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Isolation of putative FOXO1 genomic elements using an improved *in vitro* technique to isolate genomic regulatory sequences

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Abstract

The regulation of gene expression drives many biological processes and alterations in normal regulation are integral in the development of the diseased state. Therefore, the ability to screen genomic DNA for direct targets of DNA binding proteins (DNA-BP) would provide valuable information about the mechanisms underlying these processes. At present chromatin immunoprecipitation (ChIP) and its variants are the primary methods for identifying regulatory elements. However, some DNA-BPs, such as the winged-helix transcription factor FOXO1, are difficult to ChIP thereby detracting from the use of this technique as a nonbiased screen to isolate regulatory sequences. In this report we use an improved *in vitro* method to Pull Out Regulatory Elements (PORE), which uses purified protein with a stable genomic library to isolate regulatory elements directly bound by a DNA-BP, to identify putative FOXO1 genomic regulatory sequences. We first validate this technique using two known DNA-BP (FOXO1 and Pax3) by demonstrating their ability to bind and amplify identified promoter elements when present in a genomic DNA context or when present in the context of our stable genomic library. Subsequent use of this technique with FOXO1 isolated regulatory elements associated with several genes known to be regulated in a FOXO1-dependent manner and multiple genes whose biological functions are consistent with the known biological functions of FOXO1 proving that the *in vitro* PORE is a valuable and easy to use alternative to ChIP for the isolation of genomic regulatory elements.

Keywords

FOXO1; Genomic regulatory elements; DNA binding; Transcriptional regulation; SELEX

1. Introduction

FOXO1 is a member of the FOXO family of winged-helix transcription factors and was first identified in humans through its fusion to the myogenic transcription factor Pax3 in the childhood solid muscle tumor Alveolar Rhabdomyosarcoma (Shapiro et al., 1993; Fredericks et al., 1995). FOXO1 is a ubiquitously expressed protein and as such it has been demonstrated to be a key transcription factor in various cellular processes including the regulation of glucose homeostasis, apoptosis, cell cycle control, muscle differentiation, the oxidative stress response, and angiogenesis (Salih and Brunet, 2008). FOXO1 is involved in

these different cellular processes through its ability to bind to and regulate the expression of genes such as IGFBP-1 (Durham et al., 1999), the proapoptotic gene bim and the cell cycle regulatory protein p27^{Kip1} (Stahl et al., 2002), the anti-oxidant protein MnSOD (Kops et al., 2002), and the angiogenic gene Ang2 (Potente et al., 2005). Although the identification of these FOXO-regulated genes made important contributions to the understanding of the role of FOXO proteins in the various cellular functions, many of these genes were identified as the result of logical conclusions based on previous literature evidence and not from a non-biased genomic screen. Therefore, the ability to perform a non-biased genomic screen would assist in the identification of new FOXO1-regulated genes to better understand the role that FOXO1 plays in different cellular processes.

At present, chromatin immunoprecipitation (ChIP) and its variants (ChIP-PET and ChIP-on-Chip) are the favored methods to identify direct transcriptional targets (Hudson and Snyder, 2006). Although these techniques examine interactions that occur within a cellular context and can provide valuable information, they have several significant limitations. First, some proteins are not amenable to ChIP analysis due to the lack of a suitable antibody or because the epitope is inaccessible when bound to DNA. Alternatively, as is the case with FOXO1, although ChIP analyses were successfully demonstrated (Motta et al., 2004; Matsuzaki et al., 2005; Hall et al., 2007), the process is technically difficult and not readily reproducible (data not shown and personal communication, Lisa Cirillo, Medical College of Wisconsin). Second, ChIP analysis requires extensive cellular manipulations that must be optimized for each individual DNA-BP. Third, because ChIP is performed in a cellular context, it is limited to identifying regulatory elements activated in that particular cell type. Finally, analysis of the DNA fragments in ChIP-on-Chip requires expensive microarray chips, experienced personnel, and is limited by the regions of genomic DNA present on the microarray chip. Therefore, a simple, cost-effective method that can circumvent these limitations would greatly facilitate the isolation of genomic regulatory elements.

One such method is a modification of the Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Bouvet, 2001). Standard SELEX, which is commonly used to identify DNA recognition sequences, uses a randomly generated mixture of short oligonucleotides containing PCR primer recognition sequences engineered on each end. The oligonucleotide library is bound to the protein, the unbound species are removed, bound species are eluted from the protein, and subsequently PCR amplified. The partitioning and amplification procedure is repeated multiple times until a significant enrichment of species that specifically bind to the protein of interest is obtained. Since standard SELEX utilizes the basic concept of equilibrium binding *in vitro*, it is conceivable that the process could be modified for application to genomic DNA.

Several genomic variations of SELEX have been reported (Singer et al., 1997; Kim et al., 2003; Wen and Gray, 2004; Itou et al., 2005; Shimada et al., 2005). Although these studies successfully identified novel regulatory elements, each of these techniques suffers from significant drawbacks. First, genomic libraries are inherently unstable when cloned into standard DNA vectors making the creation of a genomic library difficult (Godiska et al., 2005b). Second, in attempts to increase stability, the libraries oftentimes have small insert sizes (100–300 bp) (Singer et al., 1997; Ferrieres et al., 2004; Wen and Gray, 2004; Itou et al., 2005), which require significantly larger amounts of starting material for the initial screen. Finally, to remove the necessity of a vector library altogether, some methods use a linker ligation step after the initial selection to provide PCR primer sites to the sheared genomic DNA (Shostak et al., 2004) or include a complex PCR amplification to create the initial library and for each subsequent round of selection (Williams et al., 2006). The use of these additional steps introduces unnecessary levels of complexity and uncertainty into the technique, which may bias the overall results.

In this report we describe the use of an improved *in vitro* method to Pull Out Regulatory Elements (*in vitro* PORE) to identify putative FOXO1 genomic regulatory elements. Unlike other existing methods, the *in vitro* PORE uses a stable genomic library with large insert sizes (0.75–2.5 kb) for use in the initial binding and amplification step, thereby removing the need for additional complex procedures. In addition, our method uses a simple PCR method for amplifying the bound genomic DNA allowing a more efficient and reproducible amplification of a genomic DNA population (Williams et al., 2006). We validated this technique using two known transcription factors and their known target regulatory elements. The use of this technique to screen a mouse genomic library with FOXO1 isolated several sequence elements associated with genes regulated in a FOXO1-dependent manner and multiple additional sequence elements associated with genes whose functions are consistent with the known biological functions of FOXO1.

2. Materials and methods

2.1. Creation of a stable mouse genomic library

The mouse genomic DNA library was prepared by Lucigen Corp. (Middleton, WI). Female mouse genomic DNA was sheared by sonication, end-repaired, and separated on a 1% agarose gel. DNA fragments between 0.75 and 2.5 kb were gel purified and cloned into the pSMART-LC-KanTM vector (Lucigen Corp.), which was previously blunt ended and dephosphorylated. The resulting ligated DNA was electroporated into *Escherichia coli* DH10B. The library was expanded by determining the titer of the library, plating the bacteria containing the library on 24.5×24.5 cm plates, and incubating the plates at 37 °C overnight. The resulting colonies were scraped from the plate and the DNA was isolated using a Qiagen Maxiprep kit (Valencia, CA). The resulting DNA was aliquoted and stored at –80 °C.

