to inhibit activation than restore lost function to a target [11]. Studies involving the selective elimination of Akt isoforms in knockout mice have revealed that each may play a unique role in cancer progression: Akt2 has been implicated in motility and invasion [13], amplified in pancreatic, breast and ovarian tumors; Akt3 in hormone-insensitive prostate and breast cancers, implicating involvement in hormone independence [14]. Akt1 abnormalities are observed much less frequently. Beyond cancer, Akt knockouts have elucidated the kinase's role in diabetes [15, 16], brain and nervous system [17, 18], skin, muscle and bone development [19]. Akt1 knock-out mice and normal for Akt2 shows unaffected glucose homeostasis but smaller animals, whereas Akt2 null mice (with normal Akt1) show diabetes-like phenotypes and only mild growth deficiencies. This supports previous theories that Akt2 plays a greater role in insulin signaling and Akt1 in growth [20].

THE PENTOSE PHOSPHATE PATHWAY, TKT AND TAL - In most tissues, the process of glycolysis accounts for the majority of glucose oxidation and production of ATP. Unlike glycolysis, the PPP does not result in the production of ATP and uses glucose for a different purpose. It can be divided into two separate branches; the first being the oxidative branch which serves the purpose of producing

NADPH, an important reducing agent in many metabolic pathways, and the second being the non-oxidative branch, converting six-carbon sugars to five-carbon compounds, namely fructose 6-phosphate to ribulose 5-phosphate. Unlike the irreversible reactions of the oxidative branch of PPP, the non-oxidative branch consists of a series of reactions which are reversible and reconstitute glucose 6-phosphate from pentose phosphates. The flow of PPP is dependent upon the needs of NAPDH, ATP and ribose 5-phosphate [21]. Under normal conditions, the non-oxidative branch of the PPP accounts for majority of production of ribose 5-phosphate from glucose 6-phosphate [22]. Ribose 5-phosphate is required for biosynthesis of nucleic acids as it represents the sugar component of RNA and DNA (in the form of deoxyribose) and can therefore serve to feed the pathways of nucleotide metabolism. Ribose is also necessary for, but not limited to, the production of other intermediates such as cAMP, FAD and coenzyme A [21].

One of the two enzymes unique to the non-oxidative branch of PPP is transketolse (the other being transaldolase), a homodimer with two active sites located at the interface between the two protein monomers. It is the rate-limiting enzyme of the pathway and a thiamine diphosphate-dependent enzyme, requiring one of these molecules per subunit [23]. As a primary function of the PPP is to produce NADPH, levels of TKT are much higher in tissues requiring large amounts of fatty acid synthesis such as adipose tissue and mammary glands. Conversely, tissues requiring greater amounts of ribose for the production of steroids form acetyl CoA also experience higher levels of TKT expression [24]. This can be seen in tissues such as the adrenal cortex. As can be seen by figure 2, TKT is responsible

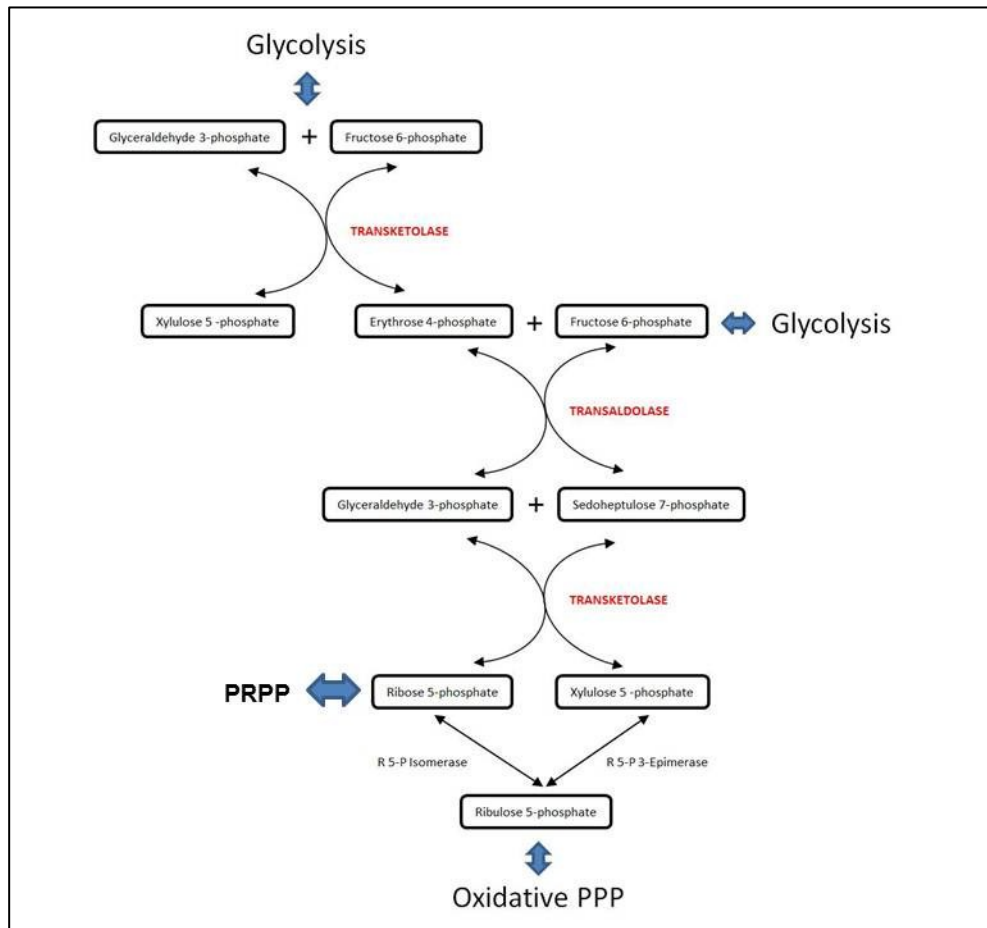for catalyzing two reactions within the non-oxidative PPP: the first converting

FIGURE 2. **Schematic diagram of the non-oxidative branch of the pentosephosphate pathway.** The non-oxidative branch of the PPP serves as a link between glycolysis and the oxidative branch of the PPP and produces the majority of ribose-5-phosphate (R5P) under normal conditions. In the first step of the completely reversible non-oxidative branch of the PPP, glyceraldehyde-3-phosphate (G3P) and fructose-6-phosphate (F6P) are converted into xylulose-5-phosphate (X5P) and erythrose-4-phosphate (E4P) by the thiamine pyrophosphate (TPP)-dependent enzyme TKT. The second reaction is carried out by TAL, which converts E4P and F6P into G3P and sedoheptulose-7-phosphate (S7P) by removing a three-carbon fragment from F6P and condensing it with the four-carbon sugar E4P. TKT catalyzes the third reaction in the pathway by removing a two-carbon fragment from S7P and condensing it with G3P, producing ribose-5-phosphate (R5P) and X5P respectively. R5P can then be used in nucleotide synthesis after being converted into phosphoribosylpyrophosphate (PRPP) by PRPP synthase, or follow the fate of X5P, being converted into R5P by R5P isomerase and R5P-3 isomerase, respectively. TKT requires the cofactor TPP in order to stabilize its carbanion intermediates, whereas TAL stabilizes its intermediate through the formation of a Schiff base between a lysine side chain and the substrate's carbonyl group.

glyceraldehyde 3-phosphate (G3P) and fructose 6-phosphate (F6P) into xylulose 5-phosphate (X5P) and erythrose 4-phosphate (E4P), and the second converting sedoheptulose 7-phosphate (S7P) and G3P into X5P and Ribose 5-phosphate (R5P) [25].

In humans, three TKT genes have been discovered: TKT, transketolase-like-1 (TKTL1) and transketolase-like-2 (TKTL2). Evidence has shown overexpression of TKTL1 exists in colon and urethral cancers, with poor patient survival [26]. Another study revealed an overexpression of TKTL1 in breast cancer: 86% expression in neoplastic tissue versus 29% expression in normal, non-neoplastic tissue [27]. An approximately 2.5-fold increase in TKT activity in neoplastic tissue seems to be a general consensus across many studies [28]. In addition, *in vitro* studies show treating tumor cells with specific TKT inhibitors reduces cell proliferation [29] and amplifying TKT activity via thiamine supplementation stimulates tumor growth [30], further suggesting a relationship between TKT and cancer. Gene specific studies to date seem to suggest that TKT and TKTL2 are not overexpressed in neoplastic tissue to the extent that TKTL1 is, although this is one area that needs to be pursued further.

