

Cloning and Expression of Forkhead Box O1 (Foxo1): Wildtype and Site Directed Mutagenesis at T24A, S256A, S319A

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Introduction:

Forkhead Box O (FoxO)

DNA transcription factors come in many forms and are categorized by characteristic DNA-binding domains. The FoxO1 transcription factor belongs to the Forkhead family characterized mainly by their distinct forkhead domain named after the discovery of the *Drosophila* forkhead protein required for the proper formation of the terminal structure of the *Drosophila* embryos.^{1,3} This DNA-binding region consists of a conserved 110-amino acid residue span. Certain classes of the forkhead family have significant roles in multiple biological processes including: cell-cycle, cell death, DNA repair, metabolism and resistance towards oxidative stress. These members fall into the O class, termed forkhead box O (FoxO).³ Recently, FoxO factors, specifically FoxO1, have become evident in being insulin-sensitive transcription factors. Transcriptional functions that effect an array of downstream targets and interacting partners is regulated by the translocation of the FoxO factor. Translocation of FoxO from the nucleus to the cytosol is a form of insulin-mediated inhibition. In effect, through a specialized shuttling mechanism, transcriptional function is eliminated.^{1,4,6}

Regulation of FoxO factors is mediated by several signal transduction cascades, including chaperones and more importantly, phosphorylation by kinases, mainly in the PI3K pathway. Within the FoxO gene, there are several intramolecular domains containing phosphorylation sites necessary for the shuttling efficiency. A two step mechanism involving phosphorylation and acetylation controls the transcriptional output of FoxO. Mutations specifically at the phosphorylation sites have been shown to alter correct shuttling resulting in constitutive localization in the nucleus and *trans*-activation of target gene expression.¹

Below is a schematic of intracellular domains in the FoxO gene (Figure 1). The total number of residues is indicated at the far right. Phospho-residues are indicated along with accompanying kinases. FoxO 1, 2, and 3 contain similar domains, FoxO6 however is missing the C-terminal stretch and has a separate, individually distinct phosphor-residue site at T338.

Figure 1: Protein structure of FoxO schematic¹
(*Biochem. J.* 2004. **380**, 297–309)

Conserved regions on the FoxO gene include (Figure 1): I) the N-terminal Protein Kinase B (PKB) motif. This is involved in the shuttling by phosphorylation through 14-3-3 protein binding. II) the Forkhead domain containing the conserved 110 amino acid residue span which mediates DNA interactions and binding of 14-3-3. Within the span is a PKB motif. Phosphorylation at this site is required for the subsequent phosphorylation of the N- and C-terminal PKB motifs. III) The third PKB motif located at the C-terminal. Following are two CK1 sites and a DYRK1A which regulates nuclear export. This stretch is missing in FoxO6 which may indicate its permanence within the nucleus.¹ IV) The NLS (nuclear localization sequences) and NES (nuclear export sequences) are regions needed for recognition by importin and exportin nuclear transporters for shuttling into and out of the Nucleus with the aid of RanGTP (RanGDP) and RanGAP. V) The conserved LXXLL (X denotes any residue) motif is involved in recruitment of nuclear receptors.¹

Conserved residues involved within the intramolecular domains are found to be serines, threonine, and lysine. These residues are involved in the regulation of the transcriptional activity due to their ability to be phosphorylated (Ser, Thr) and acetylated (Lys).^{1,8} Phosphorylation occurs through highly conserved putative PKB recognition motifs with a recognizable sequence of RXXRXXS/T, (X denotes any residue). As mentioned above, the PKB motifs are located at the N- and C-terminal sites, and one within the forkhead domain.¹

FoxO Shuttling Mechanism

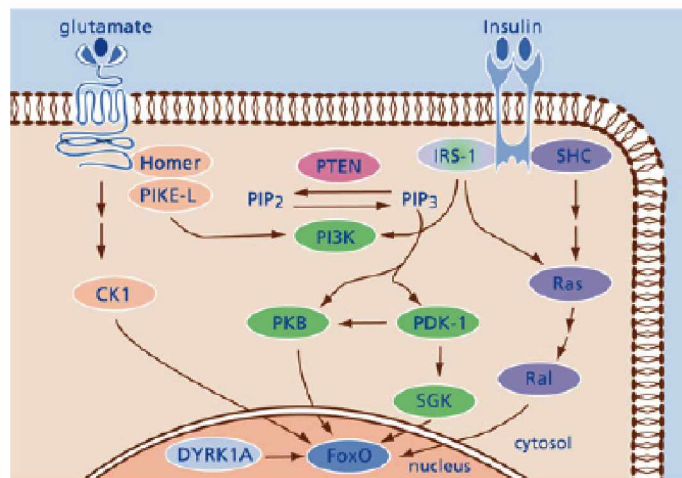


Figure 2: Signal cascade from extracellular to intercellular influencing FoxO activity.¹
(*Biochem. J.* 2004. **380**, 297–309)

