Retinoid-Mediated Regulation of *NR6A1*, *Prickle1* and *Ror2* During Development of the Mouse Embryo

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ABSTRACT

Vitamin A and its derivatives, collectively termed retinoids, are essential for proper growth and development as well as maintenance of homeostasis in the adult. Retinoic acid (RA), the major biologically active vitamin A metabolite, is well characterized for its crucial roles in gene activation during embryogenesis. Our lab had previously performed a microarray analysis to identify genes induced by exogenous RA in the tailbud of early mouse embryos. Three genes were chosen from the microarray results for further investigation; *Germ Cell Nuclear Factor* (*GCNF*/*NR6A1*), *Prickle1* (*Pk1*) and *Ror2,* the latter of which are known members of the planar cell polarity (PCP) pathway. These genes were further examined for RA regulation by embryo culture and RT-PCR, which strongly supported a direct regulatory mechanism of *NR6A1* by RA. Further analysis aiming to identify a functional response element in the promoter of the targets was attempted, including chromatin immunoprecipitation (ChIP), made possible by the generation and characterization of a highly specific antibody against RARγ. This antibody was used in a ChIP promoter walk, which identified regions on target gene promoters that are occupied by RARγ *in vivo*, and therefore likely harbor RA response elements.

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LIST OF ABBREVIATIONS

αMEM	Alpha Modification of Eagle's Medium
ADH	Alcohol dehydrogenase
AF	Activation function
APC	Adenomatous polyposis coli
βgal	β-galactosidase
BAC	Bacterial artificial chromosome
BDB1	Brachydactyly type B
CDRE	Cdx response element
Cdx	Caudal-related homeobox
CE	Convergent extension
ChIP	Chromatin immunoprecipitation
CN-Br	Cyanogen bromide
CNS	Central nervous system
CPRG	Chlorophenol Red-β-D-Galactopyranoside
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
Cyp26A1	Cytochrome P450, Family 26, Subfamily A, Polypeptide 1
DBD	DNA binding domain

DepC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Direct repeat
Dvl	Dishevelled
E	Embryonic day
EMSA	Electrophoretic mobility shift assay
ES	Embryonic stem
FGF	Fibroblast growth factor
Fmi	Flamingo
Fz	Frizzled
GCNF	Germ cell nuclear factor (NR6A1)
GFP	Green fluorescent protein
GSK-3	Glycogen synthase kinase 3
GST	Glutathione S-transferase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HI-DBS	Heat-inactivated donor bovine serum

HI-FBS	Heat-inactivated fetal bovine serum
HRE	Hormone response element
IP	Immunoprecipitation
ISH	*In situ* hybridization
Kb	Kilobase
LBD	Ligand binding domain
LEF	Lymphoid enhancer binding factor
LIM	LinII, Isl-1, Mec-3
LRP	Low-density lipoprotein receptor-related protein
MBP	Maltose binding protein
mRNA	Messenger ribonucleic acid
NCoR	Nuclear receptor co-repressor
NR	Nuclear receptor
NR6A1	Nuclear receptor subfamily 6, group A, member 1
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PET	Prickle, Espinas, Testin
Pk	Prickle
RA	Retinoic acid

RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinol binding protein
RDH	Retinol dehydrogenase
RNA	Ribonucleic acid
Ror2	Tyrosine kinase-like orphan receptor 2
RRS	Robinow Syndrome
RTK	Receptor tyrosine kinase
RT-PCR	Reverse-transcriptase polymerase chain reaction
RXR	Retinoid X receptor
Stan	Starry night
Stbm	Strabismus
TCF	T-cell factor
TESS	Transcription element search software
VAD	Vitamin A-deficiency
Vangl	Van Gogh-like
WCLB	Whole cell lysis buffer
Wnt	Wingless-related

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CHAPTER 1

INTRODUCTION

Correct regulation of embryonic development is predicated on the highly coordinated spatial and temporal expression of a variety of signaling molecules. One such molecule is retinoic acid (RA), the active metabolite of vitamin A, which has long been established as a crucial signaling molecule essential for the proper development, differentiation and homeostasis of vertebrate organisms.

1.1 VITAMIN A AND ITS RETINOID DERIVATIVES IN DEVELOPMENT

1.1.1 Vitamin A

Retinoids, such as vitamin A, encompass a variety of molecules displaying the biological activity of retinol, a lipophilic molecule essential throughout life and for the maintenance of vision and reproduction in the adult, as well as for normal growth and embryonic development. Primary sources of retinol include vegetables (in the form of carotenoids such as α-carotene, β-carotene, γ-carotene and β-cryptoxanthin) and animal products including meat and dairy products, which provide retinol in the form of retinyl esters that are metabolized in the enterocytes of the intestinal tract (reviewed in Blomhoff *et al.,* 1992).

1.1.2 Vitamin A in Development

Early work on the importance of vitamin A during development began as nutritional studies examining the consequence of a vitamin A-deficient (VAD) diet on the offspring of pregnant rats. In such studies, congenital abnormalities were reported in offspring deprived of vitamin A including ocular defects and malformed genito-urinary tracts (Wilson and Warkany, 1948; mouse, McCarthy and Cerecedo, 1952). These phenotypes could be rescued by vitamin A supplementation in a time dependent manner,

demonstrating that vitamin A was most crucial around the time of organ formation (Wilson *et al*., 1953). Subsequent studies investigated the effects of vitamin A excess, through *in utero* treatment with exogenous vitamin A, which also caused a number of teratogenic defects (Kalter and Warkany, 1961). These pregnancies were notable for their high rate of embryo reabsorption, with those surviving until fetal stages displaying gross abnormalities in many structures, especially the head and central nervous system. Taken together, these findings indicate an essential role for vitamin A in regulation of development through a strictly controlled concentration- and time-dependent manner.

The field of retinoid signaling advanced significantly after a study performed by Shenefelt (1972) determined that one particular derivative of vitamin A, retinoic acid (RA), when administered to pregnant dams had greater impact than vitamin A itself. Exogenous RA was found to be absorbed more rapidly while causing significant malformations in almost all major organs including the brain and nervous systems, eyes, heart, lungs, gastrointestinal tract, reproductive organs and limbs (Shenefelt, 1972). Again, temporal dosage studies revealed specific windows of time where RA produced the most significant defects, suggestive of its critical role in organ morphogenesis. In addition, rescue studies found that RA was capable of reversing all of the phenotypes associated with vitamin A deficiency, with the exception of night blindness, suggesting that RA is the principal active form of vitamin A (Sucov and Evans, 1995).

1.1.3 Derivation of Retinoic Acid in the Embryo

Vitamin A, in the form of retinol, is available to the embryo via maternal circulation as it passes through the placental barrier (or yolk sac). Transport through circulation can be achieved through association with a retinol binding protein (RBP)

while entry into the cell is facilitated through the Stra6 cell-surface receptor (Kawaguchi *et al.,* 2007). Once inside the cell, retinol may bind to cellular retinol binding proteins (CRBPs) before being metabolized into RA through two sequential reactions (reviewed in Donovan *et al.,* 1995 and Noy, 2000). In the first step, retinol is reversibly converted by alcohol dehydrogenases (ADHs) and/or retinol dehydrogenases (RDHs) forming retinaldehyde, which is further metabolized in an irreversible reaction to the biologically active RA by retinaldehyde dehydrogenases (RALDHs) [Fig 1.1] (reviewed in Duester, 2000, 2008; Niederreither and Dollé, 2008; Ross *et al.,* 2000). Active RA either associates with cellular retinoic acid binding proteins (CRABPs) or nuclear receptors within the cell, or it can diffuse to impact neighboring cells in a paracrine manner (Gorry *et al.,* 1994).