TKT deficiency has also been implicated in diabetes. In one specific study, vitamin B1 (a thiamine derivative) supplementation activated TKT and prevented diabetic retinopathy. This retinal damage may lead to blindness and affects 80% of all patients afflicted with diabetes for ten years or more [31].

Transaldolase (TAL), the second unique enzyme in the non-oxidative PPP, functions without a cofactor and catalyzes the conversion of E4P and F6P into G3P and S7P [25]. Like TKT, its expression is increased in tissues requiring a large

amount of lipid synthesis; it increases upon adipocyte cell division and peaks during the development of the nervous system [32], most likely attributable to the high demand for myelination [33] of nervous tissue. The most common structure seen is that of a homodimer [34] consisting of 310-350 amino acids per subunit [35] and forms a complex with two other PPP enzymes (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) localized to cellular glucose transporters [36]. Several studies suggest that TAL activity is moderated by the post-translational modification of phosphorylation with a great amount of conservation throughout all three domains of life [25].

Unlike TKT, humans only possess a single gene for TAL, organized into 8 exons on chromosome 11 [25], but similar to TKT, TAL overexpression has been associated with cancer. Breast, colon, kidney, liver and lung tumors and leukemia have shown high levels of TAL expression [25]. Even non-neoplastic lung epithelium of smokers has exhibited higher expression levels, most likely attributed to the need for oxidation-reduction balance within intracellular environments commonly exposed to the chemicals present in cigarettes [37].

TAL deficiency is seen far less commonly. TAL antibodies are present in the cerebrospinal fluid of some patients suffering from multiple sclerosis [38, 39] and other cases of deficiency carry symptoms of hemolytic anemia, liver cirrhosis and hydrops fetalis [40].

DE NOVO SYNTHESIS OF PURINE NUCLEOTIDES – Most obviously nucleotides serve as the precursor to DNA and RNA, but they also provide components for second messengers such as cGMP and cAMP within signal transduction, cofactors like NAD and FAD and chemical carriers of energy such as

ATP [25]. Two purine synthesis pathways exist; one being the salvage pathway in which nucleic acids are catabolized to yield free nucleosides and bases to be recycled [41]. This is a very energy-conserving method. Salvage requires only the energy from one ATP when compared to the primary synthetic pathway, *de novo* synthesis, which demands 5 to 7 nucleotides for the constitution of AMP and GMP respectively [42]. This expense is due to the process itself, in which the purine ring structure is not salvaged from an existing nucleotide, but built up slowly by the sequential addition to ribose of a few atoms at a time [25]. Importantly, cellular pools of free bases that can be utilized by salvage account for approximately one percent of what is actually needed for DNA synthesis, suggesting that the great majority of nucleotides during DNA synthesis must result from the *de novo* pathway.

The *de novo* process begins by the conversion of R5P (a product of non-oxidative PPP) to PRPP. The first committed step of the pathway occurs next, with the donation of an amino group by glutamine to PRPP, catalyzed by PRPP synthetase. The purine structure is then sequentially built up by the donation of molecules to the backbone by glycine, glutamine, aspartate and formate to yield inosinate (IMP), which serves as the intermediate for both adenylate (AMP) and guanylate (GMP). Differing enzymes and processes are carried out to produce AMP and GMP from IMP. The same holds true for the subsequent conversion of AMP and GMP to ADP and GDP respectively, however the non-specific enzyme nucleoside diphosphate kinase works to convert not only both purine nucleoside diphosphates to triphosphates, but pyramidine nucleosides as well. These ribonucleotides are then reduced at the 2' carbon atom by ribonucleotide reductase to ultimately provide the corresponding deoxyribonucleotides used in the synthesis of DNA molecules. The detailed synthesis of purine ribonucleotides can be seen in Figure 3.
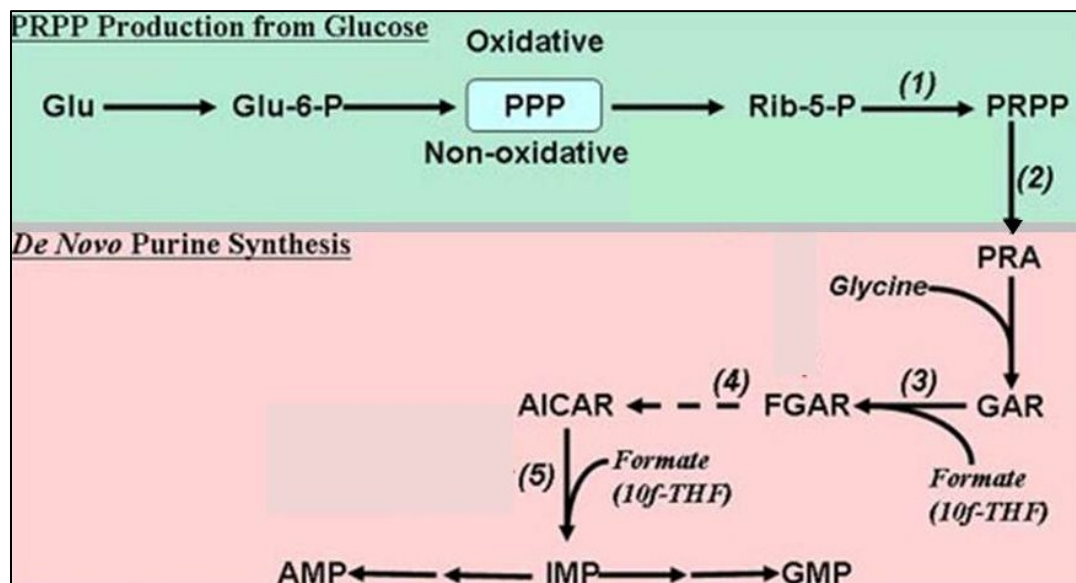
FIGURE 3. **Schematic diagram of the *de novo* synthesis of purine nucleotides from ribose 5-phosphate.** The product of the non-oxidative PPP, R5P, is converted to PRPP by ribose phosphate pyrophosphokinase *(reaction 1)*. In the first committed step of the *de novo* synthesis pathway *(reaction 2)*, glutamine donates an amino group to PRPP at the C-1 position, a reaction catalyzed by amidophosphoribosyl transferase (PRPP transferase), to produce 5-phosphoribosylamine (PRA). One ATP is consumed in the activation of the glycine donating group, in which GAR synthetase catalyzes a three atom addition resulting in the creation of glycinamide ribonucleotide (GAR). In *reaction 3*, GAR is converted to formylglycinamide ribonucleotide (FGAR) via formylation by N^{10}-formyltetrahydrofolate (10*f*-THF), catalyzed by GAR transfomylase. *Reaction 4* consists of multiple steps in which glutamine contributes nitrogen, a ring closure to form the imidazole ring of the purine nucleus occurs and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) results. The conversion of AICAR *(reaction 5)* into Inosate (IMP), occurs in two steps in which the final carbon is contributed by a second N^{10}-formyltetrahydrofolate. Inosate (IMP), which symbolizes the creation of the first complete purine ring and the initial intermediate of purine nucleotide synthesis, results from the dehydration and ring closure by IMP cyclohydrolase. Adenylate (AMP) and guanylate (GMP) are synthesized from IMP by a separate series of reactions and enzymes, requiring aspartate and GTP as an energy source for AMP. NAD^+, glutamine and ATP as an energy source yield GMP. Nucleoside monophosphate kinases, enzymes discriminatory toward bases but not sugars, catalyze the AMP/GMP conversion to the respective diphosphate. All nucleoside diphosphates (both purines and pyrimidines) are phosphorylated by nucleoside diphosphate kinase to produce the corresponding nucleotides. Conversion of monophosphates to triphosphates are not pictured. Schematic diagram adopted from Wei et al., 2008 [38].

Several points of regulation by feedback inhibition are known to exist within the purine biosynthesis pathway. For example, accumulation of ADP and GDP result in inhibition of PRPP synthetase. In addition, PRPP amidotransferase is inhibited by IMP, AMP and GMP [25].

