THE ROLE OF PROTEIN-PROTEIN INTERACTIONS IN CONTROLLING PROX1 FUNCTION

by

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ABSTRACT

Prox1 is a homeodomain transcription factor that is important for controlling lens, liver, pancreatic, and lymphatic system development. Prox1 has also been implicated in the regulation of tumor suppressor genes and the onset of cancer. Despite this wide variety of functions, it is currently unknown as to how Prox1 is modulated during its role in vertebrate development. Previously, Arid2 (also called BAF200 and zipzap) was isolated from a yeast-two-hybrid assay as a potential Prox1 interacting protein. Arid2 belongs to the ARID family of proteins that are important for cell development, gene expression, and cell growth regulation. It is also a vital component of SWI/SNF complexes that function in chromatin remodeling. This work seeks to test the hypothesis that Prox1 and Arid2 actually interact and to determine the biological relevance of this interaction. First, a GAL4 yeast two-hybrid assay was used to confirm the protein interaction by the activation of reporter genes that are transcribed if the two proteins are able to bind. The plasmids Prox1-pGBKT7 and Arid2-pACT2, the yeast two-hybrid shuttle vectors, were transformed into two different yeast strains of two different mating types. These were mated and plated on selectable media lacking nutrients that the reporter genes are responsible for making. Yeast colonies did grow on selectable media, suggesting that Prox1 and Arid2 do interact. Furthermore, fluorescent immunohistochemical analysis was performed on four-week C57BL/6HAR mouse lens, which showed that Arid2 and Prox1 colocalize

in the mouse lens fiber cells. After preliminary transfection analyses using the CAT reporter gene, it has been shown that Arid2 does not significantly affect Prox1-mediated activation of the βB1-crystallin promoter. These findings suggest that although Arid2 is able to bind Prox1, the functional consequences of this interaction may be difficult to decipher. Further research on other Prox1-interacting proteins may serve to elucidate the regulation of Prox1 in development.

Chapter 1

INTRODUCTION

The vertebrate lens is a transparent tissue within the eye that functions to focus images onto the retina. Like all vertebrate systems, its development is a complex, tightly controlled process. For instance, in lens formation the cephalic ectoderm responds to inductive signals from the optic neuroepithelium, leading to a thickening of the induced ectoderm (Piatigorsky 1981). This lens placode invaginates and pinches off to form the lens vesicle. Cells in the anterior portion of the lens become cuboidal epithelial cells while cells in the posterior portion of the lens differentiate into fiber cells.

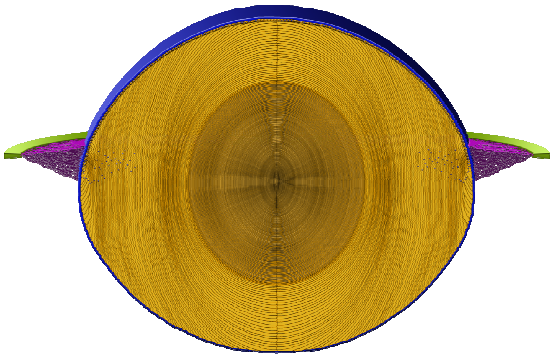

Figure 1. **Diagram of the vertebrate lens, which is composed of monolayer of cuboidal epithelial cells in the anterior portion of the lens that then differentiates into fiber cells at the transition zone. Fiber cells comprise the bulk of the lens and contribute to the focusing power of the lens. Taken from Danysh et al. 2009.**

These complex processes that occur during development are under strict control by various transcription factors that are necessary for proper lens formation. One such transcription factor, Prospero-related homeobox 1 (Prox1), is initially expressed within the lens placode and is essential for lens fiber cell differentiation (Tomarev et al. 1996; Wigle et al. 1999). Prox1 is the vertebrate homolog of the *Drosophila* Prospero, containing a divergent homeodomain and novel Prospero domain at the carboxy-terminus, as well an amino-terminal nuclear localization signal. Furthermore, Prox1 is also a critical transcription factor for other tissue systems during vertebrate development, which include the pancreas, liver, lymphatic system, and retina.

Biochemically, Prox1 has the ability to activate and deactivate transcription through direct binding to DNA (Chen et al. 2008). Prox1 can also deactivate transcription through indirect interactions with nuclear hormone receptors (Qin et al. 2004). As an example, Prox1 mediated transcriptional activation can allow cells to leave the cell cycle while Prox1 mediated transcriptional deactivation can lead to down-regulated expression of cell cycle inhibitors (Shimoda et al. 2006). These diametrically opposing actions make Prox1 such a fascinating protein because the exact method of Prox1 regulation is currently unknown.

1.1 Prox1 is the vertebrate homolog of the Prospero protein

For complex organisms, the ability to activate certain genes while repressing others is an essential function that enables the correct sequence of events during development to occur, thereby ensuring tissue system integrity. This important function is carried out by transcription factors. In *Drosophila*, one such transcription factor is the homeodomain protein Prospero, which has shown to be critical for central nervous system (CNS) and lens development (Chu-Lagraff et al. 1991). This protein is encoded from the pros gene that contains a divergent homeodomain sequence, suggesting the importance of this protein for controlling developmental processes. Prospero is found to be expressed in neuroblasts, which are the CNS progenitor cells in *Drosophila*. Interestingly, a loss of function of Prospero leads to aberrant gene expression in the neuroblasts, suggesting the importance of this transcription factor in neuronal cell fate.

Studies conducted by Oliver et al. (1993) provide convincing evidence for the possibility of Prox1 being the vertebrate homolog of the Prospero protein. Both Prox1 and Prospero contain divergent homeodomains, with 65% similarity between the amino acid sequences of these two proteins. This high sequence similarity suggests that there are many structural similarities between Prox1 and Prospero. Furthermore, Prox1 expression patterns within the CNS are quite similar to that of Prospero, suggesting a functional similarity between these two proteins, as well.

1.2 Prox1 structure

Regarding protein structure, Prox1 contains an amino-terminal nuclear localization signal and a carboxy-terminal homeoprospero domain. Within the protein are three nuclear receptor boxes, a Q-rich region, and a nuclear export signal. This structure supports the function of Prox1 as a transcription factor since specific signals are present that allow the protein to enter the nucleus and bind DNA. Furthermore, the presence of a homeodomain further indicates that Prox1 is a transcription factor, since those proteins with this domain act as major regulators for development (Christensen et al. 2008).

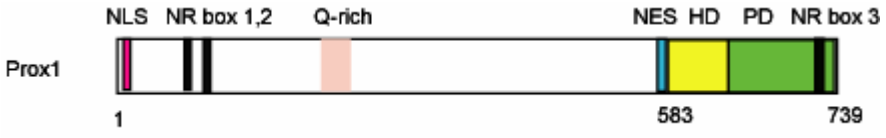

Figure 2. Linear diagram of Prox1. Prox1 contains an N-terminal nuclear localization signal (NLS), a C-terminal divergent homeodomain (HD) and novel Prospero domain (PD), and three nuclear receptor boxes (NR1-3). Taken from Dr. Xiaoren Chen.

1.3 Prox1 function during development

1.3.1 Pancreas development

Previous studies have found Prox1 to be expressed in the mouse pancreatic region, suggesting a potential role for this transcription factor in pancreas organogenesis (Burke and Oliver 2002). Interestingly, it was determined that Prox1 is essential for maintaining the cellular complexity that is characteristic of the pancreas

(Wang et al. 2005). Prox1 knockout mice showed hindered epithelial growth as well as abnormal endocrine cell differentiation, providing supportive evidence for the role of Prox1 in pancreas integrity.

1.3.2 Liver development

Prox1 also shows to be an important transcription factor during liver development. Mouse embryo immunostaining has localized Prox1 in the hepatic bud, gall bladder, and dorsal and ventral pancreatic primordial (Sosa-Pineda et al. 2000). Furthermore, Prox1 is responsible for hepatocyte migration, since Prox1 null mice develop a smaller liver with a reduced number of clustered hepatocytes. It was suggested that this may be due to an overexpression of E-cadherin that occurs in Prox1 knockout mice, which has the effect of maintaining strong cell-cell contacts that should otherwise be broken down for hepatocyte migration.

1.3.3 Lymphatic system development

Moreover, Prox1 appears to regulate lymphatic system development. Studies have demonstrated that Prox1 null embryos do not acquire lymphatic vasculature since budding endothelial cells maintain a blood vascular phenotype (Harvey et al. 2005). This provides evidence supporting Prox1's essential role in cell differentiation. In a study by Harvey et al. (2005), mice heterozygous for Prox1 showed abnormal lymph leakage, leading to adult-onset obesity. Mice were also cyanotic, showed respiratory

distress before death, and had accumulated clear fluid within the thoracic cavity. These symptoms resemble those for pulmonary lymphangiectasia. Overall, the data suggest that Prox1 is necessary for proper lymphatic system development since mice heterozygous for Prox1 sacrifice lymphatic vascular integrity, allowing for slight lymph leakage that results in adipogenesis and obesity.

1.3.4 Retina development

Since the *Drosophila* transcription factor Prospero plays an important role in central nervous system development, it is likely that Prox1 can also regulate gene expression during vertebrate development of the central nervous system. This observation has been supported specifically by research studies on mammalian retina development. In an investigation by Dyer et al. (2003), Prox1 was shown to cause retinal progenitor cells to exit the cell cycle and differentiate into specific cell types. Moreover, Prox1-null mouse retinas were much larger in size as compared to wild-type and lacked appropriate cell differentiation. Interestingly, Prox1-null mice lacked horizontal cells, which could subsequently be promoted to develop with ectopic Prox1 expression. Prox1 expression was found within differentiating horizontal cells, amacrine cells, and bipolar cells, which provides further supporting evidence for the role of Prox1 in retinal development.

