To Mend a Broken Heart:
Searching the Human Heart for
Novel Binding Partners of Mox
Utilizing the Yeast-2-Hybrid System

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Abstract

Strict regulation of cellular proliferation, cellular differentiation, and apoptosis is required during embryonic cardiogenesis to ensure the proper development of the cardiovascular system. Regulation of these processes is also important during the injury response of the diseased adult heart and blood vessels. Homeobox genes, such as the Mox family, have been found to play important roles in both of these processes. The Mox2 homeobox gene has been implicated in negatively regulating the cell cycle of both vascular smooth muscle cells and cardiomyocytes, but the effect of a related protein Mox1 has not been extensively studied. Although Mox1 and Mox2 are 98% identical at the amino acid level in their homeodomains, this degree of homology is not maintained in adjacent regions of the proteins. Through the use of a yeast two-hybrid (Y2H) system, a human heart cDNA library will be screened to search for proteins that interact with Mox proteins and modulate their function. Testing of the Gal4-based Matchmaker3 Y2H system has revealed Mox1 to be a much stronger transcriptional activator of Gal4 reporter genes relative to Mox2, but weaker than that of a Gal4 positive control. This is shown by activation of the GAL4 dependent reporter genes *Ade2*, *His3*, *Mel1*, and *LacZ* in the AH109 yeast strain.

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Introduction

Proliferation, migration, differentiation, and apoptosis of embryonic cells must be tightly regulated both spatially and temporally to ensure the proper morphogenesis of major organs and the survival of the embryo (Treisman *et al.* 1989). Embryonic development involves the concerted and controlled action of many gene products particularly the homeobox transcription factors, which, if they fail to function, may result in congenital diseases, uncontrolled cell growth or death of the organism (Gorski *et al*. 1993).

The **m**esenchyme/**m**esoderm homeob**ox** (*Mox*)[*] family of genes has been implicated in the proper construction and differentiation of the mesoderm (Candia *et al*. 1992; Candia and Wright 1995). The mesoderm gives rise to the vascular system (Epstein and Buck 2000), limb muscles (Ridgeway and Skerjanc 2001), and the axial skeleton (Stamataki *et al*. 2001) in both human and mouse (Minguillon and Garcia-Fernandez 2002) among other organisms (Candia and Wright 1995; Candia and Wright 1996; Stamataki *et al*. 2001; Minguillon and Garcia-Fernandez 2002). The phenotype of *Mox2* knockout mice is fused ribs and vertebrae (Mankoo *et al*. 2003), whereas *Mox2* knockout mice have defects in limb muscle development (Mankoo *et al*. 1999). Interestingly, mice null for both genes demonstrate an extremely severe phenotype, where most skeletal muscles and axial skeleton fail to form (Mankoo *et al*. 2003). This implies that joint action of these genes is not redundant and necessary for proper development of the embryo.

[*] To distinguish between genes and proteins, the former will be italicized, whereas the latter will be capitalized.

Mox2 has been implicated in negatively regulating the cell cycle of both vascular smooth muscle cells (VSMCs) and cardiomyocytes (Gorski *et al*. 1993; Fisher *et al.* 1997). Upon mitogen stimulation in adult VSMCs (Yamashita *et al.* 1997) or injury due to balloon angioplasty (Maillard *et al.* 1997), both the Mox2 gene and protein are down-regulated (Smith *et al.* 1997) allowing cells to proliferate. Forced overexpression of MOX2, on the other hand, ceases cell proliferation. It has been shown that this occurs through upregulation of the cyclin dependent kinase inhibitor, $p21^{CIP1}$, where the cell cycle is stopped at the G_o/G_1 phase (Smith *et al.* 1997) of VSMCs. The effect of the related protein, MOX1, on these cell types has not yet been examined.

The development of the vascular system, like that of any other in the organism, is a complex process involving the concerted action of many genes. The cardiovascular system is the first to begin functioning in the developing embryo in order to keep up with the nutritional requirements due to its rapid growth. One of the early steps in the development of the vascular system is the identification of the cardiac primordia at human embryonic day 18 (hE18), the murine equivalent of 7.5 days post coitum (dpc). By hE22 (8.5dpc), these structures fuse to form a two-cell layer heart tube which must undergo looping (Figure 1) in order to form the 4 chambers of the adult heart (Epstein and Buck 2000). It is believed that prior to this series of events the fate of the cells involved in this cascade is predetermined (Fisher *et al*. 1997) by a set of genes known as homeodomain genes.

Homeodomain (HD) genes were first discovered in *Drosophila melanogaster* (McGinnis *et al*. 1984). *Antennapedia* was one of the first HD containing proteins. If mutated in the HD, this protein resulted in the substitution of the fly's antennae for its

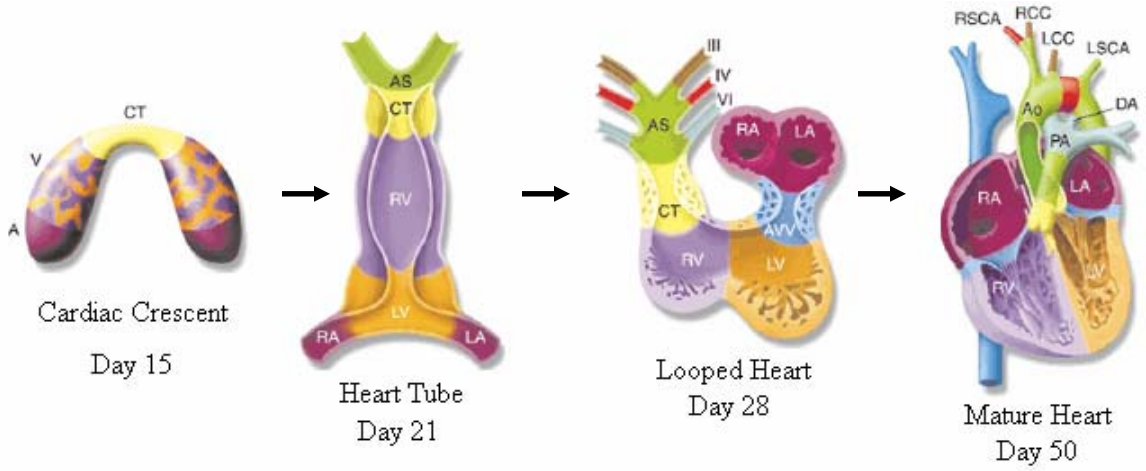

Figure 1: Looping of the heart tube to form the adult four-chambered heart. At embryonic day 15 cells are predetermined to form various structures of the adult heart. The conotruncal (CT), atrium (A), and cells that will comprise the ventricle (V) are seen. By day 21, cells from the former V are now segregated to either become part of the left or right ventricle (LV or RV). The primitive right and left atria (RA and LA) as well as the aortic sac (AS) are visible but not distinguishable from each other due to the symmetry of the heart tube. The looped heart is formed by day 28 with features such as the atrioventricular valve (AVV) and aortic arch arteries III, IV, and VI. By embryonic day 50, the heart resembles that of the adult heart. Other structures shown are aorta (Ao), ductus arteriosus (DA), left common carotid (LCC), left subclavian artery (LSCA), pulmonary artery (PA), right common carotid (RCC), and right subclavian artery (LSCA). (Adapted from Srivastava *et al.* 2000)

legs (Patel *et al*. 1992; Fisher *et al*. 1997; Gorski and Walsh 2000; Gorski and Walsh 2001; Gorski and Walsh 2003). This striking change demonstrates the important role HD genes play in development (LePage *et al.* 1994; Wigle and Oliver 1999; Bergwerff *et al.* 2000; Quinn *et al.* 2000; Delfini and Duprez 2004). The homeobox is composed of 60 amino acids which are highly conserved between organisms (Gehring *et al.* 1994a). The homeobox domain codes for a helix-turn-helix motif (Wolberger *et al*. 1991; Gorski *et al*. 1993), similar to that of *Escherichia coli* regulatory proteins CRO and CAP (Scott *et al*. 1989; Gorski and Walsh 2000; Gorski and Walsh 2003), which binds to the major groove of DNA (Gehring *et al.* 1994a). This binding may activate or repress the transcription of a gene (Treacy *et al*. 1991), and, hence, homeobox proteins are classified as transcription factors (Gehring *et al.* 1994b).

One of the major clusters of HD genes in vertebrates are the *Hox* genes that have been shown to control pattern formation in the developing embryo of many species ranging from mice (Chisaka and Capecchi 1991; Gorski and Walsh 2000) to humans (Patel *et al*. 1992; Miano *et al*. 1996) The *Mox* family of genes belongs to a group of "diverged" (Candia *et al*. 1992) or "extended" (Minguillon and Garcia-Fernandez 2002) HD genes, and, to date, contains two members, *Mox1* and *Mox2* for which homologues in humans (Gorski *et al*. 1993), mice (Candia *et al*. 1992), *Xenopus laevis* (Candia and Wright 1995), chicken (Fisher *et al.* 1997), and zebrafish (Candia and Wright 1996) have been found.

MOX1 and MOX2 share 98% amino acid sequence identity in their homeobox (Stamataki *et al*. 2001) , the sequence believed to be critical for protein and DNA binding, and the 2% differences are conservative substitutions (Figure 2). In regions

```
mMox2 MEHPLFGCLRSPHATAQGLHPFSQSSLALHGRSDHMSYP----ELSTSSSSCIIAGYPNE 56
mMox1 MDPVANSCVRNPQPPAPVWGCLRNPHSEDSSASGLSHYPPTPFSFHQKSDFPATAAYPD- 59
      *:    .*:*.*:..*     : :.     . *.   **    .:  .*.    *.**:

mMox2 EGMFASQHHRGHHHHHHHHHHHHQQQQHQALQSNWHLPQMSSPPSAARHSLCLQPDSGGP 116
mMox1 --FSASCLAATPHSLPRTERIFNEQHPAFPQTPDWHFP-----ISEAGQRLNLGP-AGSA 111
        : **      *   : .: .::*:   .  .:**:*      * * : * * * :*..

mMox2 PELGSSPPVLCSNSSSLGSSTPTGAACAPGDYGRQALSPADVEKRSGSKRKSDSSDSQE- 175
mMox1 REMGAGSPGLVDGTAGLGEDCMVLGTIAN----------ETEKKS-SRRKKERSDNQEN 159
       *:*:..* * ..::.**..  . .: *            :.**:* *:**.: **.**

mMox2 GNYKSEVNSKPRKERTAFTKEQIRELEAEFAHHNYLTRLRRYEIAVNLDLTERQVKVWFQ 235
mMox1 GGGKPEGSSKARKERTAFTKEQLRELEAEFAHHNYLTRLRRYEIAVNLDLSERQVKVWFQ 219
      *. *.* .**.**********:**************************:*********

mMox2 NRRMKWKRVKGGQQGAAAREKELVNVKKGTLLPSELSGIGAATLQQTGDSLANEDSRDSD 295
mMox1 NRRMKWKRVKGGQ---------------PVSPQEQD-------REDGDSAASP------ 250
      *************             .: *.* .      :: *** *.

mMox2 HSSEHAHL 303
mMox1 -SSE---- 253
       ***
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Figure 2: Sequence alignment of murine MOX1 and MOX2 (mMOX1 and mMOX2). The histidine/glutamine (H/Q) rich region in mMOX2 is shown in blue. The 60 amino acid homeodomain is 98% homologous in the two proteins, as shown in boldface. (Wigle 2003).