The regulation of transcription of the FoxO class as mentioned above involves the PI3K pathway. Other than mediate intracellular traffic, this signaling pathway has implications in cell survival, regulation of the cycle cell, and cell differentiation.^{1,4} PI3K are heterodimers of a catalytic subunit and either a regulatory or adaptor subunit. It is activated by G proteins and tyrosine kinases and involved in phosphorylating the 3-hydroxy group of the inositol ring of phosphatidylinositol, generating phosphoinositide phosphates PIP₂ and PIP₃. In response, PIP₂

and PIP3 trigger downstream serine and threonine kinases PKBs and PDK1 (phosphoinositide-dependent kinase-1) by recruitment to the plasma membrane.¹ PKBs are downstream mediators within the PI3K pathway and to have full catalytic activation, it must be phosphorylated at two sites before detaching from the plasma membrane. Afterward, it translocates throughout the cytosol and within the nucleus. In the nucleus, FoxO factors are targeted along recognizable motifs where the PKBs phosphorylates serine or threonine residues (Figure 2).^{1,2} The PKBs motif sites at the N-terminus and forkhead domain have been shown to be required for the FoxO factors to translocate. These two sites also induce function of the NLS as well as influence association with the 14-3-3 protein and to p300. The third phosphorylation site at the C-terminal only accelerates the exportation of the FoxO factor out of the nucleus and into the cytosol.

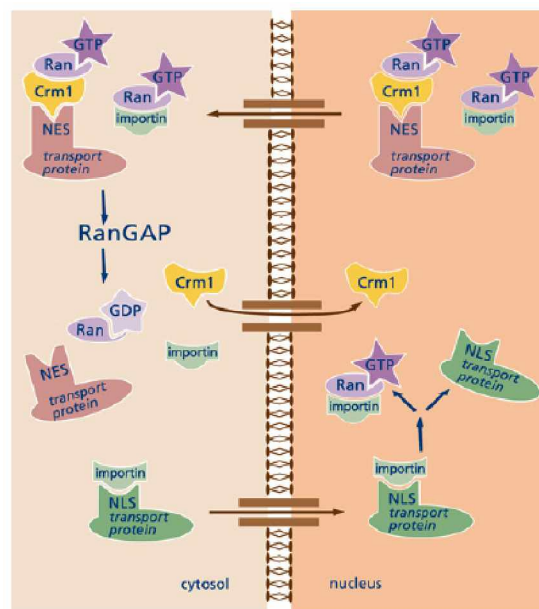


Figure 3: Protein translocation mechanism.¹
(*Biochem. J.* 2004. **380**, 297–309)

Along with the site of PKB phosphorylation, the export and import of FoxO through the nuclear pore depends on mechanism of active-transport influenced by adaptor proteins importin

and exportin as well as Ran, a small GTPase needed to interact with the nuclear pore complex.¹ Since FoxO molecules are larger than 50 kDa or 9 nm in diameter a gradient created by the phosphorylation and subsequent dephosphorylation of RanGTP to RanGDP is needed for transport. The GDP-bound Ran is abundant in the cytosol while the GTP-bound is present in the nucleus. The gradient drives transport across the nuclear membrane and its maintenance involves cytosolic Ran-GAP which hydrolyses Ran-GTP to RanGDP while in the nucleus, the conversion is the opposite. RCC1, a chromatin-associated Ran-GEF converts Ran-GDP to Ran-GTP.¹ While the gradient is provided by Ran, the transport across the pore requires the adaptor proteins importin (for import into nucleus) and exportin (for export) which recognize NLS and NES sites on the protein being transported.¹

Forkhead Box O1 (FoxO1): Wildtype

The previously described complex, yet sensitive shuttling mechanism has pivotal effects on FoxO transcription factors especially in the control of one member of the family, FoxO1. Forkhead Box O1 (FoxO1) is shown to have involvement in pathological processes which include development of cancer, organismal aging, and most notably, type 2 diabetes.^{4,6} Although the true function of the FoxO1 gene is not clearly elucidated, previous studies has implicated that it has an important role in the control of the insulin/insulin-like signaling pathway. In glucose homeostasis, FoxO1 regulates expression of gluconeogenic factors, and plays a key role in insulin regulation of glucose levels.^{4,5,6} Foxo1, like all members of the FoxO, is considered a substrate of protein kinase B (PKB) and serum- and glucocorticoidinducible kinase (SGK) which play a role in its phosphorylation which influences an important role in insulin signaling. When insulin is absent, Foxo1 resides in the nucleus, actively binding to DNA as well as acting as a *trans*-activator to

enhance promoter activity. In response to the presence of insulin, Foxo1 is phosphorylated in a PI3K-dependent manner, resulting in shuttling out of the nucleus ceasing transcriptional activity.^{5,8,11}