EXPLORING THE REGULATORY RELATIONSHIP BETWEEN PI3K/AKT, PPP AND DE NOVO PURINE SYNTHESIS – Boss et al. published an extremely important study that addressed the relationship between amino acid starvation and decreased rates of purine biosynthesis, hypothesizing that the decreased rate was due to the attrition of one or more enzymes involved in the pathway that possessed an extremely fast turn-over rate. This theory was supported by the dramatic reconstitution of synthesis activity as soon as 15 minutes after return of the deficient amino acid to starved cells. Secondly, *de novo* synthesis was almost completely silenced with the addition of protein synthesis inhibitors [43]. Finally, it was observed that purine synthesis falloff in amino acid starved cells mimicked first-order kinetics similar to that of enzyme decay [43, 44].

Other theories exist as well. It is conceivable that an increase in the intracellular purine pool (due to decreased rates of synthesis) would result in feedback inhibition of *de novo* synthesis enzyme PRPP-amidotransferase as discussed earlier [lenihnger], however several studies have shown that amino acid starvation results in no change in the free nucleotide pool. One study even noted a small *decrease* in the AMP concentration which would, if anything, *increase* the rate of synthesis [43, 45, 46].

Secondly, one cannot overlook the fact that rates of synthesis are typically measured by radiolabeled formate incorporation into the nucleotides. Formate is

attained through cellular folate pools as previously discussed (Figure 2). If amino acid starvation were to affect folate enzymes to a greater extent, then it follows less labeled formate would be incorporated, giving the illusion that levels of synthesis had decreased. To address this, Boss et al. compared formate incorporation levels to radiolabeled leucine incorporation within cellular protein. A proportionate decrease between the two was observed, therefore supporting the hypothesis that folate and *de novo* synthesis enzymes are affected equally by amino acid starvation [43].

Considering that the protein kinase Akt has a role in the regulation of numerous vital cellular processes, including glucose, lipid and protein metabolism [15, 16], Akt would be a strong candidate for modulating nucleotide synthesis. As Boss et al. points out, even before the detailed study of Akt as a regulator of purine nucleotide synthesis, several studies supported the possibility. Cells expressing constitutively activated Akt expressed ATP at levels three times higher than that of control cells [47, 48], whereas cells lacking Akt demonstrate diminished ATP levels [47]. Also, treating cells with growth factors stimulating PI3K and Akt activity increase expression levels of enzymes involved in purine synthesis [49].

Recently, regulation of *de novo* purine synthesis was shown to be PI3K/Akt mediated within the biosynthetic pathway at several points in mouse mesenchymal cells. One such point of regulation was observed to exist at the non-oxidative branch of the PPP by the variability of PRPP production, the precursor molecule of the first committed step in the purine synthesis pathway [22].

Elucidation of this relationship is of particular importance to the medical field. For example, several forms of cancer have been shown to contain elevated levels of expression and activity for Akt as well as the two non-oxidative PPP enzymes TKT

and TAL. Due to the regulatory role of Akt within several processes found to be altered (in most cases elevated) in malignant cells such as increased cell proliferation, cycle progression, metabolism, inhibition of this enzyme and its targets has been and continues to be extensively studied. Should TKT and TAL prove to be targets as well, new possibilities for attacking Akt's influence within cancerous cell lines can be explored. Taking this into consideration, this project evaluates the viability of *de novo* purine synthesis regulation via Akt-mediated control of the two unique PPP enzymes, TKT and TAL, as induced by lysine starvation. The proposed general mechanism is illustrated in Figure 4.

Lysine, a basic amino acid, was chosen for several reasons: (1) Lysine is an essential amino acid. The inability of cells to synthesize it *de novo* ensures that any loss of enzymatic activity cannot be rescued by the natural synthesis of lysine by the cell. (2) It does not participate as a donor amino acid in the *de novo* synthesis of purine nucleotides and will therefore not directly inhibit rates of biosynthesis by lack of cellular concentrations of a necessary precursor molecule. (3) Starving cells of lysine in particular has demonstrated effectiveness in reducing levels of *de novo* synthesis in prior studies [43]. (4) Media lacking lysine supplementation was readily available and relatively inexpensive to obtain. It should also be mentioned that initial trials exploring both leucine and methionine starvation were performed as well but yielded much less notable results and were therefore not pursued.

This study was conducted with two specific aims in mind: to investigate the role of Akt as a regulator of *de novo* purine synthesis during essential amino acid starvation and to assess the role of Akt in amino acid-mediated TKT/TAL regulation. We hypothesize that Akt regulates TKT/TAL, leading to the regulation of *de novo*

purine biosynthesis during amino acid starvation. To accomplish this, all experiments
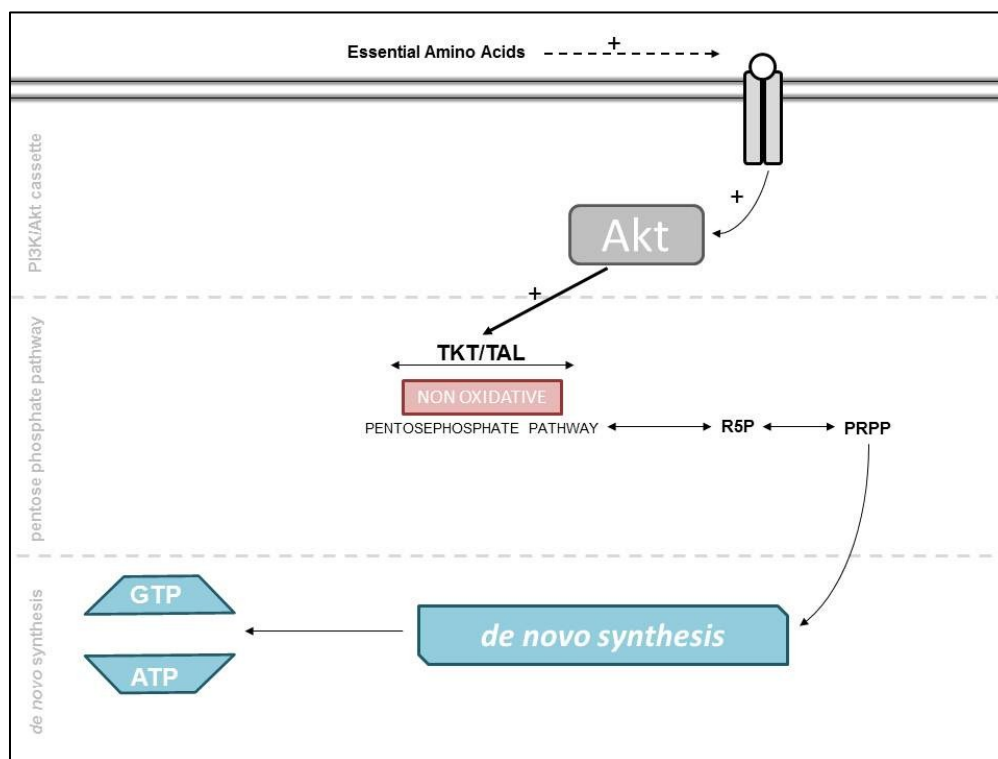

FIGURE 4. **Proposed mechanism of Akt-mediated regulation of transketolase and trandaldolase pyrophosphate pathway enzymes.** Exogenous essential amino acids allow the normal function of the PI3K/Akt signaling pathway. Receptor tyrosine kinases activate the master kinase Akt which in turn mediates the activity of transketolase (TKT) and transaldolase (TAL), regulating the available amount of ribulose 5-phosphate (R5P) from glucose 6-phosphate (G6P) available for conversion to phosphoribosyl pyrophosphate (PRPP). PRPP serves as the precursor to the first committed step of the *de novo* synthesis of purine nucleotides. Amino acid starvation greatly limits the expression and activity of Akt, thereby reducing TKT and TAL activity levels, leading to the decrease of PRPP and purine nucleotides. Hash-marks represent the distinction between each pathway.

followed the same general model in which HeLa cells were starved of the single

essential amino acid lysine for three hours and rates of *de novo* synthesis, TKT and

TAL enzymatic activity levels were measured. It is important to note that amino acid

starvation experiments have been conducted with several different amino acids with

other cell lines previously. Secondly, essential amino acid starvation studies typically

involve simultaneous deprivation of all essential amino acids for upwards of 24 hours.

