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Akt phosphorylates transketolase: regulatory step of de novo purine synthesis

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Tony Minh Phan

Committee in charge:

Professor Gerard R. Boss, Chair
Professor Immo E. Scheffler, Co-Chair
Professor Gen-Sheng Feng

2013

The thesis of Tony Minh Phan is approved, and it is acceptable

in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2013

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ABSTRACT OF THE THESIS

Akt phosphorylates transketolase: regulatory step of de novo purine synthesis

by

Tony Minh Phan

Master of Science in Biology

University of California, San Diego, 2013

Professor Gerard R. Boss, Chair
Professor Immo E. Scheffler, Co-Chair

Akt is a major regulatory protein involved in many cellular processes, particularly cell growth, proliferation, and progression. The objective of this study is to elucidate Akt regulation of de novo purine synthesis. Rates of de novo purine synthesis and phosphoribosyl pyrophosphate availability are subject to regulation by Akt. In this study, I show that rates of de novo purine synthesis and phosphoribosyl pyrophosphate availability decrease with Akt knockout and single essential amino acid starvation (lysine) in mouse embryonic fibroblasts.

I also show that Akt phosphorylates and activates transketolase, a key enzyme of the non-oxidative pentose phosphate pathway. This was shown by a series of transketolase activity assays conducted under Akt wild type and Akt knockout, phosphorylated and de-phosphorylated transketolase, and mutated transketolase conditions. Overall, Akt phosphorylation of transketolase is an important regulatory step of de novo purine synthesis.

I:

Introduction

The PI3K/Akt signaling cascade is a key regulator of many cellular functions related to cell growth, proliferation and survival [10]. The pathway is responsive to insulin, mitogens and a variety of other growth factors [5]. Stimulation of the pathway results in the phosphorylation and activation of PI3K, and subsequently the recruitment and activation of Akt by PIP3 cofactor [9]. Akt, also known as protein kinase B, is a Serine-Threonine protein kinase [9]. It has been implicated as a "master regulator," and activated Akt phosphorylates a variety of substrates involved in many different cellular processes [10]. Specifically, activated Akt plays a role in the regulation of cellular metabolism, proliferation, growth, survival, glucose uptake and angiogenesis [6]. The primary focus of this paper will be the role of Akt in the regulation of purine nucleotide synthesis and its relation to cellular growth, proliferation and progression through the G1 cell cycle phase. Cellular proliferation and progression past the G1 phase requires large amounts of purine nucleotides [8]. A depletion of nucleotides results in arrest of cellular progression past the G1 phase, directly affecting cellular division [8].

Nucleotides are precursors that make up nucleic acids RNA and DNA. They are essential components for genome replication and transcription as well as critical components of cell signal transduction pathways as cyclic AMP or cyclic GMP, secondary messengers that facilitate cellular communication [13]. Adenine and guanine nucleotide are core energy sources for metabolism. Purine nucleotides can be generated in two possible manners: de novo purine synthesis, in which the purine nucleotides are generated from simple components such as amino acids, or through the salvage pathway, in which purine nucleotides are generated from purine bases and nucleosides from catabolism of nucleic acids by hydrolytic degradation [7]. The salvage pathway is more

energy efficient than the de novo pathway, requiring only one ATP compared to the six ATP cost of the de novo pathway to form inosinate (IMP) [12]. The focus of this study will be the de novo pathway.

In de novo purine synthesis, purine nucleotides are synthesized directly onto a ribose-ring attached structure, contributed by phosphoribosyl pyrophosphate, a phospho-ribose [7]. The initial commitment step toward purine biosynthesis in de novo purine synthesis involves pyrophosphate displacement from phosphoribosyl pyrophosphate by ammonia, catalyzed by glutamine phosphoribosylpyrophsphate amidotransferase, resulting in a 5-phosphoribosyl-1-amine [7]. Following this, a series of activation, phosphorylation and displacement steps occur to yield inosinate (IMP), a purine nucleotide and precursor to AMP and GMP [7].

In previous studies, it was observed that three hours of essential amino acid starvation resulted in a decreased rate of de novo purine synthesis, up to 90% in cultured lymphoblasts [3]. The decrease in de novo purine synthesis was reversed upon amino acid restoration [1]. In addition to a decrease in purine production, a 55% decrease in production of phosphoribosyl pyrophosphate was also observed in essential amino acid starved human lymphoblast [3]. Phosphoribosyl pyrophosphate is a critical component in de novo purine synthesis, as it contributes a phospho-ribose towards purine production [7]. Phosphoribosyl pyrophosphate is formed from ribose-5-phosphate, a product of the pentose phosphate pathway, as catalyzed by ribose-phosphate diphosphokinase [5].

The pentose phosphate pathway is an alternative pathway to glycolysis, yielding higher energy compounds from the breakdown of glucose-6-phosphate [11]. The

primary purpose of the pentose phosphate pathway is production of NADPH and ribose-5-phosphate [11]. NADPH is a reducing agent for many oxidation-reduction enzymatic reactions. Ribose is essential for the synthesis of DNA, RNA and enzymatic co-factors. The pentose phosphate pathway is divided into two parts, the oxidative and non-oxidative branches [7]. The initial commitment step for the oxidative pentose phosphate pathway involves processing of glucose-6-phosphate by glucose-6-phosphate dehydrogenase. The main products from the oxidative pentose phosphate pathway are NADPH and ribulose-5-phosphate [7]. The non-oxidative branch consists of a series of reversible reactions, ultimately allowing for the synthesis of carbon chain molecules, ranging from five to seven carbons [7]. Reactions within the non-oxidative branch require two essential enzymes: transketolase and transaldolase, responsible for catalyzing two carbon transfers three carbon transfers, respectively [7]. Transketolase activity requires thymine pyrophosphate and a divalent cation to catalyze the transfer of two carbons from a ketose sugar to aldose sugar [14]. Specifically, transketolase acts as a shuttle for ribose-5-phosphate and glycolytic intermediates, thereby creating a connection between glycolysis and the pentose phosphate pathway [15].