Foxo1 consists of two structural domains, the amino domain, binds DNA binding to target promoters, and the carboxyl transactivation domain, functions to stimulate promoter activity. The forkhead transcription factor Foxo1 has specifically been implicated as a mediator of insulin action in regulating hepatic gluconeogenesis. Hepatic glucose is a major source of endogenous glucose.^{6,8} This production is crucial in the effort to counterbalance hypoglycemia and normalcy in blood glucose levels. Insulin deficiency (type 1) or insulin insufficiency (type 2) creates an unrestrained production of hepatic glucose attributing to the pathogenesis of postabsorptive hyperglycemia in diabetes.^{4,6} Using the insulin-like growth factor-binding protein-1 (IGFBP-1) promoter-directed expression system, Foxo1 mediates insulin action and regulates target gene expression. FoxO1 controls the expression of two gluconeogenic enzymes important in hepatic glucose production, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) which controls a key step in glycogen breakdown. Expression of these enzymes is inhibited by insulin, while stimulated by glucagon.⁶ PEPCK and G-6-Pase are both insulin response elements (IRE) that are similarly down regulated by insulin due to a common. These promoters share a distinctive structural motif: [T(G/A)TTT(T/G)(G/T)]. In the absence of insulin, Foxo1 binds to IRE in target promoters and stimulates the promoter activity.⁶ However, in presence of insulin, FoxO1 undergoes phosphorylation and is excised out of the nucleus eliminated transcriptional activity.

Forkhead Box O1 (FoxO1): Mutant

Previous studies looking at the structure of the FoxO1 transcription factor try to answer how regulation, important intramolecular domains, and insulin hormone levels truly effect the translocation and function in its role of glucose homeostasis. By inducing mutagenesis, experimentation of the FoxO1 factor at particular functional domains helps elucidate the true nature and biological activity. A particular mutant is devoid of its carboxyl domain leaving the truncated amino domain (Foxo1- Δ 256).¹⁰ This mutant interferes with normal FoxO1 function by inhibiting gluconeogenic gene expression. Due to the relative small size (27 kDa) of Foxo1- Δ 256 it readily diffuses easily through the nuclear-pore, therefore localization cannot be easily controlled.¹⁰ Even though its phosphorylation sites are intact, there is no need for these sites due its size, therefore, insulin levels have no effect thus, there is no control on gluconeogenic gene expression. Realizing that the truncation of the carboxyl domain creates a FoxO1 molecule free of localization, but devoid of real mechanistic function contributing to cellular homeostasis, how can this property be exploited.¹⁰ The only attribution leading to the delocalization seems to be the smaller molecular size, leading to freedom of movement; however, if FoxO1 is localized within the nucleus, it would become insensitive to fluctuation insulin levels. FoxO1 would stay within the nucleus, having greater contribution to gluconeogenic gene expression to help control glucose levels especially in pathological issues involving diabetes.^{6,8,10}

It is known that the shuttling of the FoxO family is due to conserved phosphorylation motifs. In FoxO1 one, the conserved sites are at the N- and C-terminal and in the forkhead domain. One member of the family, FoxO6 has the first two conserved sites, N-terminal and forkhead domain, but lack the C-terminal. The third site is segregated downstream after the NES domain (Figure 1).¹ This difference has shown that even after PKB activation, the location of FoxO6 is predominatly in the nucleus whereas FoxO1 would be translocated to the cytosol.

Therefore, further investigation on the phosphorylation sites is imperative in understanding the shuttling mechanism and how to stabilize the location of FoxO1 within the nucleus to have a more profound influence gluconeogenic gene expression attributing on the controlled aspect of glucose homeostasis.^{1,4} This gives implications on site-directed mutagenesis of phospho-residues attributing to phosphorylation activation by PKB. Thus, controlled localization of FoxO1 can be achieved.

Objective and Proposed Goals:

As mentioned above, a better understanding in the regulation mechanism of the FoxO1 transcription factor at its phosphorylation sites may give great insight on its effects glucose homeostasis by influencing gluconeogenic gene expression. For better understanding of the natural characteristics of FoxO1 shuttling, mechanism of transcription, and overall structure, the objective of the laboratory rotational project will be to have successful cloning and expression of the FoxO1 gene. The FoxO1 (1-334) will be expressed in two forms. The first form is as the original wildtype constructed in the Ligation-Independent Cloning (LIC) vector system. The second form will be the mutagenesis at Threonine 24 to Alanine, Serine 256 to Alanine, and Serine 319 to Alanine. These phospho-residues attributing to phosphorylation sites for PKB activation will be mutated to residues (Alanine) unable to be phosphorylated. In effect, the localization of the FoxO1 gene will hopefully be controlled and retained within the nucleus, thus reinforcing its transcriptional activity. The mutant will be constructed containing vectors from the Invitrogen Gateway® System along with using the QuickChange® Site-Directed Mutagenesis kit. After sequencing to ensure correct insertion, the mutant FoxO1 gene will be further expressed within the LIC system such as the original FoxO1 gene. Eventual goals are to obtain sufficient amounts of purified proteins to perform crystal test screenings in hopes of forming a crystal for

further analysis to solve the structure. The screening kits offering various crystallization conditions are: Crystal Screen I & II, Wizard I & II, and Index 1-96.