1.3.5 Lens development

The Duncan lab uses the vertebrate lens as a model for studying vertebrate development, and Prox1 has been implicated in lens development, as well. Immunochemistry staining within the embryonic lens confirms the expression of Prox1 throughout development.

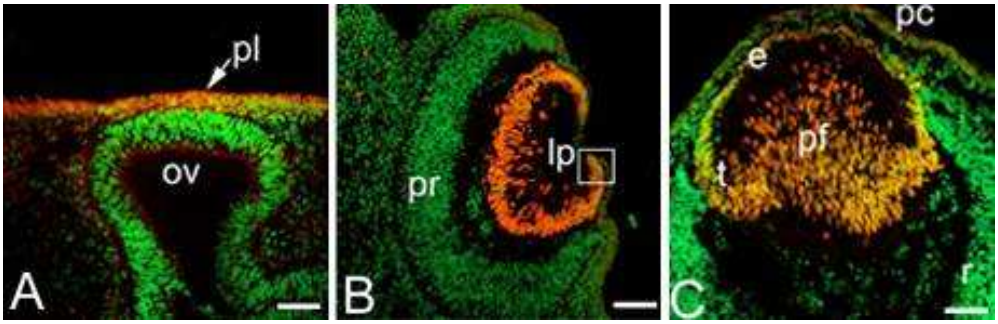

Figure 3. Prox1 is expressed throughout lens development in mice. Panel A is taken from 9.5 dpc, panel B is taken from 10.5 dpc, and panel C is taken from 15 dpc. Red signifies staining against Prox1 and green staining signifies the location of the nucleus. pl – lens placode; e – lens epithelial cells. Taken Duncan et al. 2002.

Crystallins are water soluble proteins found in the lens fiber cells that are responsible for the clarity and refractive qualities of the lens (Piatigorsky 1989). They encompass approximately 90% of all water-soluble proteins within the lens (Hejtmancik et al. 2004). There are three main types of crystallins, α-, β-, and γ-crystallins. The βγ-crystallins, such as βB1-crystallin, are known to play important roles in maintaining lens structure (Jobby and Sharma, 2007).

It has been previously shown that Prox1 can bind the chicken βB1-crystallin promoter and that this interaction upregulates βB1-crystallin expression in both lens and non-lens cells (Cui et al. 2004). For instance, in transfection tests using N/N1003A cells, the βB1-crystallin promoter was cloned upstream of the gene for chloramphenicol acetyltransferase (CAT) and this construct was cotransfected with a vector containing Prox1. This resulted in increased CAT activity by 7.0 ± 0.3-fold. Since the βB1-crystallin promoter sequence (-432/+30) contains Prox1 binding sites, this promoter has been used to study the effects of Prox1 activity on gene regulation (Cui et al. 2004). Specifically, the carboxy-terminal of Prox1 binds to the consensus sequence C(A/t)(c/t)NNC(T/c). This sequence is found in the PL2/OL2 element of the βB1-crystallin promoter, and subsequent research has shown Prox1 to bind to these elements.

Figure 4. βB1-crystallin promoter fused upstream of the reporter gene for chloramphenical acetyltransferase (CAT). Previous research shows that Prox1 can bind to this promoter and activate the transcription of CAT. This construct can be used in studies regarding Prox1 regulation by protein-protein interactions.

In studies by Wigle et al. (1999), Prox1 knockout mice were used to analyze the effect Prox1 could have on lens development. It was shown that in these knockout

mice abnormal cell proliferation occurred, which could be attributed to the downregulation of cell cycle inhibitors Cdkn1b and Cdkn1c, mixexpression of E-cadherin, and inappropriate apoptosis. Therefore, it appears that Prox1 is vital for lens cells to leave the cell cycle and differentiate into elongated fiber cells.

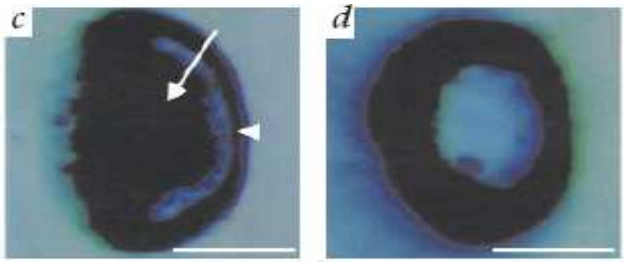

Figure 5. Comparison of WT mouse lens with Prox1-knockout lens. In the right panel, which demonstrates the Prox1-knockout condition, inappropriate apoptosis of the lens has occurred that lead to hollow lens formation. Taken from Wigle et al. 1999.

1.4 Prox1 and cancer progression

Interestingly, Laerm et al. (2006) found that Prox1 expression is downregulated within carcinomas of the bilary system. Prox1 could thus possibly act as a tumor suppressor protein since cells lacking functional Prox1 exhibited hyperproliferation, with normal cell growth occurring when Prox1 was reintroduced into the cells.

Likewise, Prox1 is linked to hepatocellular carcinoma (HCC) (Shimoda et al. 2006). This illness is the third cause of death from cancer and inactivation of Prox1 has shown to generate atypical cellular proliferation, suggesting its role in

tumorigenesis. Studies further showed that a decrease in Prox1 activity led to a recurrence of HCC cells. If overexpression of Prox1 can inhibit tumorigenesis, this protein can be studied as an appealing candidate for diagnosis and treatment for HCC.

While a loss-of-function of Prox1 in hepatocytes had previously shown to promote liver cancer, a gain-of-function of Prox1 can promote invasive tumor invasion in Kaposi's sarcoma (Dadras et al. 2008). Prox1 cDNA was stably expressed in two mouse hemangioendothelioma cell lines, resulting in an increased ability of tumors to migrate at a faster rate and invade the muscular layers.

1.5 Regulation of Prox1 by protein-protein interactions

Due to the diversity of functions that Prox1 appears to be responsible for, it is hypothesized that multiple protein-protein interactions may serve to modulate Prox1 activity. One such study that researched this hypothesis determined that Prox1 may act as a corepressor for the transcription factor liver receptor homologue 1 (LRH1) (Steffensen et al., 2004). It was found that LRH1, which normally binds to and activates transcription for the *shp* promoter, actually repressed SHP transcription when coexpressed with Prox1. LRH1 and Prox1 are colocalized throughout liver, pancreas, and intestine development in mice and humans, which further supports the possibility that these two proteins interact in vivo.

Recent research has also discovered that Prox1 function may be affected by sumoylation, which occurs when a small ubiquitin-related modifier (SUMO)

covalently attaches to the target protein via lysine residues (Shan et al. 2008). Sumoylation can affect future protein-protein interactions that occur with the target protein. In transfection assays when the expression of Prox1 repressed reporter gene transcription, the sumoylation of Prox1 served to downregulate this repressor activity. Furthermore, Prox1 has been shown to recruit HDAC3, a histidine deacetylase, to gene promoters, aiding in Prox1-mediated repression. With sumoylation of Prox1, this recruitment significantly decreased, as exhibited by co-immunoprecipitation assays of Prox1 and HDAC3.

These findings support the hypothesis of Prox1 regulation by protein-protein interactions. Therefore, a yeast two-hybrid assay was performed by Dr. Xiaoren Chen in Dr. Melinda Duncan's laboratory to determine potential proteins that can bind Prox1. Dr. Chen identified and isolated a novel protein, Arid2, which we believe to be a prospective Prox-1 interacting protein.

1.6 Arid2 structure

Arid2 contains an amino-terminal ARID (AT-rich interactive domain), an RFX domain, and two zinc finger domains located at the carboxy-terminus. Arid2 belongs to the ARID (AT- Rich Interaction Domain) family of proteins that are important for cell development, gene expression, and cell growth regulation (Patsialou, et al. 2005). All proteins of the ARID family contain the helix-turn-helix motif DNA-binding domain, which favorably bind to sequences rich in AT nucleotides.

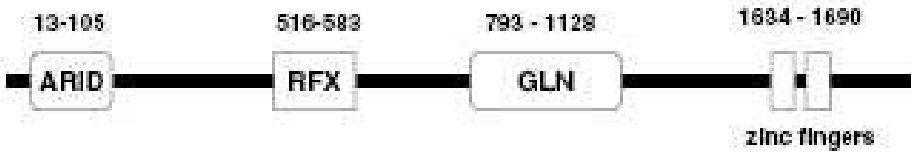

Figure 6. Linear diagram of the Arid2 protein. Arid2 contains an N-terminal ARID domain, two C-terminal zinc finger domains, an RFX domain, and a glycine-rich domain. Taken from Zhang et al. (2006).

1.7 Arid2 function

1.7.1 Arid2 as a cofactor for cardiac gene regulation

Studies have shown that Arid2, or zipzap/p200, is a serum response factor (SRF) cofactor that could possibly be used to enhance transcriptional activation (Zhang et al. 2006). SRF is a transcription factor that regulates cardiac genes during vertebrate development, in addition to controlling cell proliferation, cell size, and cell survival. This interaction between Arid2 and SRF was confirmed using a mammalian two-hybrid system, which demonstrated that there was a weak interaction among these two proteins. Additional studies using a yeast two-hybrid assay isolated the Arid2 protein, which was then found to enhance the effect of myocardin (another regulator of cardiac genes) on the atrial natriuretic factor (ANF) promoter.

1.7.2 Role of the RFX domain

Major histocompatibility complex class II (MHC-II) molecules play an important role within the adaptive immune system since they are responsible for

presenting antigens to CD4 T-helper cells. In humans, there are three main isotypes of the MHCII protein that are controlled by the activity of four gene promoters: the S-box, the X1-box, the X2-box, and the Y-box (Garvie et al. 2007).