2.2. Cloning of positive and negative controls

The control regulatory elements that were used for the development of the *in vitro* PORE technique were cloned into the pSMART-LC-Kan vector. The promoter sequences for Tyrosinase related protein-1 (Trp-1, 0.65 kb) and Msx2 homeobox 2 (Msx2, 0.75 kb), two promoter elements bound and activated by Pax3 (Galibert et al., 1999; Kwang et al., 2002), and ceruloplasmin, which is directly bound and regulated by FOXO1 (data not shown), were PCR amplified from 100 ng of mouse genomic DNA using the Trp-1 forward and reverse, the Msx2 forward and reverse, or the Cp forward and reverse primers, respectively (Table 1). Each primer was engineered to contain unique *EcoRV* restriction sites to facilitate subsequent cloning into the vector. The final clones were confirmed by sequencing. Two additional sequences, Clone #14 (1.3 kb), which contains a two-element FOXO1 DNA recognition sequence (Sidhu et al., 2008), and Clone #16 (1.0 kb), which contains no recognizable FOXO1 binding sequences, were used as FOXO1 positive and negative controls, respectively. GST-Pax3 and GST-FOXO1 were bacterially expressed, purified and immobilized on the paramagnetic substrate MagneGSTTM Glutathione affinity resin (Promega, Madison, WI) as previously described (Miller et al., 2008; Sidhu et al., 2008).

2.3. In vitro PORE technique

To determine if we can bind and amplify known genomic regulatory elements in a purified defined system, 100 ng DNA each from Trp-1, or Msx2 was bound to GST-Pax3 and Clone #14, Clone #16, or Cp was bound to GST-FOXO1 in a 100 µl binding volume containing 10 µl of the immobilized protein (approximately 0.5 µg), *in vitro* PORE binding buffer (25 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 10 mM MgCl₂, and 5% glycerol), 10 µg of poly (dI•dC), and 200 µg of bovine serum albumin. An equivalent amount of GST alone (approximately 0.5 µg) prepared in a similar manner was used in parallel binding reactions

as a negative control. Samples were gently agitated at room temperature for 30 min, unbound DNA was removed by washing the resin three times with 1 ml of the *in vitro* PORE binding buffer for 5 min with end-over-end shaking, after which the resulting resin was resuspended in 50 μ l of water. The bound DNA was eluted from the protein by boiling for 5 min, and 10 μ l of the supernatant was used as a template for PCR amplification using 10 μ M each of the PORE forward and reverse primers (Table 1), which are specific for the pSMART-LC-Kan vector. Amplification was performed with ExTaqHS DNA polymerase (TakaRa Biosciences, Shiga, Japan) in a 50 μ l reaction volume for 25 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 1 min followed by a final extension of 10 min at 72 °C. The amplified PCR product from Round 1 was visualized on a 1% agarose gel, purified by gel extraction and eluted in 50 μ l of water. A 10 μ l of the eluted fraction was used for subsequent rounds of selection and amplification.

To confirm that the *in vitro* PORE technique can isolate known FOXO1 genomic regulatory elements within the context of a genomic library, the Cp promoter sequence present in the pSMART-LC-KanTM vector was diluted 1:10 and 1:1000 with our stable mouse genomic library. A 10 μ g of the library/Cp mixture was then used as the starting material with GST-FOXO1 for the *in vitro* PORE technique as described above with the following modification: In order to efficiently amplify the genomic DNA mixture, the PCR reaction was created in a 260 μ l volume containing 10 μ M each of the PORE forward and reverse primers, 2.5 mM dNTPs, and 10 μ l of the eluted bound DNA. The mixture was aliquoted into five individual PCR tubes and amplification was performed as described above with a final extension at 72 °C for 2 min. The amplified product was visualized on a 1% agarose gel, DNA between 0.65 and 3.0 kb was purified by gel extraction, and 10 μ l of the eluted DNA was used for the next round of binding and amplification. In order to confirm the presence of the Cp promoter region in the bound and amplified DNA, 2 μ l of the gel purified DNA was PCR amplified using primers specific for the Cp promoter region.

Finally, we used GST-FOXO1 to screen 10 μ g of the stable mouse genomic library using four rounds of binding, selection, and amplification as described above for the Cp dilution experiment. The number of cycles for amplification in each round was empirically determined by visualizing one PCR reaction on a 1% agarose gel. If DNA was visible between 0.65 and 3.0 kb the remaining reactions were separated and the DNA in this range was gel purified. If no DNA were visible five additional rounds of amplification were performed and the process was repeated as described. The PCR-amplified DNA from Rounds 3 and 4 were cloned, sequenced, analyzed using the NCBI database for the mouse genome, and the identified surrounding genomic regions were examined for known genes.

2.4. Transcriptional assays

HepG2 cells (2×10^6) were plated in 60 mm dishes and transfected the following day by the FuGENE6 method (Roche, Indianapolis, IN), according to the manufacturer's specifications. The FuGENE6/DNA precipitate consisted of a combination of 1 μ g of either pcDNA3.1 vector alone or pcDNA3.1-FOXO1, 500 ng of the renilla luciferase (RL) control reporter gene under control of the thymidine kinase promoter (pRL-TK, Promega), and 1 μ g of each of the individual pGL3-Basic firefly luciferase reporter constructs containing the isolated sequence elements associated with Tnfrsf5, RACK1, Col4a2, MKL1, or Phlpp1. Forty-eight hours post transfection the cells were harvested and analysis of the luciferase activity was performed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's specifications with light output recorded on a Veritas Microplate luminometer (Tuner Biosystems, Sunnyvale, CA). All samples were corrected for the transfection efficiency using the RL activity, reported as fold activation (Tnfrsf5 and RACK1) or percent maximal activity (Col4a2, MKL1, and Phlpp1) relative to the luciferase activity of the empty pcDNA3.1 construct, and performed in triplicate.

3. Results and discussion

3.1. Creation of the stable mouse genomic library

The creation of genomic libraries is difficult due to the inherent instability in the cloning of genomic DNA fragments into standard vectors (Godiska et al., 2005a). To circumvent this problem we used the transcription-free pSMART-LC-KanTM vector system from Lucigen Corp. to create a mouse genomic library. This vector contains strong transcriptional terminators flanking each vector element, which increases the stability of the resulting clones by minimizing unintended transcription out of the DNA inserts by authentic pseudo transcriptional promoters in *E. coli* (Godiska et al., 2005b).

To confirm the stability and completeness of our mouse genomic library, we isolated DNA from 100 individual colonies and examined the inserts by sequence analysis to determine the size, composition, and identity of each clone. The sequences were analyzed using the NCBI database for the mouse genome and the presence of repeat elements was determined using the Institute for Systems Biology RepeatMasker search engine (<http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker>). Of the 100 clones examined, 95 contained inserts. Sequence analysis determined that each of the individual clones derived from an independent genomic sequence and contained insert sizes ranging from approximately 650 to 2500 bp. We found that 7/95 of the clones had a GC content greater than 50% and 67/95 of the clones had a GC/AT content between 50% and 60%. In addition, we found that 19/95 of the clones contained an AT content greater than 60% and that multiple clones contained at least one repetitive element (SINE, LINE, LTR, and/or Small Sequence Repeats), both of which are sequence elements commonly considered difficult to clone (Godiska et al., 2009). Finally, the 19 autosomal chromosomes and the X chromosome were each represented by at least two independent clones that derived from distinctly different regions of each chromosome. Therefore, these results demonstrate that we created a mouse genomic library having a full representation of the mouse genome with 4.6×10^6 independent clones having 95% insert stability, which is at least a 2.5 fold over-representation of the entire mouse genome.