Lysine starvation within HeLa cells has never been studied, and the results we present can be seen by depriving cells of only one essential amino acid over an extremely short time period of three hours.

EXPERIMENTAL PROCEDURES

CELL CULTURE AND DNA TRANSFECTION – HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with dialyzed 10% fetal bovine serum (FBS). Experiments requiring transfection were transfected 48 hours prior to use with cDNA plasmid using Lipofectamine 2000 (Invitrogen) as manufacturer's protocol suggested.

MEASURING THE RATE OF DE NOVO PURINE SYNTHESIS – Cells were plated in 6 well culture dishes at 3.5 x 10^5 cells/well in DMEM supplemented with 10% FBS. After approximately 18 hours of incubation, cells were washed twice with phosphate-buffered saline (PBS) and the media was replaced with 1 ml fresh DMEM with 10% dialyzed FBS for control cells. Cells that were lysine starved (Lys -) were replaced with 1 ml DMEM devoid of lysine and supplemented with dialyzed 10% FBS for three hours. Cells subjected to lysine return (Lys -/+) had lysine deficient media replaced with DMEM for one hour prior to extraction. After three hours of incubation in either control, Lys - or Lys -/+ conditions, 10 μCi [^{14}C]formate (specific activity 57 mCi/mmol) was added to all samples for 90 minutes. Cells were then washed twice with PBS and extracted *in situ* in 0.4 N perchloric acid to produce lysate. Lysates were then boiled for 70 minutes to release purine bases, cooled for five minutes on ice and then centrifuged for five minutes at 1,500 rpm. Lysates were then loaded onto 0.5 x 5 cm columns preloaded with AG 50 resin and equilibrated in .1 N HCl. Each column was washed twice with 10 ml of 0.1 N HCl to remove unreacted/unincorporated [^{14}C]formate. Purine bases were eluted in 5 ml of 6 N HCl. 1 ml of eluent was then added to a scintillation vial containing 10 ml of equal parts Triton 100X and liquifluor,

and radioactivity was measured by liquid scintillation counting. Results are expressed in units of [C^{14}]formate purine incorporation/h/10^5 cells.

MEASURING PRPP AVAILABILITY - Cells were plated in 6 well culture dishes at 3.5 x 10^5 cells/well in DMEM supplemented with 10% FBS. After approximately 18 hours of incubation, cells were washed twice with phosphate-buffered saline (PBS) and the media was replaced with 1 ml fresh DMEM with 10% FBS for control cells. Cells that were lysine starved (Lys -) were replaced with 1 ml DMEM devoid of lysine and supplemented with 10% FBS for three hours. After three hours of incubation in either control or Lys- [8-^{14}C] adenine (0.2 µCi, specific activity 56 mCi/mmol) was added to each sample. After 90 minutes incubation, cells were washed twice with ice cold PBS and then lysed in water. 200 µl of each lysate was individually applied to 2 x 2 cm squares of DE-81 Whatman cellulose paper. To remove all unincorporated [8-^{14}C] adenine, these samples were washed for 10 minutes four times in 1 mM ammonium formate. Cellulose paper with radioactive nucleotides still bound was then dried until crisp and submerged in scintillation vials containing liquifluor. Radioactivity was measured by liquid scintillation counting.

DETECTING PROTEIN EXPRESSION THROUGH WESTERN BLOT – [107] Cells were plated as described for *de novo* synthesis and PRPP availability assays. After treatment and incubation, cells were washed twice with ice cold PBS, then extracted in 1X SDS sample buffer containing β-mercaptoetanol (BME). Samples were then heated to 95° C for seven to eight minutes and run on a 9% polyacrylamide gel. Samples were then transferred for two hours at 25 mV to polyvinylidene difluoride membranes. After blocking for one hour in 10% non-fat milk solution, primary and

secondary antibodies were incubated and washed for the appropriate times according to manufacturers' protocol.

MEASURING TKT AND TAL ENZYME ACTIVITY – Cells were cultured and plated on 10 cm petri dishes for treatment. After treatment, cells were washed twice in ice cold PBS and then centrifuged at 500 x g for five minutes. Cells were then extracted in 400 µl buffer containing 1X phosphatase and protease inhibitor cocktails, 0.1 mol Tris-HCl (pH 7.6), 0.05 mol BME and 2 mmol EDTA. Sonication was followed by centrifugation at maximum speed for 15 minutes. This lysate would be used to measure protein content via Bradford assay (described below) and TKT/TAL enzyme activity assays.

A cell free system that promoted TKT activity was created by adding the cell lysate to an equal volume of mix containing 0.1 mol Tris-HCl (pH 7.6), 0.01 mol $MgCl_2$, 2 mM NADH, 0.15 mol NaCl, 2 mmol each of R5P and F6P and TPP. Just before measuring the absorbance at 365 nm, the enzyme glucose 6-phosphate dehydrogenase (GD6P) with activity of 1.03 KU/ml was added. Units are expressed in mol NADH formed/hr/mg of protein.

A cell free system that promoted TAL activity was created by adding the cell lysate to an equal volume of mix containing 0.1 mol Tris-HCl (pH 7.6), 0.01 mol $MgCl_2$, 2 mM NADH, 0.15 mol NaCl, 2 mmol each of X5P and E4P. Just before measuring the absorbance at 365 nm, the enzyme glucose 6-phosphate dehydrogenase (GD6P) with activity of 1.03 KU/ml was added. Units are expressed in mol NADH formed/hr/mg of protein.

PROTEIN ESTIMATION BY BRADFORD ASSAY - Standard curves were generated to normalize differing protein concentrations between samples in TKT and TAL enzyme activity assays via Bradford protein assay. 5 µl of cell lysate sample was added to 995 µl Bradford reagent, as were standards of BSA concentrations 0.0, 0.25, 0.5, 1.0 and 2.0 µg/ml. Absorbance shifts were measured at 595 nm.

STATISTICAL ANALYSIS – All error bars are represented as standard error using Microsoft Excel 2007 software.

RESULTS

LYSINE STARVATION DECREASES RATES OF *DE NOVO* PURINE

NUCLEOTIDE SYNTHESIS IS HELA CELLS - Initially it was essential to verify that

starving HeLa cells of lysine would in fact result in the reduction of *de Novo* purine

nucleotide synthesis. Because formate is a carbon donor at two steps of the *de Novo*

synthesis of purine nucleotides, supplementing media with radio-labeled [^{14}C]formate
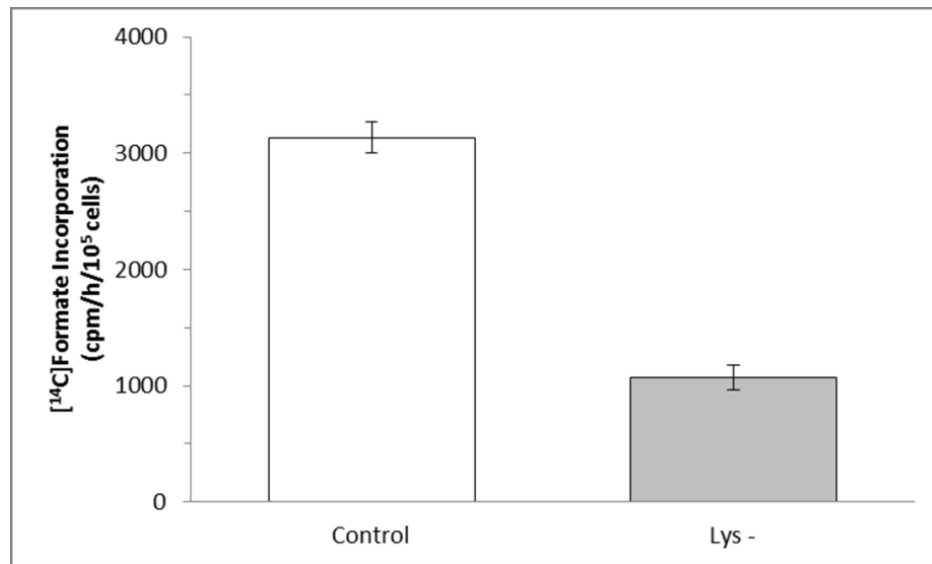