The retinoid metabolic machinery has several members which often exhibit overlapping function, and display restricted spatiotemporal patterns of expression. During early organogenesis in the mouse, ADH1, 5 and 7 (Deltour *et al.*, 1999) as well as RDH5 and 10 (Cunningham *et al.*, 2011; Driessen *et al.*, 2000) are responsible for the first oxidative reaction, converting retinol to retinaldehyde; elimination of any one of these enzymes typically results in only minor abnormalities consistent with a high degree of functional overlap. Only the RDH10 knockout mouse displays a lethal phenotype, likely due to lung agenesis (Sandall *et al.,* 2007). In contrast, the three RALDHs (1, 2 and 3), each display distinct expression patterns during embryogenesis. RALDH2 expression begins around embryonic day (E) 7.5 and is seen throughout the somites (precursors to the vertebrae), while it is notably excluded from the tailbud region (Niederreither *et al.*,

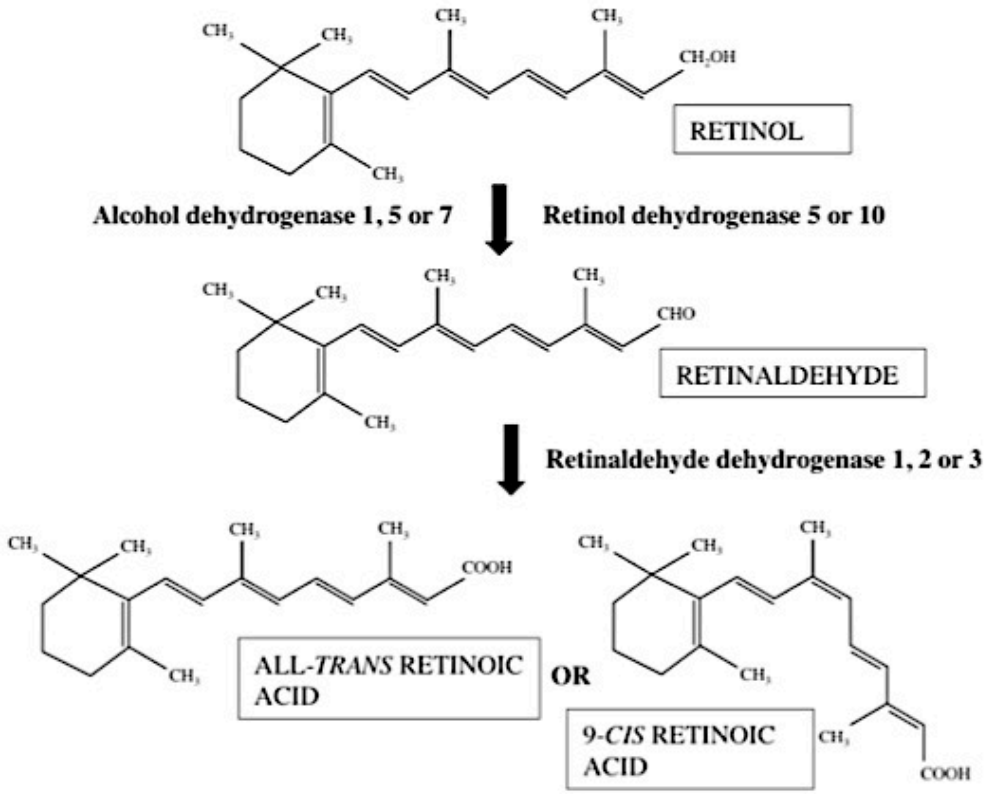

Figure 1.1: Derivation of retinoic acid from retinol.

Metabolism of retinoic acid in the developing embryo is dependent on two sequential reactions. First, retinol is reversibly oxidized into retinaldehyde by a member of the aldehyde dehydrogenase family or alcohol dehydrogenase family. The rate-limiting second reaction, from retinaldehyde into retinoic acid, is mediated by retinaldehyde dehydrogenases in a strictly controlled fashion.

1997; Ribes *et al.*, 2006). RALDH1 and 3 have more limited expression domains, mainly in the eye and forebrain regions (Suzuki *et al.*, 2000).

Knockout phenotypes reflect the key functions of these aldehyde dehydrogenases. RALDH2$^{-/-}$ mice do not survive beyond E9.5 and have severe hindbrain and spinal cord defects, along with heart, lung and pancreatic abnormalities (Niederreither *et al.,* 1999). Homozygous RALDH3 null mutation is perinatal lethal, due to blockage of nasal passages, with mutants also displaying malformed retinas, indicative of the requirement for RA in eye development (Dupé *et al.,* 2003). RALDH1 is the only non-lethal knockout of this gene family producing no obvious phenotype, which is likely attributed to functional overlap among the enzymes (Fan *et al.,* 2003).

1.1.4 Catabolism of Retinoic Acid

Work in retinoid biology has clearly demonstrated that temporal and spatial regulation of RA production is essential for correct embryogenesis. As RA is freely diffusible, the effective breakdown of RA in tissues where it is not required is also essential. This catabolism is mediated by a class of cytochrome P450 enzymes, specifically Cyp26A1, Cyp26B1 and Cyp26C1 (MacLean *et al.,* 2001; Ray *et al.*, 1997; Tahayato *et al.*, 2003). These enzymes convert retinoic acid into oxidized derivatives, some of which are also biologically active, but are likely subsequently rapidly metabolized into inert products [Fig 1.2]. This pathway is thought to be regulated by a biofeedback mechanism where RA directly regulates the degradation machinery. For example, Cyp26A1 is a well characterized direct RA target, and exposure to exogenous RA promotes its expression (Loudig *et al.*, 2000). This allows RA to regulate its own degradation, an essential property to maintain low levels of RA in tissues where retinoid

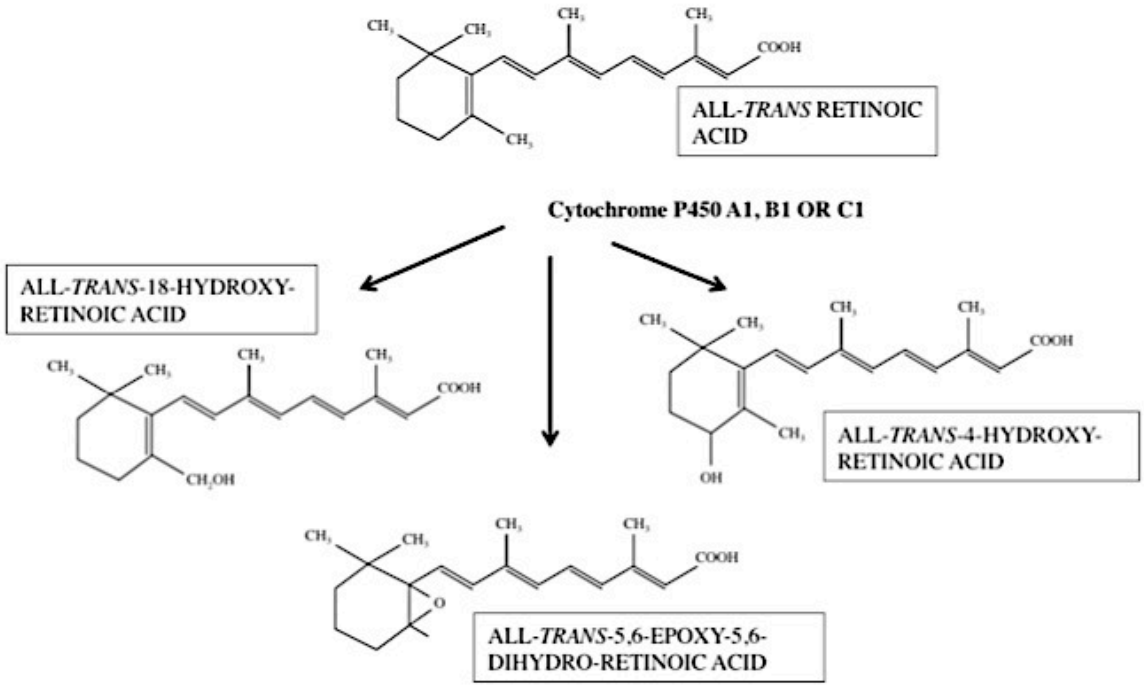

Figure 1.2: Catabolism of retinoic acid.