The primary product from the non-oxidative pentose phosphate pathway relevant to this study is ribose-5-phosphate, formed from ribulose-5-phosphate as catalyzed by ribose-5-phosphate isomerase [3]. A glucose starvation study on human lymphoblast observed a decrease in ribose-5-phosphate production [4]. More significantly, as a result of the decrease in ribose-5-phosphate production, a decrease in de novo purine synthesis and intracellular phosphoribosyl pyrophosphate was also observed [4]. Reduced phosphoribosyl pyrophosphate availability during amino acid

starvation could be a result of decreased ribose-5-phosphate availability [2]. It has also been shown that most of the ribose-5-phosphate utilized during purine synthesis comes from the non-oxidative pentose phosphate pathway [5].

Phosphoribosyl pyrophosphate has been implicated as a regulator of de novo purine synthesis [3]. Essential amino acid starvation decreases rates of de novo purine synthesis by decreasing production and availability of intracellular phosphoribosyl pyrophosphate [2]. A study in which phosphoribosyl pyrophosphate availability was specifically targeted and decreased by 40% resulted in a direct decrease of de novo purine synthesis by 80-90% [2]. Under amino acid deficient conditions, cells treated with super-active phosphoribosyl pyrophosphate synthetase observed a 9% decrease in phosphoribosyl pyrophosphate availability [2]. More significantly, de novo purine synthesis only decreased by 48% [2].

It has been found that the PI3K/Akt cassette plays a major role in the regulation of de novo purine synthesis [5]. A study in which Akt1 Akt2 was knocked out in mouse embryo fibroblasts resulted in a decrease in intracellular ATP concentrations [5]. When Akt was constitutively active, intracellular ATP concentration was found to be 3 times larger compared to control [5]. Cells treated with PI3K inhibitor LY294002 resulted in a significant decrease in de novo purine synthesis [5]. Similar results were observed when Akt was knockout by siRNA. Akt knockout and PI3K inhibition resulted in decrease of phosphoribosyl pyrophosphate availability [5]. Enzyme activity assays for ribose-phosphate diphosphokinase, the key enzyme for phosphoribosyl pyrophosphate formation from ribose 5 phosphate, established that there is no difference in activity rates in control and Akt knockout (treated with LY294002) cells [5]. Akt knockout by

LY294002 was observed to result in a decrease in carbon flow through the non-oxidative pentose phosphate pathway [5]. No such change was observed in the oxidative pentose phosphate pathway. This strongly implies that Akt does not regulate de novo purine synthesis by regulation of ribose-phosphate diphosphokinase [5]. Rather, de novo purine synthesis is regulated by phosphoribosyl pyrophosphate availability as a result of ribose-5-phosphate availability dictated by the non-oxidative pentose phosphate pathway [5].

The purpose of this study is to investigate Akt regulation of de novo purine synthesis. It has been established that Akt regulates de novo purine synthesis by two mechanisms: phosphoribosyl pyrophosphate availability and IMP cyclohydrolase [5]. The primary focus of this study will be phosphoribosyl pyrophosphate availability. The direct mechanism as to how Akt regulates phosphoribosyl pyrophosphate availability, and subsequently de novo purine synthesis has yet to be established. As noted in previous studies, a decrease in carbon flow in the non-oxidative pentose phosphate pathway was observed in Akt knockout cells. It has also been shown that most of the ribose-5-phosphate utilized during purine synthesis comes from the non-oxidative pentose phosphate pathway [5]. Transketolase, one of the primary enzymes of the non-oxidative pentose phosphate pathway will be studied. We hope to investigate the role of Akt in transketolase regulation. We also hope to investigate the effects of Akt knockout and lysine starvation on de novo purine synthesis and phosphoribosyl pyrophosphate availability. We hypothesize Akt directly phosphorylates and activates transketolase, thereby regulating de novo purine synthesis.

II:

Results

Rate of de novo purine synthesis decreases with Akt knockout and lysine starvation

A de novo purine synthesis assay was conducted to measure rates of de novo purine synthesis activity under Akt knockout and lysine starvation, as shown in figure 1. The assay was conducted with mouse embryonic fibroblasts (MEF) which were either Akt wild type or Akt knockout. A western blot was conducted to show that Akt was knocked out. The MEFs were subjected to either 3 hours of complete medium or lysine deficient medium. Rates of de novo purine synthesis were quantified by rate of ^{14}C-Formate incorporation into purines, expressed as counts per minute/hour/10^5 cells.

Rate of de novo purine synthesis for the control, wild type Akt under complete medium, averaged 2414 counts per minute/hour/10^5 cells. Wild type under lysine starvation resulted in a significant decrease of 56% compared to the control, with average rates of 1069 counts per minute/hour/10^5 cells. Akt knockout under complete medium had average rates of 1173 counts per minute/hour/10^5 cells. Akt knockout under lysine starvation had average rates of 1141 counts per minute/hour/10^5 cells, relatively little difference compared to the Akt knockout under complete medium. Rate of de novo purine synthesis for Akt knockout under complete medium relative to the control indicates a decrease by 51%. Overall, it is shown that rate of de novo purine synthesis decreases with Akt knockout and significantly with lysine starvation.

Phosphoribosyl pyrophosphate availability decreases with Akt knockout and lysine starvation

A phosphoribosyl pyrophosphate availability assay was conducted to observe availability of phosporibosyl pyorophosphate under Akt knockout and lysine

starvation, as shown in figure 2. The assay was conducted with mouse embryonic fibroblasts (MEF) which were either Akt wild type or Akt knockout. The MEFs were subjected to either 3 hours of complete medium or lysine deficient medium . Phosphoribosyl pyrophosphate availability was measured by rate of ^{14}C-Adenine incorporation into adenylate, expressed as counts per minute/hour/10^5 cells.

Phosphoribosyl pyrophosphate availability for control, wild type Akt under complete medium, averaged 24271 counts per minute/hour/10^5 cells. Wild type under lysine starvation averaged 15945 counts per minute/hour/10^5 cells, a significant decrease of 34% relative to the control. Akt knockout under complete medium averaged 5696 counts per minute/hour/10^5 cells and Akt knockout under lysine starvation averaged 4662 counts per minute/hour/10^5 cells, a decrease by 77% and 81% respectively. From these results, it is shown that phosphoribosyl pyrophosphate availability decreases with Akt knockout and lysine starvation.