Methods:

Ligation-Independent Cloning System

Efficiency of PCR cloning has always been an issue when using conventional methods involving restriction sites present in the PCR product or blunt end cloning of these products. Clones lacking correct insertions seem to always be in abundance. The Ligation-Independent Cloning of PCR product is a method developed for efficient cloning of complex PCR mixtures. What is unique about the procedure is that LIC does not require restriction enzymes, T4 DNA ligase, or alkaline phosphates normally used as preventative measures during PCR cloning/amplification to prevent high non-recombinant backgrounds traces of undesired product.¹³ The LIC system will be used to incorporate pMCSG vectors V7, V9, V10 into the FoxO1 (1-334) gene (the sequence given in Appendix A). Vector 7 contains a His-tag, 9 contains BMP-His-tag, and 10 His-GST-tag. After successful cloning, the recombinant vector and FoxO1 PCR product will be expressed and purified through a Nickel column.

Generation of cloneable PCR fragments uses a 5'-end primer containing additional 12 nucleotide (nt) sequence which lacks dCMP. These primers were designed and ordered through the company, Integrated DNA Technologies, Inc. The amplification results in the inclusion of 12-nt sequences at the 3'-end lacking dGMP.¹³ Through the use of T4 DNA polymerase, the

exonuclease activity (3'-5') will remove the 3'-terminal sequence in the presence of dGTP. A single-strand tail will result at the 5'-end of the PCR fragment. Conversely, the same method is applied to the vectors (V7, V9) in the presence of dCTP forming 12-nts ss-ends complementary to the tails formed on the amplified PCR product (FoxO1). Furthermore, before the recombinant plasmids are transformationed into bacterial cells, circulatization via the cohesive 12-nt ends occurs between vectors molecules and PCR fragments through non-covalent bi-molecular associations.¹³ This step does not require in vitro ligation, therefore increasing the efficiency of recombinant transformation into the *E. coli* cells. This method has greater efficiency due to the transformation of circulized recombinants instead of conventional linear strands. In essence, the LIC method will reduce single molecule (non-recombinant) transformation into target cells.

Primers containing the 12-nucleotide tail for cloning the FoxO1 (1-334) was designed using the Invitrogen Vector NTI Advance™ 10. The sense and anti-sense primers will contain the 12-nt tail so that when induced with vectors 7, 9, and 10 containing complementary tails will circularize.¹³ Primers for the vectors 7, 9, and 10 were previously designed and provided. Previous T4 DNA polymerase was also performed, therefore were ready for annealing to the T4 treated FoxO1 PCR product. The circularized recombinant plasmid is then transformed into XL Blue supercompetent cells.¹⁶

Gateway Vector System

The site-specific recombination properties of bacteriophage lambda is exploited in a universal cloning method developed by Invitrogen in their Gateway® Technology system. The bacteriophage T7 RNA polymerase allows regulated expression of heterologous genes in *E. coli* from the T7 promoter.¹² This method provides a rapid and highly efficient means for functional

analysis of interest genes by the possible insertion of multiple vectors. The Gateway®-adapted destination vectors are designed for recombinant expression in E.coli using the pET system. Designation of pDEST™ vectors allows production of native N- or C-terminal tagged recombinant proteins which allows for purified expression.¹²

Along with the T7 promoter and either N- or C-terminal fusion tags, the pDEST™ (14, 15, 17, 24) vectors have been designed containing the following: *attR1* and *attR2*, chloramphenicol resistance gene (Cm^R), *ccdB*, ampicillin resistance gene, and pBR322 origin for low-copy replication and maintenance. The *attR1* and *attR2* sites are two recombination sites having importance in the LR recombination reaction. The purpose of the Cm^R site is for counterselection while the *ccdB* is for negative selection. Both sites are located in between the two *attR* sites.¹²

Before any vectors can be recombined with the FoxO1 gene of interest, an entry code containing the gene of interest must be generated into the Gateway®entry vector. The FoxO1 gene within the entry code should also contain an initiation codon, ATG, to ensure proper PCR reaction and a Shine-Dalgarno sequence (RBS) with optimal spacing for proper translation initiation into *E. coli*. A previously designed entry code for the FoxO1 (1-334) was provided containing the PENTR/TEV/D-TOPO entry vector.¹²

Quick Change® Site-Directed Mutagenesis

The mutagenesis of FoxO1 (1-334) gene will be performed using the QuickChange® site-directed mutagenesis kit. This kit will allow site-specific mutagenesis of the previously cloned FoxO1 (1-334) gene containing the PENTR/TEV/D-TOPO entry vector by the insertion of the amino acid Alanine in place of the three original phospho-residue sites: Threonine 24, Serine 256,

and Serine 319. This method is performed using *PfuTurbo*® DNA polymerase in a PCR temperature cycler will Incorporation of the complementary sense and anti-sense primers containing the desired mutant amino acid insertion.¹⁵ Oligonucleotide primers were designed using the Invitrogen Vector NTI Advance™ 10 to contain the desired mutant amino acid insertion and ordered through Intergrated DNA Technologies. The product is then treated with Dpn I endonuclease to digest the parental DNA template.¹⁵ This is achieved because Dpn I is specific for methylated and hemimethylated DNA having a target sequence of: 5'-Gm6ATC-3'. The nicked vector DNA containing the desired mutations is then transformed into XL1-Blue supercompetent cells.