Retinoic acid is quickly degraded in tissue where retinoid signaling is not required, or potentially destructive. This is mediated by members of the cytochrome P450 enzymes (Cyp26A1, B1 or C1). Many derivatives can be produced including all-*trans*-18-hydroxy-retinoic acid, all-*trans*-5,6-epoxy-5,6-dihydro-retinoic acid and all-*trans*-4-hydroxy-retinoic acid, some of which may exert additional metabolic effects.

signaling needs to be restricted.

As expected by the requirement for tight spatial regulation of retinoid activity, the Cyp26 enzymes are not expressed uniformly throughout the embryo, but rather their expression patterns are indicative of their essential role in the control of RA biodistribution throughout embryogenesis. Accordingly, activity of these enzymes is generally complementary to that of the RALDH enzymes. For example, Cyp26A1 expression is high in the tailbud but excluded from the somites which express RALDH2 and have high RA levels (Abu-Abed *et al.*, 2000; Fujii *et al.,* 1997; Sakai *et al.*, 2000; White *et al.,* 1996). Cyp26B1 is found in the skin and areas of the brain which are also devoid of RA activity (MacLean *et al.,* 2001).

The knockout phenotypes of Cyp26 mutants vary but ablation of either Cyp26A1 or B1 produce phenotypes reminiscent of RA-induced teratogenicity. For example, deficiency of Cyp26A1 causes the most overt phenotype, with embryos dying at mid to late gestation and displaying posterior trunctation as well as sironomelia, a fusion of the hindlimbs together forming a 'mermaid-like' tail structure (Abu-Abed *et al.*, 2000; Sakai *et al.*, 2000). Cyp26C1 deficient animals are viable, however compound mutation with other Cyp26 deletions causes patterning defects (Uehara *et al.*, 2007).

1.1.5 Molecular Mechanisms of Retinoic Acid Function

RA signaling is mediated by two families of nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which function as regulators of transcription. Each family of receptors has three members; RAR/RXR-α, -β and -γ, which can be further classified into various isoforms generated via differential promoters and

alternate splicing of the 5' region (see Giguère *et al,* 1987; Petkovich *et al,* 1987; Brand *et al,* 1988; and Zelent *et al,* 1989).

The RARs belong to the steroid-thyroid hormone receptor superfamily of nuclear receptors (NRs), which bind various ligands including steroid and thyroid hormones, retinoids and vitamin D, among other ligands (Laudet *et al.*, 1992). Together they are responsible for transmission of key signaling cascades resulting in a wide variety of downstream activities.

The nuclear receptors share high sequence similarity and typically consist of 6 functional regions of varying homology designated A through F [Fig 1.3]. The C and E domains contain the DNA binding domain (DBD) and ligand binding domain (LBD) respectively, and are the two most highly conserved regions between nuclear receptors (for review see Bastien and Rochette-Egly, 2004; Samarut and Rochette-Egly, 2012). The DBD contains two zinc finger-like regions, termed CI and CII, which are responsible for recognizing unique response element (RE) motifs, and proper receptor dimerization, respectively. This region is highly conserved among the RARs, sharing 94-97% sequence homology, and 91-94% to that of the RXRs (Chambon, 1996). The E domain, necessary for binding of appropriate ligands, is composed of 11 or 12 (depending on the NR) alpha helices, which organize into a hydrophobic ligand binding pocket specific to each receptor. LBDs also contain conserved consensus phosphorylation sites for modulation of activation, and a major ligand-dependent transcriptional activation function (AF).

The A/B domain is more variable among NRs and contains a second, ligand-independent, AF site, as well as conserved phosphorylation sites, which impact on transcriptional activity. The D domain appears to function as a hinge between the DBD

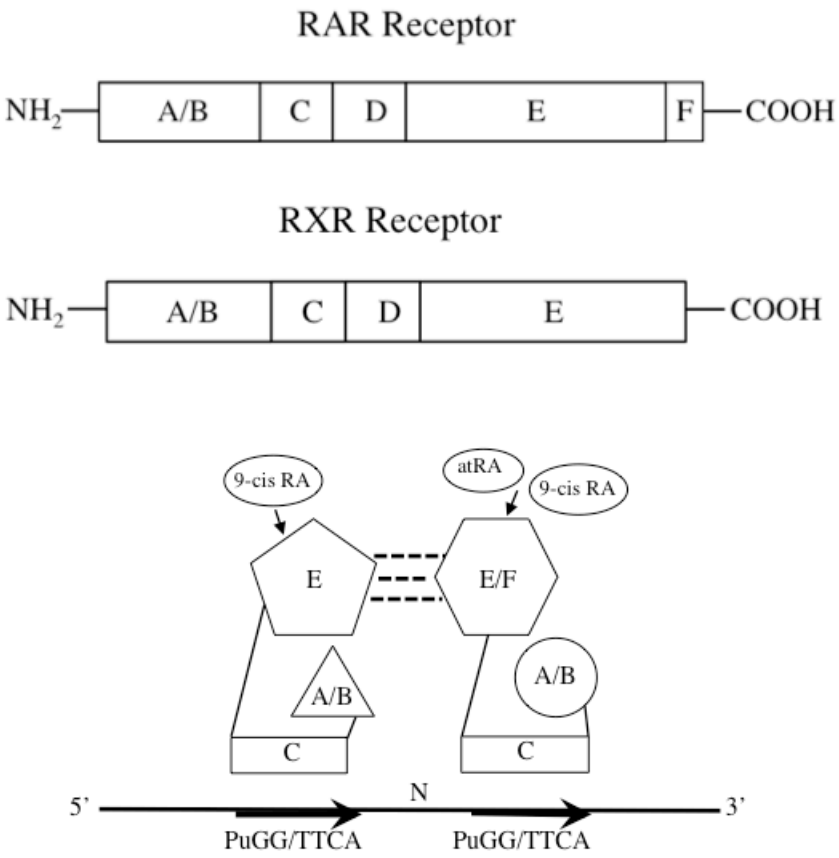

Figure 1.3 Structural domains of RARs and RXRs.

Retinoic acid receptors and retinoid X receptors are members of the nuclear receptor superfamily, and thus share high structural homology. Of closest similarity are the C (DNA-binding) and E (ligand-binding) domains. For RA signaling to occur, the receptors heterodimerize on a retinoic acid response element within a target gene sequence. Upon ligand binding, they permit a conformational change, which facilitates access for other transcription factors and basal transcription machinery necessary for gene activation.