Endogenous transketolase activity decreases with lysine starvation

A transketolase activity assay was conducted in HeLa cells to observe endogenous transketolase activity under control and lysine starvation, as shown in figure 3. Transketolase activity was expressed as an increase in change of absorbance at 365 nm. HeLa cells under control conditions exhibited a linear increase in activity with time. Under assay conditions of 30 minutes, endogenous transketolase has yet to reach an activity saturation point. HeLa cells under lysine starvation exhibited lower levels of activity compared to its control counterpart, reflected by a linear increase in activity with a lower slope compared to wild type. The results from the endogenous

transketolase activity assay shows that under assay conditions, transketolase activity is unrestricted. It also shows that transketolase activity decreases with lysine starvation.

Transketolase activity decreases with Akt knockout and lysine starvation

A transketolase assay was conducted to observe the effects of Akt knockout and lysine starvation on transketolase specific activity, as shown in figure 4. The assay was conducted with mouse embryonic fibroblast (MEFs) cell lysate. The MEFs were either Akt wild type or Akt knockout. The MEFs were subjected to 3 hours of treatment, under either complete medium or lysine deficient medium. Transketolase specific activity was quantified by an enzyme coupling reaction and expressed as nmol of NAD+/minute/mg of protein.

Transketolase specific activity for control, Akt wild type under complete medium, averaged 24.5 nmol NAD+/minute/mg of protein. Transketolase specific activity for Akt wild type under lysine deficient medium averaged 8.27 nmol of NAD+/minute/mg of protein, a significant decrease by 66%. Akt knockout under complete medium averaged 6.15 nmol of NAD+/minute/mg of protein and Akt knockout under lysine deficient medium averaged 6.41 nmol of NAD+/minute/mg of protein, a decrease by 75% and 74% respectively. From these results, it is shown that transketolase activity in MEFs decreases with Akt knockout and lysine starvation.

Phosphorylation of transketolase essential for activity

A transketolase activity assay was conducted to observe the significance of the phosphorylation state of transketolase in regards to its specific activity, as shown in figure 5. The assay was conducted with HeLa cells transfected with wild type transketolase and treated with complete medium. Generally, during cell lysis, the lysis

buffer is comprised of phosphatase and protease inhibitors. The presence of phosphatase and protease inhibitors was essential to preserve in-cell conditions and prevent de-phosphorylation of transketolase by endogenous phosphatases. Here, we tested transketolase specific activity in regards to its phosphorylation state. The cell was lysed in the presence or absence of phosphatase inhibitors. The cell lysate was also treated with either G protein tagged beads or alkaline phosphatase tagged beads.

The lysate extracted with phosphatase inhibitor, control, averaged 23.45 nmol of NAD+/minute/mg of protein. Transketolase specific activity was observed in lysate extracted without phosphatase inhibitor, averaging 2.38 nmol of NAD+/minute/mg of protein, a significant decrease by 90% relative to control. Lysate extracted without phosphatase inhibitor and then treated with alkaline phosphatase averaged 1.93 nmol of NAD+/minute/mg of protein, similar specific activity to lysate extracted without phosphatase inhibitor. This shows endogenous phosphatases results in near complete de-phosphorylation of transketolase. A transketolase activity assay conducted for lysate extracted with phosphatase inhibitor, then treated with alkaline phosphatase averaged 27.5 nmol of NAD+/minute/mg of protein, showing the efficacy of phosphatase inhibitors in mitigating phosphatase de-phosphorylation. Overall, these series of transketolase activity assays show that phosphorylation of transketolase is essential for its activity.

Transketolase is phosphorylated and activated by Akt

A transketolase activity assay was conducted to observe the relationship between Akt and transketolase, as shown in figure 6. The assay was conducted with immunoprecipitated samples of Akt and transketolase and under complete medium.

The immunoprecipitated samples were extracted from HeLa cells transfected with either flag tagged-Akt or flag tagged-transketolase, then individually extracted by co-immunoprecipitation. The samples were also treated with either G protein tagged beads or alkaline phosphatase tagged beads.

Transketolase specific activity of immunoprecipitated transketolase, the control, averaged 37.20 nmol NAD+/minute/mg of protein. A transketolase assay was then conducted with the addition of immunoprecipitated Akt. The resulting assay averaged 45.5 nmol of NAD+/minute/mg of protein, a slight increase relative to control. This increase in transketolase activity could be the result of increased phosphorylation, and subsequently increased activity of transketolase. When immunoprecipitated transketolase was assayed after alkaline phosphatase treatment, it averaged 3.83 nmol of NAD+/minute/mg of protein, a significant decrease by 90% relative to control. This significant decrease in transketolase specific activity correlates with the previous experiment and the notion that transketolase activity is regulated by its phosphorylation state. Immunoprecipitated transketolase was assayed after alkaline phosphatase treatment, with the addition of immunoprecipitated Akt. The levels of transketolase activity averaged 42.7 nmol of NAD+/minute/mg of protein, indicating a significant return of transketolase activity. Alkaline phosphatase treatment of transketolase resulted in near baseline levels of transketolase activity. The return in transketolase specific activity with the addition of Akt after alkaline phosphatase treatment shows that Akt directly phosphorylates and activates transketolase.