Regarding Arid2, studies have shown that an RFX complex binds to the X box DNA sequence in the promoter of MHC-II genes (Nagarajan et al. 2004). RFX is also shown to bind with a Y-box transcription factor called NF-Y to form an RFX-NF-Y-DNA complex, as well as bind with X2bp, an X2-box binding protein, to form an RFX-X2bp-DNA complex. Since Arid2 contains an RFX domain, this could potentially allow Arid2 to bind to other proteins associated with the RFX complex, and thus enhance or repress MHCII gene transcription.

1.7.3 Arid2 as a component of SWI/SNF complexes

Finally, Arid2 could also serve to affect gene transcription through interaction with chromatin. Chromatin is structured into units called nucleosomes that consist of 146 base pairs of DNA wrapped around eight core histone proteins (Trotter and Archer 2008). A final histone protein, H1, assists in the tight condensation of the chromatin into 30-nm fibers. As such, DNA in this state contains genes not undergoing transcription due to this compression.

For genes to be transcribed, transcription factors must be able to bind DNA elements of the promoter of a gene. With energy from ATP hydrolysis, SWI/SNF complexes break DNA-histone interactions to give transcription factors access to gene

promoters. One subfamily of the SWI/SNF complex, PBAF, contains a subunit termed BAF200, which is necessary for PBAF to regulate interferon-responsive gene expression (Yan et al. 2005). BAF200 is a 200-kDa polypeptide, contains 1835 amino acid residues, and is confirmed to be Arid2. The term "BAF" stands for BRG1-associated factor. BRG1, or "Brahma-related gene-1," is the main catalytic subunit of the SWI/SNF complexes that function in chromatin remodeling (Trotter and Archer 2008). BRG1 also associates with other proteins that serve to recruit the SWI/SNF complexes to target genes, and BAF200/Arid2 could possibly act as one of these related proteins.

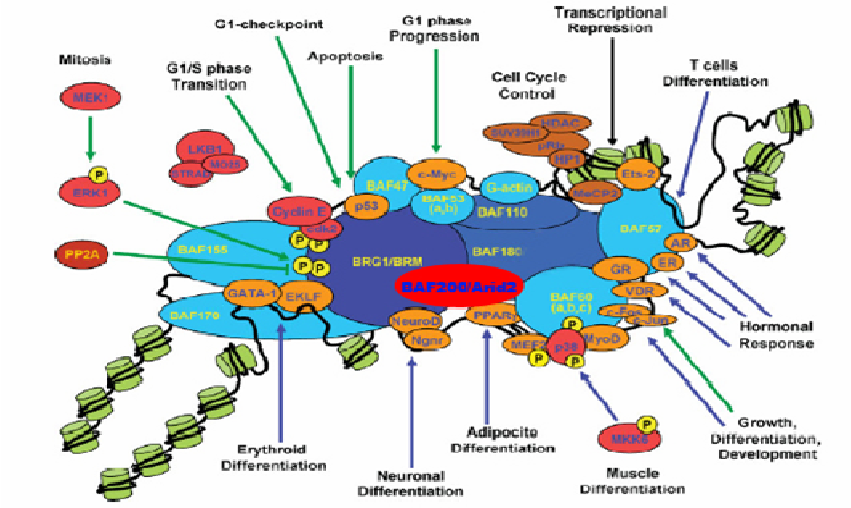

Figure 7. **PBAF subfamily SWI/SNF chromatin remodeling complex. Arid2, also known as BAF200, was recently found to be a component of this complex. This machinery breaks DNA-histone interactions, making gene promoters available for transcription factors to bind. Taken and modified from Simone (2006).**

Chapter 2

MATERIALS AND METHODS

2.1 Yeast two-hybrid assay

2.1.1 Preparing the constructs

Copies of the plasmid constructs used for the yeast two-hybrid assay were obtained from Dr. Xiaoren Chen. Two vectors were used, one containing the homeo-Prospero domain of Prox1 (HDPD, amino acids 547 to 737) cloned into the pGBKT7 vector, and the other containing a region of the Arid2 protein cloned into a pACT2 vector. I then determined which region of the Arid2 protein was cloned into the plasmid and confirmed that this clone was inserted in frame.

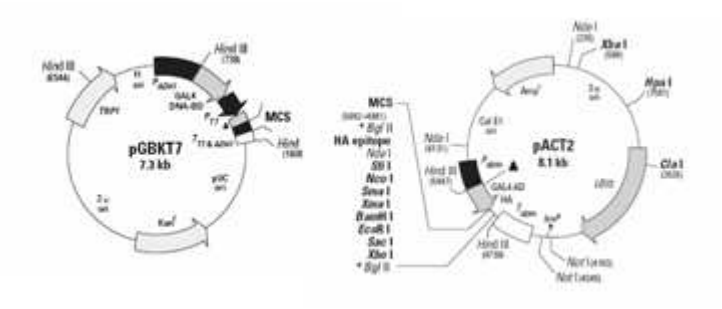

Figure 8. **The above plasmids are representations of the shuttle vectors used to transform Prox1 and Arid2 into *E. coli* and *S. cerevisiae*. Prox1 was inserted into the pGBKT7 vector, which contains an ampicillin-resistance gene, as well as a gene for making tryptophan. Arid2 was inserted into the pACT2 vector, which contains an ampicillin-resistance gene and a gene for producing leucine. Taken from Clontech.**

2.1.2 Preparation of broth and agar plates for *Escherichia coli* and *Saccharomyces cerevisiae*

To prepare LB broth and LB agar plates, the following ingredients were added in a 1 L Erlenmeyer flask: 5 g NaCl, 5 g Yeast extract, and 10 g Tryptone. The flask was filled with distilled water until 1 L total fluid was obtained. Five hundred ml of this broth was transferred to another glass bottle while 10 g of agar was added to the remaining 500ml and stirred with a magnetic stir bar.

To prepare YPDA medium and agar plates, 50 g of YPD medium, 15 ml of 0.2% adenine hemisulfate solution, and 1 L of distilled water were added to a 1 L

Erlenmeyer flask. Five hundred ml of this broth was transferred to a glass bottle with 10 g of agar, and then stirred with a magnetic stir bar.

Dropout media for yeast two-hybrid matings were prepared using the following chart:

Table 1. Minimal media ingredients for preparing agar plates used during the yeast two-hybrid assay.

Dropout type	Amount of Minimal SD base	Amount of dropout supplement	Amount of water	Amount of agar
Trp	13.35g	0.37g	480ml	10g
Leu	13.35g	0.35g	480ml	10g
Leu/Trp	13.35g	0.32g	480ml	10g
Ade/His/Leu/Trp	13.35g	0.30g	480ml	10g

All bottles containing media were then sterilized using the Wolf Hall second floor autoclave. After sterilization, the glassware was set to cool to approximately 55°C. Glassware containing media with agar was then poured into Petri dishes and set to solidify.

2.1.3 *E. coli* transformation

Escherichia coli cells were first transformed with the Prox1 plasmid and Arid2 plasmid according to the protocol provided by Invitrogen for One Shot TOP10 Chemically Competent *E. coli* cells. Two 50 µl vials of *E. coli* cells were thawed on ice and then 5 µl of each plasmid added to a vial. The vial was next set to incubate for 30 minutes on ice. After incubating on ice, the vial was placed in a 42°C water bath for 30 seconds and then immediately transferred to ice. 250 µl of pre-warmed S.O.C.

media provided by Invitrogen was added to the vial, which was next placed in a 37°C incubator with shaking at 225 rpm for one hour.

Meanwhile, LB agar plates modified with ampicillin were prepared. To prepare fresh ampicillin, 0.04 g of ampicillin was added to 10 ml of distilled water to create a final concentration of 4 mg/ml. Next, 100 µl of distilled water and 1 µl of the fresh ampicillin solution were added to the LB plate using a heated glass streaker to spread solution over the entire plate. The plates were placed in the 37°C incubator until transformation reaction is ready.

After incubation, transformation reaction was added to LB plates that had been spread with ampicillin. For one plate, 100 µl of SOC medium and 1 µl of the cells were placed on the top of the agar and spread with a heated streaker. For the second plate, 100 µl of SOC medium and 10 µl of the cells were placed on top of the agar and subsequently spread with a heated streaker. Both plates were placed in the 37°C incubator overnight, which were later stored at 4°C for future use.

2.1.4 DNA preparation

The following protocol for extracting the DNA from the transformed *E. coli* cells was obtained from Wizard Plus SV Minipreps DNA Purification System from Promega. 5 ml of LB broth was added to a 15 ml round-bottom tube. To obtain a final ampicillin concentration of 100 µg/ml, 125 µl of the ampicillin stock (4 mg/ml) was added to the LB broth. Next, one isolated colony from the LB plate was added to

the test tube and vortexed. The tube was incubated for 16-18 hours overnight in the 37°C shaking incubator. After incubation, the tube was centrifuged for 5 minutes at 4°C, 2000 rpm. Excess media was poured out of the test tube. Cells were transferred to 1.5 ml microfuge tubes.

Eluting the DNA was performed as described in the protocol provided by Promega (Figure 9). DNA concentrations were determined using the Nanodrop in Wolf Hall. Sequencing of the Arid2-pACT2 plasmid was performed at the Allen Biotechnology Laboratory.

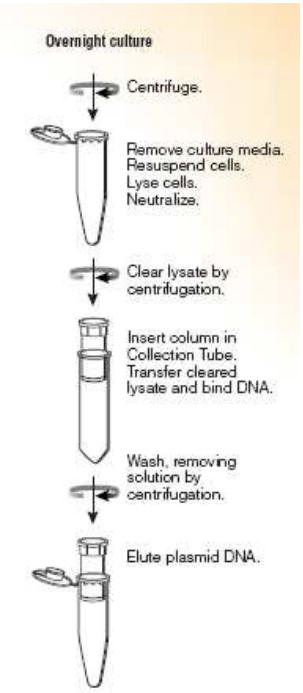

Figure 9. Purification steps to elute plasmid DNA from cultured *E. coli* cells. Taken from Promega.