(Adapted from Chambon, P., *FASEB J*, 1996, 10: 940-54)

and LBD, necessary for rotation of the protein between alternate active and inactive states. The F domain is the least conserved region between NRs, with no known function, however some contain phosphorylation sites which may contribute to the activation functions of AF-1 and AF-2, as has been observed for the estrogen receptors (Bastien and Rochette-Egly, 2004; Montano, 1995).

RA signaling relies on RAR-RXR heterodimers bound to retinoic acid response elements (RAREs) (Depoix, 2001; Kastner *et al.,* 1997). Canonical RAREs are often found in the proximal promoter region of target genes and consist of two core hexamers in the same orientation separated by a variable number of spacer nucleotides, and are referred to as direct repeats (DRs) (for reviews see Chambon, 1996; Duester, 2008; Rhinn and Dollé, 2012). The putative DR half-site is a PuG(G/T)TCA motif, typically separated by five nucleotides as observed in the promoters of the retinoid-target genes RARβ (de Thé *et al.,* 1990), Cyp26A1 (Loudig *et al.,* 2000) and several Hox genes (Langston and Gudas, 1992; Ogura and Evans, 1995; Packer *et al.,* 1998). In this scenario, the RXR occupies the more 5' half site with the RAR partner found on the 3' element. However, functional DR2s as well as an appreciable number of non-canonical retinoid-responsive motifs have also been described (reviewed in Balmer and Blomhoff, 2005; Gronemeyer and Miturski, 2001). Thus, the cryptic nature of RAREs has made identification of specific RA target genes difficult.

In the absence of ligand, RARs interact with nuclear receptor co-repressor (NCoR) complexes, which are associated with histone deacetylases (HDACs) leading to an increase in histone-DNA interactions, thereby repressing gene transcription (reviewed in Weston *et al.,* 2003). Transcription of retinoid target genes is induced when RA binds to

the RAR of the heterodimeric complex, inducing a conformational change in the receptor, resulting in a displacement of co-repressor complexes and recruitment of histone acetyltransferase (HAT) activity to the target promoter causing acetylation of the lysine residues on histone tails. This promotes a more relaxed chromatin state, which facilitates access to the DNA by transcription factors and basal transcription machinery (see Altucci and Gronemeyer, 2001 for review).

1.1.6 Expression of Retinoic Acid Receptors During Embryogenesis

Examination of transcripts of RARs and RXRs has shown that each receptor displays unique expression patterns that vary both by tissue and developmental stage, beginning from gastrulation and continuing through adult life. Generally, *RARα* is relatively ubiquitously expressed during all stages of murine development while *RARβ* and *RARγ* show distinct and complementary expression patterns (Ruberte *et al.,* 1999, Dollé, 2009). *RARβ* transcripts are found primarily in the hindbrain and also in the neural tube/plate between rhombomere 7 and the caudal end of the embryo, as well as the mesonephros, foregut endoderm and proximal limb bud mesenchyme. *RARγ* is more prominently expressed in caudal tissues such as the pre-somitic and tailbud mesenchyme as well as precartilaginous cell populations.

Gene knockout studies have been fundamental to understanding the roles of the RARs. Embryos with a singular knockout of one of these receptors do not show major developmental defects (Ghyselinck *et al.,* 1997; Lohnes *et al.,* 1993; Lufkin *et al.,* 1993). This has been attributed to the receptors overlapping expression and functional relatedness. Consistent with this, compound RAR mutants exhibit significant developmental abnormalities which include abnormal hindbrain patterning, defects in the

heart outflow tract, severe lung hypoplasia, craniofacial and limb skeletal defects, and abnormalities affecting the male and female urogenital tracts (Lohnes *et al.,* 1994; Mendelsohn *et al.,* 1994).

It should also be noted that the RXRs show their own distinct expression patterns, which create additional complexity and control of retinoid signaling. Briefly, *RXRα* and *RXRβ* are relatively ubiquitously expressed while *RXRγ* is highly restricted to pre-myogenic and chrondogenic areas in the caudal-most region of the extending embryo (Dollé *et al.,* 1994). Interestingly, and in contrast to the RAR single null animals, RXRα$^{-/-}$ is embryonic lethal at around E13.5-16.5 due to heart defects (Kastner *et al.,* 1994; Sucov *et al.,* 1994), while RXRβ$^{-/-}$ and RXRγ$^{-/-}$ single mutants display much subtler phenotypes. Again, compound mutation of the RXRs result in increased severity which is exemplified by RXRα;RXRβ double null embryos which do not survive beyond E10.5 and show truncation of the posterior body with abnormal lung, heart and craniofacial development (Wendling *et al.,* 1999). However, due to the nature of RXRs as promiscuous partners with other NRs, these null phenotypes could be related to defects in pathways other than retinoid signaling.

1.1.7 Signaling Domains in the Posterior Embryo

A number of studies clearly demonstrate that RA plays critical roles in embryogenesis, including the elongating axis. As described above, RA biodistribution is highly regulated by both RALDHs, which dictate its synthesis, and the Cyp26 enzymes, which function in its degradation. This is especially apparent during somitogenesis, when RA is being actively produced in the newly formed somites and degraded in the more

posterior tailbud region, a relationship that is believed to be fundamental to establishing somite formation (reviewed in Duester, 2007).

The tailbud region of an E8.5 mouse embryo is composed of undifferentiated, highly proliferative tissue. This region gives rise to paraxial mesoderm at the anterior side of the growth zone which eventually condenses at regular intervals to form new somites. Cells in this highly proliferative caudal "stem cell" region express *Cyp26A1* at high levels to ensure against exposure to RA, while expression of RALDHs is repressed in this region. The net result is a zone of low retinoid signaling juxtaposed to a region with RA bioactivity in the more anterior somite region (Ribes *et al.,* 2009).

There are also several other signaling networks at play which are intertwined with RA signaling and contribute to somitogenesis [Fig 1.4]. These include the fibroblast growth factor (FGF) and canonical Wnt signaling pathways, which oppose the RA gradient (reviewed in Aulehla and Pourquié, 2010). It has been shown that an *Fgf8* expression gradient is produced along the posterior-anterior axis, formed by *de novo* transcription in the caudal-most cell populations and decreasing anteriorly through an mRNA decay mechanism (Dubrulle and Pourquié, 2004). Together with this is a posterior-high Wnt/β-catenin protein gradient which decreases towards the somites (Aulehla *et al.,* 2003). These two signaling pathways act to maintain the caudal stem cell pool in an undifferentiated state. As cells exit this region and move towards the RA-high regions of the embryonic axis, they arrive at the determination front where exposure to RA is presumed to prompt changes in gene expression which promote the molecular and morphological changes associated with somitogenesis.