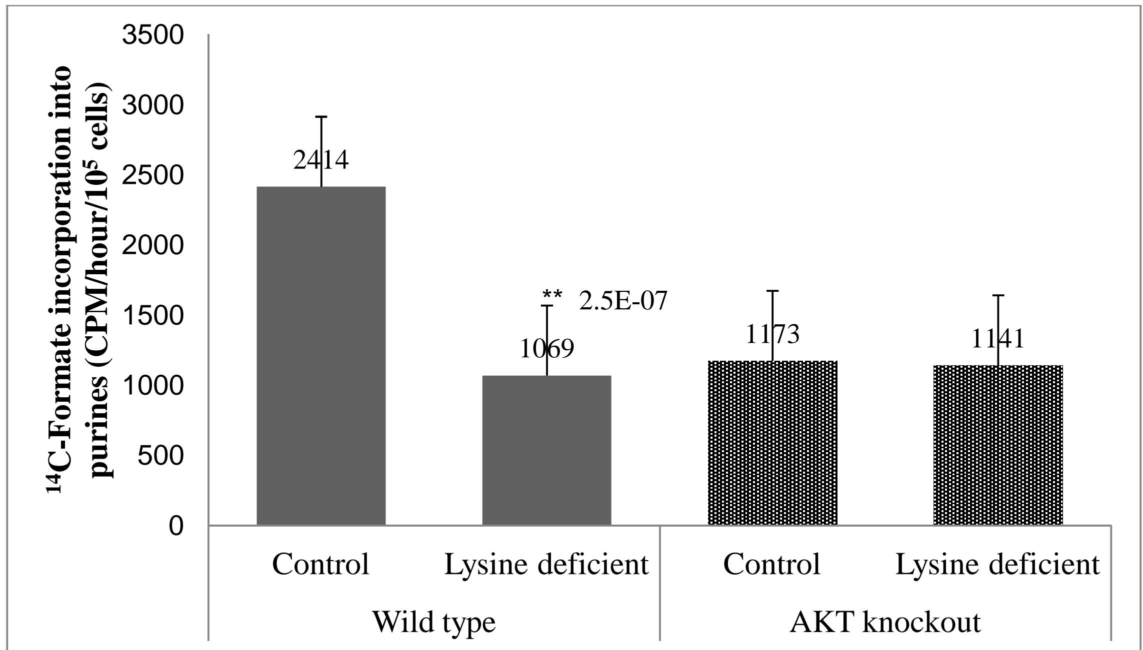
Serine 387 and Threonine 382 point mutation on transketolase results in a significant decrease in activity

A transketolase activity assay was conducted to observe the significance of residues serine 387 and threonine 382 in regards to transketolase activity, as shown in figure 7. These residues were found by Dr. Arindam Saha to be phosphoryled in transketolase. The transketolase activity assay will be conducted with immunoprecipitated samples of transketolase from HeLa cells. The HeLa cells were transfected with flag tagged wild type transketolase or mutated transketolase. The mutant transketolase were S387A, T382A, or double mutant.

Transketolase activity was expressed as a change in absorbance at 365nm. An increase in change of absorbance relates to increasing transketolase activity. Wild type transketolase expressed the greatest transketolase activity, expressed by its high slope. The mutants, T382A, S387A and double mutant, had relatively low slopes with near baseline levels of activity. From this, it can be inferred that phosphorylation of threonine 382 and serine 387 residues are essential for transketolase activity.

Figure 1: Rate of de novo purine synthesis decreases with Akt knockout and lysine starvation

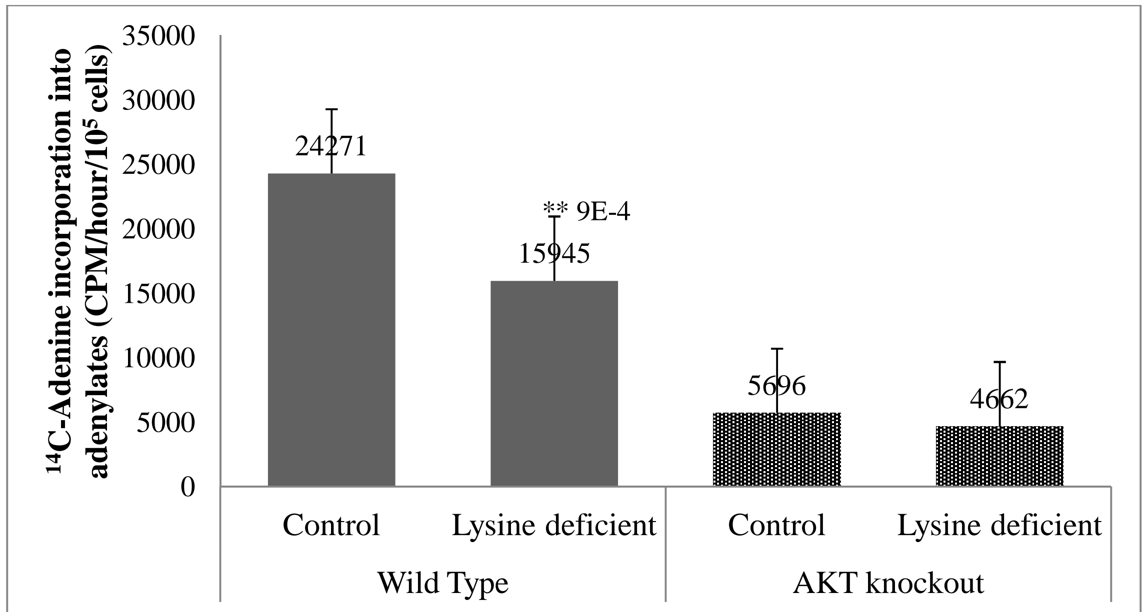
De novo purine synthesis assay in mouse embryonic fibroblasts, expressing Akt wild type or Akt knockout, under 3 hour treatment of control or lysine starvation. Rate of de novo purine synthesis quantified by ^{14}C-Formate incorporation into purines, expressed as counts per minute/hour/10^5 cells by scintillation counting. Double asterisks "**" denote t-test values for statistical significance of that experiment compared to control.



^{14}C-Formate incorporation into purines (CPM/hour/10^5 cells)
3500
3000
2500
2000
1500
1000
500
0
2414
** 2.5E-07
1069
1173
1141
Control
Lysine deficient
Control
Lysine deficient
Wild type
AKT knockout

Figure 2: Phosphoribosyl pyrophosphate availability decreases with Akt knockout and lysine starvation