2.1.5 Transformation of DNA plasmids into *S. cerevisiae*

Once a suitable amount of plasmid had been prepared, a GAL4 yeast two-hybrid assay could be performed to detect a protein interaction. GAL4 is a transcription factor found in yeast that has two domains necessary for function, the DNA-binding domain and the activation domain. In this assay, a 'prey' protein (Prox1 in this study) is fused to the DNA-binding domain of GAL4 and a 'bait' protein (Arid2) is fused to the activation domain.

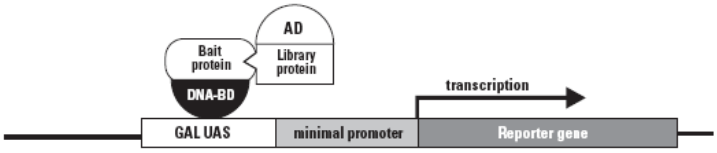

Figure 10. Representation of the GAL4 yeast two-hybrid system. Prox1 acted as the bait protein and was fused to the DNA-binding domain of GAL4. Arid2 acted as the prey protein and was fused to the activation domain of GAL4. This diagram shows that if the bait and prey protein can interact, the DNA-binding domain of GAL4 will allow the activation domain to activate the transcription of reporter genes. Taken from Clontech.

These two constructs are then transformed into yeast. If a true interaction between the bait and prey protein occur, this will bring the DNA-binding domain and activation domain of GAL4 in close enough proximity to function correctly as a transcription factor and activate the transcription of reporter genes. Yeast are grown

on minimal media lacking the amino acids that are coded by the reporter genes, such that only a true protein-protein interaction will allow yeast to grow on this media.

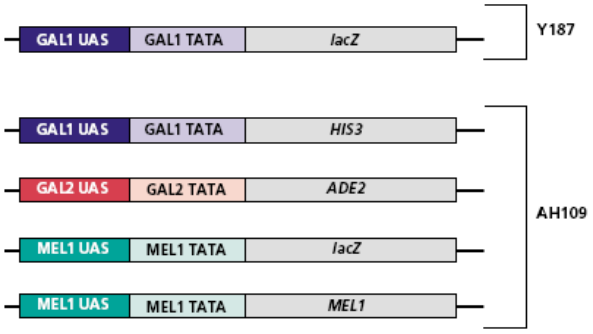

Figure 11. Promoters and reporter genes from the AH109 and Y187 strains used for the GAL4 yeast-two hybrid procedure. The lacZ gene codes for β-galactosidase, which can cleave X-α-gal to form a blue color. The HIS3 gene and the ADE2 gene code for the amino acids histidine and adenine, which are two of the amino acids that are lacking in minimal media used to confirm a positive interaction.

The first step in conducting the yeast two-hybrid assay was to transform Prox1-pGBKT7 and Arid2-pACT2 into yeast strains AH109 and Y187, respectively, according to the protocol provided by Clontech. Frozen yeast stocks of the two strains were plated on a YPDA plate prior to transformation. Once colonies had grown, the following procedure was employed to make these cells competent such that they would take up plasmid DNA.

For each strain, one colony was inoculated into 3 ml of YPDA media and incubated at 30°C with shaking (225 rpm) for 10 hours. The next day, 5 µl of this culture was transferred to 50 ml of YPDA media in a 250 ml flask. This culture was

then incubated overnight for 18 hours at 30°C with shaking. Following incubation, these cells were centrifuged at 700g for 5 minutes at room temperature. The supernatant was discarded and 100 ml of fresh YPDA media was added to the cell pellet. These cells were then incubated at 30°C with shaking for 4 hours. Cell cultures were next split into two 50 ml tubes and centrifuged at 700g for 5 minutes at room temperature. The supernatant was discarded and each cell pellet was resuspended in 30 ml of deionized water. Centrifugation was repeated and after discarding the supernatant, 1.5 ml of 1.1xTE/LiAc was added to the cell pellet. Cells were transferred to 1.5 ml microfuge tubes and centrifuged for 15 seconds at top speed. Cells were resuspended in 600 µl of 1.1xTE/LiAc and at this point considered ready for transformation.

For the actual yeast transformation, 100 ng of plasmid DNA, 5 µl of denatured Yeastmaker Carrier DNA, 50 µl of competent cells, and 500 µl of PEG/LiAc were added and mixed in a 1.5 ml microfuge tube. This mixture was then incubated at 30°C for 30 minutes. After incubation, 20 µl of DMSO was added to the mixture and placed in a 42°C water bath for 15 minutes. The mixture was then centrifuged at top speed for 15 seconds to pellet cells. Supernatant was removed from the microfuge tubes and cells were resuspended in 1 ml of YPD Plus Medium. Finally, cells were pelleted by centrifugation for 15 seconds at top speed and then resuspended in 1 ml of 0.9% NaCl Solution. AH109 cells transformed with Prox1-pGBKT7 were streaked on SD/-Trp

plates and Y187 cells transformed with Arid2-pACT2 were streaked on SD/-Leu plates. Plates were incubated at 30°C until colonies appeared.

2.1.6 Mating yeast strains

Once large colonies had been grown up on SD/- plates with the respective plasmids, mating between the two strains could be performed. To do this, 50ml of SD/-Trp media and 50ml of SD/-Leu media were each added to two sterilized 250ml flasks. One AH109 colony (containing Prox1-pGBKT7) was transferred to the flask containing SD/-Trp and one Y187 colony (containing Arid2-pACT2) was transferred to the flask containing SD/-Leu. These two flasks were incubated overnight at 30°C with shaking.

The following day, cell cultures in each flask were transferred to separate 50 ml tubes. These tubes were centrifuged for 5 minutes at 1000g and room temperature. The cell pellets were then resuspended in about 5 ml of residual media. Finally, a sterile 250 ml Erlenmeyer flask was filled with 48 ml of 2xYPDA/Kan media, 1 ml of the AH109 cells, and 1 ml of the Y187 cells. This mixture was stored overnight in the 30°C incubator with shaking.

For plating these cells, SD/-Leu/-Trp plates were prepared by spreading 100 µl of X-α-gal on each plate to be used. The mixture containing AH109 cells and Y187 cells was removed from the incubator and transferred to a 50 ml test tube. This tube was centrifuged for 10 minutes at 1000g and room temperature. The cell pellet was

then resuspended in 10 ml of 0.5xYPDA/Kan media. The prepared double-dropout plates were finally streaked with 150 µl of the mating mixture, and then set to incubate at 30°C until colonies grew.

Colonies grown on double-dropout plates were transferred to quadruple-dropout plates to confirm a positive interaction between Prox1 and Arid2. To do this, one colony from the double-dropout plate was placed in a 1.5 ml microfuge tube containing 200 µl of YPD media. The plate used was the SD/-Ade/-His/-Leu/-Trp plate spread with 100 µl X-α-gal. For each of these plates, 100 µl of the mating mixture was streaked and set to incubate at 30°C until colonies appeared.

2.2 Immunochemistry

2.2.1 Sectioning of C57Bl/6har mouse lenses

Sectioning of frozen C57Bl/6har mouse lens tissue, prepared by Dr. Yan Wang, was performed using the Leica CM3050 S Cryostat in Wolf Hall. The cryostat was set at -20°C if sectioning newborn lenses or -15°C if sectioning adult lenses. Chamber temperature was typically set for -18°C. Lens tissue was cut in 16 µm sections and then placed on cover slips for later viewing or staining.

2.2.2 Immunostaining for Prox1 and Arid2 proteins

Immunohistological staining on C57Bl/6har lenses was performed using the protocol developed by Reed, Oh, Czymmek, and Duncan (2001). First, slides were

fixed in coplin jar with 1:1 acetone:methanol solution for 20 minutes at -20°C. Slides were then subsequently blocked with 1% BSA in 1XPBS for 1 hour at room temperature. After blocking, 75 µl of primary antibody was directly added to the slides, which were then set to incubate for 1 hour in a humid chamber. Washing occurred after incubation, in which slides were placed in coplin jars with 1XPBS for 10 minutes. Secondary antibody (Alexa Fluor 568-conjugated goat anti-rabbit IgG or goat anti-mouse IgG, 1:200) and counter-stain (DraQ5, 1:2000) were prepared in 1%BSA in 1XPBS to make a total volume of 75 µl, which was then added to the slides. This was incubated in dark conditions for 1 hour in the humid chamber. Slides were washed again in 1XPBS. Finally, a drop of mounting media was placed on each slide, which was subsequently covered with a cover slip. Images were taken using the Zeiss LSM 510 Confocal microscope.

Table 2. Description of primary antibodies

Target Protein	Antibody	Vendor and catalog #	Dilution
Prox1	Rabbit polyclonal	In house, as described in Duncan et al. 2002	1:400
Arid2/BAF200	Rabbit polyclonal	National Institute on Aging, as described in Yan et al. 2005	1:50, 1:100
Arid2/BAF200	Rabbit polyclonal	Abcam ab56082	1:50, 1:100
Arid2/BAF200	Mouse monoclonal	Santa Cruz Biotechnology sc-81050	1:50, 1:100

2.3 Transfection analyses

2.3.1 Culturing of CHO cells

Cells used in this procedure were Chinese Hamster Ovary (CHO) cells. These cells were grown in T75 flasks and split at 1:8 dilutions every four days. Media used for these cells was prepared by combining 350 ml DMEM, 4 ml penicillin/streptomycin, and 4 ml L-glutamine in a Stericup filter unit from Millipore. After vacuum filtration, 40 ml of fetal bovine serum (FBS) was added to the filtered media.