FIGURE 5. **Lysine Starvation Reduces *de Novo* Purine Nucleotide Synthesis.**
HeLa cells were incubated for 21 hours with DMEM supplemented with 10% FBS,
then media was replaced with either lysine supplemented or deficient media for 3
hours.   Before extraction, all cells were incubated for 90 minutes further with
[^{14}C]formate. [^{14}C]formate incorporation was measured to determine relative rates of
de novo purine synthesis. Control condition is represented by the white bar (labeled
control). Lysine starved condition is represented by grey bar (labeled Lys -). Error
bars represent standard error.

and measuring levels of incorporation via liquid scintillation counting would allow us tt

measure relative rates of *de Novo* purine nucleotide synthesis. After culturing, HeLa

cells were incubated for three hours with DMEM supplemented with, or devoid of,

lysine. Incubation was followed by the addition of [^{14}C]formate for an additional 90

minutes before cells were extracted for scintillation counting. Three hours of lysine

starvation resulted in HeLa cells synthesizing purine nucleotides *de Novo* at a rate 65% that of control cells, with [^{14}C]formate incorporation values of 2976.75 SE ± 189.44 and 4559.75 SE ± 190.48 cpm/h/10^5 cells, respectively (Figure 5). A 35% decrease in rate of synthesis is a significant difference within a relatively short period of time, and although HeLa cells were also starved of lysine for time periods of 30, 60, 90 and 120 minutes (data not included), these time points yielded less appreciable results and were therefore not included in further experiments.

LYSINE STARVATION DECREASES AKT PHOSPHORYLATION AND INHIBITS DE NOVO SYNTHESIS THROUGH PI3K SIGNALING – To determine the effect of lysine starvation on expression levels of Akt, cells were either starved for three hours or supplemented with lysine and a western blot of the cell lysate was
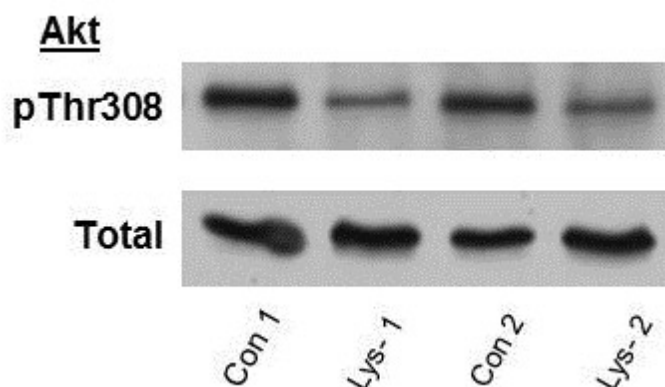

FIGURE 6. **Lysine Starvation does not Alter Expression Levels of Akt, but Reduces Akt Phosphorylation/Activity.** Cells were incubated in duplicate under two conditions: a control condition in which cells were incubated for three hr with DMEM + 10% FBS (containing lysine), or a lysine starvation condition in which cells were incubated for the same amount of time with lysine deficient media. After being extracted in 1X sample buffer containing β-Mercaptoethanol and heated to 95°C for seven minutes, western blots were carried out. After transfer, membranes were blocked and initially probed with primary antibody specific for phosphor-Threonine 308, one of two amino acid residues that become phosphorylated for Akt activation, at a dilution of 1:1000. Secondary antibody used was goat anti-rabbit, diluted 1:5000. Total Akt 1:1000 dilution, secondary goat anti-rabbit 1:5000.

performed. As seen in figure 6, a western blot of the lysate sample demonstrated no decrease in total Akt expression under lysine starvation. However, a probe for activated Akt (characterized by a phosphorylation at the Threonine-308 position of the Akt amino acid sequence) yielded an appreciable decrease in expression levels between supplemented and starved conditions. These results suggest that lysine starvation relegates purine synthesis by most directly affecting Akt activity, not expression, which in turn may mediate pentose phosphate pathway enzymes.

To elucidate a possible mechanism of *de Novo* synthesis regulation, HeLa cells were subjected to a potent PI3K inhibitor, LY294002. In cells exposed to the inhibitor, we added 20 µM LY294002 to cells at the time cells were also subjected to either lysine supplementation or starvation (figure 7). What we observed was a 31% decrease in rates of synthesis in cells exposed to inhibitor when compared to control values (2175.17 SE ± 117.80 and 3135.33 SE ± 130.77 cpm/h/10^5 cells, respectively). Notably, cells subjected to lysine starvation in combination with inhibitor showed a 62.5% decreased rate of synthesis compared to control levels; a value almost identical to cells starved of lysine exclusively, which was a 65.8% decrease (1176.44 SE ± 53.40. and 1071.67 SE ± 105.13 cpm/h/10^5 cells, respectively). Were lysine starvation and PI3K inhibition to retard purine synthesis via different mechanisms, we would expect to see a cumulative decrease within cells both lysine deprived and inhibited by LY294002. Because this condition shows a decrease equal to but not greater than that of cells solely starved of lysine, this result suggests that lysine starvation works to inhibit purine synthesis through the mechanism of PI3K signaling. In addition, restoring lysine to starved cells for only an hour almost completely restores rates of synthesis as seen in condition Lys +/-, in which [^{14}C]formate incorporation levels were 86.1% (2698.29 SE ± 64.88 cpm/h/10^5
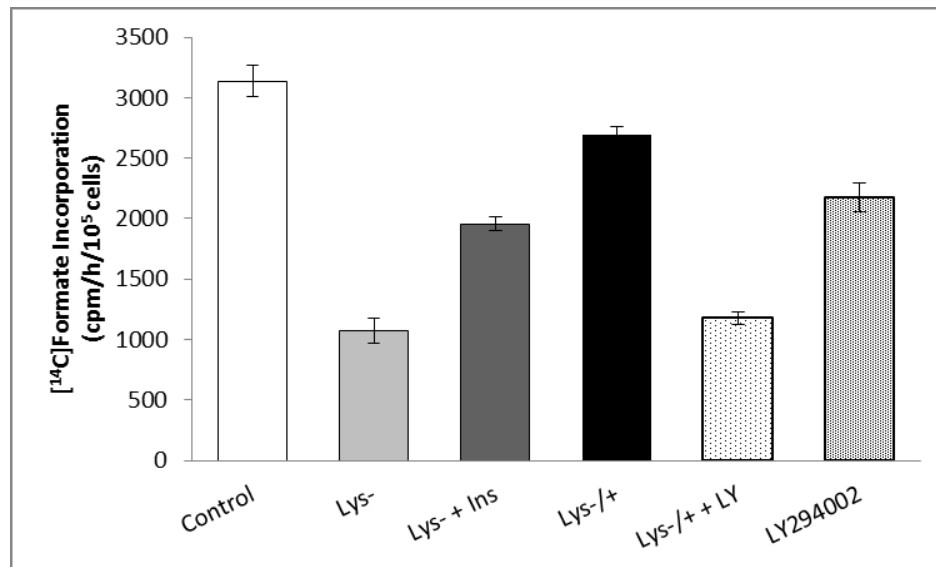
FIGURE 7. **Lysine Starvation Inhibits *de Novo* Synthesis through PI3K Signaling.** HeLa cells were incubated for 21 hours with DMEM supplemented with 10% FBS, then media was replaced with either lysine supplemented or deficient media for 3 hours. Before extraction, all cells were incubated for 90 minutes further with [^{14}C]formate. [^{14}C]formate incorporation was measured to determine relative rates of *de Novo* purine synthesis. *Control condition* is represented by the white bar (labeled control). After 21 hr incubation, media was replaced with lysine supplemented media for 3 hr before radiolabeled formate addition. *Lysine starved condition* is represented by grey bar (labeled Lys-). After 21 hr incubation, media was replaced by lysine deficient media for three hours before addition of radiolabeled formate and extraction. *Lysine starved cells supplemented with insulin* are represented by a dark grey bar (labeled Lys- + Ins). Insulin was added at the time media was replaced by lysine deficient media. *Lysine return condition* is represented by a black bar (labeled Lys -/+). After three hr of lysine starvation, media was replaced by lysine supplemented media for one hr before formate addition and extraction. *PI3K inhibited condition* is represented by dotted bar (labeled LY294002). Cells were incubated for 21 hr and replaced by fresh lysine supplemented media and the addition of PI3K inhibitor for three hr. *PI3K inhibited lysine return condition* is represented by the heavily dotted bar (labeled Lys -/+ + LY). After 21 hr incubation, media was replaced by lysine deficient media and PI3K inhibitor was added as well. After three hr incubation, media was replaced by lysine supplemented media. Error bars represent standard error.

cells) of control incorporation levels. This result concurs with earlier amino acid starvation studies performed by Boss et al, in which synthesis rates were almost completely restored upon return of the absent amino acid and further supports the

theory presented that synthesis may be mediated by an enzyme with an extremely rapid turnover rate. Such a condition would explain the rapid ability of cells to rescue synthesis activity. Lastly, lysine starved cells treated with 10 µM insulin, the major hormone responsible for receptor tyrosine kinase (RTK) activation within the PI3K/Akt signaling pathway, showed an increased level of synthesis (1957.56 SE ± 55.69 cpm/h/10^6 cells; 62.4% of control value) when compared to that of lysine deprived cells alone, but could not rescue rates as efficiently as lysine return could. This result further suggests that the mechanism of lysine starved inhibition works through the PI3K/Akt signaling pathway.