1.1.8 A Novel Strategy for Identification of RA Target Genes

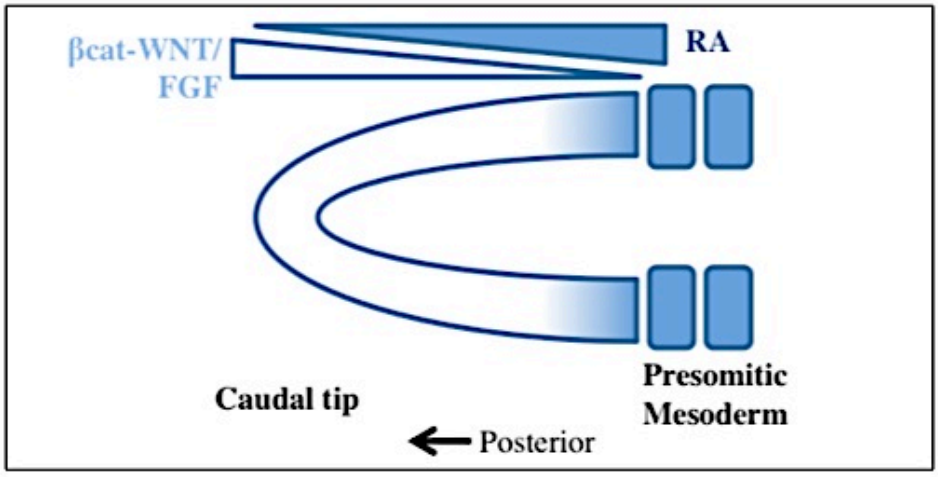

Figure 1.4 Signaling gradients in the tailbud of somitogenesis stage embryos.

The caudal region of somitogenesis stage embryos (around E8.5) is composed of undifferentiated cells that are maintained in a state of pluripotency by FGF and Wnt/β-catenin signals. This region is highly dynamic; as the trunk region expands, cells from the tailbud are incorporated into pre-somitic tissue, which, upon exposure to RA signaling begin the differentiation process.

The undifferentiated caudal population of precursor cells is of particular interest, as they represent a pool of undifferentiated tissue that expresses the necessary machinery for RA signaling, and are therefore primed for retinoid-mediated differentiation. They therefore appear to be a viable population to exploit for identification of RA targets *in vivo*. To this end, pregnant dams were dosed with RA or vehicle and caudal sections from stage-matched E8.5 embryos used to amplify representative RNA for subsequent analysis by microarray. These data were filtered by three algorithms, and genes which showed a 1.5-fold (log 2) or higher induction were considered potential RA targets. This preliminary list included several known RA targets, such as *Hoxa1* and *Cyp26A1*, suggesting enrichment for novel direct targets [Table 1]. The microarray results were further validated by whole-mount *in situ* hybridizations (ISH) as well as reverse-transcriptase polymerase chain reaction (RT-PCR) analyses. Among the genes identified as potential RA targets were several belonging the non-canonical planar cell polarity (PCP) Wnt signaling pathway, suggesting a potential link between RA and PCP signaling. A brief summary of PCP signaling will be presented below.

2.1 WNT SIGNALING IN DEVELOPMENT

2.1.1 Wnt Signaling

Wnt signaling is comprised of a large group of highly conserved secreted proteins, membrane-bound receptors and intracellular signaling machinery. The Wnt pathway plays critical roles in a number of processes, including stem cell fate decisions, tissue polarity, convergent extension and axon guidance. Wnt signaling can be divided into several arms which impact via different downstream effectors [Fig 1.5].

Gene	Observed fold induction
Abtb2 (Ankyrin repeat and BTB (POZ) domain containing 2)	2.53
Cyp26A1 (Cytochrome P450, family 26, subfamily A, polypeptide 1)	5.29
Dgkz (Diacylglycerol kinase zeta)	1.68
F11r (F11 receptor)	1.62
Fgf4 (Fibroblast growth factor 4)	1.85
Fut4 (fucosyltransferase 4)	2.00
Grsf1 (G-rich RNA sequence binding factor 1)	1.77
Hoxa1 (Homeobox A1)	2.88
Irx3 (Iroquois related homeobox 3 (Drosophila))	2.15
Lhx1 (LIM homeobox protein 1)	3.71
Msi2h (Musashi homolog 2 (Drosophila))	2.06
NR6A1 (Nuclear receptor subfamily 6, group A, member 1)	2.26
Pk1 (Prickle-like 1 (Drosophila))	1.90
Ptprz (protein tyrosine phosphatase, receptor type Z, polypeptide 1)	1.97
RET (Ret proto-oncogene)	1.54
Ror2 (Receptor tyrosine kinase-like orphan receptor 2)	1.51
Sdccag33 (serologically defined colon cancer antigen 33)	2.61

Table 1: List of selected genes shown to be induced by RA in the tailbud by microarray analysis.

Induction of gene expression by exogenous RA in the tailbud of E8.5 embryos was assessed by microarray analysis. Potential candidates were filtered by three algorithms, and genes which showed a 1.5-fold or higher increase in gene expression by all three were considered putative targets. This list contains some of the genes considered to be potential targets, including *NR6A1, Prickle1* and *Ror2*, as well as known RA targets such as *Cyp26A1*.

The first pathway to be discovered, termed the canonical Wnt pathway, remains the best understood (reviewed by Gordon and Nusse, 2006; van Amerongen and Nusse, 2009). In the absence of a Wnt ligand, the critical Wnt effector cytoplasmic β-catenin is associated with a destruction complex consisting of Axin, Adenomatous polyposis coli (APC) and Glycogen synthase kinase 3 (GSK-3), where it is phosphorylated, ubiquitinated, and targeted for degradation by the proteasome (Aberle *et al.,* 1997). When a Wnt ligand binds to a member of the Frizzled (Fz) family of transmembrane receptors, in association with its co-receptor LRP-5/6, the destruction complex is inhibited. This allows β-catenin to accumulate and translocate to the nucleus, where it can associate with members of the LEF/TCF family of transcription factors to induce the expression of Wnt target genes.

2.1.2 The Planar Cell Polarity (PCP) Pathway

Morphogenesis inherently requires precise positional information among cell populations. They must recognize different axes of growth - anterior from posterior, ventral from distal – and to interpret polarity cues across groups of cells to coordinate large-scale movements. PCP, mediated by noncanonical Wnt signaling, has recently gained considerable attention in this area for its role in cell polarity, linking information across the plane of an epithelial sheet to specify cues for cytoskeletal changes which in turn lead, for example, to coordinated cell movement.

Wnt-mediated PCP has been linked to the activation of several downstream pathways including Rho, Rac and JNK, which can all contribute to changes in gene expression (reviewed by Veeman *et al,* 2003). These pathways, however, also contribute

