The Role of Glycogen Synthase Kinase 3 beta
In Regulating the Function of PAX3-FOXO1 by Phosphorylation

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ABSTRACT

The Role of Glycogen Synthase Kinase 3 Beta in Regulating the Function of PAX3-FOXO1 by Phosphorylation

by

Alexander Andrew Tong

Alveolar rhabdomyosarcoma (ARMS) is a type of rhabdomyosarcoma, which is the most common soft tissue sarcoma in pediatric patients. Nearly 70% of ARMSs express the fusion protein PAX3-FOXO1, which is linked to poor prognosis and increased tumor aggressiveness. Previous studies have shown that the glycogen synthase kinase 3 beta (GSK3β) inhibitor TWS119 can inhibit cell proliferation in alveolar rhabdomyosarcoma cells, and that GSK3β can phosphorylate PAX3-FOXO1 in vitro. However, the specific nature of these phosphorylation events and physiological relevance of these events for TWS119 activity are not known. In this study, site-directed mutagenesis is used to evaluate a putative phosphorylation site located at the junction of the PAX3 and FOXO1 domains in the fusion protein for the site’s importance in PAX3-FOXO1 functional activity. Our results show that this site can regulate PAX3-FOXO1 functional activity and strongly suggests it may be a phosphorylation site for GSK3β. These studies provide insight to the role of PAX3-FOXO1 function in ARMS cells, important since this fusion protein is indicative of a more aggressive cancer phenotype that is resistant to conventional chemotherapy and radiotherapy. Novel strategies in treating these aggressive ARMS types might include modulating the activity of the fusion protein’s target genes as well as the fusion protein’s transcriptional activity, particularly through inhibiting GSK3β.
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Background

Glycogen Synthase Kinase 3 beta (GSK3β) is a well characterized serine/threonine kinase that is known to affect glycogen metabolism by phosphorylating and inhibiting glycogen synthase [1]. GSK3β is also involved in classic pathways such as the Wingless Integration protein (Wnt) and insulin signaling pathways that respond to various physiological changes [2]. However, GSK3β has more recently been shown to play novel roles in cancer. For example, it has been reported by Wang et al. that GSK3β supports Mixed Lineage Leukemia (MLL) leukemia cell proliferation via destabilization of the protein p27^Kip1, a cyclin-dependent kinase inhibitor [3]. Supporting GSK3β’s role in cancer, it has been shown that GSK3β inhibitors have also been shown to inhibit cell proliferation in alveolar rhabdomyosarcoma (ARMS) cells. Taken together, these and other studies implicate GSK3β as a potential novel target for cancer therapies [4]. To recognize its target, GSK3β requires a downstream “primed” phosphorylation site on a substrate that is usually phosphorylated by another kinase before GSK3β itself can phosphorylate that substrate at a different site. GSK3β recognizes the (S/T)-X-X-X-(S/T) motif on a given substrate, wherein the first serine/threonine on the substrate is the GSK3β phosphorylation site and the latter serine/threonine is the primed phosphorylation site [5].

PAX3-FOXO1 is a fusion protein that is expressed after the translocation of chromosome 2 and 13, denoted as t(2;13)(q35;q14) [6]. About 70% of ARMSs contain this translocation, and expression of the fusion protein is typically indicative of a more aggressive cancer phenotype that is resistant to conventional chemotherapy and radiotherapy. Novel strategies in treating these aggressive ARMS types include modulating the activity of the fusion protein’s target genes as well as the fusion protein’s transcriptional activity [4].

Zeng et al. [4] have shown that the GSK3β inhibitor TWS119 can inhibit fusion cell proliferation and induce apoptosis in fusion protein positive ARMS cell lines. They have also shown that GSK3β can phosphorylate PAX3-FOXO1 in vitro. However, the specific nature of
these phosphorylation events and physiological relevance of these events for TWS119 activity are not known. The objective of our study was then to identify putative GSK3β phosphorylation sites on PAX3-FOXO1 and see whether mutants of those sites would affect fusion protein transcriptional activity.