2.3.2 Preparation of plasmids for transfection

High plasmid concentrations were required for transfecting plasmid DNA into CHO cells. This was achieved using the EndoFree Plasmid Maxi Kit from Qiagen. The following diagram (Figure 12) represents the procedure that was employed to purify plasmids from *E. coli*. First, plasmids were transformed into *E. coli* and a fresh colony was used to produce a starter culture of cells in 5 ml of LB media containing ampicillin. This starter culture was incubated for 8 hours at 37°C with shaking. After incubation, the starter culture was diluted by inoculating 250 ml of LB-Amp media with 500 µl of starter culture, which was subsequently set to incubate for 16 hours at 37°C with shaking. Bacterial cells were harvested by centrifugation at 6000g for 15 minutes at 4°C. Pellets were resuspended in 10 ml of Buffer P1, and then an additional 10 ml of Buffer P2 was added to the pellet. The mixture was vigorously

mixed and set to incubate at room temperature for 5 minutes. Next, 10 ml of chilled Buffer P3 was added to the tube and the lysate was immediately poured into a QIAfilter Cartridge to incubate at room temperature for 10 minutes. After incubation, the lysate was filtered into a 50 ml tube. 2.5 ml of Buffer ER was next added to the lysate, which was then incubated on ice for 30 minutes. Meanwhile, a QIAGEN-tip 500 was equilibrated with 10 ml of Buffer QBT. The lysate was then applied to the QIAGEN-tip 500 and allowed to enter the resin by gravity flow. The QIAGEN-tip 500 was next washed twice with 30 ml of Buffer QC, and the DNA was finally eluted with 15 ml of Buffer QN.

The following steps were used to purify the DNA. The eluted DNA was precipitated by adding 10.5 ml of room temperature isopropanol and then centrifuging at 15,000g for 30 minutes at 4°C. The supernatant was decanted and the DNA pellet was washed with 5 ml of endotoxin-free room temperature 70% ethanol and again centrifuged at 15,000g for 10 minutes. The supernatant was decanted and the DNA pellet was resuspended in endotoxin-free Buffer TE. DNA concentrations were determined using the Nanodrop in Wolf Hall.

Figure 12. Flowchart for performing the maxi DNA preparation. This procedure was employed in order to obtain large concentrations of plasmid DNA to be used for the transient transfections. Taken from Qiagen.

Table 3. Description of plasmid vectors used in CHO transfections

Plasmid	Vendor and catalog #
Arid2-pCEP4	National Institute on Aging, as described in Yan et al. 2005
Prox1-pCMV	Dr. Xiaoren Chen
SC35	Tapan Patel
CRP2-pCMV6	Origene SC322165
βGal-pCMV	Dr. Xiaoren Chen
pcDNA 3.1	Dr. Xiaoren Chen
pBasic -432/+30 bB1/CAT	Dr. Xiaoren Chen

2.3.3 Transient transfection of CHO cells with plasmid DNA

Transient transfections were performed using Lipofectamine and Plus Reagent from Invitrogen. Prior to the transfection, CHO cells were plated such that there would be approximately 3.5 x 10^5 cells per plate in 3 ml of media. Cells were then incubated at 37°C until they achieved 50-90% confluency. This typically took 48 hours of incubation. On the day of transfection, fresh media was given to the cells, which were then set to incubate for four more hours at 37°C.

After incubation, microfuge tubes were labeled A, B, C, D, and Z; 2058 tubes (set 1) were labeled A, B, C, D, and Z; and 2058 tubes (set 2) were labeled 1-15. To all microfuge tubes were added 300 µl of Optimem, 24 µl of Plus reagent, and the designated amounts of plasmid (Table 3). These contents were mixed well and set to incubate for 15 minutes at room temperature. For the next step, to all 2058 tubes (set 1) were added 300 µl of Optimem, 36 µl of Lipofectamine reagent, and the contents from the corresponding microfuge tubes. These contents were again mixed and set to incubate for 15 minutes at room temperature. Finally, to all 2058 tubes (set 2) were added 2.8 ml of Optimem and 200 µl of the corresponding mixture from set 1 (tube A from set 1 is distributed to tubes 1, 2, and 3 from set 2). At this point, the CHO cells were removed from incubation and old media was aspirated. Excess serum was washed off by adding 3 ml of Optimem to the plates, and then aspirating off the Optimem. Each tube from set 2 was then added to the corresponding plate of CHO cells. The cells were incubated for 3 hours at 37°C and then after incubation,

transfection media was removed and replaced with 3 ml of fresh media. The cells were then incubated at 37°C for 48 hours.

Table 4. Plasmid DNA and corresponding amounts to be transfected into CHO cells. This set of plasmids was used to determine the functional significance of an Arid2 interaction with Prox1. Taken from Abby Grabitz.

DNA	µg/µl	µg per plate	plates per set	µl of DNA per set
pCMV b-gal	0.24387	0.5	3	6.2
pcDNA 3.1	0.36297	0.25	3	2.1
pCMV prox1	0.07868	0.25	3	9.5
pCEP4 arid2	0.565	0.25	3	1.3
pB -432/+30 bB1/CAT	0.402	2.5	3	18.7

Table 5. Set up for plating CHO cells for the transfection. There were a total of five conditions, with each condition performed in triplicate. Condition A lacked expression plasmid, condition B had only the Prox1 expression plasmid, condition C had both the Prox1 and Arid2 expression plasmids, condition D had only the Arid2 expression plasmid, and condition Z lacked any transfected plasmid. Taken from Abby Grabitz.

Plate #	set	name of experimental plasmid	amount of experimental plasmid	expression plasmid 1	amount of expression plasmid 1	expression plasmid 2	amount of expression plasmid 2	amount of pCMV b-gal	*Total volume*	H2O
1	A	pB -432/+30 βB1/CAT	18.7	pc DNA 3.1	2.1	pc DNA 3.1	2.1	6.2	*28.9*	7.5
2										
3										
4	B	pB -432/+30 βB1/CAT	18.7	pCMV prox1	9.5	pc DNA 3.1	2.1	6.2	*36.4*	0.0
5										
6										
7	C	pB -432/+30 βB1/CAT	18.7	pCMV prox1	9.5	pCMV arid2	1.3	6.2	*34.3*	2.1
8										
9										
10	D	pB -432/+30 βB1/CAT	18.7	pCMV arid2	1.3	pc DNA 3.1	2.1	6.2	*28.2*	8.2
11										
12										
13	Z	none	none	none	none	none	none	none	*none*	36.4
14										
15										

Cells were next harvested after the 48 hours incubation period. First, the old media was aspirated and cells were washed with PBS. 1 ml of PBS was added to the cells, and cell scrapers were used to scrape cells off of the bottom of the plates. These cells were transferred to microfuge tubes and set on ice until all cells from each plate had been scraped into separate tubes. All tubes were then centrifuged at 3000 rpm for 5 minutes, excess PBS was removed, and tubes were centrifuged again at 3000 rpm for 5 minutes. The remaining PBS was removed and to each tube was added 100 µl of 0.25M Tris (pH = 7.8). Cells were vigorously resuspended and stored at -80°C until future use.

2.3.4 Determining transfection efficiency using the ONPG assay

For the ONPG assay, 5 µl of cell extract was placed in a microfuge tube. The ONPG solution was made by combining 1.6 ml of 4 mg/ml ONPG in 0.1M PB (pH 7.5), 1.98 ml of 0.4M PB (pH 7.5), and 5.83 ml of deionized water in a 15 ml test tube. Using a timer, 495 µl of ONPG solution was added to each test tube in 15 second intervals and placed in a 37°C water bath. Reactions were stopped with 500 µl of 1M Na_2CO_3 upon the appearance of a yellow color in the reaction mixture. Once all reaction mixtures had been stopped, tubes were centrifuged at 1100g for 5 minutes. The reactions were then transferred to Fisherbrand Disposable Plastic Cuvets so that optical density could be measured at 420 nm.

2.3.5 Determining reporter gene activity by ELISA

The reporter gene used in the βB1-crystallin construct was chloramphenicol acetyltransferase (CAT). This protein was quantified in each cell extract using the CAT ELISA Kit from Roche Applied Science. First, 500 µl of lysis buffer was added to 20 µl of cell extract and incubated at 30 minutes at room temperature. Meanwhile, five CAT enzyme standards such that a standard curve for optical density versus enzyme concentration could be produced in later analyses. The concentrations used for CAT enzyme standards were 0 ng/ml, 1.0 ng/ml, 0.5 ng/ml, 0.25ng/ml, and 0.125 ng/ml. After sufficient incubation with lysis buffer, 200 µl of each sample was added to a 96-well plate pre-coated with polyclonal antibody to CAT (from sheep) according to the following table (Table 4). Standards were also added to this plate.

Table 6. Representation of the 96-well plate used during the ELISA procedure. Standards and samples were added in duplicate.

	1	2	3	4	5	6
A	Blank	Blank	Sample 3	Sample 3	Sample 11	Sample 11
B	Standard 0	Standard 0	Sample 4	Sample 4	Sample 12	Sample 12
C	Standard 1	Standard 1	Sample 5	Sample 5	Sample 13	Sample 13
D	Standard 2	Standard 2	Sample 6	Sample 6	Sample 14	Sample 14
E	Standard 3	Standard 3	Sample 7	Sample 7	Sample 15	Sample 15
F	Standard 4	Standard 4	Sample 8	Sample 8		
G	Sample 1	Sample 1	Sample 9	Sample 9		
H	Sample 2	Sample 2	Sample 10	Sample 10		

After standards and samples had been added to the microplate, a self-adhesive plate cover foil was placed over the microplate, which was then incubated for 1 hour at 37°C. For washing steps, wells were rinsed 5 times with 250 µl of Washing buffer for 30 seconds each. After Washing buffer had been carefully removed, 200 µl of Anti-

CAT-DIG working dilution was added to each well. The microplate was covered with foil and set to incubate for 1 hour at 37°C. The plate was subsequently washed as before. Next, 200 µl of Anti-DIG-POD working dilution was added to each well, and the microplate was covered with foil and set to incubate for 1 hour at 37°C. The microplate was then washed a final time, and after removing all Washing buffer, 200 µl of POD substrate was added to each well. At this point, reactions within each well turned green according to the concentration of CAT protein that had been in each sample or standard. Optical density for the entire microplate was read using the Dynex plate reader at 405 nm.