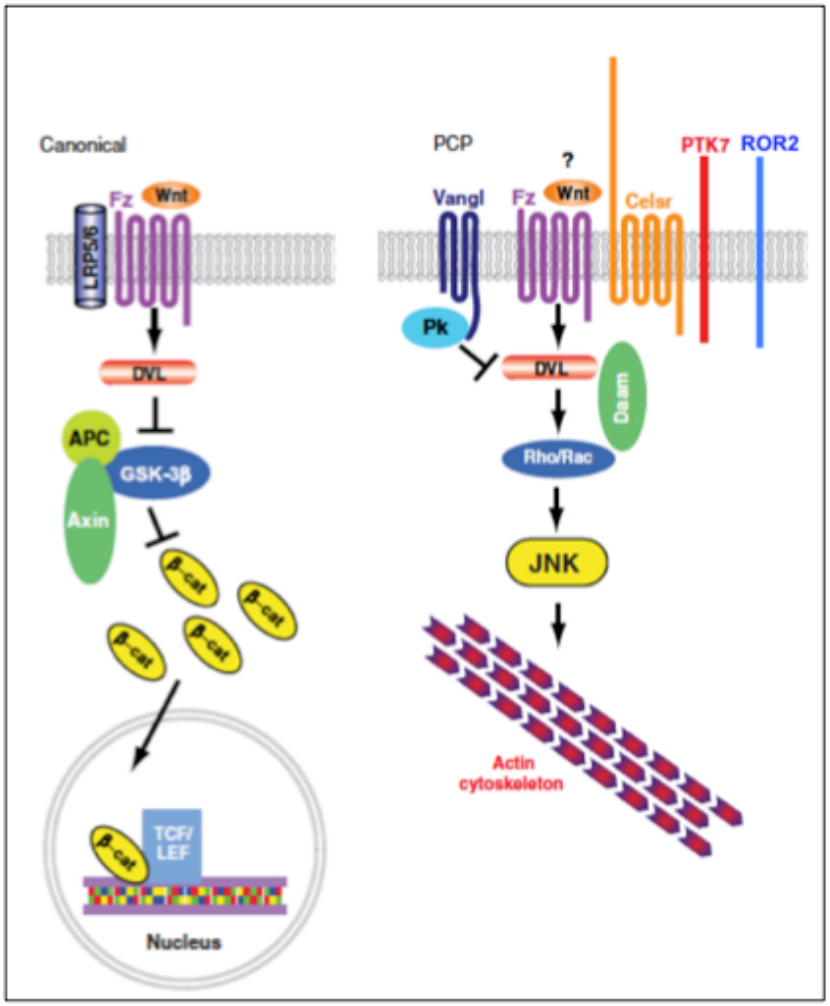

Figure 1.5 Canonical and non-canonical/PCP Wnt signaling pathways.

Wnt signaling can be divided into two groups, canonical or non-canonical, where the former depends on β-catenin for gene activation and the latter does not. Canonical Wnt signaling is activated when a Wnt ligand binds to a Frizzled receptor which allows cytoplasmic Dishevelled to inhibit the β-catenin destruction complex. This permits β-catenin to translocate to the nucleus for target gene activation. Non-canonical Wnt signaling comprises several different pathways, one of which is planar cell polarity (PCP). PCP also involves Frizzled and Dishevelled, which work in association with several other transmembrane and cytoplasmic proteins to produce an asymmetric protein gradient across cells and thus conveying the positional information necessary for cell movement.

(Adapted and reproduced with permission from Montcouquiol, M. *et al., Annu. Rev. Neurosci,* 2006, 29:363-86)

to cytoskeletal changes which are essential to PCP-dependent processes such as convergent extension (CE). CE is a specialized cell movement causing tissue to, for example, narrow and elongate as cells intercalate along the medio-lateral axis (converge), allowing the tissue to extend along the antero-posterior axis (extension); this process is critical to proper neural tube closure among other morphogenic events (Ciruna *et al,* 2006; Nishimura *et al.,* 2012; Wallingford and Harland, 2001).

Although PCP signaling is still relatively poorly understood, a rudimentary signaling cascade has been established in *Drosophila melanogaster*. As in the canonical pathway, PCP can be triggered by Wnt ligand binding to a Fz receptor, which then signals to a Dvl protein. The pathway then diverges from the canonical arm, by unknown means, to impact on several other membrane-associated proteins including Flamingo/Starry night (Fmi/Stan), Van Gogh-like/Strabismus (Vangl/Stbm), and tyrosine kinase-like orphan receptor 2 (Ror2) (for review see Adler, 2002; Simons and Mlodzik, 2008; Wansleeben and Meijlink, 2011). It is currently believed that the relative location of these proteins on the cell membrane establishes polarity across the plane of the cell thus dictating positional information. Additional cytoplasmic proteins including Prickle (Pk) and Diego also interact with the Dvl complex and have been found to inhibit or promote (respectively) Fz-Dvl activity in the PCP pathway (Jenny, A. *et al,* 2005). There is also evidence to suggest that there may be two PCP signaling cassettes working either in parallel or sequentially. While the above group of proteins make up the "core" PCP members, a second group has been described which includes the protocadherins Fat and Dachsous as well as the transmembrane protein Four-jointed and the transcriptional repressor Atrophin. It appears that this Fat/Dachsous system works upstream of the

Frizzled/Flamingo pathway, setting up the groundwork to allow the Frizzled-Flamingo group to polarize the cell (Saburi, S. & McNeill, H., 2005). Although current research is still identifying novel factors relating to the pathway, many major questions still remain, including activation and inhibitory signals involved in PCP.

2.1.3 Mammalian Planar Cell Polarity

Many of the PCP-related *Drosophila* genes have been conserved in vertebrates, although there are differences in the conservation of the pathway as well as the processes it controls. In mammals, homologues of each of the core PCP genes have been identified, frequently represented by multiple members such as *Fz3* and *Fz6* (Wang J. *et al*., 2006), *Dvl1* and *Dvl2* (Wang Y. *et al*., 2006), *Pk1* and *Pk2* (Katoh and Katoh, 2003), *Vangl1* and *Vangl2*/*Ltap* (Kibar *et al*., 2001; Kibar *et al*., 2007). Mutation of these genes has been linked to defects in neural tube closure, misalignment of stereocilia in the ear, and hair patterning, as well as malformation of the cardiac outflow tract, kidneys, and the central nervous system (CNS) (Simons and Mlodzik, 2008). Virtually all PCP members are also implicated in axial development, especially events believed to rely on CE movements.

2.1.4 Planar Cell Polarity and RA

The microarray described above revealed two PCP pathway members, *Prickle1* and *Ror2*, suggesting a role for RA signaling in regulating PCP-related events in the embryo. Prickle, considered one of the core PCP genes, is a membrane-bound protein characterized by its PET (*Prickle*, *Espinas* and *Testin*) domain, 3 LIM (*Lin11*, *Isl-1* and *Mec-3*) domains and a C-terminal prickle homologous (PKH) domain (Katoh and Katoh, 2003). Prickle co-localizes with Vangl to the proximal edge of cells in opposition to Fz-

Dsh activity (Jenny *et al*., 2003; Jenny *et al*., 2005) - this asymmetric localization of proteins is one of the foundations of PCP. Gain- and loss-of-function studies in zebrafish have implicated Prickle in convergent extension movements (Carreira-Barbosa *et al*., 2003), while perturbation of the *Xenopus* Prickle homologue is associated with disrupted gastrulation movements and failure of neural tube closure. In mice, two orthologs of the *Drosophila Prickle* gene have been identified; *Pk1* and *Pk2*. Knockout of either *Prickle* gene causes severe growth arrest and embryonic lethality. Both of these phenotypes are likely attributed to a defect in the ability of cells to control the orientation of mitotic cell divisions. In $Pk1^{-/-}$ mutants, pregastrulation movements are arrested as blastocyst stage mutants fail to define a proper apical-basal axis (Tao *et al*., 2009), while $Pk2^{-/-}$ knockout mice fail to thrive past preimplantation stages (Tao *et al*., 2012).

Ror2 is an orphan receptor belonging to the receptor tyrosine kinase (RTK) family, and has been found to play several roles in mammalian development. It is well documented to participate with Wnt5a in non-canonical signaling while concurrently inhibiting canonical/β-catenin–dependent Wnt activity (Mikels *et al.*, 2009; Oishi *et al.,* 2003). Ror2 null mice survive until birth but die shortly thereafter and display severe skeletal abnormalities, most notably shortened limbs and tails, and misshapen skull bones (DeChiara *et al.,* 2000, Takeuchi *et al.,* 2000). Disruption in the polarity of hair stereocilia, a hallmark feature of many PCP mutations, was also observed. Mutation of ROR2 is also associated with two congenital human diseases, autosomal dominant brachydactyly type B (BDB1) and autosomal recessive Robinow syndrome (RRS), both of which are forms of skeletal dysplasia (Afzal and Jeffery, 2003). Precise interactions with PCP members have yet to be confirmed, but it has been proposed that Ror2 may act

as a co-receptor with Wnt5a and Frizzled in the PCP pathway, acting much in the way LRP-5/6 functions as co-receptor in the canonical Wnt pathway, both for signal transduction and reciprocal pathway inhibition (Wang *et al.,* 2011).