There are several putative GSK3β phosphorylation sites on PAX3-FOXO1 that follow the unique (S/T)-X-X-X-(S/T) motif. However, by analyzing the sequence of the fusion protein, we have identified a matching motif with the amino acid sequence S-P-Q-N-S that occurs at the junction of the PAX3 and FOXO1 proteins and that is unique to the fusion protein. Because of its rather unique positioning, and because it is highly possible that the last serine is a phosphorylation site for mammalian sterile20-like 1 (MST1) kinase [7] and therefore possibly serves as a required “primed” phosphorylation site for GSK3β, we chose this site as the starting point for our mutagenesis and phosphorylation studies. We created phosphomimetic and phosphodeficient mutants at the GSK3β and MST1 kinase putative phosphorylation sites.

We have shown by western blot that our mutants are able to be expressed in NIH3T3 cells alongside wild type protein. In reporter assays, we have shown that a phosphomimetic mutation at either of the putative phosphorylation sites significantly increases fusion protein activity compared to wild type protein. Unexpectedly, phosphodeficient mutations at either of the sites also increase fusion protein activity, but not nearly as much as phosphomimetic mutations increase fusion protein activity. Therefore, we have determined that these sites are important for regulating fusion protein activity, and that they are particularly strong candidates for GSK3β phosphorylation.
Methods

Site-Directed Mutagenesis. The Quikchange II XL Site-Directed Mutagenesis Kit (Cat. #200521, Stratagene/Agilent Technologies) was used to generate fusion protein mutants in the original GFP-tagged vector. The GFP-PAX3-FOXO1 wild-type plasmid used for mutagenesis was previously made by Dr. David Bouck, originally cloned from PAX3-FOXO1 in a pCDNA3 vector provided Dr. Frederic Barr, University of Pennsylvania. The wild type sequence is provided in the Appendix. Previous western blot and reporter assay studies have confirmed the function and stability of the fusion protein with the GFP tag (data not shown). Sites of interest were mutated to Alanine (A) and Aspartate (D) following the guidelines in the kit manual (primer sequences provided in the Appendix). The putative GSK3β phosphorylation site mutants were denoted S389A and S389D, and the putative primed phosphorylation site mutants were denoted S393A and 389D. Double site mutants were denoted S389A/S393A and S389D/S393D. Cycling parameters on the BioRad PCR machine were used following the kit guidelines, with 9 minutes used for the variable 68°C step in segment 2. PCR products were digested with *Dpn I* for 1 hour at 37°C and transformed into XL-10 Gold Ultracompetent cells provided with the kit, then streaked on LB agar plates treated with 50 µg/ml Kanamycin antibiotic (Invitrogen) overnight at 37°C. Individual colonies were then picked and plasmids purified using the Sigma Aldrich GenElute Plasmid Miniprep Kit (Catalog #PLN350-1). Plasmids with correct sequences were then purified using the Qiagen EndoFree Plasmid Maxiprep Kit (Catalog #12362).

