2. SPECIFIC AIMS

Alveolar Rhabdomyosarcoma (ARMS) is an aggressive childhood tumor derived from striated muscle that responds poorly to conventional treatments[1,2]. ARMS is primarily characterized by a t(2;13) chromosomal translocation that fuses two myogenic transcription factors, Pax3 and FOXO1, to create the oncogenic fusion protein Pax3-FOXO1[1]. This fusion protein has altered biological activities relative to wild-type Pax3, including increased transcriptional activity[3] and protein stability[4], both of which alter myogenesis and are believed to contribute to the development of ARMS. Although some of the molecular mechanisms regulating the biological activities of Pax3 and Pax3-FOXO1 are known, at present the exact role of post-translational modifications, particularly phosphorylation, in regulating both proteins has yet to be elucidated. Therefore, it is *the long-term goal of this lab* to understand how phosphorylation of Pax3 regulates its biological activity, how this regulation contributes to normal myogenesis, and how this regulation is altered when Pax3 is fused to FOXO1 to contribute to the development of ARMS

In accord with the stated long-term goal, the ***central hypothesis of this proposal is that differences in the phosphorylation of Pax3 and Pax3-FOXO1 throughout early myogenic differentiation contributes to alterations in gene expression and protein stability, thereby affecting myogenesis and subsequently contributing to ARMS phenotypes.*** This hypothesis is formulated on the basis of results published by this lab and preliminary data presented in this proposal. We published the identification of serines 201, 205, and 209 as the only residues that are phosphorylated on Pax3 in physiologically relevant primary myoblasts[5,6]; we have determined that phosphorylation of these sites is dynamic and changes throughout early myogenic differentiation[5,6]; and we demonstrated that distinct differences exist between the phosphorylation patterns observed between Pax3 and Pax3-FOXO1[5,6]. Preliminary data presented in this proposal shows that the phosphorylation of Pax3 affects its ability to interact with the transcriptional co-regulatory protein hDaxx. Phosphorylation at serine 201 promotes the stability of Pax3 throughout differentiation. We also show that expression of Ser205-phosphorylated Pax3 enhances the proliferation rate of primary myoblasts, while phosphorylation at Ser209 enhances the expression of MyoD, a gene critical for myogenic differentiation. In this proposal, we will use mouse primary myoblasts as a model system to test our hypothesis by pursuing two *specific aims:*

Specific Aim 1: To examine the role of the phosphorylation of Pax3 and Pax3-FOXO1 phosphorylation in myogenesis and as a contributor to the pathology of ARMS. We will use the physiologically relevant mouse primary myoblasts or ARMS tumor cell lines stably expressing Pax3, Pax3-FOXO1, or mutants in which the identified sites are mutated to be phospho-incompetent (Ser to Ala) or phospho-mimetic (Ser to Asp). We will determine how phosphorylation at these sites contributes to normal myogenesis and the development of ARMS, examining cellular functions such as growth, migration, myogenic differentiation, and by performing an unbiased survey to analyze changes in the transcriptome profiles during early myogenesis.

Specific Aim 2: To determine how phosphorylation regulates the molecular activities of Pax3 and Pax3-FOXO1 in early myogenesis. Primary myoblasts or ARMS tumor cell lines stably expressing the Pax3 or Pax3-FOXO1 phospho-mutants described in Aim 1 will be used to examine the role of phosphorylation in regulating Pax3 and Pax3-FOXO1 DNA binding, transcriptional activity, endogenous gene expression, protein-protein interactions, and protein stability.

The research accomplished in Specific Aim 1 will provide an understanding for how changes in the phosphorylation of Pax3 and Pax3-FOXO1 contribute to both normal myogenesis and the development of ARMS. Completion of Specific Aim 2 will provide an understanding of the role that phosphorylation plays in regulating the transcriptional and biological activities of Pax3 and Pax3-FOXO1. The *rationale* for the proposed research is that elucidation of the role of phosphorylation in regulating Pax3 biological activity will provide understanding of a molecular mechanism that, when dysregulated by the presence of Pax3-FOXO1, contributes to the development of ARMS. This knowledge will provide evidence for the identification of the phosphorylation of Pax3 and Pax3-FOXO1 as novel molecular targets for the potential development of new therapeutic treatments of ARMS.