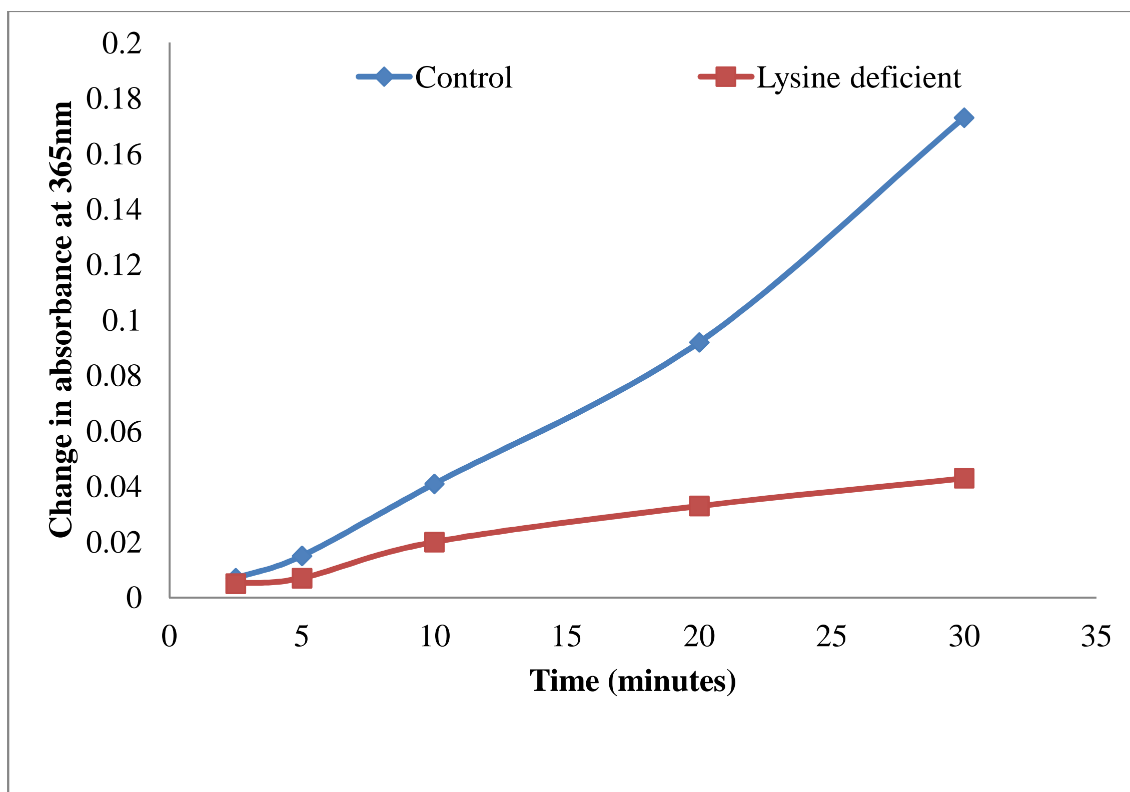
Phosphoribosyl pyrophosphate availability assay in mouse embryonic fibroblasts, expressing Akt wild type or Akt knockout, under 3 hour treatment of control or lysine starvation. Phosphoribosyl pyrophosphate availability quantified by ^{14}C-Adenine incorporation into adenylate, expressed as counts per minute/hour/10^5 cells by scintillation counting. Double asterisks "**" denote t-test values for statistical significance of that experiment compared to control.



^{14}C-Adenine incorporation into adenylates (CPM/hour/10^5 cells)
35000
30000
25000
20000
15000
10000
5000
0
24271
** 9E-4
15945
5696
4662
Control
Lysine deficient
Control
Lysine deficient
Wild Type
AKT knockout

Figure 3: Endogenous transketolase activity decreases with lysine starvation

Transketolase activity assay conducted in HeLa cells with endogenous transketolase under 3 hour treatment of control or lysine starvation. Increasing transketolase activity expressed as an increase in change of absorbance at 365nm.



Control
Lysine deficient
Change in absorbance at 365nm
Time (minutes)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
25
30
35

Figure 4: Transketolase activity decreases with Akt knockout and lysine starvation

Transketoalse activity assay in mouse embryonic fibroblasts, expressing Akt wild type or Akt knockout, under 3 hour treatment of control or lysine starvation. Transketolase specific activity quantified by an enzyme coupling reaction and expressed as nmol NAD+/minute/mg of protein. Double asterisks "**" denote t-test values for statistical significance of that experiment compared to control.

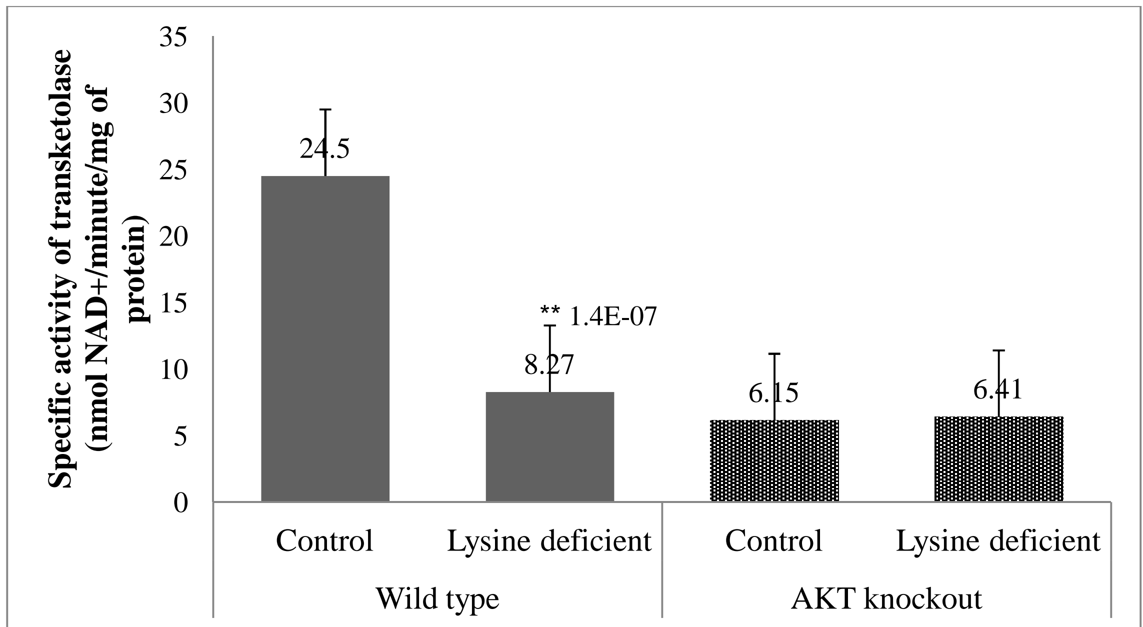
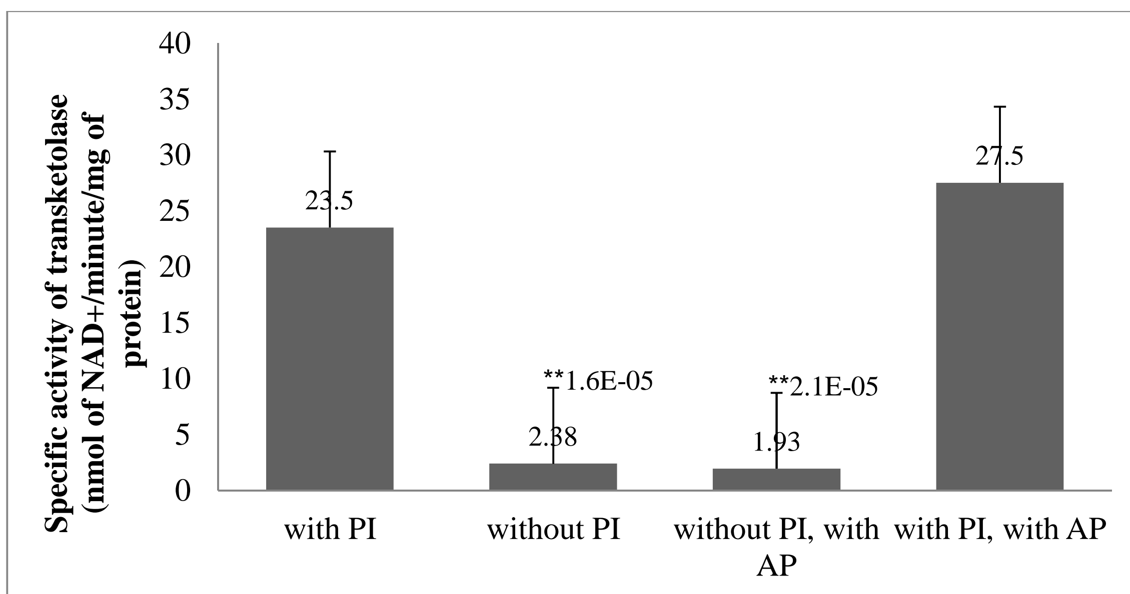