Cell Culture. Following standard guidelines [8], the NIH3T3 cell line was grown in RPMI 1640 (GIBCO) supplemented with 10% FBS (Hyclone), 1X sodium pyruvate (GIBCO), 2mM L-glutamine (GIBCO), and 1X penicillin/streptomycin (GIBCO). An incubator maintaining a humidified atmosphere at 5% CO₂ and 95% air at 37°C was used to culture cells. For luminescence assays [9,10], phenol-red free RPMI 1640 media (GIBCO) was used.
Western blot analysis. Whole cell lysate was collected from NIH3T3 cells 24 hours post-transfection with Fugene 6 (Roche) and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Thermo Scientific) for 20 minutes. Lysates were subsequently inverted for 20 minutes, then centrifuged in the Eppendorf Centrifuge 5417R at 20,800xg for 20 minutes to clear cellular debris. Equal amounts of cleared lysate were mixed with 1X loading buffer made from 4X Loading Buffer and 10X Reducing Buffer (Invitrogen) and boiled at 99°C for 5 minutes to denature protein. The samples were then loaded onto a 4-12% Bis-Tris acetate gel (Invitrogen) alongside a Kaleidoscope Protein Ladder (BioRad) as a marker for SDS-PAGE gel electrophoresis at 150V. Protein from the gel was transferred to a nitrocellulose membrane using an iBlot transfer stack (Invitrogen), after which the membrane was blocked with Blocking Buffer (Odyssey) for 1 hour. Membranes were probed with anti-GFP full length antibody (Santa Cruz Biologicals) overnight to maximize signal. After washing thrice with 1X TBST, fluorescent secondary antibodies (Odyssey) reactive against the respective primary antibody animal were used. A LiCor Odyssey Scanner was used to image the membrane after washing thrice again with 1X TBST. An anti-β-actin antibody (Sigma-Aldrich) was used to probe the same membrane in a similar fashion to check approximately equal loading in each well. Bands were quantified using the LiCor Odyssey software.

Luciferase reporter assays. NIH3T3 cells were transiently transfected using Fugene 6 at a plating density of approximately 3.0X10^5 cells per each well of a 6-well plate. 6XPRS9 reporter, a widely used reporter for PAX3-FOXO1 [4,10], and TK-Renilla as a transfection control were included in each transfection reaction. Both plasmids were gifts from Dr. Lingling Liu, St. Jude Children’s Research Hospital. After 24 hours incubation, cells were trypsinized and homogeneously resuspended in phenol-red free RPMI 1640 media. Cells were then counted using the Auto T4 Cellometer (Nexcelom) and diluted such that 75 µl of resuspended cells contained approximately 1.5X10^4 cells. 75 µl of cells were then aliquoted per well in a 96-well white
bottom CulturPlate (PerkinElmer), then incubated for another 24 hours to allow cells to adhere to the bottom of the plate. Luciferase activity was measured using the Dual Glo/Stop & Glo Luciferase Assay System (PerkinElmer), according to manufacturer’s instructions. Luminescence was quantitated using the PerkinElmer Envision plate reader.
Results

Development of Mutants (see Appendix sequence map)

In order to define the specific sites phosphorylated, GFP-tagged Alanine (A) and Aspartate (D) mutants were generated for each site using the Stratagene Quikchange II XL Kit. We denoted the first serine as S389 and the second serine as S393, marking S389 as our putative GSK3β phosphorylation site and S393 as our putative MST1 kinase primed phosphorylation site. We expected that an S to A mutation would render PAX3-FOXO1 resistant to phosphorylation (phosphodeficient), whereas an S to D mutation would mimic phosphorylation (phosphomimetic). We denoted our alanine mutants as S389A and S393A, and we denoted our aspartate mutants as S389D and S393D. In order to investigate the unique GSK3β (S/T)-X-X-(S/T) phosphorylation motif, we also created GFP-tagged double mutants at both sites, denoted as S389A/S393A and S389D/S393D.

Mutants in reporter assays

The function of PAX3-FOXO1 was evaluated by using a “reporter assay” in which the expression of the reporter gene is controlled by a promoter that is regulated by PAX3-FOXO1. In our reporter assays, we used the 6 x PRS9 reporter, which has routinely been used to detect the activity of PAX3-FOXO1 [4,10]. Using this system, we sought to determine the functional activity of our mutants as compared to wild type.

Although an ARMS cell line would be the ideal system to test mutant expression and functional activity, we were not able to use an ARMS cell line to confirm our fusion protein mutants’ expression because of the endogeneous PAX3-FOXO1 expression. We selected the NIH3T3 cell line to express our mutants, as NIH3T3 is a standard cell line for studying oncogenes, including PAX3-FOXO1, since the fusion protein is not endogeneously expressed in this cell line [8].
If the S389 site were a critical site for the activity of the fusion protein, we expected to see a statistically significant decrease in activity for the phosphodeficient S389A mutant and an increase in activity for the phosphomimetic S389D mutant as compared to the wild type. Since the S393 site was our putative primed phosphorylation site, we expected to see similar results for the S393A and S393D mutants, as well as for our S389A/S393A and S389D/S393D double mutants as compared to the wild type.