Chapter 3

PROX1 HD/PD CAN BIND TO C-TERMINUS OF ARID2 PROTEIN

3.1 Confirmation that Arid2 clone was inserted in frame into pACT2 vector

Our study of Prox1 regulation suggests that protein-protein interactions assist in controlling Prox1 activity. Using a yeast two-hybrid assay, we were able to demonstrate that the homeo-Prospero domain of Prox1 has the ability to interact with an Arid2 clone. First, Arid2-pACT2 vectors were successfully transformed into *E. coli* and subsequently eluted from the DNA preparation. Successful transformation was confirmed since the LB agar plates had been supplemented with ampicillin, which under normal circumstances would inhibit growth of any *E. coli* cells. However, Arid2-pACT2 plasmid contains a gene for the production of an ampicillin-resistant product. Thus, successful transformants would be able to grow on the LB-ampicillin plates since the Arid2-pACT2 plasmid allows the cells to make a product that blocks the effect of ampicillin.

Sequencing results confirmed that the Arid2 clone had been inserted into the pACT2 vector in frame, such that upon transformation into *S. cerevisiae*, Arid2 protein would be produced. By comparing the sequencing results with the known Arid2 DNA sequence, we were able to determine that the C-terminal portion of the

Arid2 clone had been inserted into the pACT2 vector. These results are demonstrated in Figures 13 and 14.

Figure 13. Colonies of *E. coli* that had been transformed with Arid2-pACT2 grew on LB agar plates supplemented with ampicillin.

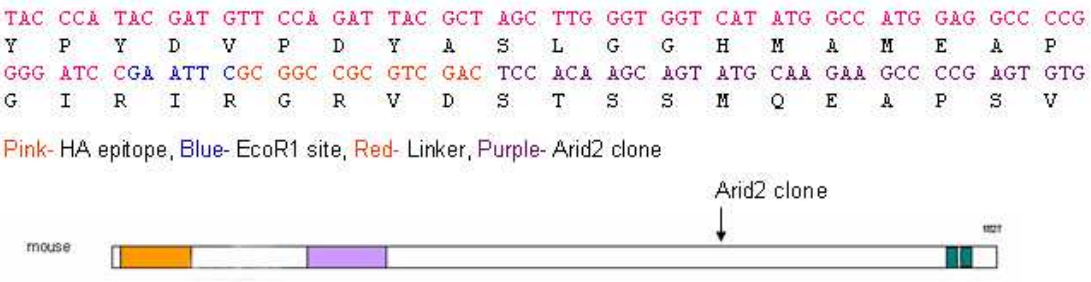

Figure 14. Results of sequencing the Arid2-pACT2 vector. This confirms that the Arid2 clone was inserted in frame, such that the C-terminal segment as illustrated in the protein figure would be successfully formed when plasmid was transformed into *S. cerevisiae*.

3.2 Positive interaction between Arid2 and Prox1 found with yeast two-hybrid assay

Furthermore, successful yeast transformations occurred, due to the presence of colonies on minimal media. Transformation was confirmed because in addition to containing genes that code for antibiotic resistance, there are also genes on each plasmid vector that code for making an amino acid product that is necessary for cell growth. Thus, when these plasmid vectors were transformed into yeast, they allowed the yeast to make an amino acid product that was lacking on media where they had been plated to grow. The transformation efficiency for Prox1-pGBKT7 was much higher than that for Arid2-pACT2, as can be seen in Figure 15. These colonies were then used in the mating procedure.

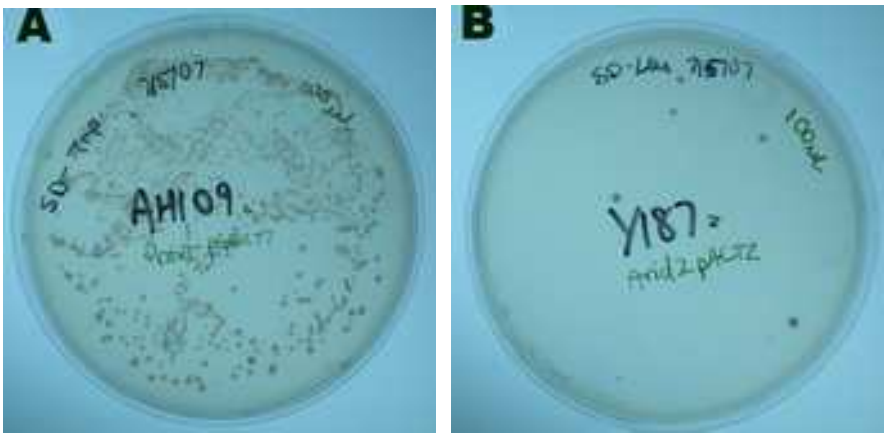

Figure 15. Panel A shows AH109 yeast colonies transformed with Prox1-pGBKT7 growing on minimal media agar plates that lack tryptophan. Panel B shows Y187 yeast colonies transformed with Arid2-pACT2 growing on minimal media agar plates that lack leucine.

As can be seen in Figure 16, successful mating of yeast strains occurred. The purpose of the yeast matings was to make sure each yeast cell contained both Prox1-pBGKT7 and Arid2-pACT2 vectors. Since yeast colonies did grow on minimal media lacking tryptophan and leucine (the two amino acids that are produced by the pGBKT7 and pACT2 plasmids, respectively), it is highly suggestive that each cell must contain each plasmid, and hence, successful mating occurred.

Figure 16. Mated yeast strains growing on minimal media that lacks both tryptophan and leucine.

In order to fully confirm a positive interaction between Arid2 and Prox1, a method for reporter gene detection was implemented. This was performed by plating mated cells on minimal media agar plates that lacked adenine and histidine. Furthermore, the chemical X-α-gal was spread on these plates to act as a third method for reporter gene detection.

The reporter genes for this construct code for adenine, histidine, and β-galactosidase. By plating mated yeast on minimal media that lack tryptophan, leucine, adenine, and histidine, one can confirm that an actual protein interaction occurred if cells grew, as this would be made possible by the production of adenine and histidine by the cell. If minimal media had been used that did include adenine and histidine, one could not be sure if a true interaction occurred, or if the two plasmids were just simultaneously being expressed within the same yeast cell. Furthermore, positive interactions can be confirmed by a blue appearance when the plate had been spread with X-α-gal. The enzyme β-galactosidase, normally found on the lac operon of *E. coli*, is able to cleave X-α-gal to form a blue product. Thus, yeast colonies that are able to grow on media lacking adenine and histidine, as well as turn blue on media containing X-α-gal, support a positive interaction between proteins.

As described above, blue colonies did grow on minimal media lacking the adenine and histidine, suggesting a positive interaction between Prox1 and Arid2. To make sure that the proteins were truly interacting with each other and not a by-product of the plasmid vector, negative controls were spread on plates. These negative controls were the Prox1-pGBKT7 vector mated with the pACT2 empty vector, and the Arid2-pACT2 vector mated with the pGBKT7 empty vector. No yeast colonies were able to grow on this media for each of these matings. A positive control was also used between two proteins known to have an interaction, p53 and T-antigen. This mating did produce yeast colonies on the minimal media with X-α-gal, but no blue product appeared, suggesting a possible mutation in the Y187 strain that contains the β-galactosidase reporter gene. This plate with all four matings can be seen in Figure 17.

Figure 17. Positive interaction was confirmed between Arid2 and Prox1 in the yeast two-hybrid assay. Panel 1 is the positive control between two proteins known to interact, p53 and T-antigen. Panel 2 demonstrates the positive interaction between Arid2 and Prox1. Panels 3 and 4 are negative controls, with Panel 3 being Arid2 with the empty pGBKT7 vector and Panel 4 being Prox1 with the empty pACT2 vector.

3.3 Discussion of positive interaction between Arid2 and Prox1

These results suggest that the Prox1 homeo-Prospero domain can interact with the C-terminus of the Arid2 protein, since these were the segments that had been cloned within each of their respective vectors. Thus, the level of regulation that Arid2 could impose on Prox1 may be by an interaction of Prox1 with the two zinc finger domains of Arid2. Interestingly, previous studies have shown that Arid2 can modulate serum response factor (SRF) via the two zinc finger domains (Zhang et al. 2006). Arid2, therefore, may be able to interact with Prox1 in a similar manner.

Future studies with this system would be to perform a yeast two-hybrid assay using varying domains of the Prox1 proteins that have been cloned into plasmid. This could further specify which segment of the Prox1 protein can bind to Arid2. Additionally, since no blue colonies appeared for the positive control between p53 and T antigen, this experiment should be studied to determine why this did not occur. For instance, the p53 and T antigen plasmid vectors could be reintroduced into new AH109 and Y187 yeast strains, such that the yeast two-hybrid assay could be repeated to obtain the desired blue colonies.

Chapter 4

PROX1 AND ARID2 ARE COLOCALIZED IN C57BL/6HAR LENS FIBER CELLS

4.1 Developmental study of Arid2 localization in the C57Bl/6har mouse lens

Once it had been determined that Prox1 and Arid2 have the potential to interact *in vivo*, immunochemistry staining was performed to localize Prox1 and Arid2 within the mouse lens. Prox1 expression is already known to be present within the mouse lens throughout development (Duncan et al. 2002), making this a suitable model for determining Prox1 and Arid2 colocalization. In a developmental study of Arid2 expression in the C57Bl/6har mouse lens, it was found that Arid2 is not expressed in the lens fiber cells until adulthood (Figure 18, middle panel). Arid2 was not expressed in embryonic lenses (data not shown), nor was expression found in WT newborn lenses. Interestingly, Prox1 and Arid2 are colocalized in the fiber cells in WT adult lenses, suggesting the possibility for post-developmental control of Arid2 over Prox1.