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3. RESEARCH STRATEGY

Significance. Rhabdomyosarcoma accounts for nearly half of all soft tissue sarcomas in children under 15 years of age, 30% of which are classified as Alveolar Rhabdomyosarcoma (ARMS). ARMS is associated with an unfavorable prognosis, highlighted by the finding that patients with metastatic ARMS have a four-year survival rate of only 8%, due in part to an increased resistance to chemotherapy[1,2]. One of the primary characteristics of ARMS is a t(2;13) chromosomal translocation that fuses the 5' sequence of the myogenic transcription factor Pax3 to the 3' sequence of FOXO1[1] (Figure 1). The oncogenic Pax3-FOXO1 fusion protein is a more potent activator of transcription than Pax3[3] and its expression persists throughout differentiation in contrast to the normal degradation seen with wild-type Pax3[4]. There are only three sites of phosphorylation on Pax3, all identified by our laboratory. We demonstrated that phosphorylation at these sites varies during myogenesis[5,6]. In proliferating myoblasts, there are two independent species of Pax3 individually phosphorylated at Ser201 and Ser205. Upon the induction of differentiation, phosphorylation at Ser201 persists. However, phosphorylation at Ser205 is rapidly lost with a concomitant increase in phosphorylation on Ser209. In contrast, we found that Pax3-FOXO1 is simultaneously phosphorylated on Ser201 and Ser205, and this status remains unaltered throughout myogenesis[5,6]. These results support the idea that differences in phosphorylation of Pax3 and Pax3-FOXO1 contribute to normal myogenesis and the development of ARMS, respectively. However, the mechanistic role of phosphorylation in regulating Pax3 and Pax3-FOXO1 biological activities remains unknown. Therefore, the focus of this proposal is to determine the mechanism by which phosphorylation regulates Pax3 and Pax3-FOXO1 and examine how phosphorylation contributes to normal myogenesis and the development of ARMS. *This contribution is significant because it will be the first study to determine the role that phosphorylation has in regulating Pax3, determine how altered phosphorylation contributes to the altered activities observed for Pax3-FOXO1, and how both contribute to normal myogenesis and the development of ARMS.* By understanding the molecular mechanism by which phosphorylation at specific sites regulates Pax3 and Pax3-FOXO1, we will identify phosphorylation as a potential target to exploit for the development of pharmaceutical therapies for improved treatment of ARMS.

Approach

Specific Aim 1: To examine the role for phosphorylation of Pax3 and Pax3-FOXO1 in myogenesis and as contributing to the pathology of ARMS.

Rationale: Pax3 is crucial for the proliferation and early commitment of myoblasts to the myogenic lineage. The exogenous expression of both Pax3 and Pax3-FOXO1 leads to increases in cellular proliferation[7,8,9,10]. Additionally, Pax3-FOXO1 activates the transcription of MyoD similarly to Pax3, contributing to the commitment of cells to the myogenic lineage[11,12]. Interestingly, expression of a dominant negative Pax3 or Pax3-FOXO1 prevents terminal differentiation by blocking myotube formation and inhibiting the expression of myosin heavy chain (MyHC), both markers for terminal differentiation[713]. The similarity between Pax3-FOXO1 and an aberrant Pax3 suggest that dysregulation of Pax3 activity is partially responsible for ARMS phenotypes, potentially as a result of our observed differences in phosphorylation. ARMS cells also display increased invasiveness and metastasis compared to other rhabdosarcomas[14,15], due in part to the ability of Pax3-FOXO1 to aberrantly activate c-MET[9,15], an established marker for cellular motility and a known target of Pax3 and Pax3-FOXO1[16]. Taken together, this evidence shows that changes in Pax3 and Pax3-FOXO1 biological activity can affect cellular proliferation, myogenesis, and migration. Despite this knowledge, the contribution that phosphorylation on Pax3 and Pax3-FOXO1 make to affect these biological processes are unknown. ***Therefore, the objective of this aim is to test the working hypothesis that phosphorylation at specific sites contributes to the ability of Pax3 and Pax3-FOXO1 to regulate myogenesis and contributes to the pathological phenotypes of ARMS.*** This hypothesis is based, in part on our published results demonstrating that Pax3-FOXO1 has altered phosphorylation relative to wild-type Pax3[6,17], suggesting that these differences in phosphorylation contribute to known ARMS phenotypes.