In Dual-Glo/Stop & Glo reporter assays, we saw a significant change in the functional activity of our S389A, S389D, S393D, and S389A/S393A mutants, as shown in Figure 1. Contrary to expectation, S389A and S389A/S393A mutants did not show statistically significant changes in activity as compared to wild type. However, S389D and S393D mutants showed 2-fold and 1.5-fold decreases in activity, contrary to our hypothesis that an aspartate mutant at these sites should mimic phosphorylation and therefore activation of the fusion protein. In addition, the S393A and S389D/S393D mutants did not show any significant change in activity, contrary to our expectation that a phosphodeficient mutation and a double phosphomimetic mutation should respectively decrease and increase PAX3-FOXO1 activity.

**PAX3-FOXO1 protein expression in NIH3T3 cells**

The S389D mutant showed nearly 2-fold decrease in reporter activity, however its expression as measured by western blot showed a nearly 6-fold reduction, indicating the decreased reporter activity may be due to reduced expression levels. Similarly, the S393D mutant showed a 5.2-fold decrease in activity that might explain the unexpected 1.5-fold decrease in its reporter activity. Although the S393A and S389D/S393D mutants did not show significant change in reporter activity, their western blot expression levels were reduced nearly 6-fold and 2-fold respectively. Finally, compared to fusion protein wild type, the S389A and S389A/S393A mutants showed 2.9-fold and 2.3-fold reductions in expression respectively, which would indicate
that their reporter activities that initially appeared to be reduced compared to wild type may actually be higher than expected.

*Normalization of reporter assay data to western blot expression*

When luciferase data was normalized to that of western blot expression quantification data, all of the mutants showed significantly increased reporter activity compared to wild type (Figure 3). The S389A and S393A mutants showed a 3.7-fold and 3-fold induction respectively, contrary to expectations. More consistent with our hypothesis, the S389D and S393D mutants showed a 13.6-fold induction and 7.8-fold induction respectively. Finally, the S389A/S393A and S389D/S393D double mutants exhibited a 6.2-fold and 3.8-fold induction respectively.
Discussion

ARMS cells expressing PAX3-FOXO1 show increased metastatic potential and chemotherapeutic resistance, as the fusion protein more potently activates transcription of several oncogenic factors [4]. Patients who have fusion protein positive ARMS have decreased event-free survival rates due to the aberrant transcriptional regulation of genes by PAX3-FOXO1 [6]. Therefore, new therapies that target PAX3-FOXO1 are critical for tackling these aggressive ARMSs.

Zeng et al. [4] have shown that siRNA knockdown of GSK3β results in a decrease in PAX3-FOXO1 reporter activity as compared to non-targeting siRNA. They have also shown that GSK3β phosphorylates PAX3-FOXO1 in vitro, suggesting that the activity of the fusion protein can be modulated by phosphorylation at a given site(s). We have shown that S389 and S393 are important sites for PAX3-FOXO1 activity, and that phosphomimetic or phosphodeficient mutations at one or both sites significantly alters fusion protein activity in NIH3T3 cells.