AKT ACTIVATION OVERCOMES LYSINE STARVATION-INDUCED *DE NOVO* PURINE SYNTHESIS REGULATION* – To determine whether Akt plays a role in the regulation of lysine starvation-mediated *de novo* purine synthesis, cells were transfected with either a plasmid containing constitutively activated Akt or an empty vector.   Figure 8 shows cells supplemented with lysine and transfected with overexpressed Akt plasmid showed 112.4% the value of [^{14}C]formate incorporation when compared to control cells transfected with empty vector (2999.78 SE ± 121.90 and 2663.48 SE ± 88.17 cpm/h/10^5 cells, respectively). Interestingly, lysine starved cells overexpressing Akt resulted in 256.9% of the rate of synthesis that lysine deprived cells transfected with an empty vector showed (2723.46 SE ± 103.78 and 1063.90 SE ± 78.52 cpm/h/10^5 cells, respectively).   This suggests that overexpressing Akt more than overcomes any relative decrease in synthesis rates lysine starvation may induce. Additionally, constitutively activated Akt restores synthesis rates to near empty vector control levels after starvation.
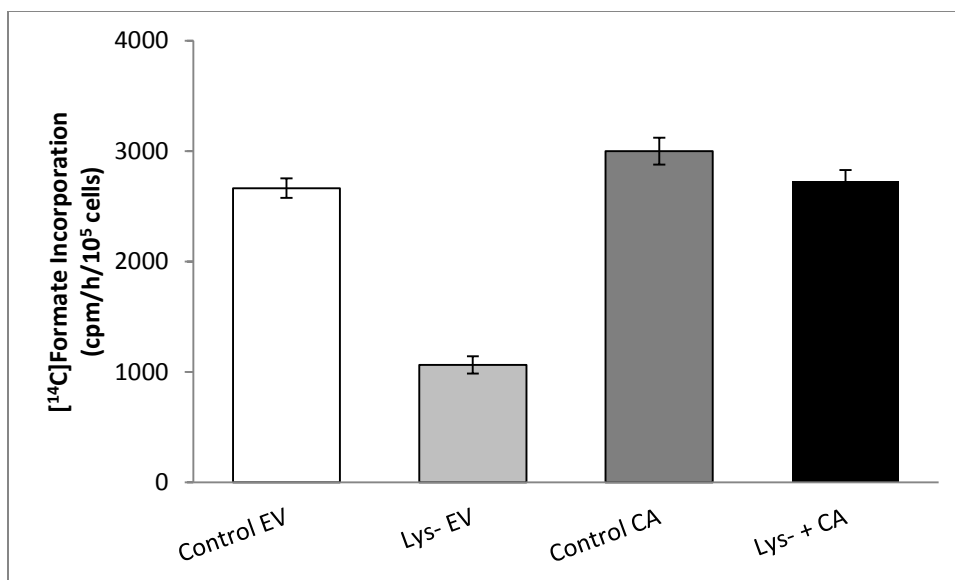
FIGURE 8. **Akt Activation Overcomes Lysine Starvation-Induced *de Novo* Purine Synthesis Regulation.** Cells were transfected with either empty vector plasmid or constitutively activated Akt overexpression vector. After approximately 21 hr incubation, cells subjected to control conditions had media replaced with fresh lysine supplemented media for three hr before addition of [^{14}CFormate. Lysine starved conditions were incubated for the same initial 21 hr after transfection, but media was then replaced with lysine deficient media for three additional hr of incubation before addition of [^{14}C]formate. Samples transfected with empty vector under control conditions are represented by a white bar (labeled Control EV). Lysine starved condition transfected with empty vector is represented by a light grey bar (labeled Lys- EV). Control condition transfected with Akt overexpression vector is represented by a dark grey bar (labeled Control CA), and samples subjected to lysine starvation after Akt overexpression vector transfection is represented by a black bar (labeled Lys- + CA). Error bars represent standard error.

LYSINE STARVATION DECREASES PRPP AVAILABILITY IN HELA CELLS

– To test if the resulting decrease in purine synthesis rates by lysine starvation can be observed prior to the *de novo* synthesis pathway itself, levels of available PRPP were measured by liquid scintillation of [^{14}C]adenine incorporation. PRPP is the product of the non-oxidative pentose phosphate pathway and the protein catalyzed during the first committed step of *de novo* synthesis. Cells starved of lysine demonstrated rates

of synthesis 40.1% lower than that of control cells (5518.75 SE ± 55.31 and 9220.25 SE ± 817.40 cpm/h/10^5 cells, respectively), indicating that the decreased rate of purine synthesis can be attributed to the reduction of PRPP availability, further suggesting that lysine mediated regulation of purine synthesis most likely occurs at a point prior to the *de novo* synthesis pathway, quite possibly in the non-oxidative branch of the pentose phosphate pathway.
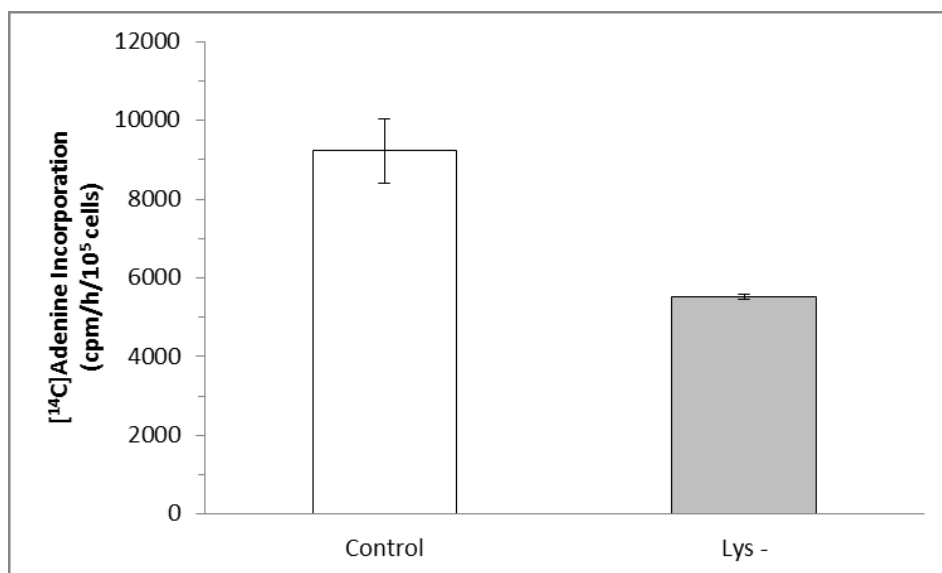

FIGURE 8. **Lysine Starvation decreases PRPP Availability in HeLa Cells.** Control condition is represented by a white bar. Cells were incubated for 21 hr before media was replaced by fresh lysine supplemented media for three additional hr of incubation before addition of [^{14}C]adenine. Lysine starved condition is represented by a grey bar. Cells were incubated for 21 hr before media was replaced by lysine deficient media for three additional hr of incubation before addition of [^{14}C]adenine. Error bars represent standard error.