adjacent to the HD there is no conservation of sequence. The two proteins are expected to interact with the same, yet also unique proteins and DNA sequences due to the nearly identical homeobox region and the adjacent non-conserved regions, respectively. It is the non-conserved regions that most likely account for any potential differences in function between the two proteins (Chauvet *et al*. 2000; Stamataki *et al*. 2001). Of interest is a region upstream of the homeodomain of MOX2, that codes for histidine (H) and glutamine (Q) residues (LePage *et al.* 1994). This region is also termed CAX, and has been found to appear in many transcription factors (Casacuberta and Pardue 2003; LePage *et al.* 1994). Very little is known about this H/Q rich region, but Perutz *et al.* (1994) believe that extended CAX repeats are linked to neurodegenerative diseases such as bulbar and spinal muscular atrophy and Huntington disease.

Some functional overlap between the two MOX family members is expected (Desplan *et al*. 1988; Candia and Wright 1996; Gorski and Walsh 2000) and has recently been implied to occur (Mankoo *et al*. 2003). This data is consistent with that of double knockouts for many isoforms of the *Hox* clusters (Gorski and Walsh 2000; Gorski and Walsh 2003), and other homeobox containing protein pairs such as PRX1/2 (Bergwerff *et al*. 2000; Gorski and Walsh 2003), PAX3/7 (Ridgeway and Skerjanc 2001), and PAX1/3 (Stamataki *et al*. 2001).

Pax1 and *Pax3* are a group of **pa**ired-bo**x** genes containing a "paired" homeobox 128 amino acids in length, which mediate its DNA and protein interacting abilities (Bopp *et al*. 1986; Wilm *et al*. 1998). PAX1 is involved in the development of the axial skeleton (Stamataki *et al*. 2001), including the sternum and vertebral column

(Wilm *et al*. 1998), and PAX3 in the development of limb muscles (Tajbakhsh *et al*. 1997; Stamataki *et al*. 2001). To date, MOX1 and MOX2 have been shown upstream of PAX1 and PAX3 *in vivo* (Rodrigo *et al.*2003) and interact with PAX1 and PAX3 *in vitro*, respectively (Stamataki *et al*. 2001). Although weaker interactions between MOX1 and PAX3 as well as MOX2 and PAX1 have also been observed, Stamataki *et al.* (2001) hypothesize that only the stronger interactions take place *in vivo* during embryogenesis, and that the weaker *in vitro* interactions merely occur due to the homology in the homeobox regions of MOX1 and MOX2.

The interactions between MOX and PAX proteins as well as novel interacting partners of the *Mox* genes may be found utilizing a yeast two-hybrid (Y2H) system. Two types of systems with their respective properties are discussed below. Both rely on the interaction of two proteins of interest which leads to a distinguishing phenotype of the host cell, which can be either mammalian (Finkel *et al*. 1993) or yeast (Fields and Sternglanz 1994).

The first Y2H system developed was reported by Fields and Song (1989) and although many types of reporter genes have been employed in the system, most utilize the *LacZ* reporter gene and assay for β-galactosidase (β-gal) activity (Fields and Sternglanz 1994). The most commonly used protein for regulating transcription of reporter genes is GAL4 (Fields and Sternglanz 1994) within *Saccharomyces cerevisiae* and causes transcription of various galactose metabolizing proteins (Johnston 1987), one of them being β-gal. Although the full length, 881 amino acid, GAL4 protein (Laughon and Gesteland 1984) induces optimal β-gal activity (Fields and Song 1989), significant β-gal activity is seen when only the N-terminal DNA binding domain (BD) – amino acids

1-147 – and the C-terminal transcriptional activating domain (AD) – amino acids 768-881 – are reconstituted via protein-protein interactions (Fields and Song 1989).

Fusion of a bait protein (MOX) and target protein (from cDNA) to the BD and AD located on separate vectors, respectively, with subsequent introduction into the host cell, is the basis for the Gal4 Y2H (Fields and Song 1989; Fields and Sternglanz 1994). When a protein-protein interaction occurs between the bait and target, the Gal4-BD and Gal4-AD, respectively, are brought into proximity on a Gal4 upsteam activating sequence (UAS) followed by transcription of one or more reporter genes that will give rise to phenotypically distinct cells from those who do not harbour a bait-target interaction (Figure 3, Fields and Song 1989).

The Clontech Matchmaker3 Y2H system will be used to screen a human heart cDNA library for MOX binding proteins. This system utilizes two vectors: pGBKT7 and pGADT7 (Clontech 1999). The former (Figure 4A and 4B) contains the bait protein in frame with the BD, whereas the latter (Figure 4C) is a fusion product of the AD with the target cDNA.

The second Y2H system from Cytotrap, termed the son of sevenless (Sos) recruitment system (SRS), allows for growth of yeast at a restrictive temperature if the *ras* survival cascade (Reviewed in Agell *et al.* 2002) is activated by interaction of SOS (the human homologue of the yeast CDC25) with RAS (Aronheim *et al.* 1997). The bait protein is fused in frame with *Sos*, and the target in sequence with a myristoylation domain that allows for anchorage of the target to the cytoplasmic membrane. Cells with bait and target fusion proteins contain a point mutation in the native *Cdc25-2*, which does not allow for the inherent interaction of this protein with ras, therefore resulting in death

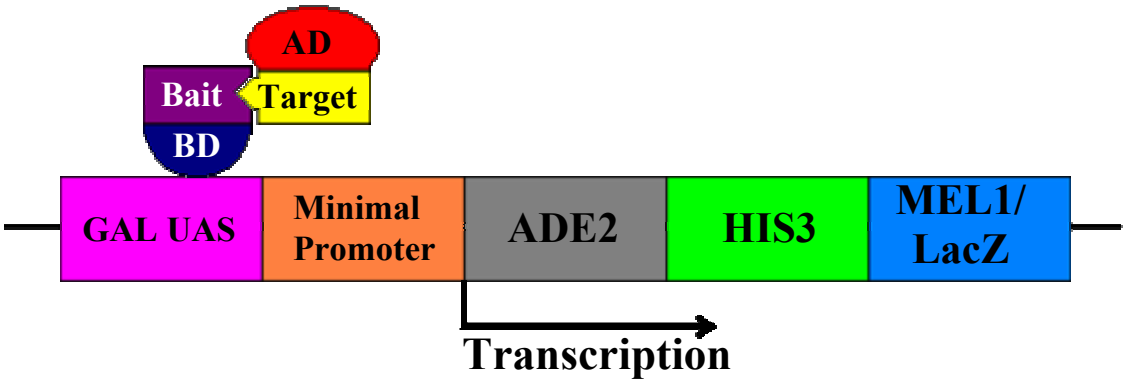

Figure 3: Gal4 based activation of four reporter genes. Matchmaker3 yeast two hybrid system (Y2H) is based on the transcription of four reporter genes: *Ade2*, *His3*, *Mel1*, and *LacZ*. The bait protein in sequence with the Gal4 binding domain (BD) is bound to the Gal4 upstream activating sequence (GAL UAS), which is next to a minimal promoter TATA box. Each reporter gene is under the control of a unique minimal promoter, simplified for illustrative purposes. When there is interaction of the bait with the target, the Gal4 activating domain (AD) in frame with the target protein, comes into proximity with the BD. This close interaction causes transcription of the four reporter genes (Adapted from Clontech 1999).

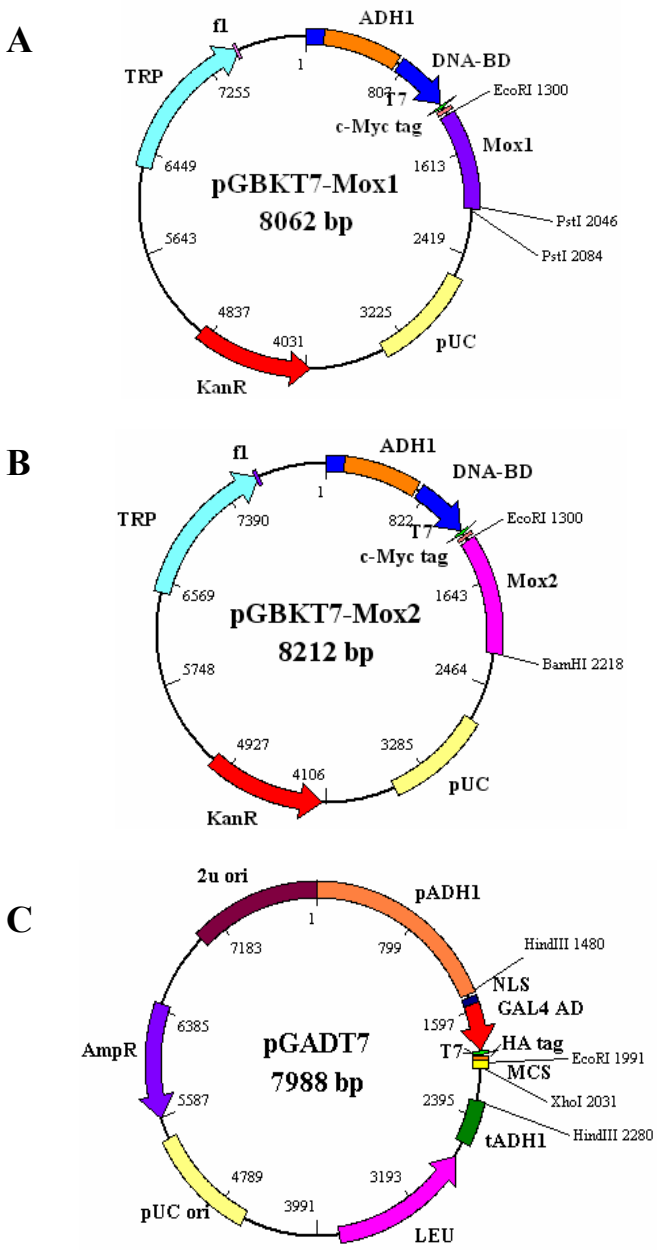

Figure 4: (**A**) pGBKT7-Mox1, (**B**) pGBKT7-Mox2, and (**C**) pGADT7 plasmid maps. Both pGBKT7 Mox constructs contain the Gal4 DNA binding domain (BD) under the constitutive control of the alcohol dehydrogenase (ADH1) promoter with c-myc epitope tag for binding anti-c-myc antibody. Kanamycin resistance in bacteria and tryptophan nutritional selective marker in yeast allow for selection of the respective organisms that contain this construct. The pGADT7 plasmid (**C**) has the Gal4 activating domain (AD) also under the control of the constitutively active ADH1 promoter. A hemagglutinin epitope tag, ampicillin resistance in bacteria, and a leucine nutritional selective marker are features of this plasmid, which will contain cDNA from a heart library within the multiple cloning site (MCS).