These contradictory results may be partly explained by the inconsistent levels of expression of our mutants in NIH3T3 cells as detected by western blot, shown in Figure 2. The varying expression levels of the mutants could be attributable to the point mutations affecting the stability and therefore expression of the fusion protein by an unknown mechanism. Since our mutants show varying levels of expression western blots in NIH3T3 cells, normalizing the reporter assay luciferase readings by western blot expression is necessary to more accurately assess each mutant. In this case, we can normalize Dual-Glo values to the quantified expression levels of our mutants from western blot. This approach is valid since the cells that were used for reporter assay and western blot came from the same transfection. As shown in Figure 3, Dual-Glo values normalized in this manner yield aspartate mutant activities that more closely correspond to our hypothesis, which is that phosphomimetic mutants should show increased functional activity relative to wild type. In this case, our S389D, S393D, and S389D/S393D mutants show
statistically significant fold inductions and therefore follow expected results. Contrary to expectation, the phosphodeficient mutants S389A, S393A, and S389A/S393A show significantly increased activity relative to wild type. In these cases, through an unknown mechanism, an alanine mutant at these sites appears to actually increase activity. In either case, we have shown that the S389 and S393 sites are important for the function of PAX3-FOXO1, and that phosphomimetic mutations especially increase PAX3-FOXO1 activity. Our findings strongly suggest that S389 in particular might be an important GSK3β phosphorylation site, although further studies are needed to specifically identify GSK3β’s association with this site.
Future Directions & Conclusion

Future studies should include performing \textit{in vitro} kinase assays to determine whether the PAX3-FOXO1 mutants can be phosphorylated by GSK3β as compared to the wild type. If the S389 site is the most critical site for phosphorylation by GSK3β, we expect to see decreased levels of phosphorylation for all of our mutants in \textit{in vitro} kinase assays as compared to the wild type, as mutating either the putative GSK3β phosphorylation site or the putative primed phosphorylation site to either alanine or aspartate should confer resistance to phosphorylation by GSK3β. We could then compare phosphorylation resistance to reporter activity and expression levels for each of our mutants to confirm that a phosphorylation defect results in reduced fusion protein activity.

Overexpression of GSK3β with fusion protein mutants in reporter assays would also help confirm that GSK3β specifically phosphorylates the S389 site. Since the S389 mutants would be resistant to phosphorylation by GSK3β, we would that the mutants would not increase in activity as much as wild type when co-transfected with GSK3β. Similarly, the putative MST1 kinase phosphorylation site mutants S393A and S393D should be resistant to phosphorylation by MST1 kinase.

By better elucidating how proteins like GSK3β modulate PAX3-FOXO1 transcriptional activity, we hope to identify potential targets for drug discovery and therapy in ARMS. TWS119 has already been shown by Zeng et al. [4] to selectively inhibit GSK3β, resulting in decreased cell proliferation and increased apoptosis in ARMS cell lines, possibly through a decrease in the phosphorylation and functional activity of PAX3-FOXO1. Identification of the functional site(s) of GSK3β on the fusion protein will confirm regulation of the fusion protein by GSK3β, and GSK3β would become a leading target for modulating fusion protein activity in aggressive ARMSs.
Figure 1. The S389D and S393D mutants show significant changes in reporter activity when luciferase activity is normalized to Renilla count. Dual-Glo values were normalized by dividing by Stop & Glo values. Each reaction was performed six times. One asterisk (*) indicates p-value less than 0.05. Two asterisks (**) indicates p-value less than 0.0005. NS indicates not significant.
Figure 2A-B. Figure 2A shows a western blot of GFP-PAX3-FOXO1 constructs in NIH3T3 cells. The membrane was probed with anti-GFP (upper scan), then anti-actin (lower scan). Lanes were loaded as follows: (1) No transfection, (2) pEGFP-C3 vector, (3) GFP-PAX3-FOXO1 wildtype, (4) S389A, (5) S389D, (6) S393A, (7) S393D, (8) S389A/S393A, (9) S389D/S393D. (4.1), (6.1), and (6.2) were transfections with increased concentration of S389A and S389D mutants, but were not used in subsequent experiments. The upper intense band in lane 2 is pEGFP-C3 vector, while the lower intense band in lane 2 is likely pEGFP-C3 vector degradation product. LiCor quantification values are shown in Figure 2B.
Figure 3. All of the mutants show an increase in reporter activity when luciferase activity is normalized to protein expression. Dual-Glo values were normalized by dividing by respective western blot quantifications. Two asterisks (**) indicates p-value less than 0.0005.
Appendix