As discussed above, alteration in RA signaling can have profound effects on development. Retinoid deficiency or excess, or RAR knockout, have been linked to spina bifida, cardiac outflow defects and kidney malformations (Shenefelt, 1972; Ratajska *et al,* 2009), all of which are reminiscent of the defects associated with aberrant PCP signaling. Both *Pk1* and *Ror2* both were identified in the microarray as potential retinoid targets, and partially phenocopy atypical RA signaling, suggesting that RA may impact on PCP through direct regulation of these genes.

3.1 ADDITIONAL CANDIDATE RA TARGET GENES

3.1.1 Germ Cell Nuclear Factor/NR6A1

Also identified in the microarray was *Germ Cell Nuclear Factor (GCNF)/NR6A1* (Chen *et al.,* 1994; Hirose *et al.,* 1995). As part of the nuclear receptor superfamily, NR6A1 shares the same structural makeup of these receptors, with a short N-terminal A/B domain, a well-conserved DNA binding domain (C domain), a hinge D domain, and the C-terminus E domain containing the putative ligand-binding region (Chung and Cooney, 2001). NR6A1 shares the highest similarity with the RXR receptors (32-34%) (Chen *et al.,* 1994). Similar to retinoid receptors, NR6A1 binds DR0 response elements to mediate transcriptional activation (Chen *et al.,* 1994), however, NR6A1 homodimerizes on DNA, as opposed to RXR-dependent heterodimers (Borgmeyer, 1996; Schmitz *et al.,* 1999).

NR6A1 transcripts are detected in the mouse as early as E6.5 and by E8.5 are found

throughout the neurectoderm, with expression stronger in posterior regions (Chung *et al.,* 2001). *NR6A1* knockout in the mouse is embryonic lethal; mutants exhibit trunk and posterior defects, including an open neural tube and failure of axis rotation (Chung *et al.,* 2001). While no link has been formally demonstrated, the open neural tube phenotype and its overlapping expression pattern with PCP members suggests a potential link between *NR6A1* and PCP signaling.

Previous work in PCC7-Mz1 and P19 embryonic carcinoma cell lines, both models of retinoid-induced differentiation, showed RA-mediated induction of *NR6A1* transcripts within 3 hours of treatment, consistent with direct regulation (Bauer *et al.,* 1997; Heinzer *et al.,* 1997). Recently, NR6A1 has also been under investigation for its role in repression of pluripotency markers, including direct regulation of *Oct4* and *Nanog,* in embryonic stem (ES) cells upon RA treatment (Gu *et al*., 2005). Silencing of these genes is achieved through differential recruitment of methyl CpG-binding factors and DNA methyltransferases recruited to target gene promoters (Gu *et al.,* 2011). While research has focused on the action of NR6A1 in this process, more thorough characterization of the regulation of *NR6A1* as a direct RA target is currently lacking.

4.1 HYPOTHESIS AND OBJECTIVES

I hypothesize that three genes necessary for proper development, the planar cell polarity members *Prickle1* and *Ror2,* as well as *NR6A1,* are directly regulated by retinoic acid.

This hypothesis will be addressed through (1) analysis of transcription induction by exogenous RA, and (2) functional studies of candidate gene promoters.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

CD-1 wild-type mice were obtained from Charles River. Timed matings were set up overnight; successful matings were determined by the presence of a vaginal plug the following morning and noon of the day of plug detection was termed E0.5. Pregnant females were sacrificed by cervical dislocation at E8.5 and embryos harvested in diethylpyrocarbonate (DepC)-treated phosphate buffered saline (PBS) unless otherwise noted.

RAR mutant skin samples were obtained from E18.5 fetuses from intercrosses between RAR$\alpha^{+/-}\gamma^{+/-}$ animals (Lohnes et al., 1993; Lufkin et al., 1993). Dorsal skin samples were harvested in PBS, snap-frozen, and stored at -80°C for later use.

2.2 Cell Lines

P19 embryonic carcinoma cells were maintained in Alpha Modification of Eagle's Medium (αMEM; Multicell, Wisent) supplemented with 7.5% heat-inactivated donor bovine serum (HI-DBS), 2.5% heat-inactivated fetal bovine serum (HI-FBS) and penicillin/streptomycin in 5% CO_2 at 37°C. For routine maintenance, cells were passed at a 1:10 ratio every other day.

COS7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Multicell, Wisent) supplemented with 10% HI-FBS and penicillin/streptomycin in 5% CO_2 at 37°C. For routine maintenance, cells were passed at a 1:4 ratio every other day.

F9 embryonic carcinoma cells were grown on gelatin-coated plates and maintained in DMEM supplemented with 10% HI-FBS and penicillin/streptomycin in 5% CO_2 at 37°C. For routine maintenance, cells were passed at a 1:15 ratio every other day.

2.3 Analysis of Gene Expression

2.3.1 Embryo Culture

E8.5 embryos were harvested in pre-warmed PBS supplemented with 10% HI-FBS, then transferred to DMEM supplemented with 25% HI-FBS at 37°C until all embryos were collected. Embryos were stage-matched by somite number and divided into 4 groups in a 6-well tissue culture plate. Embryos were subsequently cultured in DMEM, 25% HI-FBS and 3µL/mL cycloheximide (Sigma) or vehicle (EtOH) where appropriate, in 5% CO_2 at 37°C. After 30 minutes, 10^{-6} M RA (Sigma), or vehicle (DMSO), was added and embryos cultured for an additional 4 hours. Embryos were then washed twice with PBS and transferred into 4mL snap cap tubes and stored at -80°C in Trizol reagent (Invitrogen).

2.3.2 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA from embryos or cells was used to make cDNA using standard procedures. cDNA was subsequently amplified by PCR using *β-Actin* as an input control. Oligonucleotide sequences used for PCR were:

***β-Actin*:**

5'- AGCCATGTACGTAGCCATCC & 5'- CTCTCAGCTGTGGTGGTGAA

***Cyp26A1*:**

5'- AAGATCCGCCGGCTTCAGGCTA & 5'- TTGCAAACTCTTTGCCTACACAGCTCC

***NR6A1*:**

5'- GGTATGCTGTGACACAGGCAGAACT & 5'- GGGGCACATTCACCATCTTGCCT

***Prickle1*:**

5’ - CTGTGGGGAGCATATTGGTGTGGA & 5’ - CGCCGAACTTGAGGAGGAGCTG

***Ror2*:**

5’ – CGTGTAGTCCCCGAGATGGCAG & 5’ CACGAAGTCCGGGGGCTCCA

2.3.3 RARE Analysis

30 kilobase (kb) pairs of sequence upstream of the transcriptional start sites of *NR6A1*, *Ror2* or *Pk1* was scanned using Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess) to identify canonical response element sequences for diverse transcription factors.