of the cells at a restrictive temperature. If, on the other hand, the bait and target interact, SOS fused to the bait comes in proximity of RAS, and these two proteins interact as well (Figure 5). RAS is activated and survival of the yeast at a restrictive temperature occurs (Aronheim 1997).

A comparison of the two Y2H systems reveals each of their advantages and limitations. One of the main advantages of Matchmaker3 is its use of four reporter genes: *Ade2*, *His3*, *Mel1*, and *LacZ*. This set of reporter genes allows for a stringent screening process that minimizes the incidence of false positive bait-target interactions (Clontech 1999). The weakness of this Y2H system occurs when the bait protein utilized for screening is itself a transcriptional activator. In this case, the AD is not needed to exert the distinguishing phenotype(s) of the cells (Fields and Sternglanz 1994). Additional steps can be taken during the screening in order to minimize the weak activation by the bait, such as supplementing media with 3-amino-1,2,4-triazole (3-AT) to suppress leaky histidine expression. A major advantage of the Cytotrap Y2H is that it allows for the extranuclear interaction of the bait and target (Stratagene 2004). As well, it does not rely on the transactivation of a protein in order to exhibit the yeast phenotype, therefore a transcriptional activator can still be used as bait. Finally, post-translational modifications of the bait protein can be attained with this system, due to the cytoplasmic localization of the bait, where these modifications occur. Since yeast survival is dependent on only a point mutation in *Cdc25*, revertants that lose this phenotype are common and cause a high background in this assay.

It is the purpose of this study to find novel partners of the Mox family members by screening a human heart cDNA library through the use of a GAL4 based Y2H.

Discovering these new binding partners will bring science one step closer to elucidating the exact mechanism by which this transcription factor exerts its function and, in turn, a step closer to controlling acceleration and suppression of vascular tissue growth, which is necessary for maintaining homeostasis of cells comprising the tissue.

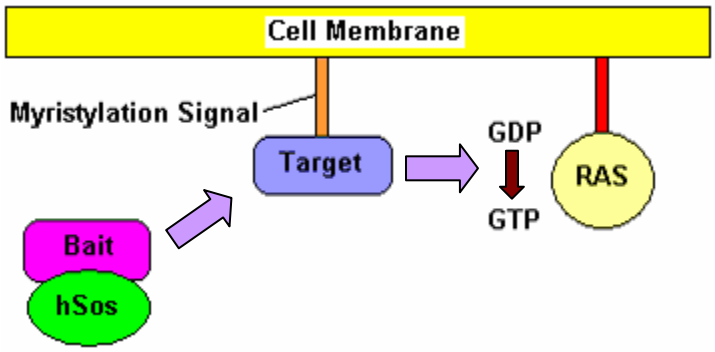

Figure 5: RAS based yeast two hybrid system. The target protein of interested is anchored to the cytoplasmic membrane through a myristoylation signal. If the bait (in frame with the hSOS protein) and target interact, hSOS will come into close proximity of the membrane-bound RAS protein. hSOS will cause the exchange of GDP for GTP, thereby activating RAS and the RAS/RAF/MEK pathway, allowing for the temperature sensitive Cdc25-2 yeast to grow at the restrictive temperature of 37^{o}C. (Adapted from Stratagene 2004).

Materials and Methods

2.1 Bacterial Transformation.

Calcium competent *Escherichia coli* DH5α strain was transformed using the heat shock method (Inoue *et al.* 1990). 5μL of supercoiled/circular or 20μL of ligated DNA were incubated on ice for 30 minutes with 250μL of DH5α cells, heat shocked at 37^{o}C for exactly two minutes, placed on ice for two minutes, followed by addition of one mL Luria-Bertani (LB) broth prior to shaking (350 rpm) at 37^{o}C for one hour. This shaking step was necessary for the expression of antibiotic resistant proteins. 200μL and 400μL aliquots of the transformants were plated on LB agar with appropriate antibiotic: kanamycin or ampicillin at 50μg/mL of agar. Plates were then incubated for 16-18 hours at 37^{o}C.

2.2 DNA Isolation and Gel Extraction.

(i) Standard Alkaline Lysis Miniprep. 5mL of LB broth containing appropriate antibiotic (50μg/mL) were inoculated with a DH5α colony from a selective plate, and incubated with shaking for 16-18 hours at 37^{o}C. 1.5mL of culture was transferred to a microcentrifuge tube and cells were pelleted (5.7krpm, 1min). Discarded supernatant and re-spun for 30 seconds, again followed by removal of supernatant. Cells were resuspended by vortexing in 200μL of GTE (0.9% glucose, 0.01M EDTA, 0.025M Tris ph 8.0) followed by a five minute incubation at room temperature. 400μL of lysis solution (0.2M NaOH, 1% SDS) and 300μL of ice cold 3M potassium acetate were added, each followed by gentle mixing and 5 minute incubations on ice. Pelleted cell lysate (13.2krpm, 5min) and carefully transferred supernatant only to fresh tube. 540μL of isopropanol was added and mixed by vortexing, followed by incubation at room

temperature for two minutes. The nucleic acid was pelleted by spinning at 13.2 krpm for two minutes and the supernatant was discarded followed by two washings with 70% ethanol (EtOH). Once dry, the pellet was resuspended in 50μL TE (pH 8.0) and 1μL RNAse (10mg/mL) for 30 minutes at room temperature.

(ii) QIAGEN Miniprep. For greater purity and concentration of recovered DNA, QIAprep Spin Miniprep Kit (QIAGEN #27106) was utilized and DNA extraction was performed as per "QIAprep Spin Miniprep Kit Protocol using a microcentrifuge" procedure. This DNA was used for sequencing and subsequent transformations into yeast.

(iii) QIAGEN Maxiprep. Large scale DNA recovery was achieved by use of QIAfilter Plasmid Maxi Kit (QIAGEN #12263) as per QIAfilter plasmid DNA purification protocol.

(iv) DNA Gel Extraction. Bands from the agarose gel were visualized by a low intensity UV light for a minimum amount of time to prevent damage of the DNA. Utilizing a scalpel, slices of the DNA bands of interest were isolated. QIAquick Gel Extraction Kit (QIAGEN #28704) was utilized for extracting DNA samples from the agarose gel slices.

(v) Protein Extraction from DNA. The total volume of the DNA solution within the centrifuge tube was brought to 500μL with double distilled water (ddH_2O) and an equivalent volume of phenol/chloroform/isoamyl alcohol (pH 6.7, FisherBiotech #BP1752) was added. After centrifuging (10krpm, 10min), the upper aqueous layer containing protein-free DNA was removed, and the lower organic layer was properly disposed of.

(vi) Ethanol Precipitation. Combined 2uL of liver glycogen, one-tenth volume of 3M sodium acetate (pH 5.2), and 2.5 volume of 100% ethanol (EtOH) to a microcentrifuge tube of DNA and mixed well. Incubated at -20^{o}C for 14-18 hours, or -80^{o}C for 20 min, then centrifuged for 30 minutes at maximum speed. The pellet was washed twice with 70% EtOH, centrifuging two minutes in between washings, then allowed to air dry. ddH_2O was used to resuspend the pellet.

2.3 Vectors.

(i) pGBKT7. pGBKT7 vector (7.3 Kb) from Clontech contains the Gal4 DNA-BD under the constitutive control of the abridged *S. cerevisiae* alcohol dehydrogenase promoter (pADH1). A human myc proto-protoncogene (c-myc) epitope tag 5' to the multiple cloning site (MCS), created a fusion with the bait protein for use in Western blotting. The pUC origin and 2μ origin allow for autonomous replication of this plasmid in *E. coli* and *S. cerevisiae*, respectively. *E. coli* transformed with this plasmid gain kanamycin resistance (kan^{r}) and the tryptophan (TRP1) nutrition marker, which is utilized for selection in yeast. Suitable yeast strains host used are AH109, Y187, Y190, SFY526, CG1945, and HF7c.

(ii) pGADT7. The Clontech pGADT7 vector (8.0 Kb) contains the Gal4 DNA-AD also under control of the pADH1 promoter. The hemagglutinin (HA) epitope tag creates a fusion with the target protein for use in Western blot analysis. Similar to pGBKT7, the pUC origin in *E. coli* and the 2μ origin in *S. cerevisiae* allow for independent replication of this vector in their respective organisms. This vector gives *E. coli* resistance to ampicillin (amp^{r}) and allows for nutritional selection in *S. cerevisiae* due to the leucine

(LEU2) marker. Suitable yeast strains host used are Y187, Y190, SFY526, CG1945, HF7c, and AH109.

(iii) pCL1. This positive control vector contains the full length sequence of the Gal4 protein and the selective markers within *E. coli* and *S. cerevisiae* same as for pGADT7.

2.4 Plasmid Constructs.

(i) pGBKT7–Mox1, –Mox2, and –Mox2ΔH/Q. The empty pGBKT7 vector was isolated by transformation of DH5α with subsequent large-scale plasmid recovery as described above. Restriction cuts were made with *Bam*HI restriction endouclease (RE) (37^{o}C, 2 hours) and *Eco*RI RE (37^{o}C, 1 hour). The cut plasmid DNA was run on a 1% agarose gel and the insert was purified.

Mox1 coding sequence was amplified by polymerase chain reaction (PCR) utilizing primers Mx11 (5'-GG**GAATTC**ATGGATCCAGTGGCCAAC-3')[†] and Mx58 (5'-GC**AGATCT**TCACTCTGAACTTGGAGAAGC-3') and pBluescript–*Mox1* as template. pBluescript–*Mox2* was used as template to PCR amplify the inserts with primers Mx12 (5'-GG**GAATTC**ATGGAACACCCGCTCTTTGG-3') and Mx57 (5'-GC **GGATCC**TCATAAGTGCGCATGCTCTG-3'). Mx11 and Mx12 primers incorporate an *Eco*RI site at the 5' ends and primers Mx57 and Mx58 incorporate *Bam*HI and *Bgl*II sites into their respective 3' ends of the PCR products. A one minute extension time was allotted for each respective reaction with Pfx DNA polymerase. PCR products were purified and isolated by phenol:chloroform and EtOH precipitated respectively. Each insert was cut with the RE for which sites were incorporated by PCR. The *Mox2* gene with the deletion in the H/Q region (ΔH/Q) was created previously by this lab with an *Eco*RI at the 5' end and a *Bam*HI RE site at the 3' end. The *Mox1* and *Mox2* PCR

[†] Restriction sites within the primers are in boldface.