TRANSKETOLASE AND TRANSALDOLASE ENZYMATIC ACTIVITY, NOT EXPRESSION, WAS DECREASED WITH LYSINE STARVATION – Western blot analysis shows that TKT and TAL expression are not directly regulated by lysine starvation (figure 9). To ensure equal levels of protein were added to each lane, an internal control using a β-Actin probe was carried out. As can be seen, no appreciable difference in expression of β-Actin can be seen between lanes.
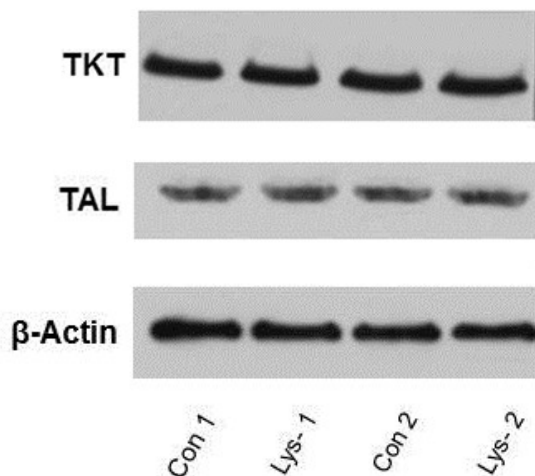
FIGURE 9. **Lysine Starvation does not Alter Expression Levels of TKT or TAL.** Cells were incubated in duplicate under two conditions: a control condition in which cells were incubated for three hr with DMEM + 10% FBS (containing lysine), or a lysine starvation condition in which cells were incubated for the same amount of time with lysine deficient media. After being extracted in 1X sample buffer containing β-Mercaptoethanol and heated to 95°C for seven minutes, western blots were carried out. Membrane was probed for TKT primary and goat anti-rabbit secondary with 1:1000 and 1:5000 dilutions, repectively. A second probe for TAL, secondary goat anti-mouse with 1:1000 and 1:5000 dilutions respectively was carried out. An internal control of β-Actin (HRP) was performed at a 1:10000 dilution. No secondary antibody was needed.

We next tested the ability of lysine starvation on enzymatic activity of two enzymes completely unique to the non-oxidative branch of the pentose phosphate pathway: transketolase and transaldolase. Activity levels of TKT and TAL cannot be measured directly, but can be coupled to a reaction that will allow one to quantitate them indirectly. By coupling the reaction to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and taking advantage of the NADH formed as a byproduct (which will absorb light at 365 nm versus NAD^+, which will not), the rate of formation of NADH or NAD^+ can be directly related to the level of enzyme activity of either TKT

or TAL. Figure 10 shows lysine starvation resulted in a 57.2% decrease in TKT activity compared to control levels, and lysine return restored TKT activity levels to that of controls (5.65 SE ± 0.39, 2.67 SE ± 0.18 and 6.55 SE ± 0.80 nmoles NADH formed/min/mg of protein respectively). Similar results were seen with TAL enzyme activity levels; lysine starvation resulted in a 49.0% decrease in enzyme activity with lysine return restoring activity completely. Therefore, lysine starvation proves to be responsible for decreased TKT and TAL enzyme activity levels.
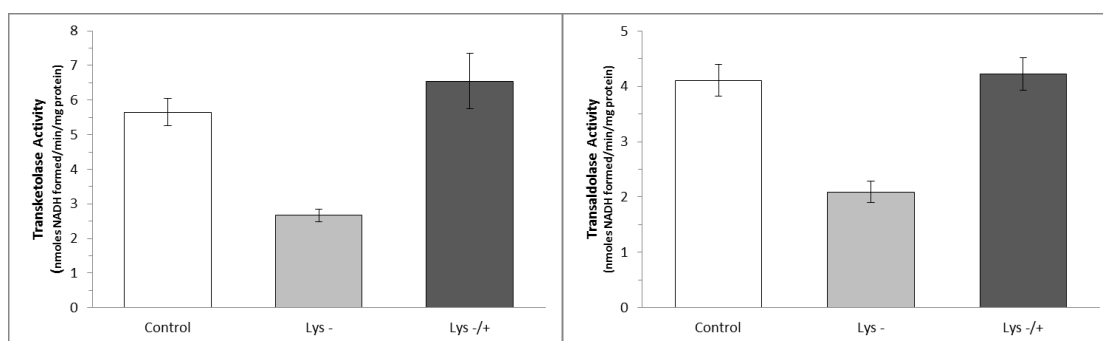

FIGURE 10. **Transketolase and Transaldolase Activity Decreases with Lysine Starvation.** Control condition is represented by a white bar. Cells were incubated for 21 hr, media was replaced by fresh lysine supplemented media for three additional hr of incubation before cells were extracted for enzyme activity assay. Lysine starved condition is represented by a grey bar. Cells were incubated for 21 hr, media was replaced by lysine deficient media for three additional hr of incubation before extraction for enzyme activity assay. Lysine return condition is represented by a dark grey bar. Protocol follows that of lysine starved condition, with one exception: lysine deficient media was replaced with lysine supplemented media for one hr before extraction. Error bars represent standard error.

AKT ACTIVATION OVERCOMES DECREASE OF TKT ACTIVITY BY LYSINE STARVATION – As a control to determine whether transfecting cells with constitutively activated Akt vectors activates its downstream targets to a greater effect than cells lacking expression vectors, cells were transfected with the Akt expression vector or empty vector. Total Akt levels were greatly increased in expression vector conditions. This overexpression resulted in a greater level of Akt substrate activation

as well. Akt substrate probes preferentially recognize peptides and proteins containing phospho-Ser/Thr preceded by Lysine/Arginine at positions -5 and -3 that Akt recognizes (figure 11).
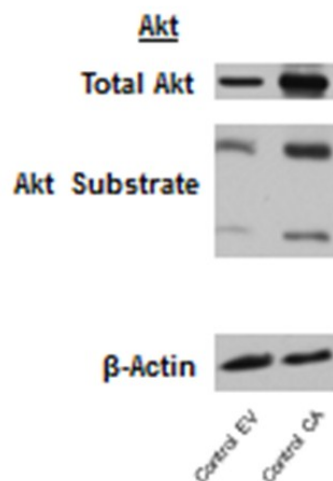

FIGURE 11. **Akt Over-Expression Yields Greater Levels of Phosphorylated Targets.** Cells were transfected with constitutively activated Akt overexpression vector (Control CA) or empty vector (Control EV), then incubated for approximately 24 hr with DMEM + 10% FBS (containing lysine). After being extracted in 1X sample buffer containing β-Mercaptoethanol and heated to 95°C for seven minutes, western blot was carried out. After transfer, the membrane was incubated with primary antibody for anti-total Akt at a 1:1000 dilution. Secondary antibody was goat anti-rabbit and diluted 1:5000. Membrane was then stripped and re-probed for anti-Akt substrate, dilution 1:1000 and goat anti-rabbit secondary with 1:5000 dilution. Bands at 70 and 40 kDa were observed. An internal control of β-Actin (HRP) was performed at a 1:10000 dilution. No secondary antibody was needed.