Preliminary Data: We created an array of Pax3 mutants in which the three identified sites were mutated to phospho-incompetent alanine, phospho-mimetic aspartic acid, or combinations of the two (Table 1). These mutants were cloned into the pGEX-5X-1 GST bacterial expression vector and a FLAG-epitope tagged construct in a retroviral expression vector. *All Pax3 mutants are stably transduced into mouse primary myoblasts, express the indicated proteins, and are ready for experimental use.* The creation of similar Pax3-FOXO1 mutants is nearly complete. We also created a luciferase reporter construct containing optimized Pax3 recognition sites. Using this construct we determined that we are capable of detecting ectopic Pax3

Table 1: Pax3 point mutant constructs. Serines (S) were mutated to either Alanine (A) or Aspartic Acid (D) at positions 201, 205, and/or 209.

transcriptional activity above endogenous Pax3 background activity (data not shown). ***These results demonstrate that we have in the reagents required to perform the experiments described this aim.***

In addition to published reports, our preliminary data supports our hypothesis that phosphorylation of Pax3 and Pax3-FOXO1 contributes to downstream myogenic and ARMS biological activities. To determine the affect of phosphorylation of Pax3 on cellular growth, we performed proliferation assays on myoblasts that were individually stably transduced with Pax3, Pax3 mutants that mimic a "permanently" phosphorylated state at a single site, or phospho-incompetent at all three sites (Table 2). We plated the cells to 100,000 cells per 60 mm collagen-coated dishes and total cell counts were determined each day for seven days. A majority of the mutants show no significant differences in proliferation rate and maximal growth density relative to wild-type Pax3. However, *consistent with the presence of phosphorylation at Ser205 only in proliferating myoblasts*, we observe a significantly enhanced growth rate of cells expressing Pax3 "permanently" phosphorylated at this site. Further, these same cells are capable of growing to a significantly higher density relative to wild-type Pax3. ***This result demonstrates that phosphorylation of Pax3 at Ser205 enhances cell growth.***

Table 2: Growth characteristics of myoblasts transduced with: Pax3, Pax3 phosphomimetic mutants at each individual site (Ser201 [DAA], Ser205 [ADA], or Ser209 [AAD]), or phopsho-incompentent [AAA]. Growth rate and maximum growth were determined in triplicate.

To determine how phosphorylation of Pax3 affects myogenic differentiation, myoblasts stably expressing Pax3 or the mutants just described were plated at an identical density, grown to approximately 80% confluency, and induced to differentiate as previously described[4]. Total cell extracts were made at 0 and 24 hours of differentiation, and the presence of MyoD was determined by Western blot analysis, quantified using ImageJ[18] software and normalized to GAPDH (Figure 2). We observed no significant differences in MyoD protein levels for mutants permanently phosphorylated at Ser201 or Ser205 during proliferation relative to wild-type Pax3. In contrast, *consistent with the presence of phosphorylation at Ser209 only in early differentiation,* we observed a nearly 2.5-fold increase in MyoD expression in myoblasts stably transduced with the Pax3 mutant "permanently" phosphorylated at Ser209, levels seen with wild-type after 24-hours of differentiation. ***This data demonstrates that phosphorylation of Pax3 at Ser209 contributes to the expression of genes essential for early myogenic differentiation.***

Figure 2. MyoD protein levels in proliferating and differentiated (24 hours) empty myoblasts (light blue) or myoblasts transduced with Pax3 (green), Pax3 phosphomimetic mutants at each individual site (Ser201 [DAA] (dark blue), Ser205 [ADA] (purple), Ser209 [AAD]) (red), or phopsho-incompentent [AAA] (orange). Normalized levels were compared relative to MyoD expression in proliferating, non-transduced myoblasts.