3.2. Validation of the *in vitro* PORE

Before screening our genomic library with FOXO1, we first needed to validate the *in vitro* PORE by determining whether a DNA-BP could bind to its cognate recognition sequence when present in the genomic context of known regulatory sequences. To validate the technique we used two transcription factors, FOXO1 a transcription factor involved in a variety of cellular mechanisms (Bober et al., 1994; Strachan and Read, 1994; Durham et al., 1999; Nakae et al., 2001; Kawano et al., 2002; Machida et al., 2003; de Candia et al., 2008; Walter et al., 2008), and Pax3 a transcription factor important for myogenic differentiation (Bober et al., 1994). We cloned the promoter regions of Trp-1 and Msx2, demonstrated to be bound by Pax3 (Galibert et al., 1999; Kwang et al., 2002), and the promoter region of ceruloplasmin (Cp), which is bound by FOXO1 (data not shown), into pSMART-LC-Kan. In addition, we utilized a genomic DNA fragment fortuitously selected from our mouse genomic library (Clone #14), which contains the two-element FOXO1 DNA binding sequence (Sidhu et al., 2008). A BLAST search of Clone #14 demonstrated that it is associated with the Gab-1 gene, a gene whose expression increases over four-fold in the presence of FOXO1 (Ramaswamy et al., 2002). Finally, we used another genomic fragment (Clone #16), which did not contain any identifiable FOXO1 DNA binding elements, as a negative control.

Bacterially expressed and purified GST-Pax3 or GST-FOXO1 immobilized on resin was used for the *in vitro* PORE using purified Trp-1 and Msx2 (Pax3) or Cp, Clone #14, and

Clone #16 (FOXO1). GST alone was used in parallel reactions as a negative control. The amplified products from Rounds 1 and 2 were visualized by agarose gel electrophoresis. We observed Pax3 specific binding and subsequent amplification for the Trp-1 and Msx2 promoters and FOXO1 specific binding and subsequent amplification for the Cp promoter and Clone #14 in Round 1, all of which were further amplified in Round 2 (Fig. 1A and B). Although we observed a small amount of non-specific binding and amplification for Clone #16 and the GST negative controls in Round 1, these non-specific interactions disappeared with Round 2, a result in stark contrast to the Pax3 and FOXO1 specific amplification of Trp-1, Msx2, Cp, and Clone #14. These results demonstrate that two independent DNA binding proteins can specifically bind and amplify their known DNA regulatory sequences *in vitro*.

We next wanted to determine whether we could selectively isolate a known genomic regulatory element when present in the context of the genomic library. We were required to modify the PCR conditions since the amplification of complex genomic libraries using standard PCR has two main drawbacks: The presence of homologous regions of genomic DNA predisposes the amplification to the generation of artifactual fragments; and shorter genomic fragments are preferentially amplified over larger ones (Meyerhans et al., 1990). Various methods have been described to overcome these problems including the use of high template concentrations, low number of PCR cycles, or complex emulsion PCR (Polz and Cavanaugh, 1998; Qiu et al., 2001; Kojima et al., 2005; Williams et al., 2006). However, these methods cannot be employed with *in vitro* PORE since the DNA that is released from the DNA-BP in each round would not provide high enough template concentrations and a low number of PCR cycles would preclude the visualization of the amplified product by agarose gel electrophoresis. In addition, emulsion PCR, although an effective procedure is a long complicated process involving DNA emulsification and extraction using organic solvents. The inclusion of these complex steps would increase the probability that putative transcriptional targets may be lost. Therefore, we modified the standard PCR conditions by using 10 μ l of the DNA eluted from the DNA-BP in a 260 μ l master reaction mixture with concentrations of reagents as described in Materials and methods. The master mixture was aliquoted into five individual tubes (50 μ l in each tube) and the amplification was performed using the conditions described above. This process provided a consistent, efficient, and highly reproducible method to amplify the complex mixture of longer genomic fragments (Figs. 2 and 3).

We utilized the Cp promoter region alone as a positive control or the Cp promoter diluted 1:10 and 1:1000 with our stable mouse genomic library along with GST-FOXO1 immobilized on the resin for the *in vitro* PORE. Binding reactions with GST only were used in parallel as a negative control and the resulting PCR amplifications from Rounds 1 and 2 were visualized on a 1% agarose gel. We observed a FOXO1 specific binding of the Cp positive control in Round 1 with an increase in this binding and amplification in Round 2, both of which are absent with the GST only negative control (Fig. 2A). In the presence of either a 1:10 or a 1:1000 dilution of Cp with the mouse genomic library, we saw a FOXO1 specific binding and amplification of DNA of approximately 0.75–2.5 kb in both Rounds 1 and 2. Although there was a small amount of non-specific binding and amplification of genomic DNA with the GST only negative control in Round 1, this non-specificity was greatly reduced by Round 2 of the procedure (Fig. 2A). The amplification of genomic DNA fragments with sizes consistent with the known insert size of the library confirms that our modified PCR procedure efficiently and accurately amplifies a complex genomic library containing large inserts. In addition, the fact that we observed a much greater binding and amplification of genomic DNA in the presence of FOXO1 relative to GST supports the conclusion that the amplification of the genomic DNA fragments is specific for FOXO1.

To confirm that the Cp promoter is present in the bound and amplified genomic population, aliquots of the gel extracted DNA from the GST-FOXO1 and the GST only samples from Round 2 were used to amplify the Cp promoter using primers specific for this region (Table 1). We observed an amplification of the Cp promoter in the presence of GST-FOXO1 with both the 1:10 and the 1:1000 dilution samples, an amplification that was greatly reduced or non-existent with the GST only negative control (Fig. 2B). Therefore, FOXO1 is able to specifically isolate one of its known genomic regulatory elements when present in the context of a stable genomic library. This result validates our technique and demonstrates that a known DNA binding protein can capture and enrich its cognate regulatory elements from a genomic library *in vitro*.

3.3. In vitro PORE screen

Having validated the technique, we used the *in vitro* PORE to screen a mouse genomic library for regulatory elements bound by FOXO1. We utilized 10 µg of the mouse genomic library for the initial starting material with four rounds of binding and amplification to GST-FOXO1. The binding, elution, and amplification conditions were as described above for the Cp dilution control. Binding reactions with GST only were used in parallel as a negative control and the PCR amplified genomic DNA from Rounds 3 and 4 were gel extracted, cloned, and sequenced. We observed a gradual increase in the specifically bound and amplified DNA in the presence of FOXO1 with minimal non-specific binding in the presence of GST (Fig. 3). We obtained similar results from three independent repetitions of the *in vitro* PORE technique using FOXO1 (data not shown), strongly supporting the idea that the *in vitro* PORE is capable of specifically and efficiently enriching a population for genomic regulatory elements in a FOXO1-dependent manner.