To assess the role of Akt on TKT activity, cells were transfected with empty vector, constitutively activated Akt plasmid or dominate negative Akt plasmid. As expected, figure 12 shows cells transfected with constitutively activated Akt plasmids demonstrated higher levels of TKT enzyme activity than control cells transfected with empty vector (6.77 SE ± 0.99 and 5.95 SE ± 0.37 nmoles NADH formed/min/mg
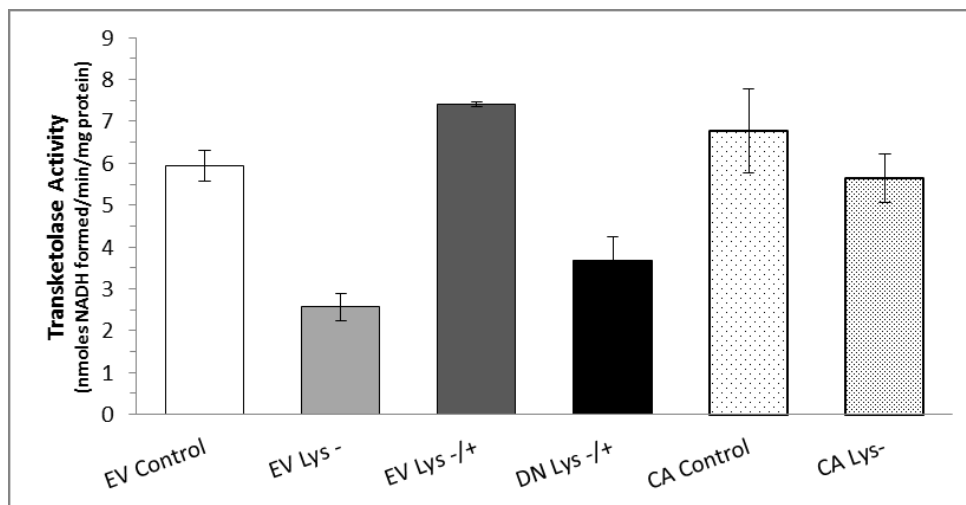
FIGURE 10. **Akt Activation is Necessary for Transketolase Regulation Mediated by Lysine Starvation.** Cells were transfected with an empty vector plasmid, a consitutively activated Akt expression vector, or a kinase-dead dominant negative Akt vector. After 21 hr incubation, DMEM supplemented with 10% FBS was replaced by fresh DMEM for three hr in control conditions, media devoid of lysine for three hr in lysine starved conditions, or three hr of lysine deficient media followed by one hr of media supplemented with lysine in lysine return conditions. Control condition transfected with empty vector plasmid is represented by a white bar (labeled EV Control). Lysine starved condition transfected ith empty vector is represented by a light grey bar (labeled EV Lys-). Lysine return condition transfected with empty vector represented by dark grey bar (labeled EV Lys -/+). Control condition transfected with Akt overexpression vector represented by black bar (labeled AKT Control). Lysine starved condition transfected with Akt overexpression vector represented by dotted bar (labeled AKT Lys-). Lysine return condition transfected with dominant negative Akt vector is represented by heavily dotted bar (labeled DN Lys -/+). Error bars represent standard error.

protein respectively; AKT control 113.9% that of EV control). Moreover, Akt overexpression in lysine deprived cells yielded enzyme activity levels 94.9% the value (5.64 SE ± 0.57 nmoles NADH formed/min/mg protein) seen in empty vector control levels; an almost complete TKT activity rescue. Additionally, cells transfected with a dominant-negative, kinase-dead Akt vector fails to restore TKT activity levels to control levels after lysine return, signifying the need for Akt to remain fully functional for TKT to function normally. Lysine return completely restores function in empty vector control cells, yet only restores TKT activity to 61.6% of control levels with

kinase-dead Akt vector transfection. These results, taken together, illustrate that Akt activation influences levels of TKT enzyme activity levels. An identical experiment for TAL enzymatic activity was performed as well (not pictured) with almost identical results.

DISCUSSION

We have shown that lysine starvation-induced reduction in Akt activity negatively regulates the activity of TKT and TAL, thereby reducing the rates of *de novo* purine nucleotide synthesis in HeLa cells. After three hours of lysine starvation, rates in *de Novo* synthesis decreased by 66%. Simultaneous starvation and introduction of a PI3K inhibitor in cells did not result in a cumulative decrease in synthesis, suggesting that lysine starvation works within the same mechanism of PI3K inhibition; also, the receptor tyrosine kinase activating hormone insulin was unable to rescue synthesis rates within lysine starved cells, suggesting further that lysine starvation does work through inhibiting PI3K signaling. Additionally, lysine return for one hour almost completely restored synthesis rates, telling behavior within enzymes possessing high turn-over rates. Akt, the major kinase within the PI3K signaling pathway which is involved in glucose and protein metabolism and most recently discovered to regulate purine nucleotide synthesis, expectedly overcomes lysine starvation when overexpressed in HeLa cells.

A concurrent 40% reduction in PRPP availability, a protein involved in the first committed step in *de novo* synthesis pathway, was observed. As the conversion of ribose 5-phosphate to PRPP is the reaction directly following the non-oxidative branch of the pentose phosphate pathway, we explored the possibility that Akt may be regulating pentose phosphate enzymes TKT and TAL. Interestingly, lysine starvation decreases TKT and TAL activity, but can be overcome upon Akt overexpression under the same conditions.

Western blots show that Akt overexpression also yields a greater concentration of phosphorylated target substrates at the 70 and 40 kDa size ranges,

TKT and TAL sizes respectively. Western blot also reveals that protein expression levels of Akt, TKT and TAL are not decreased upon lysine starvation, but instead phosphorylation at the Thr308 amino acid residue needed for Akt activation is greatly reduced. Because Akt influence on TKT and TAL does not result from a decrease in Akt protein expression but Akt phosphorylation and activation, further investigation toward lysine starvation and the effect it may have on the upstream modulators of Akt. Expression of PDK1, the kinase responsible for direct phosphorylation of Akt at Thr308, did not appear to be negatively affected by lysine starvation. In addition to the possibility that effectors upstream of PDK1 are exerting less activation upon PDK1 due to lysine starvation, another option may exist; phosphatases responsible for down-regulating PI3K/Akt signaling may play an important role as well. Several phosphatases that target silencing of Akt activity at Thr308 and Ser437 exist. Two such examples are protein phosphatase 1 (PP-1) and Protein phosphatase 2A (PP2A) has been known to directly negatively regulate Akt [50], and studies have shown that upon silencing expression of these phosphatases, Akt Thr308 phosphorylation was shown to markedly increase. PP2A showed specific selectivity toward Thr308 [51]. Perhaps lysine starvation plays a more impactful role upon the expression or activity of regulating phosphatases, allowing the accumulation of phosphorylated Akt as lysine starvation decreases intracellular phosphatase concentrations. A master regulator such as Akt would understandably have a very quick turn-over rate, as many processes, such as glucose uptake, require quick response. It is not unrealistic that the phosphatases responsible for regulation of activity would possess a quick turnover rate as well, allowing for rapid activation and deactivation of Akt. Therefore, the effect of lysine starvation on expression levels of Akt-specific phosphatases such as PP-1 and PP2A should be explored as well.

In the future, exploitation of this new discovery in a clinical capacity will most definitely require the identification of the specific amino acid sequence and residue phosphorylated within TKT and TAL by Akt. Sequence analysis of TKT and TAL genes has revealed multiple possible phosphorylation sites conserved among organisms in all domains of life [52]. Recently, promising experiments have been conducted in which sequencing of TKT and TAL have revealed probable phosphorylation sites and upon generating site directed mutants of these sites, enzyme activity and *de novo* purine synthesis was decreased and TKT and TAL exhibit insensitivity to Akt. Additionally, *in vivo* mouse models to corroborate the mechanism elucidated within this report have commenced.

Given the role of Akt in multiple cellular processes seen frequently to be constitutively activated in cancer (such as cell cycle progression, proliferation and glucose metabolism), it is no wonder that the PI3K/Akt signaling pathway is arguably the most extensively studied transduction pathway in cancer research today. A unique characteristic of PI3K/Akt lends itself to be an attractive candidate for therapeutic targeting – PI3K/Akt has to be activated in order to exert its oncogenic effect, whereas other pathways tend to lose activity upon transformation; it is much easier to inhibit activity than restore lost function.

Increased cell division within malignant cells requires an increased rate of nucleotide synthesis. Our work to further elucidate the details of the mechanism behind the recent discovery that PI3K/Akt regulates nucleotide synthesis may eventually provide another way to explore novel cancer treatments, but also provide a medium to confront tumor progression on an additional front.

A characteristic trait of all cancers, known as the Warburg effect, describes the preference of malignant cells to perform aerobic glycolysis and suppress oxidative phosphorylation, even in the presence of oxygen [53]. This method allows rapidly proliferating cells to use the most abundant extracellular macromolecule, glucose, to produce ATP at a rate that can actually exceed that of oxidative phosphorylation [54], as well as the rapid generation of macromolecules (lipids, nucleic acids and proteins) demanded by increased cellular proliferation. However, it also results in complete dependence upon the uptake of glucose and glutamine into the PPP for nucleic acid synthesis. The most direct effect is decreased R5P availability, but TKT and TAL serve as the reversible link between the oxidative branch of the PPP and glycolysis as well; dampening TKT and TAL activity may inhibit the capacity of the oxidative branch to produce NADPH, an essential cofactor for lipid biosynthesis. Regulating the activity of TKT and TAL will not only allow control of DNA, but lipid synthesis, two macromolecules whose increased concentrations are imperative for the rapid cell proliferation characteristic of malignant cells.

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