Experimental Design: Our preliminary results, along with published reports support our hypothesis that phosphorylation of Pax3 and Pax3-FOXO1 contribute to myogenesis and the pathology of ARMS. For the experiments proposed in this aim, we will use Pax3 or Pax3-FOXO1 with three distinct sets of phospho-mutant transduced myoblasts (Table 1): 1) individually mutating each site to an alanine, which will test the effects of eliminating that site; 2) individually mutating each site to an aspartic acid, which will test the effects of permanently mimicking a single site of phosphorylation; 3) combination mutants containing one S to D mutant at a specific site while the other two sites contain S to A mutations, which will allow us to test the function of one site of phosphorylation with the exclusion of the other two. We will also use myoblasts transduced with wild-type Pax3 or Pax3-FOXO1 (positive control) and a phospho-incompetent mutant containing alanines at all three sites of phosphorylation (Table 1). We will use primary myoblasts and the ARMS cell line (RH30) stably transduced with the mutants to assess the contributions that phosphorylation makes to proliferation, differentiation, and migration/invasion potential. All experiments will be performed in triplicate and the effects of the individual mutants will be determined relative to the wild-type Pax3 or Pax3-FOXO1 control.

Experiment 1-1: Determine the contribution of phosphorylation of Pax3 and Pax3-FOXO1 on myoblast growth. We demonstrated that Pax3 "permanently" phosphorylated at Ser205 enhances primary myoblast growth characteristics (Table 2). To fully characterize how phosphorylation affects proliferation, we will replicate this experiment, as described in the Preliminary Studies, using Pax3 mutants phospho-incompetent at each individual site (ASS, ASA, AAS, Table 1) or phospho-mimetic at each individual site (DSS, SDS, SSD, Table 1). These assays will be similarly repeated for myoblasts transduced with Pax3-FOXO1 mutants containing the identical mutants. To assess the role of phosphorylation in regulating Pax3-FOXO1 in ARMS cells, this experiment will be carried out in the RH30 ARMS cell line stably transduced with Pax3-FOXO1 phospho-mutants identical to those described for Pax3.

Experiment 1-2: Determine the contribution of phosphorylation on Pax3 and Pax3-FOXO1 on cellular migration/invasion. We will perform a cell invasion assay as previously described[19] utilizing the phospho-mutant transduced myoblasts and ARMS cells described in Experiment 2-1. For each sample, 100,000 cells will be plated onto 6.5 mm transwell plates (pore size 8μm; Fisher) and exposed to human hepatocyte growth factor (HGF) at a concentration of 75 ng/mL for 48 hours[9]. Migrated cells will be fixed and stained with Diff-Quick Stain set (Siemens). We will test for the expression of known markers for cellular migration, including c-MET[16] and NCAM[20], known Pax3 targets, using qRT-PCR and Western Blot analysis using antibodies and primers specific for these genes.

Experiment 1-3: Determine the influence that phosphorylation of Pax3 and Pax3-FOXO1 has on myogenic differentiation. We show that phosphorylation at Ser209 is capable of increasing the expression of the early myogenic marker MyoD (Figure 3). We will further test the expression of the mid- and late-myogenic markers myogenin and MyHC, respectively, and replicate these experiments with the full complement of Pax3 and Pax3-FOXO1 phospho-mutants described in Experiment 2-1. We will also determine the effect of phosphorylation on Pax3 and Pax3-FOXO1 on the terminal differentiation of primary myoblasts by examining the ability of cells stably transduced with the above described mutants to form multinucleated myotubes, an indicator of terminal differentiation. We will plate one million cells per 60 mm dish with one day of recovery before we induce differentiation for up to 72-hours. Every 24 hours, we will use immunofluorescence to visualize the nuclei with DAPI and the cytoskeleton by staining filamentous actin with Alexa-fluor 488-conjugated phalloidin. We will use multiple fields for each sample to obtain a minimum of 1000 nuclei and we will quantify cell fusion by determining the percentage of the total nuclei present in multinucleated myotubes.