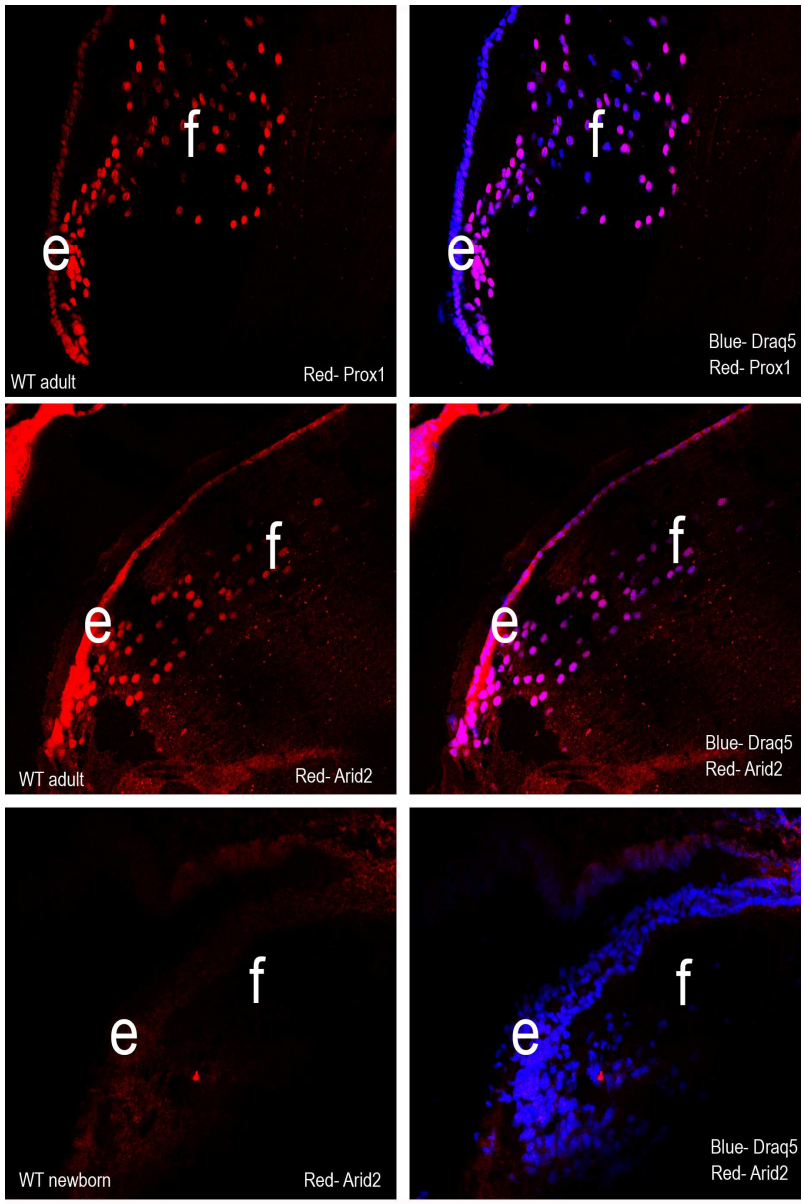

Figure 18. **Immunostaining with C57Bl/6har mouse lens. The top panel represents staining for Prox1 in the 4 week-old C57Bl/6har mouse lens. Left is Prox1 only, right is merged with nucleus staining. The middle panel represents staining for Arid2 in the 4 week-old C57Bl/6har mouse lens. Left is Arid2 only, right is merged with nucleus staining. These two panels show that Prox1 and Arid2 are colocalized in the lens fiber cells. The bottom panel represents Arid2 staining in the newborn C57Bl/6har mouse lens. There appears to be no Arid2 expression at this stage.**

4.2 Discussion of Arid2 and Prox1 colocalization

The colocalization of Arid2 and Prox1 in mouse lens fiber cells support the possibility for an interaction between these two proteins. As stated previously, lack of Arid2 expression in embryonic lenses suggests a potential role for this protein as a post-developmental modulator of Prox1.

During the summer of 2008, antibodies purchased from AbCam and Santa Cruz (see 2.2.2) were used in a developmental study of Arid2 localization within the wild-type mouse lens. These antibodies were purchased because only a small supply of antibody was received as a gift from the Wang lab. In comparing the immunostaining among the three antibodies used, it appeared that the antibodies purchased from AbCam and SantaCruz did not detect Arid2 as specifically as with the antibody received from the Wang lab. Future studies could therefore be performed to determine if these purchased may be better suited for detecting Arid2 under Western blotting conditions as opposed to immunochemistry.

Chapter 5

ARID2 MODULATION OF PROX1

5.1 Cotransfection of Arid2 and Prox1 in CHO cells

It is known that a construct containing three copies of a Prox1 binding site, cloned upstream of the β actin basal promoter and fused to the gene for chloramphenicol acetyltransferase (CAT) is activated ten-fold by Prox1 in transfection tests (Figure 19). If Prox1 is able to bind with Arid2, this assay will determine the effect of Arid2 on Prox1 by measuring protein production of CAT in Arid2 and Prox1 cotransfections compared with Prox1 transfection alone. Because both Prox1 activation and repressive sites are found within the βB1-crystallin promoter (Chen, et al. 2008), we hypothesized that cofactors such as Arid2 may be necessary for Prox1 function to occur within the lens.

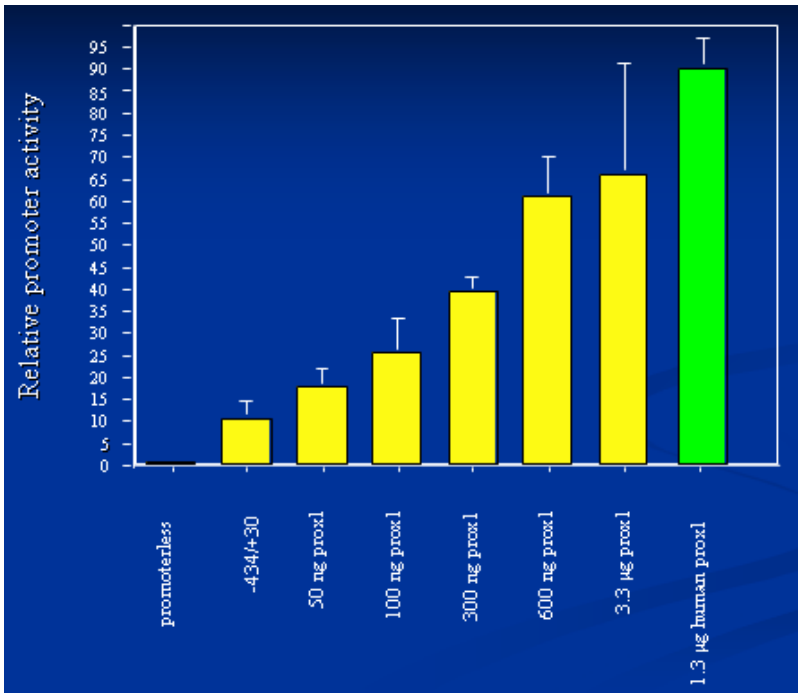

Figure 19. Increasing amounts of Prox1 transfected in the presence of the fμll βB1-crystallin promoter show increasing activation of promoter activity. Taken from Dr. Melinda Duncan.

As expected, Prox1-mediated expression of the CAT reporter gene occurred. Unfortunately, there was no apparent increase or decrease in CAT concentration when Arid2 was cotransfected into CHO cells along with Prox1. This finding caused our lab to seek other Prox1-interacting candidates that may serve to regulate Prox1 function. Table 4 exhibits a list of candidate proteins for Prox1 binding as found by Dr. Xiaoren Chen in a yeast two-hybrid assay. In my study of Prox1 modulation, I used the proteins CRP2 and SC35 for further studies in transfection tests.

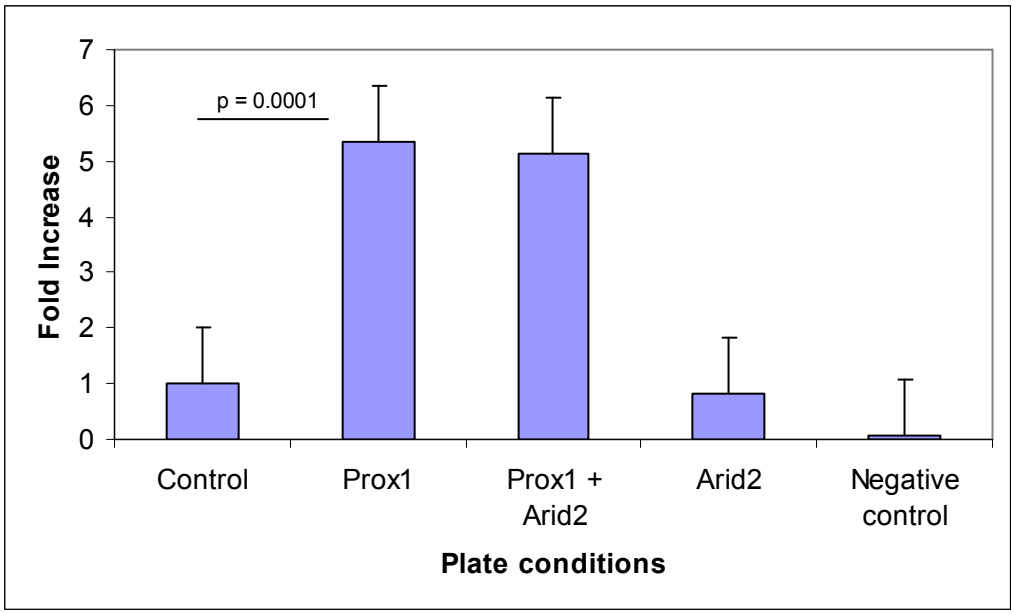

Figure 20. **Results of transient transfection to determine role of Arid2 using the entire βB1-crystallin promoter. Prox1-mediated activation was observed, but there was no apparent Prox1 regulation by Arid2.**