products, as well as the *Mox2ΔH/Q* insert were each ligated into an aliquot of the previously restriction endonuclease (RE)-cut pGBKT7 vector. This allowed for formation of the three constructs: pGBKT7–*Mox2* and pGBKT7–*Mox2ΔH/Q* constructs within *Eco*RI and *Bam*HI RE sites, and pGBKT7–Mox1 with an *Eco*RI RE site only. Due to the cohesive compatible ends of pGBKT7 *Bam*HI and *Mox1 Bgl*II, ligation was possible with concurrent elimination of the respective RE sites. Ligations were carried out by use of T4 DNA ligase at 18^{o}C for 17 hours then heat killed (65^{o}C, 20min). Constructs were amplified as above and sequence verified at the University of Calgary.

(ii) pGADT7–Mox1 and –Mox2. One aliquot of amplified pGADT7 vector was cut with *Bam*HI (37^{o}C, 2 hours) and *Eco*RI (37^{o}C, 1 hour). The second aliquot was cut with *Sal*I (37^{o}C, 1 hour) and *Eco*RI (37^{o}C, 1 hour). *Mox1* and *Mox2* were excised out of the pGBKT7 constructs from above with *Xho*I/*Eco*RI and *Bam*HI/*Eco*RI, respectively (37^{o}C, 2hours/1hour). Each vector and insert was purified from a 1% agarose gel (25Volts, 17-20 hours) by the QIAquick gel extraction method above, followed by ligation of *Mox2* (*Eco*RI/*Bam*HI) into pGADT7 cut with *Eco*RI and *Bam*HI, and Mox1 (*Eco*RI/*Xho*I) into pGADT7 (*Eco*RI/*Sal*I). Ligations were heat killed as above and transformed into DH5α for selection and amplification. DNA was isolated by the alkaline lysis miniprep method, followed by a large-scale DNA isolation for further use.

2.5 Yeast Transformation.

Small scale yeast transformations were carried out as in "Library Transformation & Screening Protocols" (Clontech 1999). 50mL of YPDA [2% Difco peptone (BD Biosciences #211677), 1% yeast extract (BD Biosciences #212750), 0.003% adenine hemisulfate salt (Sigma #A-9126)] broth were inoculated with a fresh (1-3 week old)

AH109 colony and let incubate (30°C, 16-18 hours) with shaking (250 rpm). Once stationary phase growth was established (OD_{600} > 1.5), cells were transferred to 300mL of YPDA broth (OD_{600} 0.2-0.3) and continued to shake (30°C, 2-3 hours, 250 rpm) until an OD_{600} of 0.4-0.6 was reached. Cells were pelleted (1,000 g, 5 min, room temperature), then resuspended in 25 mL of sterile water. Cells were pelleted again, then resuspended in 1.5mL of tris-EDTA/lithium acetate (TE/LiAc) [0.01M Tris-HCl and 1mM EDTA (pH 7.5), 0.1M LiAc (pH 7.5)]. 0.1µg of plasmid DNA and 0.1mg of herring sperm DNA (Promega #D181B) were added to 0.1mL of the freshly prepared competent yeast then vortexed to mix. To this mixture, 0.6mL of polyethylene glycol (PEG)/LiAc solution [40% PEG (Sigma #P-3640), 0.01M TE, 0.1M LiAc] was added, mixed, and allowed to shake (200 rpm, 30°C, 30 min). After this incubation, 70µL of DMSO were added, followed by gentle mixing. Cells were heat shocked at 42°C for 15 minutes, then chilled on ice for two minutes. Cells were pelleted (8 seconds, 13.2krpm), the supernatant was discarded, then cells were resuspended in 0.5mL of TE (pH 7.5). Transformed *S. cerevisiae* were diluted two-fold, five-fold, and ten-fold prior to plating onto appropriate synthetic dropout (SD) agar [0.67% w/v Difco yeast nitrogen base without amino acids (BD Biosciences #291940), 2% dextrose (Fisher Scientific #D14), Difco agar (BD Biosciences #214530)]. *S. cerevisiae* were transformed with all three pGBKT7 constructs, the pCL1 positive control, and empty pGBKT7 and pGADT7 as negative controls.

2.6 Galactosidase Assays.

(i) X-α-Gal. α-Galactosidase activity was assayed by plating transformed cells on appropriate SD media supplemented with 5-bromo-4-chloro-3-indoyl-α-D-

galactopyranoside (X-α-Gal, BD Biosciences #8061-1). X-α-Gal was diluted in N,N-dimethyl formamide (DMF, Fisher Scientific #D119-1) to a final concentration of 3mg/mL. Plates were incubated from 3-6 days at 30°C.

(ii) X-β-Gal Filter Assay. Assay was carried out as in "Colony-lift Filter Assay" (Clontech 2001) with slight modification. Fresh colonies (2-4 days) were imprinted onto a paper filter. The filter was frozen at -80°C for 12 minutes, then allowed to thaw at room temperature for 10-12 minutes. Once thawed, the filter was placed colonies side up onto a X-β-gal/Z buffer [1.61% w/v $Na_2HPO_4 \cdot 7H_2O$, 0.55% w/v $NaH_2PO_4 \cdot H_2O$, 0.075% w/v KCl, 0.0246% w/v $MgSO_4 \cdot 7H_2O$, 0.027% v/v β-mercaptoethanol (Sigma #M-6250), 0.167% v/v X-β-gal (20mg/mL in DMF)] presoaked filter followed by incubation of the filters for three hours at 30°C.

2.7 Suppression of Leaky Histidine Expression.

For suppression of histidine expression, 3-amino-1,2,4-triazole (3-AT, Sigma #A8056) was incorporated into the proper SD agar prior to solidifying (60°C). Varying concentrations of SD 3-AT plates were made: 2.5mM, 5.0mM, 7.5mM, 10.0mM, 12.5mM, and 15.0mM, and no 3-AT as control. pGBKT7 and constructs thereof were plated on SD/-His/-Trp for each concentration of 3-AT. pCL1, pGADT7, and pGADT7-T/pGBKT7-LamC containing AH109 were plated on each 3-AT concentration of SD/-His/-Leu. Untransformed AH109 yeast were also streaked on all plates as controls for the dropout media.

2.8 Protein Extraction.

In preparation for Western blotting, extraction of protein was accomplished by the "Urea/SDS Method" (Clontech 2001). Manipulation of the pellet from the first centrifuge step was omitted.

2.9 Western Blotting and Antibodies.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was accomplished by use of a 3% stacking gel [3% acrylamide mix, 0.38M Tris (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.08% TEMED] and 12% resolving gel [12% acrylamide mix, 0.38M Tris (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 0.08% TEMED]. Transfer of protein was achieved by the semi-dry method onto nitrocellulose membrane (Bio-Rad #162-0115) at 240mA for 2 hours. Membranes were fixed using Ponceau S stain (0.1% Ponceau S w/v in 5% acetic acid) for five minutes at room temperature with light shaking, followed by treatment with blocking buffer [5% milk protein (mp) in TBS pH 8.0] for 30 minutes. Western blotting of pGBKT7 constructs required the use of an anti-myc antibody (1:500) with 5% mp in TBS for 15-18 hours at 4^{o}C with gentle rocking. A goat anti-mouse antibody conjugated to horse radish peroxidase (1:4800, 5%mp in TBS) was utilized as secondary antibody for 60 minutes. Horseradish peroxidase chemiluminescent (Pierce #34075) substrate was added to the membrane and incubated for 5 minutes.

Results

3.1 pGBKT7 Mox Constructs

pGBKT7-Mox1 and -Mox2 vectors were each digested with restriction endonucleases to confirm the presence of the inserts within the MCS regions (Figure 6). pGBKT7-Mox2 cut with RE *Eco*RI and *Bam*HI showed the predicted 918 base pair (bp) Mox2 insert. (Figure 6) Mox1 (*Eco*RI/*Bgl*II) from PCR (758 bp) was ligated into pGBKT7 (*Eco*RI/*Bam*HI). Cohesive compatible ends between the *Bgl*II and *Bam*HI sites allow for the ligation to take place with concurrent elimination of both of these RE recognition sites. A *Pst*I RE site, 12 bp upstream of the destroyed RE recognition site, allowed for the Mox1 fragment (746 bp) to be released from the vector with a double EcoRI/PstI digest (Figure 6). Insert sequences were verified at the University of Calgary.

3.2 pGADT7 Mox Constructs

pGADT7-Mox2 was verified by cutting with *Eco*RI and *Bam*HI RE. A Mox2 insert 918 bp in length was excised, as shown by lane C of Figure 7A. Lane UC displays the uncut pGADT7-Mox2 plasmid as a control. Figure 7B, lane C shows pGADT7-Mox1 digested with *Pst*I and *Eco*RI. The presence of four *Pst*I sites and one *Eco*RI site allows for visualization of five bands with sizes of 3490 bp, 2470 bp, 1978 bp, 746 bp, and 38 bp. Due to the size of the smallest fragment, it migrated out of the gel. Mox1 is contained within the 746 bp fragment and shows an identical mobility to that of a Mox1 gel purified insert (774 bp, *Eco*RI/*Sal*I) that was loaded in lane M1 as a reference. This band is only 12 bp shorter than that of Mox1 in lane C, but this difference is too small to detect with the resolution of a 1% agarose gel.

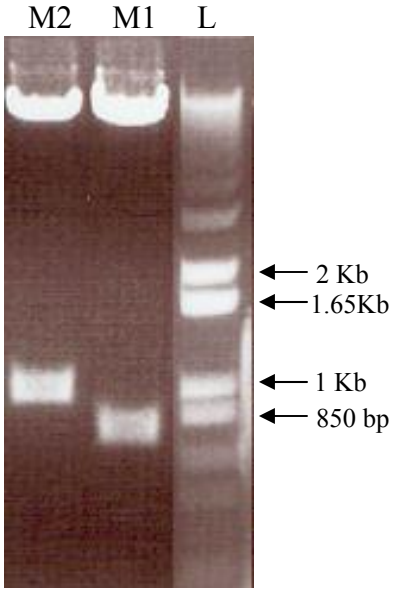

Figure 6: pGBKT7-Mox2 and pGBKT7-Mox1 constructs. 1% agarose gel (100V, 60 minutes) with lane M2 containing the pGBKT7-Mox2 cut with restriction endonucleases (RE) *Eco*RI and *Bam*HI, releasing the 918 bp fragment. Lane M1 shows a band near 800 bp, which is the pGBKT7-Mox1 cut with RE *Eco*RI and *Pst*I, releasing the 746 bp Mox1 fragment. A 1 kbp DNA ladder (lane L) marked with sizes of relevant bands is also shown.