PAX3-FOXO1 protein sequence (GenBank #AAC50053.1)

MTTLAGAVPRMMRPGBPQGQNYPRSGFPLEVSTPLGQGRVNLGGFINGRPLPNHRH
KIVEMAHHHGIRPCVIRQRLRVSHPCHVSKILCRYQETGQPTPAIGGSKPKQVTTPDVEKK
IEEYKRENPGMSWEIRDKKLLAVDCRNTVPVSSSHISRIRSFRSKFAGGEEEEADLERKE
AEESEKAKHSIDILSERASAPQSDGSEDIDSEPDLPLMKRQRRSRTTFFATEQLLEEL
RabethYPIYDITYRELAQRAKLTEARVQVWFSNRRARWRKOQAGANQLMNFNLHPG
GFPPTAMPPTLPYQLSETSYQPSIPQAVSDDSTVHRPQLPQLSTVHSTIPSNPDSS
SAYCLPSTRHGFSSYDSFVPSPGSPSNPNMPTINGNSQPNSRHNLSLHSKFIYRQNE
GTMKSSWWMLNPEGGKSKGSPRRAAASMDNNSKFAKRSRRAKAKSLRSSQEGA
GDSPGSQFSKWPASPGSSHNDFDNWSTFRPTSSNASTISGRSLSPIMEQDDLLEG
DVHSMYVPPSAAKMANTLPSLEISNPENMMLDDLNNLLSPTSLTVSTQSSPPTMM
QQTPCYSFAPMPNTLSNSPSNPYQKYTYGQSSMSPLPQMQIMLQDNKSSYGGMSQYN
CAPGLLLELLTSDDENPTVPDGPVAAQNSRVLGQNVMMGPMVSSTGYSQASH
NKMMPNPSHTHPGHQATCSSAVGRPLPHVTSTMPHTSGMNRDLTQVKTVPQVPLPHP
MQMSALGGYSSVSSSCNGYGRMLVQKLPDSLDMFIERLDLMDESIRNDLMDGD
TLDFNFDVNLQPSFHPHSVKTTHSWSVG

PAX3 sequence shown in red
FOXO1 sequence shown in blue

Underlined, bolded red S indicates putative GSK3β phosphorylation site
Underlined, bolded and italicized blue S indicates putative primed phosphorylation site

Primers for Mutagenesis

S389A Forward: 5’-CCATTGGCAATGGCCTCGACCTCAGAATTCAATCC-3’
S389A Reverse: 3’-GGTACCCCTTACCCAGGACGATGAGTCTCTAAGTTAAG-5’
S389D Forward: 5’-CCTCCATTGGCAATGGCCTCGACTCCAGAATTCAATCTCAT-3’
S389D Reverse: 3’-GGTGTTGTAACCCCTACCCAGGACGATGAGTCTCTAAGTTAAGCAGTA-5’
S393A Forward: 5’-GGCCTCTACCTACCTCAGATCGAGTCTCTAATCTG-3’
S393A Reverse: 3’-CCGTGAGTGGAGTCTCTAATCGTAAGCAGTATTAGACA-5’
S393D Forward: 5’-GGGAGGAGTGGAGTCTCTAATCGTAAGCAGTATTAGAC-3’
S393D Reverse: 3’-CCCTTGACCGGAGTGGAGTCTCTAATCGTAAGCAGTATTAGAC-5’
S389A/S393A Forward: 5’-GCCTGCACCCCTACCTCAGATCGAGTCTCTAATCTG-3’
S389A/S393A Reverse: 3’-CGGAGGAGTGGAGTCTCTAATCGTAAGCAGTATTAGAC-5’
S389D/S393D Forward: 5’-CCCACCATTGGCAATGGCTCGATCCTCAGAATGATATTGTCAT-3’

S389D/S393D Reverse: 3’-GGGTGGTAACCGTACCGTAGCTAGGAGTTACTATAAGCAGTA-5’
Bibliography