Experiment 1-4: Determine the effect that phosphorylation of Pax3 and Pax3-FOXO1 have on the transcriptome profile during early myogenic differentiation. Our preliminary data demonstrates that individual phosphorylation events on Pax3 affect myoblast growth (Table 2) and the expression of early myogenic markers (Figure 2). Further, literature evidence details how alteration of Pax3, either as a dominant negative or as Pax3-FOXO1, inhibits terminal differentiation. Taken together this evidence supports the idea that phosphorylation of Pax3 and Pax3-FOXO1 alters the transcriptome of primary myoblasts to contribute to our observed biological changes. Therefore, we will perform an unbiased survey to determine how phosphorylation of Pax3 and Pax3-FOXO1 alters the transcriptome profile during early myogenesis. We have initiated these studies and are presently performing mRNA deep sequencing on primary myoblasts, and myoblasts stably expressing wild type Pax3 or Pax3-FOXO1. We will expand this analysis to include myoblasts stably expressing corresponding phosho-mutants with which we observed distinct biological affects (DAA [Figure 4], ADA [Table 2], and AAD [Figure 2]). We will use proliferating cells, cells differentiated for one hour (a time at which we observe an increase in phosho-Ser209), and 24 hours (a time at which we observe morphological differences in differentiation.) We will convert isolated mRNA into a library of template molecules using the TruSeq™ RNA Sample Preparation kit (Illumina). The cluster generation of the template library and subsequent 36 cycles of sequencing will be performed in the LSUHSC Genomics Core facility using an Illumina Genome Analyzer 2X. The raw sequencing data will be sent to and processed by Dr. Chris Taylor, computer and bioinformatics specialist at the University of New Orleans (see Letter of Support and Biosketch), to identify the genes present in the samples, normalize the data for gene length, and determine relative expression levels of the genes. We will perform comparative analyses between the proliferative and differentiated states within each group. We will determine the effects of phosphorylation by comparing profiles within the *proliferative state* or the *differentiated state* between cells expressing wild type protein and each

individual mutant. Finally, we will perform an ontological analysis to examine the comparative gene expression between samples as they relate to the functional nature of the genes (e.g. cell cycle regulatory genes, etc.).

Expected Outcomes. Our preliminary results support our hypothesis that phosphorylation at specific sites contributes to primary myoblast growth (Table 2) and differentiation (Figure 2). Therefore, we expect cells transduced with additional mutants that mimic phosphorylation at Ser205 to show enhanced growth characteristics, including increased growth rate and higher maximum growth, while cells phospho-incompetent at this site will resemble cells transduced with wild-type Pax3 and Pax3-FOXO1. We also expect phosphorylation at Ser205 to contribute to cell invasion, evidenced by an increased migration potential since phosphorylation at Ser205 is observed in proliferating myoblasts whose migration is dependent on Pax3 *in vivo*[21]. Finally, consistent with literature evidence and our preliminary results (Figure 2), we expect cells transduced with mutants that mimic phosphorylation at Ser209 will show increased MyoD expression and myoblast elongation. Conversely, we expect cells transduced with Pax3 permanently phosphorylated at Ser209 to have hindered terminal differentiation indicated by reduced expression of MyHC and the reduced ability to form multinucleated myotubes. Mutants that are phospho-incompetent at Ser209 will resemble cells transduced with wild type Pax3 or Pax3-FOXO1. Finally, it is difficult to predict the exact changes in the global transcriptome profiles. However, consistent with our preliminary results (Table 2 and Figure 2) we minimally expect to see changes in cell cycle regulatory genes consistent with increased growth rate in primary myoblasts that stably express Pax3 “permanently” phosphorylated at Ser205. We also minimally expect to see an increase in genes essential for early myogenesis and a decrease in genes essential for terminal myogenesis in myoblasts that stably express Pax3 “permanently” phosphorylated at Ser209.