Specific activity of transketolase (nmol NAD+/minute/mg of protein)
35
30
25
20
15
10
5
0
24.5
** 1.4E-07
8.27
6.15
6.41
Control
Lysine deficient
Control
Lysine deficient
Wild type
AKT knockout

Figure 5: Phosphorylation of transketolase essential for activity

Transketolase activity assay in HeLa cells, transfected with wild type transketolase under control medium. Cells were lysed in the presence or absence of phosphatase inhibitor, and treated with either g-protein tagged agarose beads or alkaline phosphatase tagged agarose beads. Double asterisks "**" denote t-test values for statistical significance of that experiment compared to control.



Specific activity of transketolase (nmol of NAD+/minute/mg of protein)
40
35
30
25
20
15
10
5
0
23.5
**1.6E-05
2.38
**2.1E-05
1.93
27.5
with PI
without PI
without PI, with AP
with PI, with AP

Figure 6: Transketolase is phosphorylated and activated by Akt

Transketolase activity assay in HeLa cells using immunoprecipitated samples of transketolase or Akt. The HeLa cells were transfected with either flag tagged transketolase or flag tagged Akt and individually co-immunoprecipitated. The samples were treated with either g-protein tagged agarose beads or alkaline phosphatase tagged agarose beads. Double asterisks "**" denote t-test values for statistical significance of that experiment compared to control.

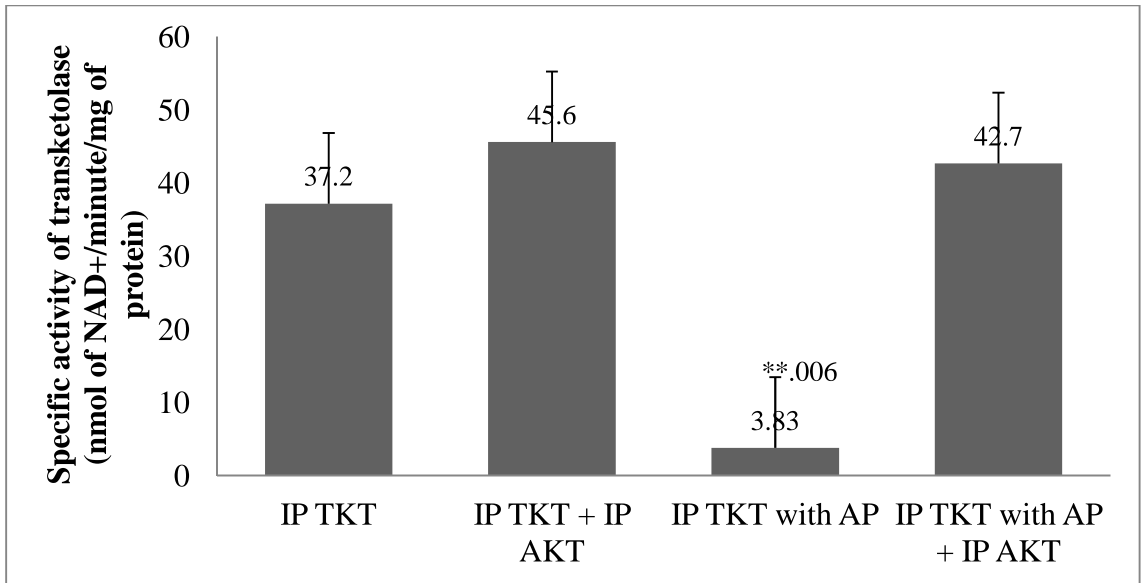
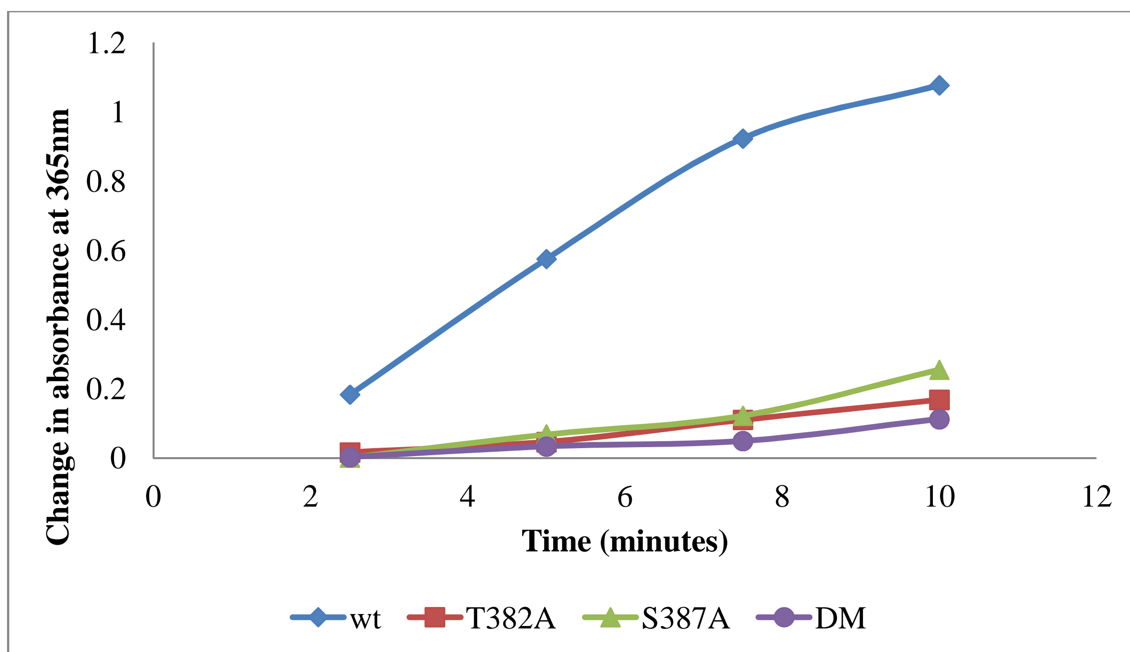