3.4. Putative FOXO1 genomic regulatory elements

We analyzed the sequences from a total of 262 individual clones, which were isolated from Rounds 3 and 4 from three independent repetitions of the *in vitro* PORE, using the NCBI database for the mouse genome. After determining the genomic location of each of the individual clones, we examined the surrounding genomic regions for potential genes or open reading frames. Thirty-one percent (82 individual clones) lie in proximity to genes that are regulated in a FOXO1-dependent manner (Table 2) and 38% (98 individual clones) are in proximity to genes whose functions are consistent with the known biological activities of FOXO1 (Table 3). In addition, 29% of the isolated elements (76 individual clones) are associated with hypothetical proteins, proteins with no known biological function, or contained sequences for repetitive elements (see below). Finally, 2% of the isolated sequences (6 individual clones) are associated with proteins whose function has no determinable relation to the known biological functions of FOXO1 (including arylsulfatase B, origin recognition complex subunit 4, HIV type 1 enhancer binding protein 2, extraembryonic spermatogenesis homeobox 1, and tyrosinase related protein 1, data not shown).

Genes regulated in a FOXO1-dependent manner (Table 2)—Fifty-three independent clones (20%) isolated from three independent screens contained a regulatory element associated with the tumor necrosis factor receptor superfamily 5 (Tnfrsf5 or CD40). Tnfrsf5 is a pro-apoptotic cell surface receptor. FOXO1, through its function as a pro-apoptotic transcription factor, upregulates a variety of tumor necrosis factor (TNF) induced gene families, including Tnfrsf5, thereby acting as a master regulator of the apoptotic response (Ramaswamy et al., 2002; Alikhani et al., 2005). Twenty-seven additional independent clones (10%) isolated from two independent screens contained a regulatory element associate with Cyclin D1. Like Cyclin D1, FOXO1 is important for cell cycle regulation. As a key player in cell cycle regulation, the downregulation of Cyclin D1 by

FOXO1 leads to the G1 arrest of the cell cycle (Ramaswamy et al., 2002; Schmidt et al., 2002). Finally, a single isolate contained genomic sequences associated with the BMP and activin membrane bound inhibitor (BAMBI), a gene important in TGF- β signaling and development (Onichtchouk et al., 1999), while a second single isolate contained sequences for a genomic region associated with the ZW10 interacting protein 1 (Zwint-1), a gene important for cell cycle regulation (Kops et al., 2005). The expression of BAMBI and Zwint-1 are increase in a FOXO1-dependent manner (Takano et al., 2007). Therefore, the isolation of genomic sequences associated with genes whose expression is regulated in a FOXO1-dependent manner provides strong evidence for the validity of our technique.

Genes whose function is consistent with FOXO1 biological activities(Table 3)

—FOXO1 has a two-fold role in myogenic differentiation. In early myogenesis, FOXO1 inhibits the entry of myoblasts into the myogenic program (Kitamura et al., 2007); however, in late myogenesis, it controls the rate of myotube fusion (Bois and Grosveld, 2003). Consistent with these facts, we isolated regulatory elements from multiple independent screens that are associated with four genes important for early and late myogenesis (Table 3). Twenty-nine independent clones (11%) isolated from three independent screens contained a regulatory element associated with Insulin like growth factor binding protein 9 (IGFBP9, also known as NOV). IGFBP9 is a secreted matrix protein capable of inhibiting the entry of myoblasts into the myogenic program through the activation of the Notch1 signaling pathway (Sakamoto et al., 2002). Conversely, five independent clones (2%) isolated from three independent screens contained genomic sequences associated with megakaryoblastic leukemia 1 (MKL1), which is required for the expression of alpha-actin and alpha-myosin heavy chain to promote myoblast fusion and terminal differentiation (Selvaraj and Prywes, 2003). Finally, our screen isolated genomic regulatory elements associated with two genes important in cardiac myocyte functioning. Three independent isolates (1%) contained genomic sequences associated with the A-kinase anchoring protein 6 (AKAP6) and two independent isolates contained an element associated with the potassium inwardly rectifying channel (Kir3.1). Although not demonstrated for Kir3.1, FOXO1 is capable of regulating the expression of Kir6.1 in cardiac myocytes (Philip-Couderc et al., 2008). Taken together, the isolation of regulatory elements associated with muscle-specific genes provides an expanding picture by which FOXO1 regulates myogenesis.

In addition to myogenesis, FOXO1 promotes apoptosis, in part through its activation of various proapoptotic genes. The ability of FOXO1 to activate these genes and promote apoptosis is inhibited by Akt, which phosphorylates FOXO1 thereby promoting its degradation. In our screen, we isolated regulatory elements associated with two genes known to promote apoptosis through their ability to inhibit Akt (Matsuzaki et al., 2003). Twenty independent isolates (8%) contained genomic sequences that are present in the genomic region important for the regulation of the expression of procollagen type 4 alpha 2 (Col4a2) (Kuhn, 1995). The c-terminal, non-collagenous domain of Col4a2, which is also known as canstatin, promotes apoptosis, in part, by inhibiting the action of Akt (Panka and Mier, 2003). Similarly, the PH domain and leucine rich repeat protein phosphatase isoform 1 (Phlpp), which was isolated two independent times, is a phosphatase that specifically dephosphorylates and inactivates Akt resulting in the promotion of apoptosis (Gao et al., 2005). It is conceivable that in addition to promoting the expression of proapoptotic genes that FOXO1 may also regulate apoptosis through a positive feedback regulation in which the expression of genes such as Col4a2 and Phlpp inhibit the activity of Akt and the subsequent stability of FOXO1.

In further keeping with the pro-apoptotic actions of FOXO1, FOXO1 has been described as the “master switch” in TNF- α -induced apoptosis (Alikhani et al., 2005). Consistent with this fact several isolated clones contained genomic sequences associated with three genes

important in TNF- α -dependent apoptosis. The Traf3-interacting Jun N-terminal kinase activating modulator (T3JAM) is an adaptor molecule that associates with the tumor necrosis factor receptor-associated factor 3 (TRAF3) in response to TNF- α stimulation. As a result of this interaction, T3JAM alters the cellular localization of TRAF3 to promote the specific activation of the Jun N-terminal kinase (JNK) signaling pathway (Dadgostar et al., 2003) to promote apoptosis. In addition, a single isolate is associated with the early growth response protein (EGR-3), which increases the expression of TNF- α through a direct action on the TNF- α promoter (Decker et al., 2003). Finally, two independent single isolates contained sequences associated with membrane associated guanylate kinase (MAGI-1) and yes associated protein 1 (Yap1), both genes that are involved in promoting apoptosis (Wu et al., 2000; Levy et al., 2007). Therefore, the isolation of regulatory elements associated with genes that promote apoptosis through a variety of signaling cascades is consistent with the pro-apoptotic functions of FOXO1 and may represent yet another mechanism by which FOXO1 promotes cell death.

Multiple additional isolates contained genomic sequences that are associated with a variety of genes whose functions are consistent with other FOXO1 biological functions. These genes included the split hand/foot deleted gene (Dss1) and nuclear transcription factor Y beta (Nfya), which are implicated in cell cycle regulation and enhanced cellular proliferation (Bhattacharya et al., 2003; Wei et al., 2003); erythrocyte protein band 4.1-like 3 (epb4.1l3), which is necessary for erythroid differentiation (Bakker et al., 2004); neuromedin S (NMS), which regulates the circadian clock in response to oxidative stress (Mori et al., 2005; Zheng et al., 2007); MAPKKK7 interacting protein 1 (Map3K7IP1 or TAK1), which is important for regulating FOXO1 activity (Kim et al., 2010); and cytotoxic T-lymphocyte associated protein 2 alpha (Ctla2a), which is required for T-cell homeostasis (Peng, 2008).