Potential Problems. We routinely perform the techniques described in this Aim and are therefore not expected to pose significant difficulties. Our published results and preliminary data supports our hypothesis that phosphorylation at distinct sites contribute to myogenesis and the development of ARMS. However, it is possible that phosphorylation at a single site may be insufficient to fully regulate Pax3 biological activities. In the event that we do not observe significant changes as a result of individual phosphorylation events, we will repeat the experiments using our combination mutants described in our Preliminary Results (Table 1).

Specific Aim 2: To determine how phosphorylation regulates the molecular activities of Pax3 and Pax3-FOXO1 in early myogenesis.

Rationale: Pax3 plays a crucial role in regulating the expression of genes essential for skeletal muscle development as seen in its ability to activate the expression of c-Met, Myf5, and MyoD, genes critical for early myogenesis[16,22-24]. The transcriptional properties of Pax3 are controlled by the Paired Domain and Homeodomain DNA-binding regions and the octapeptide domain, which mediates Pax3 interactions with other proteins[25,26], including the co-repressor hDaxx[27] (Figure 1). Pax3-FOXO1 has several altered activities relative to Pax3, including the increased ability to activate Pax3 target sequences and the ability to activate the expression of genes not normally recognized by Pax3[3,28,29]. Further, despite an interaction with hDaxx, Pax3-FOXO1 is resistant to its repressive effects[27]. Finally, Pax3-FOXO1 has a greater stability relative to Pax3 during differentiation[4]. Taken together, these altered activities may contribute to the oncogenicity of Pax3-FOXO1. Despite this knowledge, the mechanism by which phosphorylation regulates Pax3 and how differences in phosphorylation aberrantly regulate Pax3-FOXO1 to promote these altered molecular activities are not known. Therefore, ***the objective of this Aim is to test the working hypothesis that phosphorylation of specific sites on Pax3 and Pax3-FOXO1 regulates their molecular activities, including: DNA-binding, transcription, protein-protein interactions, and protein stability.*** Our working hypothesis was formulated on the following published data. We showed that phosphorylation of Pax3 is dynamic and changes upon the induction of myogenic differentiation and that phosphorylation of Pax3-FOXO1 differs from that of Pax3[5,6]. Further, a recent study showed using a non-myogenic cell line that phosphorylation at uncharacterized sites surrounding the octapeptide domain contributes to the transcriptional activity of Pax3-FOXO1[18].

Preliminary Data: In addition to published reports, our preliminary results further support our working hypothesis. We performed an *in vitro* protein interaction assay to determine how phosphorylation affects the ability of Pax3 to interact with its known co-repressor hDaxx. Proliferating mouse primary myoblast total cell extract was incubated with bacterially expressed and purified GST-Pax3 or GST-Pax3 [S201-05-09D] (3D) in

Figure 3: *In vitro* pull-down of hDaxx using GST-Pax3 and phospho-mimetic GST-Pax3 (3D).

which all three sites were mutated to the phospho-mimetic aspartic acid. After extensive washing, the proteins retained on the resin were separated by SDS-PAGE and the presence of hDaxx was detected using antibodies specific for hDaxx[27]. We observed that hDaxx was efficiently retained by the wild type GST-Pax3 and not GST-Pax3-(3D) (Figure 3). **This result demonstrates that phosphorylation of Pax3 reduces its ability to interact with endogenous hDaxx *in vitro*.**

To determine the qualitative effect of phosphorylation on the stability of Pax3 during differentiation, myoblasts stably transduced with the indicated FLAG-Pax3 phospho-mutants (Figure 4) were induced to differentiate for up to 20 hours. Total cell extracts were prepared at four-hour intervals and the presence of ectopic FLAG-Pax3 was determined by Western blot analysis using a FLAG-specific antibody. Consistent with our previous results[4], Pax3 protein levels decrease significantly over the first 20 hours of differentiation. In contrast, there were no observable differences in the levels of protein during this same time period when FLAG-Pax3 is "permanently" phosphorylated at Ser201 (S201D, Figure 4). This result is not a consequence of mutations in this region since mutations at Ser205 (S205D) or a phospho-incompetent alanine (S201A) have similar protein levels to wild type Pax3 (Figure 4). ***This result demonstrates that phosphorylation at Ser201 promotes the stability of Pax3 during early differentiation.*** Taken together, our preliminary data combined with literature evidence support our hypothesis that phosphorylation regulates the biological activities of Pax3 and Pax3-FOXO1 in early myogenesis.