Mutagenesis Primer Design

Since the site-directed mutagenesis is performed on a double stranded plasmid DNA, two mutagenic oligonucleotide primers must be design. The sense and anti-sense primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid The primers were designed by the specifications provided in the QuickChange® Manual.¹⁵ The desired mutation should be in the middle of the primer with approximately 10-15 bases of correct sequence on both sides totaling to a length of 25 to 45 bases in length. The melting temperature of the primers is recommended to greater than or equal to 78°C and contain a minimum of 40% GC bases.¹⁵

The designed sense and anti-sense primers for the T24A, S256A, and S319A mutation sites are provided on Appendix B. Mutagenesis of 3 sites of FoxO1 gene is done individually. Once the first T24A mutation site is inserted, ensured to be correct through sequencing, the second site (S256A) is inserted by using the first FoxO1 mutant (Foxo1-Mut-T24A) as the DNA

template instead of the original FoxO1 (1-334) DNA template. Consequently, once the second mutation is ensured to be correct, the FoxO1 mutant (Foxo1-Mut-T24A,S256A) is the DNA template for the third mutation site, S319A.

Transformation

Transformation for both the LIC and the site-directed mutagenesis recombinant plasmids will be done with the usage of XL1-Blue supercompetent cells. The XL1-Blue strain allows blue-white color screening for recombinant plasmids through the *lacIqZΔM15* gene on the F' episome. This strain is routinely used for cloning using plasmid or lambda vectors.¹⁶ As stated in the user's manual: XL-1 Blue cells are tetracycline resistant. Quality of miniprep DNA is improved by XL1-Blue cells being endonuclease (*endA*) deficient. Insert stability is improved by deficiency of ability for recombination (*recA*). Cleavage of cloned DNA by the *EcoK* endonuclease system is prevented by a *hsdR* mutation.¹⁶

MiniPrep Purification

MiniPrep purification is a procedure to extract plasmid DNA from bacterial cell suspensions and is based on the alkaline lysis procedure developed by Birnboim and Doly.¹⁴ Plasmids are relatively small supercoiled DNA molecules compared to bacterial chromosomal DNA; being larger and less supercoiled. The topological difference is taken advantage of for selective precipitation of the chromosomal DNA and cellular proteins from plasmids and RNA molecules. Lysis (alkaline conditions) of the cells denatures nucleic acids and proteins. Chromosomal DNA and proteins precipitate because after the solution is neutralized (usually potassium acetate), due to their larger size, it is impossible for them to re-nature correctly.

Plasmids however are able to re-nature in solution and are separated. A spectrometer using a UV lamp is used to measure the nucleic acid concentration ($\mu\text{g/ml}$) of the DNA plasmid.

MINIPREP of PCR Product		
FoxO1 S256A Mut UV Spectrometer		
Sample #	Protein ($\mu\text{g/mL}$)	Nucleic Acid ($\mu\text{g/mL}$)
1	0.0860	142.5875
2	0.1351	223.7526
3	0.0849	141.1679
4	0.0446	76.3143
5	0.0735	123.6361

Table 1: Representative sample of MiniPrep purification and nucleic acid concentration measurement through Beckman DV@640B Spectrophotometer

Sequencing

Sequencing is performed to ensure correct mutagenesis of the FoxO1 (1-334) at T24A, S256A, S319A is performed by sending a sample of the DNA plasmid to the Indiana Molecular Biology Institute (IMBI) to be analyzed. The sequencing technique is done in an automated fashion. However, correct template preparation must be observed to ensure proper sequencing and optimal performance of the ABI3730 Sequencer.¹⁸ Too much template DNA (nucleic concentration) will result in poor readings and possibly damage the capillaries of the ABI3730 machine. The recommended reaction mix and template nucleic concentration was obtain from the IMBI institute and provided below.

DNA Sequencing Reactions (final volume=10 μl)

2 μl of water
 3 μl of 5 mM MgCl_2
 2 μl of primer (2 μM stock)
 2 μl of template*
 1 μl of ABI Big Dye version 3.1

Template:	Recommended Amount
500 bp PCR product	10-20 ng
1 kb PCR product	20-40 ng
2 kb PCR product	40-80 ng
4 kb plasmid	80-160 ng
10 kb plasmid	200-400 ng

As shown above, the amount of DNA to be added to the sequencing protocol depends on the base pair size of the template. It is important to consider the molarity of the DNA template.¹⁸

Results:

FoxO1 Wildtype Clone

The cloning of the FoxO1 transcription factor with insertions of vectors 7, 9 and 10 should be straightforward through protocols tested repetitively to be successful. However, difficulties were encountered in obtaining the correct molecular mass of the recombinants (Figure 4). The agarose gel shows correct and distinctive molecular sizes of the recombinants with individual vectors, 7, 9, and 10 (Figure 4A). It seems that during Tag polymerase amplification for PCR confirmation, lower sized molecular weights were obtained than the expected 1002 base pair size of the correct FoxO1 (1-334) protein. The lower molecular weights seemed to be fragments of impurity or cleavage of the full length protein due incorrect temperature cycling causing excessive denaturation and improper annealing. Another cause, as it was later discovered, for the incorrect protein length was improper preparation of the 7 and 9 vectors (Figure 4B).