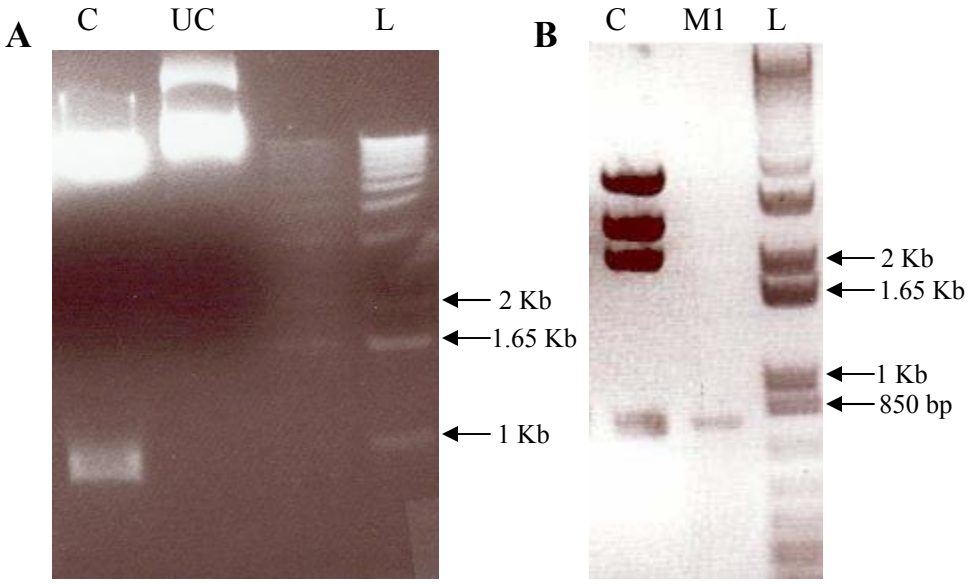

Figure 7: pGADT7-Mox2 and pGADT7-Mox1 constructs. 1 Kb DNA ladders (lanes L) are marked with relevant band sizes. Both gels are 1% agarose, ran at 100V for 60 minutes. **A.** pGADT7-Mox2 cut with *Eco*RI and *Bam*HI (lane C) and uncut (lane UC) as a control to verify that the constructs contained the Mox2 insert. **B.** pGADT7-Mox1 cut with *Eco*RI and *Pst*I (lane C), and Mox1 (*Eco*RI/*Sal*I) from a gel purification in lane M1.

3.3 X-α-Gal Hydrolysis by α-galactosidase

α-galactosidase is capable of hydrolysing X-α-gal (3mg/mL) into a blue derivative outside of the yeast cell, since this enzyme is secreted. Figures 8A and 9A do not contain X-α-gal in the medium, whereas figures 8B and 9B show the effect of α-galactosidase on X-α-gal supplemented SD/-Leu and SD/-Trp media, respectively. SD/-Leu selects for colonies containing pGADT7 constructs and the positive control vector pCL1. pCL1-containing AH109 colonies are clearly blue in color, whereas the negative control pGADT7 vector remains red, as in figure 8A. SD/-Trp media (figure 9) selects for AH109 colonies containing pGBKT7 and all constructs thereof. The empty pGBKT7 vector-containing yeast, as well as AH109 containing the Mox2 and Mox2ΔH/Q pGBKT7 constructs are negative for α-galactosidase activity, since colony colour is the same in figure 9A as in 9B. pGBKT7-Mox1 colonies are blue on X-α-gal supplemented media, but a much weaker blue than the pCL1 positive controls.

3.4 β-galactosidase Activity Measured by X-β-Gal Filter Lifts

β-galactosidase is coded for by the LacZ gene, and is not secreted by the AH109 yeast cells. Yeast cells must be lysed in order for this enzyme to hydrolyse X-β-gal into its blue derivative. The filter lift assay on for β-galactosidase activity is a more vivid blue indicator than the α-galactosidase assay (figure 10). In figure 10A, only yeast containing the positive control vector, pCL1, are dark blue. Negative controls, T-Lam (AH109 cotransformed with pGADT7-T and pGBKT7-Lamin C) and cells containing pGADT7-T alone, display no growth on the lift from an SD/-Leu plate (Figure 10A). An X-β-gal paper filter lift was also conducted from a SD/-Trp plate (Figure 10B).

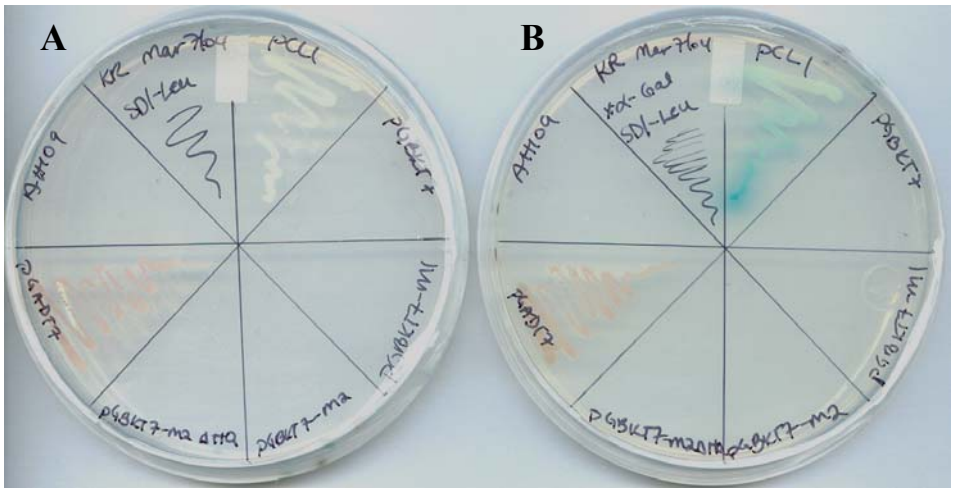

Figure 8: X-α-gal assay on SD/-Leu plates. This media selects for pCL1 positive control vector and pGADT7 constructs. Plates are without (**A**) and with (**B**) X-α-gal (3mg/mL) supplemented media, incubated at 30°C for 6 days. Cells carrying the pCL1 vector transcribe the *Mel1* gene, producing the α-galactosidase reporter, capable of hydrolyzing X-α-gal to its blue derivative. pGADT7 acts as negative control in that no blue color is observed. Untransformed AH109 were streaked as a control for the dropout media, no growth was observed. All yeast carrying the pGBKT7 constructs were streaked on this plate and no growth was observed, as this media selects for yeast capable of producing leucine.

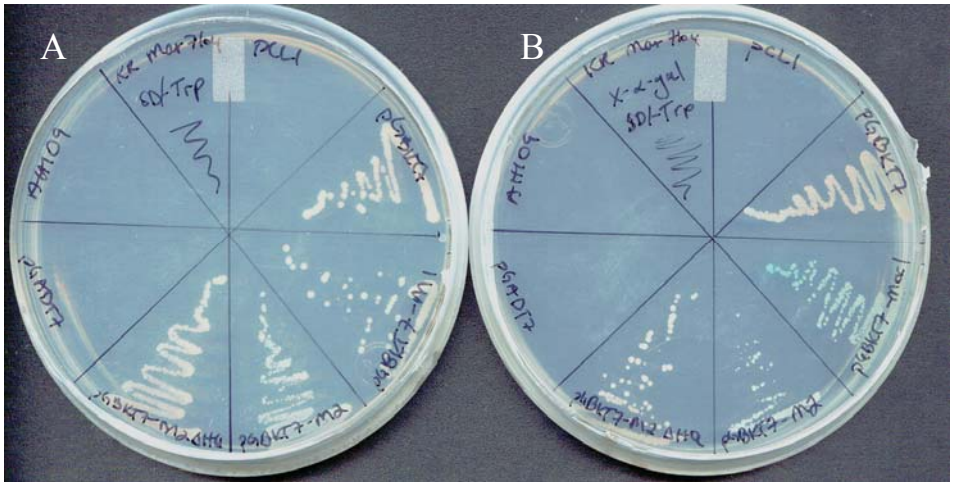

Figure 9: X-α-gal assay on SD/-Trp plates. This media selects for pGBKT7 constructs, as this vector carries a tryptophan selective marker in yeast. Plate **A** is without X-α-gal and plate **B** is with X-α-gal (3 mg/mL) supplemented media incubated at 30°C for 6 days. The negative control empty pGBKT7 vector shows no blue coloration. pGBKT7-Mox1 containing AH109 cells are faintly blue, indicating production of the *Mel1* gene product, α-galactosidase. As a control for the dropout, untransformed AH109 do not grow on media lacking tryptophan.

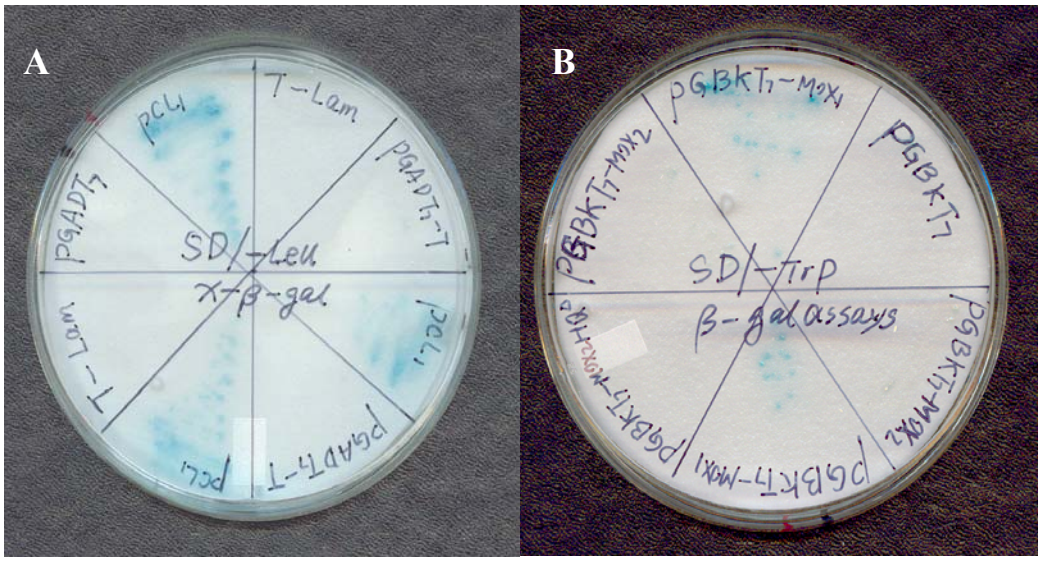

Figure 10: X-β-gal assay on nitrocellulose filters. Filter lift in **A** is from an SD/-Leu plate. All three positive control vector pCL1 sectors display heavy blue coloration in colony imprints. Negative control sectors, T-Lam (AH109 cotransformed with pGADT7-T and pGBKT7-LamC) and pGADT7-T alone, do not produce the blue derivative of X-β-gal hydrolysis. **B** was filter lifted from an SD/-Trp plate, which selects for yeast containing pGBKT7 constructs. Yeast containing the empty pGBKT7 vector, and those with the pGBKT7-Mox2ΔH/Q did not hydrolyse X-β-gal (no blue colour in these sectors). Although very faint, AH109 containing the pGBKT7-Mox2 construct was blue near streak edges. pGBKT7-Mox1 transformed yeast showed a heavier blue colour, but less than that of the pCL1 positive control. Blue in these colonies was observed near areas of heavy growth in these streaks. Imprinted plates were incubated at 30^{o}C for 6 days.