Figure 4: Stability of FLAG-PAX3 or FLAG phospho-mutants during myogenesis.

Experimental Design:

The phospho-mutants described in Aim 1 (Experimental Design) will be used for the following experiments. All experiments will be performed in triplicate and the effects of the individual mutants will be determined relative to the wild-type Pax3 or Pax3-FOXO1 control.

Experiment 2-1. Determine the effect of phosphorylation on the DNA-binding ability of Pax3 and Pax3-FOXO1. We will use AlphaScreen technology (PerkinElmer) to perform protein:DNA interaction studies[31,32] using bacterially expressed and purified GST-Pax3, GST-Pax3-FOXO1, and GST-Pax3 or Pax3-FOXO1 phospho-mutants as described in Aim 1 and biotinylated oligonucleotide probes containing two consecutive Pax3 recognition sequences[33]. The individual proteins and oligonucleotide probe will be incubated with AlphaScreen anti-GST-acceptor beads and streptavidin donor beads, respectively, in 96-well plates. Interaction between the protein and oligonucleotide probe generates an AlphaScreen signal upon excitation, which will be quantified using the EnVision 2104 Multilabel Reader (PerkinElmer). We will perform a Scatchard analysis as previously published by us[34] to determine the affinity of DNA for each GST-tagged protein. Additionally, to determine how phosphorylation of Pax3, as expressed *in vivo*, affects DNA binding, we will use the AlphaScreen analysis as just described using anti-FLAG acceptor beads with total cell extracts from primary myoblasts stably expressing FLAG-Pax3, FLAG-Pax3-FOXO1, or their corresponding mutants (described above). This procedure will be carried out in proliferating myoblasts and myoblasts that have been induced to differentiate for 1 hour, the time period where Ser209 is maximally phosphorylated.

Experiment 2-2. Determine the effect of phosphorylation on the transcriptional activity of Pax3 and Pax3-FOXO1. We will perform transcriptional assays using the Luciferase reporter vector described in Aim 1 (Preliminary data). The luciferase vector will be transiently transfected into primary mouse myoblasts that were stably transduced with FLAG-Pax3, FLAG-Pax3-FOXO1, or their respective phospho-mutants (described above). The transfected cells will be incubated for 48 hours after which we will determine luciferase activity, as published by us[35]. Each experiment will be performed in triplicate and normalized using a co-transfected constitutively active Renilla luciferase construct. All samples will be corrected for background luminescence using non-transfected cells (negative control). The transcriptional activity of each mutant will be compared to that of the wild-type FLAG-Pax3 or FLAG-Pax3-FOXO1 proteins. To determine the effect phosphorylation has on the ability of Pax3 or Pax3-FOXO1 to act on known promoters, the promoter region of c-Met, Myf5, and

MyoD, genes known to be directly regulated by Pax3[16,23,24], will be cloned upstream of luciferase in the pGL3-Basic construct. Luciferase assays, normalizations, and analyses will be carried out as described above. Finally, to determine the ability of phosphorylation of Pax3 and Pax3-FOXO1 to regulate the expression of its endogenous targets, we will use quantitative real time-PCR (qRT-PCR) to determine the level of expression for genes directly regulated by Pax3 (c-met, Myf5, and MyoD) using primers specific for each gene.