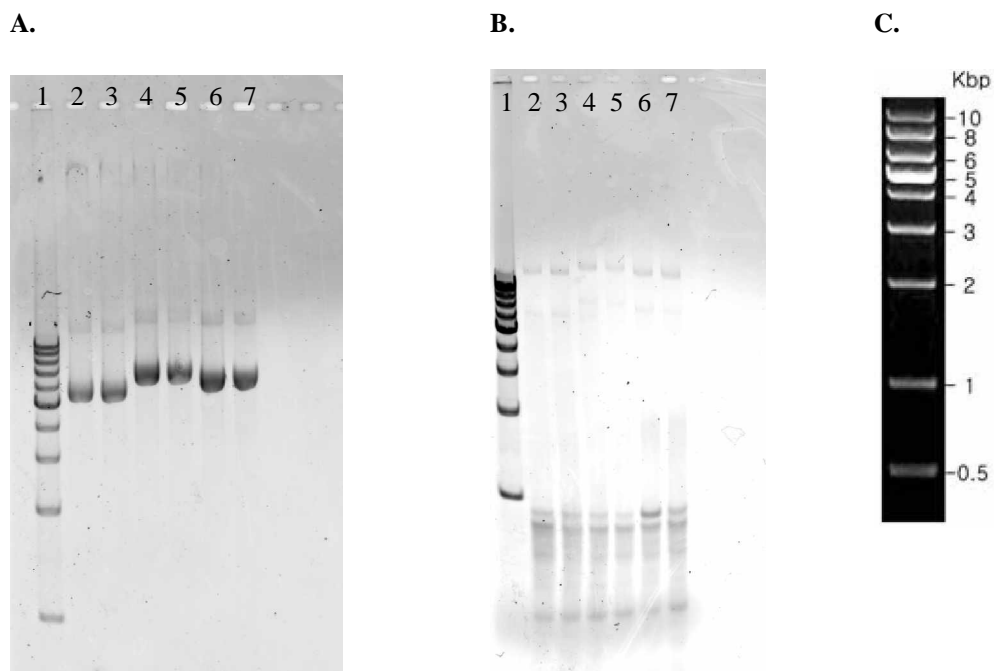


Figure 4: A. Agarose gel electrophoresis of MiniPrep coiled coiled DNA product. Lane 1: 1kbp molecular weight marker; 2,3: Recombinant FoxO1 gene with vector 7; 4,5: Recombinant FoxO1 with vector 9; 6,7: Recombinant FoxO1 with vector 10. B. Taq polymerase PCR confirmation of target FoxO1 gene clone. Lane 1: 1 kbp molecular weight marker; 2,3: Recombinant FoxO1 + V7; 4,5: Recombinant FoxO1 + V9; 6,7: Recombinant FoxO1 + V10. C. molecular weight marker sizes representative of Lane 1 of both A and B. The sizes are in Kbp units. The diagram was obtained from Mbiotech.

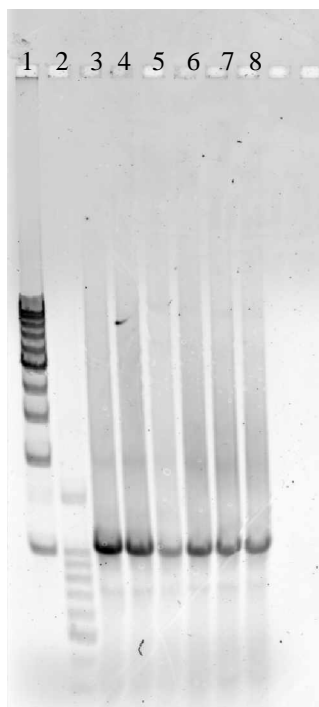


Figure 5: Tag polymerase confirmation of FoxO1 and vector recombinants. Lane 1: 1Kbp molecular weight marker; 2: 100bp molecular weight marker; 3,4: Recombinant FoxO1 + V7; 5,6: Recombinant FoxO1 + V9; 7,8: Recombinant FoxO1 + V10. Molecular weight marker size representative of Lane 1 can be found in Figure 4C. of both A and B. The sizes are in Kbp units. The diagram was obtained from Mbiotech.

The steps for LIC cloning suspected to have led to improper vector preparation could have been attributed to restriction digest with SspI. This step is crucial in leading to correct cleavage of the pMCSG vectors 7, 9, and 10 with complementary 12-nt tails for recombination and circulation with the FoxO1 PCR product. Therefore, the preparation of vectors 7, 9, and 10 were ensure to be correct before LIC cloning of FoxO1 was repeated.

Once the vectors 7, 9, and 10 were corrected, the recombination with the PCR product was seen to be correct through molecular weight analysis through agarose gel electrophoresis. Three recombinants were obtained each contain the differing vectors containing the individually specific tags. Further analysis described in the *Future Goals* section will be performed to characterized the properties of the FoxO1 transcription factor (Figure 5).