Finally, our *in vitro* PORE screen isolated 18 individual clones that contained a regulatory element associated with the gene receptor activated C-kinase 1 (RACK1). RACK1 is a ubiquitously expressed scaffold protein implicated in multiple different signaling pathways to mediate a variety of critical biological processes (McCahill et al., 2002), many of which are consistent with known FOXO1 biological activities, including cell cycle inhibition, induction of apoptosis, and increasing the expression of acute phase reactant proteins. RACK1 inhibits cell cycle progression by suppressing Src kinase activity (Mamidipudi et al., 2004); it assists protein kinase C in the induction of acute phase reactant proteins (Ivashchenko et al., 2005); and it acts as an adaptor to protein kinase C to enhance TNF- α dependent apoptosis (Tcherkasova et al., 2002).

Additional isolated sequences—Fourteen of the isolated sequences are associated with genes whose function has not yet been determined including hypothetical proteins located on chromosomes 4, 8, 16, 17 and X, a zinc finger containing protein (ZNF126), and the winged helix transcription factor FOXI2 (data not shown). In addition to these elements, 58 individual isolates (22%) contained between two and five copies of a 235 bp repetitive element. Each repetitive element contains two putative copies of the mid-affinity, two-element FOXO1 recognition sequence and at least seven sequence elements with loose homology to low affinity, single-element FOXO1 recognition sequence (Sidhu et al., 2008). At present the functional significance for the high selection frequency of a repeat element from our screen is not known. However, it was previously demonstrated that a large number of transcription factor binding sites are embedded in distinct repetitive elements, which contributes to regulatory expansion and differences between species (Bourque et al., 2008).

We visually examined a representative sequence from each of the isolates associated with the genes described above to determine whether they contained putative FOXO1 binding sites. This analysis revealed that every isolate contained at least one putative FOXO1

binding site with several of the isolates containing multiple putative binding sites. We found that the sequences containing multiple putative FOXO1 binding sites had various combinations of the low-affinity, single-element site, the mid-affinity, double-element site, and the high-affinity, triple-element site, as previously described by us (Sidhu et al., 2008). These combinations ranged from sequences with multiple copies of the low-affinity site, to sequences that contained multiple copies of the low-affinity and mid-affinity sites, to sequences containing a combination of the low-, mid-, and high affinity FOXO1 binding sites. We also observed a rough correlation between the number and composition of putative FOXO1 binding sites and the frequency with which a particular sequence was isolated. For example, the sequence associated with Yap1, which contained a single, low-affinity putative FOXO1 binding site, was isolated only once from all three independent screens. In contrast, the sequence associated with IGFBP9, which was isolated 29 independent times from 3 independent screens, contained five putative low-affinity sites and two putative mid-affinity sites. Taken together, these results demonstrate that our *in vitro* PORE technique is useful for recognizing and isolating sequence elements with a wide range of putative binding affinities.

3.5. FOXO1-dependent transcriptional regulation from select regulatory elements

Finally, we wanted to determine if select isolated genomic sequences were capable of conferring the ability to regulate transcription in a FOXO1-dependent manner. We cloned the isolated sequences associated with the genes *Tnfrsf5*, *RACK1*, *Col4a2*, *MKL1*, and *Phlpp1* into the luciferase reporter construct pGL3-Basic. This selection provided a good representation of regulatory elements associated with a gene regulated in a FOXO1-dependent manner (*Tnfrsf5*) and regulatory elements associated with genes whose functions are consistent with the biological activities of FOXO1 (*RACK1*, *Col4a2*, *MKL1*, and *Phlpp1*). In addition, they included regulatory elements that were isolated with a high frequency (*Tnfrsf5*, *RACK1* and *Col4a2*) and two regulatory elements isolated with a low frequency (*MKL1* and *Phlpp1*). HepG2 cells were transiently transfected with the individual reporter constructs with the pcDNA3.1 empty vector negative control or the pcDNA3.1-FOXO1 expression vector and the ability of FOXO1 to regulate transcription from the indicated sequences was determined using the luciferase assay as described in Materials and methods. Consistent with the FOXO1-dependent regulation of *Tnfrsf5* (Alikhani et al., 2005), we observed a nearly 7-fold increase in transcriptional activity from the genomic sequence associated with that gene (Fig. 4A). We also observed a nearly 2-fold increase in transcriptional activity from the isolated sequence associated with *RACK1* (Fig. 4A). FOXO1 is also capable of repressing the expression of genes (Ramaswamy et al., 2002). Consistent with this fact we observed between 60% and 80% repression of transcriptional activity from the isolated sequences associated with *Col4a2*, *MKL1*, and *Phlpp1*, respectively (Fig. 4B). Taken together, these results demonstrate that five of the isolated sequences, isolated with high and low frequency are capable of conferring the ability to regulate transcription in a FOXO1-dependent fashion.

4. Conclusions

In the present report, we used an improved *in vitro* technique for the isolation of putative FOXO1-dependent genomic regulatory elements. We validated the *in vitro* PORE by demonstrating that two known transcription factors, FOXO1 and Pax3, could bind and amplify several of their well-characterized regulatory elements. We further validated the technique by providing evidence that FOXO1 was able to isolate one of its known promoter elements when present in context of our stable genomic library. An *in vitro* PORE screen using FOXO1 isolated multiple genomic regulatory elements that are associated with genes whose expression is known to be regulated by FOXO1 (*Tnfrsf5*, *Cyclin D1*, *BAMBI*, and

Zwint-1) or genes whose functions are consistent with the known biological functions of FOXO1 (Table 3). An analysis of the presence of putative FOXO1 binding sites determined that low-, mid-, and/or high-affinity sites were present on all of the isolated sequences and that a rough correlation exists between the number and affinity of the sequences and the frequency with which the sequences were isolated. Finally, we demonstrated that five of the isolated sequences were capable of conferring the ability to alter transcription in a FOXO1-dependent manner. Our results with FOXO1 demonstrate that this technique will provide a simple and rapid method to screen, isolate, and analyze genomic regulatory elements specifically bound by a known DNA-BP that are not amenable to ChIP analysis.

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Abbreviations

Akap6	A kinase (PRKA) anchor protein 6
BAMBI	BMP and activin membrane bound inhibitor
ChIP	chromatin immunoprecipitation
Col4a2	procollagen type 4 alpha 2
Cp	Ceruloplasmin
Ctla2a	cytotoxic T-lymphocyte associated protein 2 alpha
DNA-BP	DNA binding protein
Dss1	split hand/foot deleted gene
EGR-3	early growth response protein 3
epb4.1i3	erythrocyte protein band 4.1-like 3
GST	glutathione-S-transferase
IGFBP9	insulin like growth factor binding protein 9
Kir3.1	potassium inwardly rectifying channel J3
MAGI-1	membrane associated guanylate kinase
Map3K7IP1	MAPKKK interacting protein 1
MKL1	megakaryoblastic leukemia 1
Nfyb	nuclear transcription factor Y
NMS	neuromedin S
Phlpp1	PH domain and leucine rich repeat protein phosphatase isoform 1
PORE	pull out regulatory elements
RACK1	receptor for activated kinase 1
SELEX	systematic evolution of ligands by exponential enrichment
T3JAM	TRAF3-interacting Jun N-terminal kinase activating modulator
TNF	tumor necrosis factor