3.5 Testing activation of His by bait plasmids

To suppress background growth on media lacking histidine, 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the HIS3 protein, is added to the media. pGBKT7-Mox2ΔH/Q and pGBKT7-Mox2 transformed AH109 displayed minimal growth on low stringency media lacking 3-AT similar to the empty vector pGBKT7 (Figure 11A). The lowest concentrations of 3-AT that suppressed growth of the each yeast containing one of the three constructs was 5mM (Figure 11B). pGBKT7-Mox1 transformed AH109 continued to grow at 15mM 3-AT after six days of incubation at 30^{o}C, and grew even at a concentration of 40 mM (not shown). At 15mM 3-AT pGBKT7-Mox1 AH109 colonies were $\leq$1mm in diameter, smaller than those observed at lower concentrations of 3-AT.

3.6 Testing for Transactivation of the Ade2 gene by the Mox baits

AH109 colonies grown on media supplemented with minimal or no adenine turn pink/red in colour, unless they are capable of producing their own adenine. This rescue occurs either when a complete Gal4 protein is present, or when the Gal4 DNA-BD and a Gal4 AD interact via the bait binding to the prey protein, which results in transcription of the *Ade2* gene allowing these cells to synthesize their own adenine. Media that includes adenine (Figure 12A) allows the growth of yeast containing all pGBKT7 constructs (empty vector, Mox1, Mox2, and Mox2ΔH/Q) as well as the positive control (AH109 cotransformed with pGBKT7-53 and pGADT7-T). Not including adenine in the media (Figure 12B), selects for only those cells which can synthesize their own. The media in figure 12B is enough to suppress the growth of cells containing pGBKT7 and the Mox2 and Mox2ΔH/Q vectors, but not those which harbour the pGBKT7-Mox1 as bait.

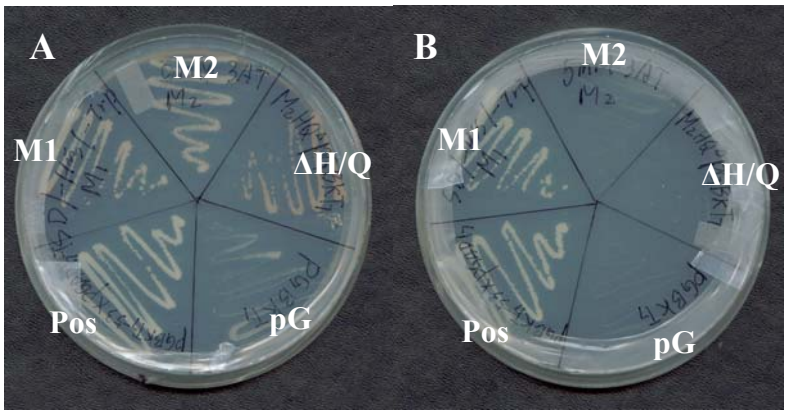

Figure 11: 3AT assay on SD/-His/-Trp plates. An unsupplemented plate (**A**) with AH109 cells transformed with an empty pGBKT7 (pG) and its constructs. Growth is observed in all sectors. As low as 5mM 3-AT (**B**) suppressed growth of yeast containing the empty pGBKT7, as well as those containing the Mox2 (M2 and ΔH/Q) constructs. pGBKT7-Mox1 (M1) yeast growth persists at this concentration, although colony size is smaller than that of the positive control (Pos). The positive control (Pos) is AH109 cotransformed with pGBKT7-53 and pGADT7-T. Plates were incubated for six days at 30°C.

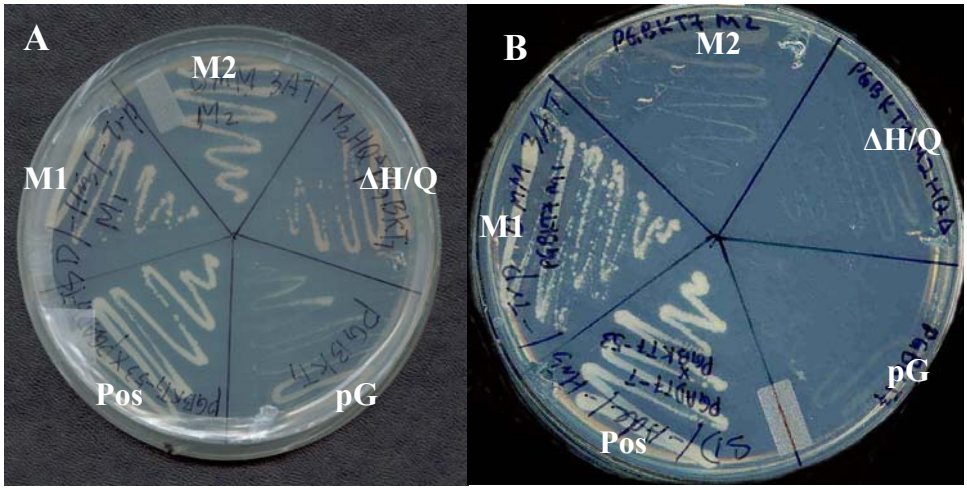

Figure 12: Adenine selection in AH109 yeast. SD/-His/-Trp plate (**A**) and SD/-Ade/-His/-Trp (**B**) after five days of growth at 30°C. The positive control (Pos), consisting of AH109 cotransformed with pGADT7-T and pGBKT7-53, fully rescues cells by transcribing the ADE2 reporter gene coding for adenine. As well, cells containing the Mox1 construct (M1) are rescued to a smaller extent by transcribing the ADE2 gene. Omitting adenine from the media stops growth of cells containing pGBKT7 (pG) and the Mox2 constructs (M2 and ΔH/Q), as adenine is not produced by these yeast.

Growth of these colonies (M1 in figure 12B) is smaller in size when compared to those of the positive control (Pos in Figure 12B).

3.7 Expression of bait proteins in AH109

c-Myc epitope is fused to Gal4 within the pGBKT7 vector. The empty vector (lane pG, figure 13) shows a band on a Western blot at 22kDa, which corresponds to the weight of the Gal4 BD. The Gal4-Mox1 fusion protein bands appear at 50kDa (lane M1), Gal4-Mox2 at 55kDa (lane M2), and Gal4-Mox2ΔH/Q at 51kDa (lane ΔH/Q).

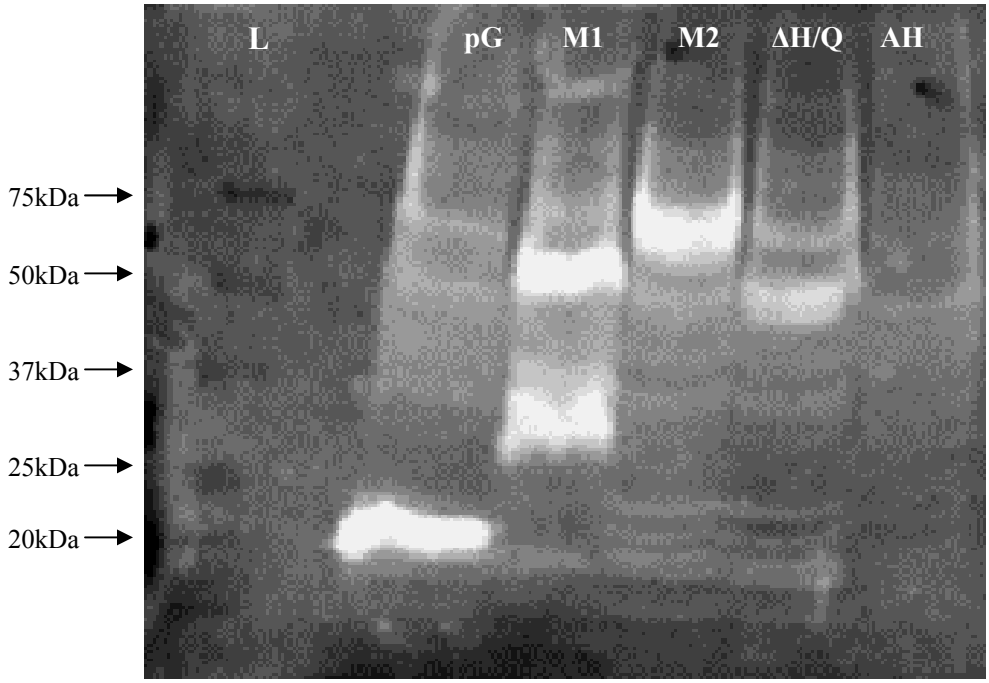

Figure 13: Western blot of AH109 yeast transformed with various constructs. AH109 are transformed with pGBKT7 empty vector (lane pG), and pGBKT7 constructs: Mox1 (lane M1), Mox2 (lane M2) and ΔH/Q. Primary antibody is c-Myc monoclonal antibody, and secondary antibody is a goat-anti-mouse antibody conjugated to horseradish peroxidase. Lane pG shows the Gal4 DNA binding domain at approximately 22kDa. M1, M2, and ΔH/Q Gal4 fusion proteins are at their respective weights of 50kDa, 55kDa, and 51kDa. Lane AH contains the untrasformed AH109 as a control to show that there is no c-myc epitope in these cells, unless they are transformed with it.