FoxO1 Mutagenesis

As mentioned above, mutagenesis of 3 sites of FoxO1 gene is done individually. Once one mutagenic site was inserted, sequenced to ensure correct insertion, the mutant template was used for subsequent mutations. The QuickChange® Site-Directed Mutagenesis kit is proven very proficient in the insertion of replacement amino acid residues with a success rate of nearly 100% if the steps in the protocol were performed correctly. Mutation of the first site, T24A, seemed to be performed correctly, however when the second mutation of S256A was performed and the protein sequenced, there was an unsuspected, extensive deletion of the N-terminal end of the protein. The first mutation site at T24A was no longer noticeable. Before further mutation of the third site, S319A, the problem of the large scale deletion at the N-terminus had to be analyzed.

The reason suspected in creating the N-terminal deletion of the FoxO1 sequence was attributed the GC rich primer regions of the original FoxO1 (1-334) template. It has been shown that the PCR amplification of targets rich in GC content can often result in little or even no yield of the expected PCR product or multiple bands of molecular weights sizes other than the expected target. In the original protocol for the PCR reaction mixture of the mutagenesis kit, there was no addition of an additive or enhancing agent. Since the FoxO1 template is rich in GC, an additive or enhancing agent would help increase yield, specificity, and consistency of overall target product.¹⁷ The enhancing agent that will be added to the PCR reaction mixture is 5% DMSO (dimethyl sulfoxide).¹⁷ DMSO disrupts base pairing, in hopes of increasing efficiency and specificity of the PCR amplification. However, although DMSO is a useful enhancing agent, it affects the overall melting temperature of the DNA which alters the optimal annealing condition for the PCR reaction.¹⁷

After the addition of 5% DMSO to the PCR reaction mixtures the problem of N-terminal deletion to the mutant FoxO1 templates seemed to be eliminated. Successful mutations of the T24A and S256A sites were conducted and a mutant FoxO1 template containing the one (T24A) and two (T24A and S256A) mutant sites was obtained and ensured to be correct through DNA sequencing. The Thr24 was converted to Ala at nucleotide numbering 70, 71, and 72. The codon changed from ACC (Thr) to GCA (Ala). The Ser256 was converted to Ala at nucleotide numbering 766, 767, and 768. The codon changed from TCC (Ser) to GCC (Ala). Therefore, the template containing the two mutagenesis sites would sequentially be used for the final third mutagenesis site, S319A.

The mutagenesis at S319A seemed to be troublesome and unsuccessful. Through three trials and sequence testing, the site was not inserted into the mutant template. When sequenced, the DNA showed the previously inserted T24A and S256A sites, but never the third, S319A. There was suspicion that the cause for the unsuccessful mutagenesis was the digestion enzyme, Dpn I, which targeted parental FoxO1 template was inactivated through denaturation. Therefore, when transformed into XL-Blue strain of *E.coli*, the original template containing only the two mutagenic site was also incorporated along with possibly correct product containing all three sites. However, the yield of the correct product was low and interspersed with incorrect product. Therefore, continuation of the project was carried on with the template carrying only the first two mutagenic sites, T24A and S256A.

The template was recombined with vectors 7, 9, and 10 as describing in the LIC cloning system. The correct recombinants was obtained and observed through agarose gel electrophoresis. Further analysis described in the *Future Goals* section will be performed to characterized the properties of the mutant FoxO1 transcription factor compared to the original

protein. The replacement of two phosphor-residues will be observed to see effects on phosphorylation by PKB activation.

Future Goals:

At present, the research of Forkhead Box O1 (FoxO1) transcription factor has been the successful cloning of the original FoxO1 (1-334) protein and the incorporation of two of the three intended mutagenesis sites, T24A and S256A. Further studies into the recombinant clones will be necessary to further analyze and extensively study its characteristics. Recombinant clones of the original FoxO1 template through the LIC system have been tested to contain the correct molecular weights with the additions of vectors 7, 9, and 10 through agarose gel electrophoresis. The mutant FoxO1 (T24A, S256A) has also been sequenced to contain the correct mutant sites, and analyzed to contain the correct vectors 7, 9, and 10 insertion by molecular weight through agarose gel electrophoresis.

Further experimentation to obtain purified individual recombinant FoxO1 with V7,9 and 10 and the recombinant (with V7, 9, and 10) mutants FoxO1 (T24A, S256A) will consist of transformation into the more competent *E.coli* strain of cells, small-scale expression tests, solubility tests, large-scale expression, purification, and final activity and crystal screenings. The transformation of the protein construct is typically done in the B834 DE3 or BL21 Rosetta expression strains of *E.coli*. Small-scale expression will be performed to verify that the construct is of desired length, the protein is soluble, and whether the protein binds to affinity beads for subsequent large-scale purification. This first expression and solubility test can also check multiple lysis conditions that the FoxO1 protein will tolerate. Variables that can be taken into

consideration include: expression temperature, pH of lysis buffer, salt concentration in lysis buffer, detergent or glycerol in lysis buffer, and the expression strain used for transformation.