Specific activity of transketolase (nmol of NAD+/minute/mg of protein)
60
50
40
30
20
10
0
37.2
45.6
**.006
3.83
42.7
IP TKT
IP TKT + IP AKT
IP TKT with AP
IP TKT with AP + IP AKT

Figure 7: Serine 387 and Threonine 382 point mutation on transketolase results in a significant decrease in activity

Transketolase activity assay conducted in HeLa cells transfected with wild type, T382A, S387A, or double mutant transketolase, using immunoprecipitated samples of transketolase. Increasing transketolase activity expressed as an increase in change of absorbance at 365nm.


1.2
1
0.8
0.6
0.4
0.2
0
Change in absorbance at 365nm
0
2
4
6
8
10
12
Time (minutes)
wt
T382A
S387A
DM

III:

Discussion

This study shows that Akt knockout and lysine starvation results in a decrease of rates of de novo purine synthesis and phosphoribosyl pyrophosphate availability in mouse embryonic fibroblasts. These findings support previous work in regards to the regulatory roles of Akt in de novo purine synthesis and phosphoribosyl pyrophosphate availability. It was shown that the PI3K/Akt cassette plays a regulatory role in de novo purine synthesis by regulation of phosphoribosyl pyrophosphate availability. It was also shown that Akt knockout resulted in a decrease of carbon flow through the non-oxidative pentose phosphate pathway [5]. Phosphoribosyl pyrophosphate is formed from ribose-5-phosphate, a product from the non-oxidative pentose phosphate pathway. These findings provided the foundation for this study.

A decrease in transketolase activity was observed with Akt knockout and lysine starvation. A transketolase activity assay based on the phosphorylation state of transketolase shows that it is activated by phosphorylation. A transketolase activity assay using immunoprecipitated Akt and immunoprecipitated transketolase shows that transketolase is phosphorylated and activated by Akt, indicating Akt as a direct regulator for transketolase activity. Transketolase mutations T382A, S387A, and double mutant resulted in minimal levels of transketolase activity, showing phosphorylation at Threonine 382 and Serine 387 residues are essential for transketolase activity. In all, it is shown that Akt regulates transketolase by phosphorylation, a regulatory step of de novo purine synthesis.

Although this study was able to elucidate the regulatory relationship between Akt and transketolase, more must be addressed to further understand the mechanism of

Akt regulation of de novo purine synthesis. This study primarily focused on transketolase of the non-oxidative pentose phosphate pathway. Although it was established that Akt phosphorylates and activates transketolase, and the phosphorylation sites Serine 387 and Threonine 382 are essential for transketolase activity, it was not shown that these sites are phosphorylated by Akt. An in vitro kinase assay must be conducted. Also, the direct mechanism as to how transketolase regulates de novo purine synthesis was not shown or expressed. This can be addressed by de novo purine synthesis assays with transketolase knock down or over-expression. Future studies involving transaldolase, the other enzyme of the non-oxidative pentose phosphate pathway, will also be beneficial.

This study is significant, as the PI3K/Akt signaling cascade is involved in many cellular pro-survival pathways. Over activity of Akt is commonly attributed as a characteristic of cancer, related to anti-apoptosis and uncontrolled cellular proliferation and replication properties [16]. De novo purine synthesis is the main purine nucleotide biosynthetic pathway. Regulation of de novo purine synthesis dictates levels of DNA and RNA synthesis, and subsequently cell proliferation and cell cycle progression. Elucidation of Akt regulation of de novo purine synthesis may present therapeutic targets for cancer.

IV:

Materials and Methods

Cell culture and transfection

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). The cell media was exchanged according to experimental conditions. The control sample was exchanged with DMEM and 10% FBS. The lysine starved sample was exchanged with lysine deficient DMEM and 10% dialyzed FBS. The samples were allowed to incubate for 3 hours prior to experimentation. Experiments requiring transgenic cells were transfected 48 hours prior to experimentation. Cells were transfected with plasmid cDNA using Invitrogen Lipofectamine 2000 according to manufacturer guidelines.

Transketolase activity assay

The cells were plated and cultured overnight on a 100mm cell culture plate. Once at 70% confluency, the cells were washed with ice cold phosphate buffered saline (PBS), collected, then centrifuged at 500x G for 5 minutes. The PBS was aspirated off, and the cells re-suspended in 400uL extraction buffer comprised of 0.1 mol Tris-HCl (pH 7.6), 2 mmol EDTA, 0.05 mol BME, and phosphatase and protease inhibitor cocktails. The cell suspension was sonicated, and then centrifuged at 13200 RPM for 15 minutes at 4 degree Celsius. The concentration of the lysate was measured by Bradford assay at 595 nm and normalized among samples.