TNFRSF5	tumor necrosis factor receptor super family 5
Yap1	yes associated protein 1
Zwint-1	ZW10 interacting protein 1

References

- Alikhani M, Alikhani Z, Graves DT. FOXO1 functions as a master switch that regulates gene expression necessary for tumor necrosis factor-induced fibroblast apoptosis. *J Biol Chem.* 2005; 280:12096–12102. [PubMed: 15632117]
- Bakker WJ, et al. FoxO3a regulates erythroid differentiation and induces BTG1, an activator of protein arginine methyl transferase 1. *J Cell Biol.* 2004; 164:175–184. [PubMed: 14734530]
- Bhattacharya A, Deng JM, Zhang Z, Behringer R, de Crombrughe B, Maity SN. The B subunit of the CCAAT box binding transcription factor complex (CBF/NF-Y) is essential for early mouse development and cell proliferation. *Cancer Res.* 2003; 63:8167–8172. [PubMed: 14678971]
- Bober E, Franz T, Arnold HH, Gruss P, Tremblay P. Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development.* 1994; 120:603–612. [PubMed: 8162858]
- Bois PR, Grosveld GC. FKHR (FOXO1a) is required for myotube fusion of primary mouse myoblasts. *Embo J.* 2003; 22:1147–1157. [PubMed: 12606579]
- Bourque G, et al. Evolution of the mammalian transcription factor binding repertoire via transposable elements. *Genome Res.* 2008; 18:1752–1762. [PubMed: 18682548]
- Bouvet P. Determination of nucleic acid recognition sequences by SELEX. *Methods Mol Biol.* 2001; 148:603–610. [PubMed: 11357617]
- Dadgostar H, Doyle SE, Shahangian A, Garcia DE, Cheng G. T3JAM, a novel protein that specifically interacts with TRAF3 and promotes the activation of JNK (1). *FEBS Lett.* 2003; 553:403–407. [PubMed: 14572659]
- de Candia P, Blekhman R, Chabot AE, Oshlack A, Gilad Y. A combination of genomic approaches reveals the role of FOXO1a in regulating an oxidative stress response pathway. *PLoS ONE.* 2008; 3:e1670. [PubMed: 18301748]
- Decker EL, Nehmann N, Kampen E, Eibel H, Zipfel PF, Skerka C. Early growth response proteins (EGR) and nuclear factors of activated T cells (NFAT) form heterodimers and regulate proinflammatory cytokine gene expression. *Nucleic Acids Res.* 2003; 31:911–921. [PubMed: 12560487]
- Durham SK, et al. FKHR binds the insulin response element in the insulin-like growth factor binding protein-1 promoter. *Endocrinology.* 1999; 140:3140–3146. [PubMed: 10385407]
- Ferrieres L, Francez-Charlot A, Gouzy J, Rouille S, Kahn D. FixJ-regulated genes evolved through promoter duplication in *Sinorhizobium meliloti*. *Microbiology.* 2004; 150:2335–2345. [PubMed: 15256575]
- Fredericks WJ, et al. The PAX3-FKHR fusion protein created by the t(2;13) translocation in alveolar rhabdomyosarcomas is a more potent transcriptional activator than PAX3. *Mol Cell Biol.* 1995; 15:1522–1535. [PubMed: 7862145]
- Galibert MD, Yavuzer U, Dexter TJ, Goding CR. Pax3 and regulation of the melanocyte-specific tyrosinase-related protein-1 promoter. *J Biol Chem.* 1999; 274:26894–26900. [PubMed: 10480898]
- Gao T, Furnari F, Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol Cell.* 2005; 18:13–24. [PubMed: 15808505]
- Godiska R, Mead D, Dhodda V, Wu C, Hochstein R, Karsi A, Usdin K, Entezam A, Ravin N. Linear plasmid vector for cloning of repetitive or unstable sequences in *Escherichia coli*. *Nucleic Acids Res.* 2009
- Godiska, R.; Patterson, M.; Schoenfeld, T.; Mead, DA. Beyond pUC: Vectors for Cloning Unstable DNA. Jones and Bartlett Publishers; 2005a. p. 1-3.

- Godiska, R.; Patterson, M.; Schoenfeld, T.; Mead, DA. Beyond pUC: Vectors for Cloning Unstable DNA. Jones and Bartlett Publishers; MA: 2005b.
- Hall RK, Wang XL, George L, Koch SR, Granner DK. Insulin represses phosphoenolpyruvate carboxykinase gene transcription by causing the rapid disruption of an active transcription complex: a potential epigenetic effect. *Mol Endocrinol.* 2007; 21:550–563. [PubMed: 17095578]
- Hudson ME, Snyder M. High-throughput methods of regulatory element discovery. *Biotechniques.* 2006; 41:673, 675, 677. passim. [PubMed: 17191608]
- Itou H, et al. The CGL2612 protein from *Corynebacterium glutamicum* is a drug resistance-related transcriptional repressor: structural and functional analysis of a newly identified transcription factor from genomic DNA analysis. *J Biol Chem.* 2005; 280:38711–38719. [PubMed: 16166084]
- Ivashchenko Y, et al. Protein kinase C pathway is involved in transcriptional regulation of C-reactive protein synthesis in human hepatocytes. *Arterioscler Thromb Vasc Biol.* 2005; 25:186–192. [PubMed: 15539624]
- Kawano T, et al. Decreased akt activity is associated with activation of forkhead transcription factor after transient forebrain ischemia in gerbil hippocampus. *J Cereb Blood Flow Metab.* 2002; 22:926–934. [PubMed: 12172378]
- Kim S, Kim Y, Lee J, Chung J. Regulation of FOXO1 by TAK1-NLK pathway. *J Biol Chem.* 2010; 285:8122–8129. [PubMed: 20061393]
- Kim S, Shi H, Lee DK, Lis JT. Specific SR protein-dependent splicing substrates identified through genomic SELEX. *Nucleic Acids Res.* 2003; 31:1955–1961. [PubMed: 12655012]
- Kitamura T, et al. A Foxo/Notch pathway controls myogenic differentiation and fiber type specification. *J Clin Invest.* 2007; 117:2477–2485. [PubMed: 17717603]
- Kojima T, Takei Y, Ohtsuka M, Kawarasaki Y, Yamane T, Nakano H. PCR amplification from single DNA molecules on magnetic beads in emulsion: application for high-throughput screening of transcription factor targets. *Nucleic Acids Res.* 2005; 33:e150. [PubMed: 16214800]
- Kops GJ, et al. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature.* 2002; 419:316–321. [PubMed: 12239572]
- Kops GJ, et al. ZW10 links mitotic checkpoint signaling to the structural kinetochore. *J Cell Biol.* 2005; 169:49–60. [PubMed: 15824131]
- Kuhn K. Basement membrane (type IV) collagen. *Matrix Biol.* 1995; 14:439–445. [PubMed: 7795882]
- Kwang SJ, et al. Msx2 is an immediate downstream effector of Pax3 in the development of the murine cardiac neural crest. *Development.* 2002; 129:527–538. [PubMed: 11807043]
- Levy D, Adamovich Y, Reuven N, Shaul Y. The Yes-associated protein 1 stabilizes p73 by preventing Itch-mediated ubiquitination of p73. *Cell Death Differ.* 2007; 14:743–751. [PubMed: 17110958]
- Machida S, Spangenburg EE, Booth FW. Forkhead transcription factor FoxO1 transduces insulin-like growth factor's signal to p27Kip1 in primary skeletal muscle satellite cells. *J Cell Physiol.* 2003; 196:523–531. [PubMed: 12891709]
- Mamidipudi V, Zhang J, Lee KC, Cartwright CA. RACK1 regulates G1/S progression by suppressing Src kinase activity. *Mol Cell Biol.* 2004; 24:6788–6798. [PubMed: 15254245]
- Matsuzaki H, Daitoku H, Hatta M, Aoyama H, Yoshimochi K, Fukamizu A. Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. *Proc Natl Acad Sci U S A.* 2005; 102:11278–11283. [PubMed: 16076959]
- Matsuzaki H, Daitoku H, Hatta M, Tanaka K, Fukamizu A. Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proc Natl Acad Sci U S A.* 2003; 100:11285–11290. [PubMed: 13679577]
- McCahill A, Warwicker J, Bolger GB, Houslay MD, Yarwood SJ. The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. *Mol Pharmacol.* 2002; 62:1261–1273. [PubMed: 12435793]
- Meyerhans A, Vartanian JP, Wain-Hobson S. DNA recombination during PCR. *Nucleic Acids Res.* 1990; 18:1687–1691. [PubMed: 2186361]
- Miller PJ, Dietz KN, Hollenbach AD. Identification of serine 205 as a site of phosphorylation on Pax3 in proliferating but not differentiating primary myoblasts. *Protein Sci.* 2008; 17:1979–1986. [PubMed: 18708529]