Once initial small scale tests have been performed, large-scale expression will be done to purify the original and mutant FoxO1 proteins. Once the correct variables tolerable by the protein were found, the optimal conditions are used to grow expression strains in abundance to obtain considerable amounts of recombinant plasmids. Once plasmids are obtained, purification is performed through affinity columns. Since the individual vectors contain His-tags (7), Bmp-His-tags (9), and His-GST-tags, a Nickel column will be used to extract the correct recombinants, while eluting out trace impurities (individual nickel columns will be used for individual recombinant containing differing vectors).

Once the recombinants are extracted, they are eluted out and the various vector tags cleaved to obtain only the original and mutant FoxO1 protein. Furthermore, after the protein is obtained and analyzed to be of correct length, crystal screening conditions will be performed to eventually obtain a crystal and solve its structure. The solved crystal structure will help understand the function and elucidate the mechanism of the FoxO1 transcription factor. The original FoxO1 protein will elucidate the natural means of conduct while the mutant FoxO1 absent of phosphor-residues will elucidate the repercussions of the removal of PKB activated phosphorylation for shuttling out of the nucleus.

References:

1. Van der Heide, Lars P.; Marco F. M. Hoekman and Marten P. Smidt. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. *Biochem. J.* 2004. **380**, 297–309
2. Morris, Brian J. A forkhead in the road to longevity: the molecular basis of lifespan becomes clearer. *Journal of Hypertension*, 2005. **23**:1285–1309.
3. Katoh, Masuko and Masaru Katoh. Human *FOX* gene family (Review). *International Journal of Oncology*, 2004. **25**: 1495-1500.
4. Nakae, Jun; Tadahiro Kitamura, David L. Silver, and Domenico Accili. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J. Clin. Invest.* 2001. **108**:1359–1367.
5. Kitamaru, Tadahiro; Yun Feng, Yukari Ido Kitamura, Streamson C Chua, Jr, Allison W Xu, Gregory S. Barsh, Luciano Rossetti and Domenico Accili. Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. *Nature Medicine*. Advance

Online Publication. 2006 Nature Publishing Group.

<http://www.nature.com/naturemedicine>

6. Kitamaru, Tadahiro; Yun Feng, Yukari Ido Kitamura, Streamson C Chua, Jr, Allsion W Xu, Gragory S. Barsh, Luciano Rossetti and Domenico Accili. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic β cell growth. *J. Clin. Invest.* 2002. **110**:1839–1847.
7. Altomonte, Jennifer ; Lin Cong, Sonal Harbaran, Anja Richter, Jing Xu, Marcia Meseck, and Hengjiang Henry Dong. Foxo1 mediates insulin action on apoC-III and triglyceride metabolism. *J. Clin. Invest.*, 2004. **114**:10
8. Matsuzaki, Hitomi; Hiroaki Daitoku, Mitsutoki Hatta, Hisanori Aoyama, Kenji Yoshimochi, and Akiyoshi Fukamizu. Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. *PNAS*, 2005. **102**:32, 11278–11283.
9. Kim, Moonil ; Joon-Woo Ahn, Kiwon Song, Kyung-Hee Paek, and Hyun-Sook Pai. Forkhead-associated Domains of the Tobacco NtFHA1 Transcription Activator and the Yeast Fhl1 Forkhead Transcription Factor Are Functionally Conserved. *The Journal Of Biological Chemistry*, 2002. **277**:41, 38781–38790.
10. Altomonte, Jennifer; Anja Richter, Sonal Harbaran, Jenny Suriawinata, Jun Nakae, Swan N. Thung, Marcia Meseck, Domenico Accili, and Hengjiang Dong. Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. *Am J Physiol Endocrinol Metab*, 2003. **285**: E718–E728.
11. Weigel, D., Jurgens, G., Kuttner, F., Seifert, E., and Jackle, H. (1989) *Cell* **57**, 645–658.
12. *E. coli* Expression System with Gateway® Technology Instruction Manual, Version E, 2004.
13. Aslanidis, Charalampos and Pieter J.de Jong. Ligation-independent cloning of PCR products (LIC-POR). *Nucleic Acids Research*, 1990. **18**:20 6069-6074.
14. Birnboim, H.C. and J. Doly. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* 1979. **7**, 1513-1523.
15. QuikChange® Site-Directed Mutagenesis Kit Instruction Manual, Catalog #200518 (30 reactions) and #200519 (10 reactions), Revision #046008r, 2005.
16. XL1-Blue Supercompetent Cells Catalog #200236, Stratagene Technical Services, 2004.
17. Frackman, Susan; Gary Kobs, Dan Simpson and Doug Storts, Promega Corporation. Betaine and DMSO: Enhancing Agents for PCR. *Promega Notes* Number 65, 1998, p. 27

18. Indiana Molecular Biology Institute: DNA Sequencing Facilities. Indiana University, Bloomington, IN. 2005. <http://imbi.bio.indiana.edu/>