Experiment 2-3. Determine the effect of phosphorylation on the ability of Pax3 and Pax3-FOXO1 to interact with the co-repressor hDaxx. In vitro: We demonstrated that phosphorylation inhibits the interaction between Pax3 and hDaxx *in vitro* (Figure 3). We will repeat this experiment, as described in the Preliminary Studies, with the full complement of GST-tagged Pax3 and Pax3-FOXO1 mutants. *In vivo:* We will use FLAG-specific antibodies to immunoprecipitate FLAG-Pax3, FLAG-Pax3-FOXO1, or the corresponding phospho-mutants (described above) from the stably transduced proliferating primary myoblasts. We will determine the ability of hDaxx to co-immunoprecipitate using Western Blot analysis with antibodies specific for hDaxx. To determine how phosphorylation of Pax3 affects its ability to be regulated by hDaxx, we will perform luciferase assays (as described above) in which myoblasts transduced with Pax3 or its mutants will be co-transfected with the luciferase construct described in preliminary data and increasing amounts of hDaxx expression vector (pcDNA3.1 hDaxx)[27]. We will determine the ability of hDaxx to repress Pax3 activity by comparing results in the presence of hDaxx relative to those observed in non-hDaxx transfected cells and determine the effects of phosphorylation by comparing the results obtained with the mutants to wild type Pax3 or Pax3-FOXO1.

Experiment 2-4. Determine the effect of phosphorylation on the stability of Pax3 and Pax3-FOXO1. We demonstrated that phosphorylation at Ser201 stabilizes Pax3 during myogenic differentiation (Figure 4). We will repeat this experiment with the full complement of our Pax3 and Pax3-FOXO1 mutants as described above. To quantify the effect of phosphorylation on Pax3 stability, we will perform a cycloheximide chase experiment[36]. Myoblasts will be plated at identical densities and allowed to rest for one day before being induced to differentiate for 16 hours, a time at which we observe qualitative differences in Pax3 protein levels (Figure 4). We will inhibit protein translation by incubating the cells with cycloheximide (100μg/mL) after which we will make total cell extract at hourly time points for up to 12 hours. The level of exogenous protein will be determined by Western blot analysis of total cell extracts using antibodies specific for FLAG. The half-life will be determined as previously described by us[4]. As a control, we will use Tubulin, a protein commonly used in cycloheximide chase experiments because of its stability.

Expected Outcomes. Our preliminary data and published reports show that phosphorylation of Pax3 contributes to its stability and transcriptional activity. Based on preliminary data showing that phosphorylation at Ser209 affects gene expression in early differentiation (Figure 2), we expect mutants that mimic phosphorylation at Ser209 will demonstrate a greater ability to bind to DNA target sequences than the wild-type protein or other mutants. Similarly, we expect cells stably transduced with Ser209 phospho-mimetic mutants to have greater luciferase activity with a concomitant increase in c-MET, Myf5, and MyoD expression as determined by qRT-PCR, relative to Pax3. In addition, we showed that phosphorylation reduces Pax3's interaction with hDaxx (Figure 3). Based on this data, it is difficult to determine how each site of phosphorylation contributes to this interaction. However, in the hypothetical situation that interaction with hDaxx is regulated by phosphorylation at Ser209, we would expect mutants that mimic phosphorylation at Ser209 to show a decreased ability to bind hDaxx both *in vitro and in vivo* and an increased resistance to hDaxx-dependent repression of Pax3. Finally, we show that Pax3 phosphorylated at Ser201 appears to be more stable upon differentiation (Figure 4). Therefore, we expect phospho-mimetic mutants at Ser201 will have a longer half-life compared to other mutants in the cycloheximide chase experiments.

Potential Problems and Alternatives. As discussed in Aim 1, it is possible that phosphorylation at a single site may be insufficient to fully regulate Pax3 biological activities. In the event that we observe no significant changes with single mutants, we will repeat the experiments using our combination mutants described in our Preliminary Results (Table 1). Also, it is a possibility that the transfection efficiency is insufficient to produce effective levels of hDaxx. In the event that we do not observe significant levels of hDaxx expression, we will stably co-transduce primary myoblasts with an hDaxx retroviral vector containing the green fluorescent protein with subsequent selection by FACS analysis. Also, in the instance that cyclohexamide is toxic to our transduced myoblasts, we will perform pulse-chase experiments using [35S]-Methionine as published by us[4].

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