- Mori K, et al. Identification of neuromedin S and its possible role in the mammalian circadian oscillator system. *Embo J*. 2005; 24:325–335. [PubMed: 15635449]
- Motta MC, et al. Mammalian SIRT1 represses forkhead transcription factors. *Cell*. 2004; 116:551–563. [PubMed: 14980222]
- Nakae J, Kitamura T, Silver DL, Accili D. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest*. 2001; 108:1359–1367. [PubMed: 11696581]
- Onichtchouk D, et al. Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. *Nature*. 1999; 401:480–485. [PubMed: 10519551]
- Panka DJ, Mier JW. Canstatin inhibits Akt activation and induces Fas-dependent apoptosis in endothelial cells. *J Biol Chem*. 2003; 278:37632–37636. [PubMed: 12876280]
- Peng SL. Foxo in the immune system. *Oncogene*. 2008; 27:2337–2344. [PubMed: 18391975]
- Philip-Couderc P, Tavares NI, Roatti A, Lerch R, Montessuit C, Baertschi AJ. Forkhead transcription factors coordinate expression of myocardial KATP channel subunits and energy metabolism. *Circ Res*. 2008; 102:e20–e35. [PubMed: 18202312]
- Polz MF, Cavanaugh CM. Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol*. 1998; 64:3724–3730. [PubMed: 9758791]
- Potente M, et al. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Invest*. 2005; 115:2382–2392. [PubMed: 16100571]
- Qiu X, et al. Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. *Appl Environ Microbiol*. 2001; 67:880–887. [PubMed: 11157258]
- Ramaswamy S, Nakamura N, Sansal I, Bergeron L, Sellers WR. A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. *Cancer Cell*. 2002; 2:81–91. [PubMed: 12150827]
- Sakamoto K, et al. The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. *J Biol Chem*. 2002; 277:29399–29405. [PubMed: 12050162]
- Salih DA, Brunet A. FoxO transcription factors in the maintenance of cellular homeostasis during aging. *Curr Opin Cell Biol*. 2008; 20:126–136. [PubMed: 18394876]
- Schmidt M, FernandezdeMattos S, van der Horst A, Klompmaker R, Kops GJ, Lam EW, Burgering BM, Medema RH. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. *Mol Cell Biol*. 2002; 22:7842–7852. [PubMed: 12391153]
- Selvaraj A, Prywes R. Megakaryoblastic leukemia-1/2, a transcriptional co-activator of serum response factor, is required for skeletal myogenic differentiation. *J Biol Chem*. 2003; 278:41977–41987. [PubMed: 14565952]
- Shapiro DN, Sublett JE, Li B, Downing JR, Naeve CW. Fusion of PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma. *Cancer Res*. 1993; 53:5108–5112. [PubMed: 8221646]
- Shimada T, Fujita N, Maeda M, Ishihama A. Systematic search for the Cra-binding promoters using genomic SELEX system. *Genes Cells*. 2005; 10:907–918. [PubMed: 16115199]
- Shostak Y, Van Gilst MR, Antebi A, Yamamoto KR. Identification of *C. elegans* DAF-12-binding sites, response elements, and target genes. *Genes Dev*. 2004; 18:2529–2544. [PubMed: 15489294]
- Sidhu A, Miller PJ, Johanson KE, Hollenbach AD. Novel flanking DNA sequences enhance FOXO1a DNA binding affinity but do not alter DNA bending. *Biochemistry*. 2008; 47:6809–6818. [PubMed: 18537265]
- Singer BS, Shtatland T, Brown D, Gold L. Libraries for genomic SELEX. *Nucleic Acids Res*. 1997; 25:781–786. [PubMed: 9016629]
- Stahl M, et al. The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J Immunol*. 2002; 168:5024–5031. [PubMed: 11994454]
- Strachan T, Read AP. PAX genes. *Curr Opin Genet Dev*. 1994; 4:427–438. [PubMed: 7919921]
- Takano M, et al. Transcriptional cross talk between the forkhead transcription factor forkhead box O1a and the progesterone receptor coordinates cell cycle regulation and differentiation in human endometrial stromal cells. *Mol Endocrinol*. 2007; 21:2334–2349. [PubMed: 17609436]

- Tcherkasowa AE, et al. Interaction with factor associated with neutral sphingomyelinase activation, a WD motif-containing protein, identifies receptor for activated C-kinase 1 as a novel component of the signaling pathways of the p55 TNF receptor. *J Immunol.* 2002; 169:5161–5170. [PubMed: 12391233]
- Walter PL, Steinbrenner H, Barthel A, Klotz LO. Stimulation of selenoprotein P promoter activity in hepatoma cells by FoxO1a transcription factor. *Biochem Biophys Res Commun.* 2008; 365:316–321. [PubMed: 17986386]
- Wei SJ, Trempus CS, Cannon RE, Bortner CD, Tennant RW. Identification of Dss1 as a 12-O-tetradecanoylphorbol-13-acetate-responsive gene expressed in keratinocyte progenitor cells, with possible involvement in early skin tumorigenesis. *J Biol Chem.* 2003; 278:1758–1768. [PubMed: 12419822]
- Wen JD, Gray DM. Selection of genomic sequences that bind tightly to Ff gene 5 protein: primer-free genomic SELEX. *Nucleic Acids Res.* 2004; 32:e182. [PubMed: 15601993]
- Williams R, Peisajovich SG, Miller OJ, Magdassi S, Tawfik DS, Griffiths AD. Amplification of complex gene libraries by emulsion PCR. *Nat Methods.* 2006; 3:545–550. [PubMed: 16791213]
- Wu Y, et al. Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase. *J Biol Chem.* 2000; 275:21477–21485. [PubMed: 10748157]
- Zheng X, Yang Z, Yue Z, Alvarez JD, Sehgal A. FOXO and insulin signaling regulate sensitivity of the circadian clock to oxidative stress. *Proc Natl Acad Sci U S A.* 2007; 104:15899–15904. [PubMed: 17895391]

Fig. 1.