Transketolase activity was observed by replication of cellular conditions in a cell free system. The lysate was added to a mix comprised of 2 mmol NADH, 0.15 mol NaCl, 0.1 mol Tris-HCl (pH 7.6), 0.01 mol MgCl2, 1 mmol thymine pyrophosphate (TPP), 2.5 mmol ribose-5-phosphate (R5P) and 2.5 mmol xylulose-5-phosphate (X5P). A glycerol-3-phosphate dehydrogenase (G3PDH) and triose phosphate isomerase (TPI)

enzyme mix with activity of 1.03 KU/mL was added. Absorbance was then monitored at 365 nm at 5 minute intervals for 30 minutes.

Transketolase activity is represented by a change in absorbance as a result of NADH oxidation by glycerol-3-phosphate dehydrogenase conversion of glyceraldehyde-3-phosphate to glycerol-3-phosphate. The glyceraldehyde-3-phosphate was created from reactions catalyzed by transketolase, in which a 2-carbon is donated from ketose xylulose-5-phosphate to aldose ribose-5-phosphate, yielding a 7 carbon ketose and the remaining 3 carbon aldose, glyceraldehyde-3-phosphate. The rate of NADH oxidation to NAD+, as observed by change in absorbance at 365 nm, is directly proportional to transketolase activity. Change in absorbance is converted to nmol of NADH converted to NAD+ per minute per mg of protein by the formula depicted as followed:

Change in absorbance

$(A_{M1} - A_{M2})_I - (A_{M1} - A_{M2})_F$

Activity

Change in absorbance/Time (minutes)

Specific Activity

Activity/Amount of protein (mg)

(Change in absorbance/minute/mg of protein) x 162.9 (conversion factor)

=

nmol NAD+/minute/mg of protein

De novo purine synthesis

5×10^5 cells were plated in a 6 well plate in 1 mL DMEM with 10% FBS at around 50-60% confluency. The cells were incubated overnight, and then washed with PBS. The medium was exchanged with control DMEM and 10% FBS or lysine deficient DMEM and 10% dialyzed FBS. The cells were allowed to incubate for 3 hours. Following incubation, 10 uCi ^{14}C-formate was added to the samples and allowed to incubate for 90 minutes. The medium was then aspirated and the cells washed twice with cold PBS. The cells were extracted in situ in 1 mL 0.4 N perchloric acid, scraped, and extracted into 16 x 100 mm glass tubes. The wells were then washed with 500 uL 0.4 N perchloric acid and collected into the glass tubes. The collections were then boiled for 70 minutes to reduce purine nucleotides and nucleosides to purine bases. After boiling, the samples were cooled on ice, and then centrifuged for 5 minutes at 1500 rpm at 4 degrees Celsius. The supernatants are isolated, and then applied to Dowex 50 columns that have been pre-equilibriated with 0.1 N HCl. The columns are washed twice with 10 mL 0.1 N HCl. The purine bases are eluted in 5 mL 6 N HCl. 1 mL of each sample is added to a scintillation vial containing 10 mL Liquifluor-TritonX. The sample was vortexed, and then counted in a scintillation counter, expressed as counts per minute per hour per 10^5 cells.

Immunoprecipitation of Flag-tagged Akt and Flag-tagged transketolase using Flag-tagged protein

Cells are collected, and then lysed in 1 mL of immunoprecipitation buffer comprised of 50 mmol Tris HCl pH 7.4, 150 mmol NaCl, 0.5% NP40, 10% glycerol, and a protease and phosphatase inhibitor cocktail. The cells were placed into an

overhead rocker for 30 minutes at 4 degrees Celsius. Following this, the lysate was centrifuged at 13200 rpm for 10 minutes at 4 degrees Celsius. Sigma Flag beads were prepared according to manufacturer guidelines, and suspended in immunoprecipitation buffer, then added to the lysate samples. The lysate with Flag beads were placed into an overhead rocker for 2 hours at 4 degrees Celsius. Following this, the sample was centrifuged at 4500 x G for 30 seconds and the lysate discarded. The beads were washed with immunoprecipitation buffer, then with PBS. The PBS was aspirated off, then 100 uL of PBS was added to each sample. Sigma 3x Flag peptide was added to each sample, then placed into an overhead rotor for 30 minutes at 4 degrees Celsius. Following this, the samples were centrifuged at 7000 rpm for 30 seconds at 4 degree Celsius. The supernatant containing the Flag-tagged protein of interest was collected.

Alkaline phosphatase treatment

Alkaline phosphatase tagged agarose beads were added to lysate samples according to manufacturer guidelines and allowed to incubate at room temperature for 30 minutes. The alkaline phosphatase tagged agarose beads and lysate sample mix were occasionally disrupted to ensure proper activity. Following the 30 minute incubation, the beads were centrifuged at 7000 rpm at 4 degree Celsius for 30 seconds and the supernatant collected.

Control samples without alkaline phosphatase treatment are instead treated with G-protein tagged agarose beads. The same procedure applies.

PRPP availability assay

The cells were plated with 3.5 x 10^5 cells per condition in DMEM and 10% FBS. The cells were allowed to incubate for 18 hours. The cells were then washed twice with

PBS. The cell media was exchanged accordingly, depending on experimental conditions. The control sample was exchanged with DMEM and 10% FBS. The lysine starved sample was exchanged with lysine deficient DMEM and 10% dialyzed FBS. The samples were allowed to incubate for 3 hours. Following incubation for 3 hours, the samples were supplemented with ^{14}C-Adenine (0.2 uCi, specific activity 56 mCi/mmol). The samples were allowed to further incubate for an additional 90 minutes. After incubation with the ^{14}C-Adenine, the samples were washed twice with cold PBS, then lysed in water. Each lysate sample was individually applied to DE-81 Whatman cellulose paper. Following application, the cellulose papers with samples were washed to remove unincorporated ^{14}C-Adenine. This was done in a bath of 1 mmol ammonium formate, 10 minutes at a time, for four times. Following the bath, the cellulose papers with samples were dried in an oven until crisp. The cellulose papers with samples were then individually placed into scintillation vials containing Liquifluor-TritonX, then vortexed well. Radioactivity due to ^{14}C-Adenine incorporated phosphoribosyl pyrophosphate was measured by liquid scintillation counting, expressed as counts per minute per 10^5 cells per hour of ^{14}C incorporation.

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