Discussion

4.1 Gal4 BD and Mox Protein Expression in AH109

The MOX proteins are all expressed within AH109 after transformation of these cells with pGBKT7-Mox1, pGBKT7-Mox2, and pGBKT7-Mox2ΔH/Q bait vectors. AH109 yeast which did not contain any DNA vector constructs displayed no bands on the Western blot. This lane contained a great deal of protein when the membrane was treated with Ponceau S stain (not shown) due to the rapid growth of these cells in YPDA media. This indicates that the AH109 cells do not intrinsically produce the c-myc epitope tag, unless it is introduced into them. In the yeast transformed with the empty pGBKT7 vector, a band of 22 kDa was detected, which corresponds to the predicted molecular weight of the Gal4 DNA BD fused to the c-myc epitope tag. In other transformed yeast, all of the baits (Mox1, Mox2, and ΔH/Q, each fused to the c-myc epitope tag and the Gal4 DNA BD) were produced with their respective weights of 50 kDa, 55 kDa, and 51 kDa. This indicates that the fusion proteins created are, in fact, in frame with one another and that they are not toxic to the yeast. One anomaly is the Mox1 bait, in that a smaller molecular weight (near 31 kDa) fragment appears along with that of the expected size of the fusion protein. Post transcriptional modification through cleavage of the Mox1 fusion protein may be a possible reason for the approximately 19 kDa decrease in molecular weight. Protein extracts from all three Mox transformed cells appear to be equally expressed but to a lesser extent relative to the pGBKT7 transformed cells.

4.2 Four Reporter Genes Point to Mox1 as the Strongest Transactivator

The four tests, one for each of the reporter genes (*Ade2*, *His3*, *Mel1*, and *LacZ*) all show Mox1 to be the strongest transcriptional activator, relative to Mox2 and Mox2ΔH/Q, yet

weaker than either of the positive controls. The full-length Gal4 protein, coded for within the pCL1 vector, is expected to activate the strongest. The binding and activating domains of the Gal4 protein, to which the bait and target are bound respectively, are not enough to exert the maximal function of the Gal4 protein (Fields and Song 1989) if the bait and target only weakly interact. The second positive control focused on the strong interaction between two proteins, murine p53 (within the pGBKT7-53 vector) and SV40 large T-antigen found within the pGADT7-T vector (Clontech 2003).

Mox2 is a slightly stronger activator than that of the H/Q deleted isoform, but weaker than that of Mox1, as shown by the X-β-gal assay. Previous studies of the H/Q region of transcription factors have shown that removal of these amino acids decreased transcriptional activity by half (Perutz *et al.* 1994). The H/Q region of Mox2 may, therefore, function to weakly mediate the transactivational potential of the protein. No blue colour was observed on X-α-gal supplemented media for Mox2-containing yeast. Since α-galactosidase is a secreted enzyme, and Mox2 acts very weakly as a transcriptional activator, the amount of this enzyme produced by these cells may not be enough to exhibit a noticeable colour change in the media. The X-β-gal assay is a more sensitive method of detecting galactosidase activity than that of the X-α-gal assay, hence blue colour is observed by the former method with the weakly transactivating Mox2 AH109 cells.

AH109 are "leaky" with respect to transcription of the *His3* gene product such that low levels of *His3* transcription occurs in these cells in the absence of any exogenous Gal4 function. This basal level of expression was suppressed by incorporating 3-amino-1,2,4-triazole (3-AT) into the media. Growth of yeast containing either of the Mox2

constructs and the empty pGBKT7 vector were suppressed by low concentrations of 3-AT (2.5 mM 3-AT). Yeast containing Mox1 were still capable of growing at concentrations of 3-AT as high as 40mM. This indicates that Mox1 is actively inducing the transcription of the *His3* gene product, thus permitting their growth, even in the presence of high levels of 3-AT.

Rescued cells also transcribe for the *Ade2* gene product, which allows these yeast to survive on media lacking this purine nucleotide base. The use of this reporter gene in conjunction with *His3* eliminates the need for supplementing 3-AT into the media. Only yeast which truly display a positive interaction between a MOX protein and a library protein will survive on SD/-His/-Ade. However, MOX1 by itself is able to activate this gene.

4.3 Modification of Mox1 to use as Bait

MOX1, being itself a strong transactivator, is unsuitable as a bait protein in the Gal4-based Y2H. MOX1 does not require binding of a target protein in order to activate transcription of the four reporter genes used in this system. Therefore, a putative binding protein could not be identified among the high background resulting from Mox1 activation.

The first step to utilizing Mox1 as a bait protein within this Y2H is to determine which part of the protein modulates the transactivation potential of Mox1. The homeodomain is not a likely candidate for this transcriptional activation domain because all three Mox proteins contain this domain, yet Mox2 and Mox2ΔH/Q do not transactivate. In order to narrow down the area of Mox1 that is important for its transcriptional function, its N and C terminal could separately be replaced by those of

Mox2 (Figure 14). If, for example, the N terminus is responsible for transactivation in Mox1, placing this domain onto Mox2 should result in the transactivation potential of Mox2 to increase. Once the transactivating region is known, the amino acids crucial to this region can be determined. This may be achieved by point mutations leading to nonconservative changes in amino acid sequence.

The transactivation potential of a mutated Mox1 protein may be decreased or even eliminated and thus it maybe suitable to use as bait in the yeast 2-hybrid system. Unfortunately, it is possible that by making these nonconservative changes, specificity of Mox1 for its interacting protein is lost or diminished. If a putative interacting partner of the modified Mox1 is found, this protein may then be tested for interaction with the unchanged Mox1.

4.4 Gal4-based vs. Ras-based Yeast Two-Hybrid

The ras-based Y2H has been used by this lab in the past. The point mutation in the yeast Cdc25 gene allows cells to survive at room temperature, but not at the restrictive temperature of 37^{o}C. Unfortunately, these cells were very prone to reverting from their temperature sensitive phenotype to that of being able to tolerate temperatures of 37^{o}C, either by back-mutation or by a new mutation which rescued the restrictive temperature phenotype. As a result, many false positive interactions were observed. The Gal4 based Y2H, with the use of a non-transactivating bait protein is a much more reliable and stringent system for detecting protein interaction, because it utilizes four Gal4- specific reporter genes. Each of these genes must be transcribed in order for cells to survive the most stringent screenings.

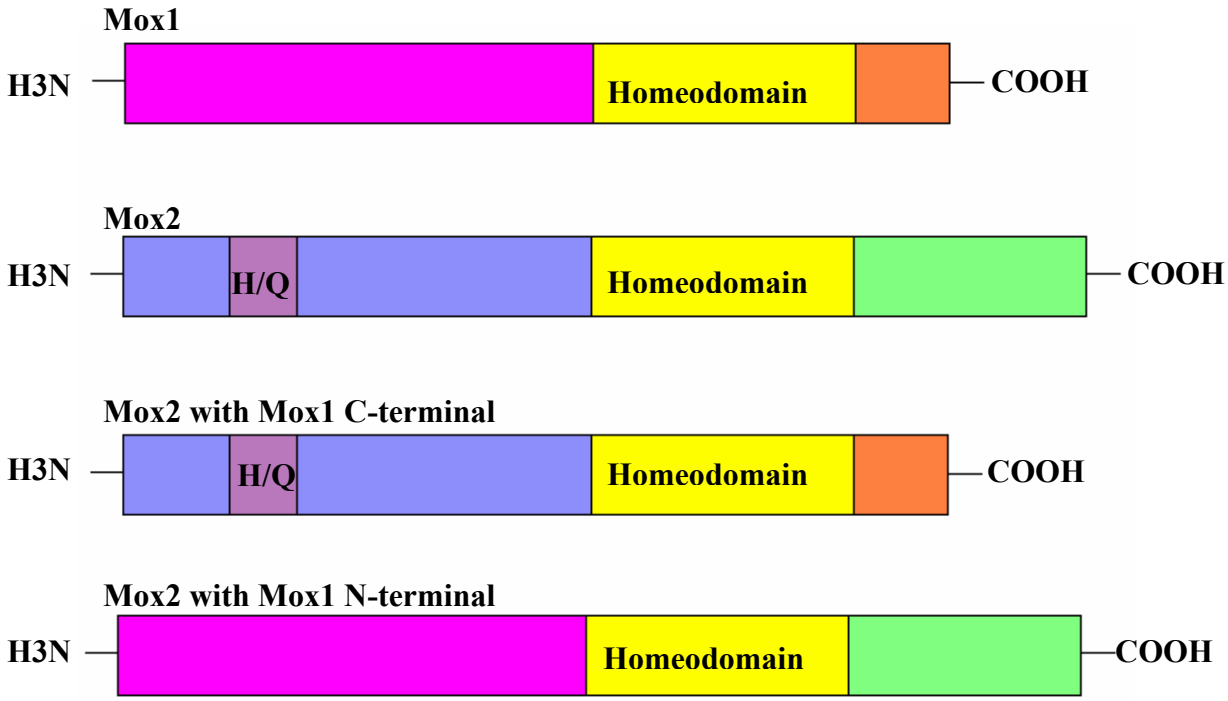

Figure 14: Modification of Mox1 to determine transactivating region. In order to elucidate the amino acids crucial for transcriptional activation in Mox1, the first step is to create various Mox1 constructs. If, for example, the transactivating region is within the amino terminal of Mox1, substituting this region into Mox2 should allow yeast harbouring this construct to survive on media selecting for the four reporter genes, *Ade2*, *His3*, *Mel1*, and *LacZ*. To further determine amino acids needed for transactivation, point mutations leading to non-conservative changes in amino acids can be done.

Conclusions

1. *Mox1* is a poor bait to utilize in the Gal4-based Y2H system, because *Mox1* acts as a strong transcriptional activator of the four reporter genes, *Ade2*, *His3*, *Mel1*, and *LacZ*.

References

Agell, N., O. Bachs, N. Rocamora and P. Villalonga 2002. Modulation of the Ras/Raf/MEK/ERK pathway by Ca(2+), and calmodulin. Cell Signal 14(8): 649-54.

Aronheim, A. 1997. Improved efficiency sos recruitment system: expression of the mammalian GAP reduces isolation of Ras GTPase false positives. Nucleic Acids Res 25(16): 3373-4.

Aronheim, A., E. Zandi, H. Hennemann, S. J. Elledge and M. Karin 1997. Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. Mol Cell Biol 17(6): 3094-102.

Bergwerff, M., A. C. Gittenberger-de Groot, L. J. Wisse, M. C. DeRuiter, A. Wessels, J. F. Martin, E. N. Olson and M. J. Kern 2000. Loss of function of the Prx1 and Prx2 homeobox genes alters architecture of the great elastic arteries and ductus arteriosus. Virchows Arch 436(1): 12-9.

Bopp, D., M. Burri, S. Baumgartner, G. Frigerio and M. Noll 1986. Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of Drosophila. Cell 47(6): 1033-40.

Candia, A. F., J. Hu, J. Crosby, P. A. Lalley, D. Noden, J. H. Nadeau and C. V. Wright 1992. Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. Development 116(4): 1123-36.

Candia, A. F. and C. V. Wright 1995. The expression pattern of Xenopus Mox-2 implies a role in initial mesodermal differentiation. Mech Dev 52(1): 27-36.

Candia, A. F. and C. V. Wright 1996. Differential localization of Mox-1 and Mox-2 proteins indicates distinct roles during development. Int J Dev Biol 40(6): 1179-84.