Validation of the *in vitro* PORE technique through binding and amplification of known genomic regulatory sequences by Pax3 (A) and FOXO1 (B). The genomic regulatory sequences were cloned into the pSMART-LC-Kan vector and used for two rounds of binding and amplification to GST-Pax3 (A) or GST-FOXO1 (B) (top panels—round #1; bottom panels—round #2). The genomic regulatory sequences are as follows: (A) Trp-1 (650 bp) and Msx2 (750 bp), two genes known to be directly regulated by Pax3; (B) Clone #14 (1.3 kb), which contains two FOXO1 DNA binding elements, and Clone #16 (1.0 kb—negative control), isolated from the genomic library; and Ceruloplasmin (Cp) (850 bp), a gene demonstrated to be directly regulated by FOXO1. Binding reactions containing GST only were carried out in parallel.

Fig. 2.

Binding and amplification of a known FOXO1 regulatory sequence from a genomic library pool. (A) Agarose gel electrophoresis demonstrating two rounds (top panel—round #1; bottom panel—round #2) of binding and amplification using the Ceruloplasmin (Cp) promoter region alone (positive control), and with the Cp promoter diluted 1:10 and 1:1000 with the mouse genomic library. Binding reactions with bacterially expressed GST were carried out in parallel as a negative control. (B) To confirm the presence of the Cp promoter region in the second round of selection, 2 μ l of the gel purified eluate was PCR amplified using primers specific for the Cp promoter region and visualized on a 1% agarose gel.

Fig. 3.

In vitro PORE screen with FOXO1. 1% agarose gel analysis demonstrating FOXO1-specific binding and amplification from 10 μ g of mouse genomic library used for the initial round of selection. Binding reactions with GST only were used in parallel as a negative control. The bracket indicates the region of the gel that was excised and gel extracted, with 10 μ l of the gel purified eluate being used for subsequent rounds of binding and amplification. The PCR amplified genomic DNA from rounds 3 and 4 were gel extracted, cloned into the pCRII TA cloning vector and analyzed by sequencing.

Fig. 4.

FOXO1-dependent regulation of transcription from select isolated genomic sequences. The isolated genomic regulatory elements associated with Tnfrs5 and RACK1 (A) or Col4a2, MKL1, and Phlpp1 (B) were cloned into the pGL3-Basic firefly luciferase reporter vector. HepG2 cells were transiently co-transfected with the reporter constructs and empty pcDNA3.1 (light gray bars) or pcDNA3.1-FOXO1 (dark gray bars). Luciferase activity was determined as described in Materials and methods. All values were normalized for transfection efficiency using the co-transfected pRL-TK renilla luciferase reporter vector and the results are reported as fold activation (A) or percent maximal activity (B) relative to the normalized luciferase activity of each individual regulatory element in the absence of FOXO1. Error bars represent the standard deviation from three independent determinations.

Table 1

Primers used in this study.

Primer name	Primer sequence
Trp-1 Forward	5'-CGGATATCAAGCTTTGTAGAGTAATCAT-3'
Trp-1 Reverse	5'-CGGATATCAGCTGTTAATTGCCCCAAGAG-3'
Msx2 Forward	5'-CGGATATCTCTACCTAAATTCCCTGCTGAGGAGCTC-3'
Msx2 Reverse	5'-CGGATATCTAACCGTGAAGCGTTGAGCACAGA-3'
Cp Forward	5'-TATAGCATTTCAGCATAGTTATAGACTGTACAGG-3'
Cp Reverse	5'-CGTGTCAACAGAAATAAGTTTCTTTTCCCC-3'
PORE Forward	5'-CGTGAAGGTGAGCCAGTGAGTTGATTGCAGTCC-3')
PORE Reverse	5'-CGTGCCGATCAAGTCAAAAGCCTCCGGTCGG-3'

Table 2
Known FOXO1 regulated genes within proximity of the isolated genomic regulatory sequences.

Gene ^a	FOXO1-related function	Replicate #1 ^b	Replicate #2	Replicate #3	Total
Tnfrsf5	Pro-apoptotic	42	9	2	53 (20%) ^c
Cyclin D1	Cell cycle regulation	20	7		27 (10%)
BAMBI	TGF-β signaling/development	1			1
Zwint-1	Cell cycle regulation		1		1

^aTnfrsf5—tumor necrosis factor receptor super family 5; BAMBI—BMP and activin membrane bound inhibitor; Zwint-1—ZW10 interacting protein 1.
^bThe numbers for each replicate indicate the frequency with which each gene was isolated from 118 (replicate #1), 94 (replicate #2), and 50 (replicate #3) individual clones.
^cThe percentage of the total number of clones from all three replicates that were comprised of each isolated gene.

Table 3

Putative FOXO1 regulated genes within proximity of the isolated genomic regulatory sequences.

Gene ^a	FOXO1-related function	Replicate #1 ^b	Replicate #2	Replicate #3	Total
IGFBP9	Muscle differentiation	20	2	7	29 (11%) ^c
Col4a2	Apoptosis, inhibition of Akt		14	6	20 (8%)
RACK1	TNF- α signaling/apoptosis, cell cycle regulation	12	4	2	18 (7%)
MKL1	Muscle differentiation	1	1	3	5 (2%)
T3JAM	TNF- α signaling/apoptosis		2	3	5 (2%)
Akap6	Muscle differentiation	2	1		3 (1%)
Dss1	Cellular proliferation		2	1	3 (1%)
Phlpp1	Apoptosis, Inhibition of Akt		2		2
epb4.1l3	Erythroid differentiation		2		2
NMS	Oxidative stress/circadian clock	1		1	2
Map3K7IP1	Regulation of FOXO1 activity		2		2
Kir3.1	Muscle differentiation/metabolism			2	2
Ctla2a	T-cell homeostasis	1			1
EGR-3	TNF- α signaling/apoptosis			1	1
MAGI-1	Apoptosis			1	1
Yap1	Apoptosis			1	1
Nfyb	Cell cycle regulation			1	1

^aIGFBP9—insulin like growth factor binding protein 9; Col4a2—procollagen type IV alpha 2; RACK1—receptor for activated kinase; MKL1—megakaryoblastic leukemia 1; T3JAM—TRAF3-interacting JNK-activating modulator; Akap6—A kinase (PRKA) anchor protein 6; Dss1—split hand/foot deleted gene 1; Phlpp1—PH domain and leucine rich repeat protein phosphatase 1; epb4.1l3—erythrocyte protein band 4.1-like 3; NMS—neuromedin S; Map3K7IP1—MAPKKK7 interacting protein 1; Kir3.1—potassium inwardly rectifying channel I3; Ctla2a—cytotoxic T-lymphocyte associated protein 2 alpha; EGR-3—early growth response 3; MAGI-1—membrane associated guanylate kinase; Yap1—yes associated protein 1; Nfyb—nuclear transcription factor Y beta.

^bThe numbers for each replicate indicate the frequency with which each gene was isolated from 118 (replicate #1), 94 (replicate #2), and 50 (replicate #3) individual clones.

^cThe percentage of the total number of clones from all three replicates that were comprised of each isolated gene.