Table 7. **Potential Prox1-interacting proteins as determined by a yeast two-hybrid assay conducted by Dr. Xiaoren Chen. Of this list, Arid2, CRP2, and SC35 have been used in this study of Prox1 regulation. Taken from Dr. Melinda Duncan.**

Putative Prox1 interacting proteins		
Protein	# of clones recovered	Amino acids
Nuclear		
FK506 binding protein 3 (FKBP25)	2	145-224
Interferon response element-binding factor IREBF-2 / SC35	7	226-277
Cysteine and glycine-rich protein 2 (Csrp2)	1	1-101
Translocated promoter region protein	2	4-55
Poly(rC) binding protein (PCBP1)1	3	1-63
Proliferating cell nuclear antigen (PCNA)	1	167-261
Proliferation-associated 2G4 (Ebp1)	1	286-394
filamin B	1	637-719
Arid2 (Baf200; zipzap)	1	1-1636
Ubiquitin/Sumoylation pathway		
Polyubiquitin C	1	94-142
Ubiquitin-conjugating enzyme E2I/Ubc9	2	1-102; 69-220
Anaphase-promoting complex subunit 10	1	39-181
Proteosome subunit alpha type 7	1	165-222
Other		
Tubulin folding cofactor B	6	32-87
Fibrillin 2	2	2687-2773
FABP7	3	57-132

5.2 Possible modulation of Prox1 by CRP2

Cysteine- and glycine-rich protein (CRP2) is an evolutionarily conserved protein with two LIM domains (Sagave et al. 2008). It is suggested that these LIM domains may serve as protein interfaces that facilitate the binding of other proteins to CRP2. Previous research has also showed that CRP2 can activate smooth muscle gene activity in adults, suggesting a role for this protein in gene control (Chang et al. 2007). Furthermore, CRP2 is heavily associated with mesenchyme and epithelia in

embryogenesis, and therefore may possibly associate with lens epithelial cells during eye development (Henderson et al. 2002).

Results from cotransfection of Prox1 with CRP2 in CHO cells showed that CRP2 has no apparent effect on Prox1-mediated expression of the CAT reporter gene. There was no significant difference in CAT concentration with the cotransfection of CRP2 and Prox1 as opposed to transfection of Prox1 alone. Therefore, CRP2 may not have a direct role in Prox1 modulation. Due to these findings, one other Prox1-interacting candidate protein was selected to be studied, SC35.

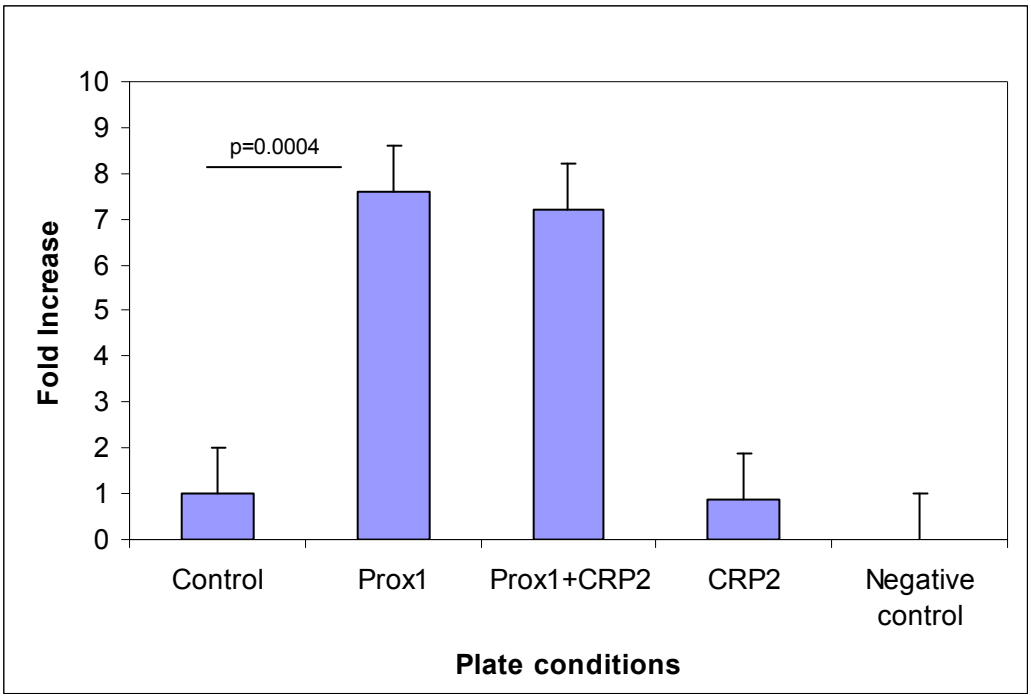

Figure 21. Results of transfection to determine the role of CRP2 using the entire βB1-crystallin promoter. Prox1-mediated activation was observed, but no apparent modulation of Prox1 by CRP2 occurred.

5.3 Possible modulation of Prox1 by SC35

SC35 is a serine/arginine-rich (SR) protein that binds RNA, targeting these sequences to enter the splicing pathway after transcription (Fu 1993). Previous studies show that alternative splicing and gene transcription may be intimately coupled to maintain mRNA integrity (Pandit et al. 2008). Interestingly, a wide defect in transcription was found in SC35 knockout systems, supporting the coupling that occurs between transcription and splicing (Lin et al. 2008). Specifically, this research deduced a possible role for SC35 in transcription elongation.

These findings from past research have prompted this lab to study the effect of SC35 on Prox1 activity. As with previous transfections, a significant increase in CAT was observed when Prox1 was transfected into this system. However, this increase in reporter gene activation was smaller than with previous transfections performed with Arid2 and CRP2. Despite this, with the cotransfection of SC35 and Prox1, a significant increase in CAT concentration was observed as compared with Prox1 transfection alone. Therefore, SC35 may perform a modulating function on Prox1, which could involve the facilitation of transcription elongation. More transfections would need to be performed to fully support the enhanced transcription of CAT with the cotransfection of Prox1 and SC35.

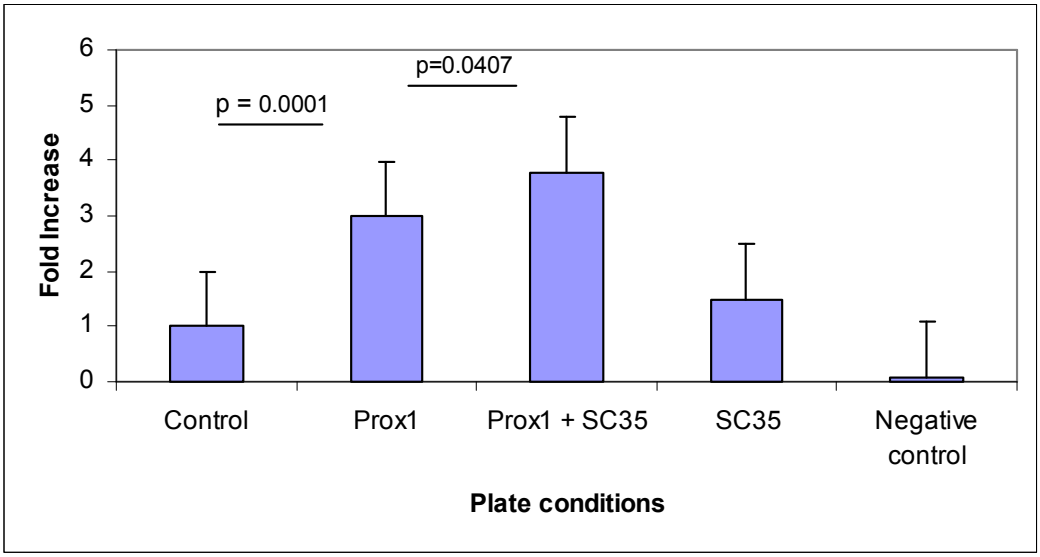

Figure 22. **Results of transfection to determine the role of SC35 using the entire βB1-crystallin promoter. Prox1-mediated activation was observed. Cotransfecting SC35 with Prox1 resulted in a significant increase in Prox1-mediated activation of the βB1-crystallin promoter.**

Chapter 6

CONCLUSIONS

With the results from this study, it appears that although Arid2 has the capacity to bind the homeo-Prospero domain of Prox1, there may be no functional significance to this interaction. Further studies on Arid2 and its possible interaction with Prox1 via a chromatin remodeling pathway may show interesting results as to how Arid2 could affect Prox1 function. Since Arid2 was previously shown to be a vital component of PBAF chromatin remodeling complexes (Yan et al. 2005), this could provide an appealing explanation as to how Prox1 could perform its diametrically opposing roles in gene expression.

Additional studies as to how Prox1 is regulated could include studying the interactions of other proteins listed in Table 4 with Prox1. As of now, my results show that SC35 may provide an essential role in mediating Prox1 function since cotransfection assays show that SC35 can enhance Prox1-mediated transcription of CAT. By studying how other proteins could affect Prox1 activity during transcription, a compelling mechanism could be discovered regarding Prox1 function. Furthermore, information gleaned from the control of developmental processes can be extremely important in understanding adult disease states such as cancer, since this may involve the expression of certain genes that mimic developmental states.

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