Casacuberta, E. and M. L. Pardue. 2003. Transposon telomeres are widely distributed in the drosophila genus: Tart elements in the virilis group. Proc Natl Acad Sci U S A. 100(6): 3363-8.

Chauvet, S., S. Merabet, D. Bilder, M. P. Scott, J. Pradel and Y. Graba 2000. Distinct hox protein sequences determine specificity in different tissues. Proc Natl Acad Sci U S A 97(8): 4064-9.

Chisaka, O. and M. R. Capecchi 1991. Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5. Nature 350(6318): 473-9.

Clontech, L. I. 1999. MATCHMAKER GAL4 two-hybrid system 3 & libraries user manual. 38.

Clontech, L. I. 2001. Yeast Protocols Handbook. 66.

Clontech, L. I. 2003. Matchmaker Co-IP Kit User Manual. 27.

Delfini, M. C. and D. Duprez. 2004. Ectopic myf5 or myod prevents the neuronal differentiation program in addition to inducing skeletal muscle differentiation, in the chick neural tube. Development. 131(4): 713-23.

Desplan, C., J. Theis and P. H. O'Farrell 1988. The sequence specificity of homeodomain-DNA interaction. Cell 54(7): 1081-90.

Epstein, J. A. and C. A. Buck 2000. Transcriptional regulation of cardiac development: implications for congenital heart disease and DiGeorge syndrome. Pediatr Res 48(6): 717-24.

Fields, S. and O. Song 1989. A novel genetic system to detect protein-protein interactions. Nature 340(6230): 245-6.

Fields, S. and R. Sternglanz 1994. The two-hybrid system: an assay for protein-protein interactions. Trends Genet 10(8): 286-92.

Finkel, T., J. Duc, E. R. Fearon, C. V. Dang and G. F. Tomaselli 1993. Detection and modulation in vivo of helix-loop-helix protein-protein interactions. J Biol Chem 268(1): 5-8.

Fisher, S. A., E. Siwik, D. Branellec, K. Walsh and M. Watanabe 1997. Forced expression of the homeodomain protein Gax inhibits cardiomyocyte proliferation and perturbs heart morphogenesis. Development 124(21): 4405-13.

Gehring, W. J., Y. Q. Qian, M. Billeter, K. Furukubo-Tokunaga, A. F. Schier, D. Resendez-Perez, M. Affolter, G. Otting and K. Wuthrich. 1994a. Homeodomain-DNA recognition. Cell 78(2): 211-23.

Gehring, W. J., M. Affolter and T. Burglin. 1994b. Homeodomain proteins. Annu Rev Biochem. 63: 487-526.

Gorski, D. H., D. F. LePage, C. V. Patel, N. G. Copeland, N. A. Jenkins and K. Walsh 1993. Molecular cloning of a diverged homeobox gene that is rapidly down-regulated during the G0/G1 transition in vascular smooth muscle cells. Mol Cell Biol 13(6): 3722-33.

Gorski, D. H. and K. Walsh 2000. The role of homeobox genes in vascular remodeling and angiogenesis. Circ Res 87(10): 865-72.

Gorski, D. H. and K. Walsh 2001. Control of vascular cell differentiation by homeiobox transcription factors. Circ Res 88(1): 7-8.

Gorski, D. H. and K. Walsh 2003. Control of vascular cell differentiation by homeobox transcription factors. Trends Cardiovasc Med 13(6): 213-20.

Hoffmann, H. M., K. M. Catron, A. J. van Wijnen, L. R. McCabe, J. B. Lian, G. S. Stein and J. L. Stein 1994. Transcriptional control of the tissue-specific, developmentally regulated osteocalcin gene requires a binding motif for the Msx family of homeodomain proteins. Proc Natl Acad Sci U S A 91(26): 12887-91.

Inoue, H., H. Nojima and H. Okayama 1990. High efficiency transformation of Escherichia coli with plasmids. Gene 96(1): 23-8.

Johnston, M. 1987. A model fungal gene regulatory mechanism: the GAL genes of Saccharomyces cerevisiae. Microbiol Rev 51(4): 458-76.

Kissinger, C. R., B. S. Liu, E. Martin-Blanco, T. B. Kornberg and C. O. Pabo 1990. Crystal structure of an engrailed homeodomain-DNA complex at 2.8 A resolution: a framework for understanding homeodomain-DNA interactions. Cell 63(3): 579-90.

Laughon, A. and R. F. Gesteland 1984. Primary structure of the Saccharomyces cerevisiae GAL4 gene. Mol Cell Biol 4(2): 260-7.

LePage, D. F., D. A. Altomare, J. R. Testa and K. Walsh. 1994. Molecular cloning and localization of the human gax gene to 7p21. Genomics. 24(3): 535-40.

Maillard, L., E. Van Belle, R. C. Smith, A. Le Roux, P. Denefle, G. Steg, J. J. Barry, D. Branellec, J. M. Isner and K. Walsh. 1997. Percutaneous delivery of the gax gene inhibits vessel stenosis in a rabbit model of balloon angioplasty. Cardiovasc Res. 35(3): 536-46.

Mankoo, B. S., N. S. Collins, P. Ashby, E. Grigorieva, L. H. Pevny, A. Candia, C. V. Wright, P. W. Rigby and V. Pachnis 1999. Mox2 is a component of the genetic hierarchy controlling limb muscle development. Nature 400(6739): 69-73.

Mankoo, B. S., S. Skuntz, I. Harrigan, E. Grigorieva, A. Candia, C. V. Wright, H. Arnheiter and V. Pachnis 2003. The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. Development 130(19): 4655-64.

McGinnis, W., R. L. Garber, J. Wirz, A. Kuroiwa and W. J. Gehring 1984. A homologous protein-coding sequence in Drosophila homeotic genes and its conservation in other metazoans. Cell 37(2): 403-8.

Miano, J. M., A. B. Firulli, E. N. Olson, P. Hara, C. M. Giachelli and S. M. Schwartz 1996. Restricted expression of homeobox genes distinguishes fetal from adult human smooth muscle cells. Proc Natl Acad Sci U S A 93(2): 900-5.

Minguillon, C. and J. Garcia-Fernandez 2002. The single amphioxus Mox gene: insights into the functional evolution of Mox genes, somites, and the asymmetry of amphioxus somitogenesis. Dev Biol 246(2): 455-65.

Newberry, E. P., T. Latifi, J. T. Battaile and D. A. Towler 1997. Structure-function analysis of Msx2-mediated transcriptional suppression. Biochemistry 36(34): 10451-62.

Otting, G., Y. Q. Qian, M. Billeter, M. Muller, M. Affolter, W. J. Gehring and K. Wuthrich 1990. Protein--DNA contacts in the structure of a homeodomain--DNA complex determined by nuclear magnetic resonance spectroscopy in solution. Embo J 9(10): 3085-92.

Patel, C. V., D. H. Gorski, D. F. LePage, J. Lincecum and K. Walsh 1992. Molecular cloning of a homeobox transcription factor from adult aortic smooth muscle. J Biol Chem 267(36): 26085-90.

Perutz, M. F., T. Johnson, M. Suzuki and J. T. Finch. 1994. Glutamine repeats as polar zippers: Their possible role in inherited neurodegenerative diseases. Proc Natl Acad Sci U S A. 91(12): 5355-8.

Quinn, L. M., S. E. Latham and B. Kalionis. 2000. The homeobox genes msx2 and mox2 are candidates for regulating epithelial-mesenchymal cell interactions in the human placenta. Placenta. 21 Suppl A: S50-4.

Ridgeway, A. G. and I. S. Skerjanc 2001. Pax3 is essential for skeletal myogenesis and the expression of Six1 and Eya2. J Biol Chem 276(22): 19033-9.

Rodrigo, I., R. E. Hill, R. Balling, A. Munsterberg and K. Imai. 2003. Pax1 and pax9 activate bapx1 to induce chondrogenic differentiation in the sclerotome. Development. 130(3): 473-82.

Scott, M. P., J. W. Tamkun and G. W. Hartzell, 3rd 1989. The structure and function of the homeodomain. Biochim Biophys Acta 989(1): 25-48.

Smith, R. C., D. Branellec, D. H. Gorski, K. Guo, H. Perlman, J. F. Dedieu, C. Pastore, A. Mahfoudi, P. Denefle, J. M. Isner and K. Walsh. 1997. P21cip1-mediated inhibition of cell proliferation by overexpression of the gax homeodomain gene. Genes Dev. 11(13): 1674-89.

Srivastava, D. and E. N. Olson. 2000. A genetic blueprint for cardiac development. Nature. 407(6801): 221-6.

Stamataki, D., M. Kastrinaki, B. S. Mankoo, V. Pachnis and D. Karagogeos 2001. Homeodomain proteins Mox1 and Mox2 associate with Pax1 and Pax3 transcription factors. FEBS Lett 499(3): 274-8.

Stratagene (2004). CytoTrap vector kit instruction manual.

Sugihara, T. M., E. I. Kudryavtseva, V. Kumar, J. J. Horridge and B. Andersen 2001. The POU domain factor Skin-1a represses the keratin 14 promoter independent of DNA binding. A possible role for interactions between Skn-1a and CREB-binding protein/p300. J Biol Chem 276(35): 33036-44.

Tajbakhsh, S., D. Rocancourt, G. Cossu and M. Buckingham 1997. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell 89(1): 127-38.

Treacy, M. N., X. He and M. G. Rosenfeld 1991. I-POU: a POU-domain protein that inhibits neuron-specific gene activation. Nature 350(6319): 577-84.

Treisman, J., P. Gonczy, M. Vashishtha, E. Harris and C. Desplan 1989. A single amino acid can determine the DNA binding specificity of homeodomain proteins. Cell 59(3): 553-62.

Wigle, J. T. and G. Oliver. 1999. Prox1 function is required for the development of the murine lymphatic system. Cell. 98(6): 769-78.

Wilm, B., E. Dahl, H. Peters, R. Balling and K. Imai 1998. Targeted disruption of Pax1 defines its null phenotype and proves haploinsufficiency. Proc Natl Acad Sci U S A 95(15): 8692-7.

Wolberger, C., A. K. Vershon, B. Liu, A. D. Johnson and C. O. Pabo 1991. Crystal structure of a MAT alpha 2 homeodomain-operator complex suggests a general model for homeodomain-DNA interactions. Cell 67(3): 517-28.

Yamashita, J., H. Itoh, Y. Ogawa, N. Tamura, K. Takaya, T. Igaki, K. Doi, T. H. Chun, M. Inoue, K. Masatsugu and K. Nakao. 1997. Opposite regulation of gax homeobox expression by angiotensin ii and c-type natriuretic peptide. Hypertension. 29(1 Pt 2): 381-7.