2.4 Transfections and Luciferase Reporter Assays

2.4.1 Plasmid Constructs

Oligonucleotides were designed to amplify two 3kb regions of the *NR6A1* promoter immediately upstream of the transcriptional start site (denoted NR6A1 Region 1 – ‘proximal’ section, and NR6A1 Region 2 – ‘distal’ section) by PCR using high-fidelity taq polymerase (Hi-Fi taq; Invitrogen) and genomic DNA isolated from a mouse tail biopsy. Amplicons were isolated using a Qiagen PCR purification kit and TA cloned into pDK101 (ATCC). Following validation by sequencing, inserts were subcloned into TK109 using restriction sites incorporated into the PCR primers (NR6A1 Region 1 utilizing BamH1 and Xho1 sites, NR6A1 Region 2 utilizing HindIII and Xho1 sites).

2.4.2 Transfections

DNA was transfected into COS7 or P19 using the calcium phosphate method in 6-well plates in triplicate. Briefly, 0.3µg of a β-galactosidase (β-gal) expression vector (for

normalization of transfection efficiency), 0.2μg each of RARγ and RXRα (where indicated), 1μg of reporter vector and pCDNA4 (where needed), to a final amount of 2μg DNA per transfection. DNA was combined with calcium chloride, mixed with HEPES-buffered saline, then added to cells. Media was replenished 24 hours later, adding 10^{-6} RA (final) or DMSO (vehicle), and cultured for an additional 24 hours. Cells were rinsed, disrupted in 150μL of lysis buffer, frozen, and analyzed using the Promega Luciferase Assay System as per the manufacturer's instructions using an LMax II microplate reader and SOFTmax PRO Software Version 4.7 (Molecular Devices). β-gal activity was quantified from cell lysates using CPRG as a substrate and assessed for absorption at 585 nm on a Spectramax M2 microplate reader using SOFTmax PRO Software Version 4.7.

2.5 RARγ Antibody Generation

A 136bp region of the F domain of *RARγ* (*RARγ-$_F$*) was amplified by PCR using the primers 5'-TATGAATTCCGAGAGATGCTGGAGAACCCG (forward); and 5'-ATAGTCGACTCAGGGCCCCTGGTCAGGTTG (reverse, restriction enzyme sequences used for subsequent cloning are underlined) using Hi-Fi taq polymerase, purified and cloned into pCR®2.1 (Invitrogen) utilizing the T/A overhangs, and verified by sequencing. The insert was then subcloned into pGEX4T-2 (GE Healthcare) or pMAL-CX-2 (New England Biolabs) via the EcoR1 and Sal1 sites.

To generate GST fusion protein, the pGEX4T-2-RARγ-$_F$ plasmid was transformed into BL21 cells, grown to an A600 reading of 0.5-0.6, induced with 0.3mM IPTG, and allowed to grow for an additional 3 hours. Cells were pelleted by centrifugation, lysed, sonicated and centrifuged again, and the GST-RARγ-$_F$ containing supernatant was bound to glutathione beads. GST-RARγ-$_F$ protein was eluted from the beads using 15mM

reduced glutathione, dialyzed in PBS, concentrated, and used for rabbit immunization by a commercial enterprise (Cedarlane).

MBP-RARγ-$_F$ protein, generated as for the GST fusion protein, but using pMAL-CX-2-RARγ-$_F$, was bound to CN-Br activated sepharose beads (GE Healthcare), and then incubated overnight with the rabbit serum. Elution of the bound α-RARγ-$_F$ was achieved with 0.2M glycine (pH 2.5), and the eluent neutralized with K_2HPO_4. The antibody was dialyzed in PBS and concentrated using Amicon Ultra spin columns (Millipore), and stored in sodium azide (0.01%, final) at -20°C.

2.6 Chromatin Immunopreciptation (ChIP)

2.6.1 Cells

P19 cells were grown to approximately 90% confluency, rinsed with PBS then fixed using 1% formaldehyde in PBS for 10 minutes at 37°C. Fixation was quenched by the addition of glycine to a final concentration of 0.125M. Cells were rinsed twice with PBS then resuspended in a ChIP lysis buffer (1% Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris-HCl [pH 8.1], 1X protein inhibitor cocktail). Samples were left on ice for 15 minutes then sonicated using a Bronson sonicator in 4 pulses of 30 seconds each. 5% of the sample was removed as an input control. The remaining lysate was divided for immunoprecipitation and brought up in volume with ChIP dilution buffer (1% Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris-HCl [pH 8.1], 1X protein inhibitor cocktail). Clearing of the lysate was carried out by adding 50µL of pre-blocked A/G sepharose beads with 2µg of sheared herring sperm DNA and 10µg of rabbit pre-immune serum and overnight rocking at 4°C. 5µg of appropriate antibody or pre-immune IgG was incubated with 50µL of A/G sepharose beads and 2µg of sheared herring sperm

DNA overnight at 4°C with gentle rocking. The next day, pre-cleared lysate was added to the antibody/bead slurry and incubated with rocking for 4 hours at 4°C. The beads were then washed sequentially for 10 minutes each in TSE I (0.1%SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl [pH 8.1], 150mM NaCl), TSE II (0.1%SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl [pH 8.1], 500mM NaCl), buffer III (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl [pH 8.1]), and $T_{10}E_1$. DNA was eluted in 300µL elution buffer (1% SDS, 0.1M $NaHCO_3$) and incubated at 65°C overnight to reverse the crosslinking. DNA was then isolated using a QIAgen PCR purification kit and analyzed by PCR.

2.6.2 Embryos

E8.5 embryos were fixed in 1% formaldehyde in PBS for 8 minutes at room temperature, and the reaction stopped through the addition of glycine to a final concentration of 0.125M. Embryos were washed twice with PBS then resuspended in RIPA buffer (10mM Tris [pH 8.0], 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 1X protein inhibitor cocktail). Embryo tissue was disrupted by passage through $26^1/_2$ gauge needle three times, then sonicated for 2 minutes using a Branson Sonifer 450 (settings: duty cycle 20%, output 2) and centrifuged to remove debris. An input sample was removed and the remainder precleared using 2µg sheared herring sperm DNA, 10µg pre-immune serum and 50µL of Protein A/G agarose beads (Santa Cruz) for 2hrs at 4°C with gentle rocking. Precleared chromatin was precipitated as above using 5µg of appropriate antibody (α-Gal4, α-Cdx2, αRARγ-$_F$) or a pre-immune serum control; 'no DNA' and/or 'no antibody' controls were also used. The next morning, 50µL Protein A/G beads plus of 2µg sheared herring sperm

DNA was added per sample and incubated with gentle rocking for 3 hours at 4°C. The beads were then washed sequentially for 10 minutes with 1mL of 1 X TSEI, 1 X TSEII, 1 X buffer III, and 3 X $T_{10}E_1$. DNA was purified and analyzed by PCR as above.

2.6.3 Oligonucleotides for ChIP Studies

Cyp26A1 RARE: 5' – GAACCCCGATCCACAACCC & 5' – ATGGGAAGCCCATGGTACCG

Cdx1 RARE/CDRE: 5' – GGTAGGTACACAATGCAACTCGGTG & 5' – GGGGGTTCCGTCTGTAAGGTAG

***Cdx2* off-target control**: 5' – GGGGGTTCCGTCTGTAAGGTAG & 5' – TAGGGTAGAAACTCCTCCTTGACG

***NR6A1*, 3kb**: 5' – GTCAGTCACTCCTTTGCATAG & 5' – CCCCCACCTCCCTTGAATCTC

***NR6A1*, 5kb**: 5' – CAGGAGTGGGGCTTGCACGC & 5' – GGGATGTCCCAAGTGCACTG

***NR6A1*, 7kb**: 5' – GCCCCTGGGACTGGCCTGCC & 5' – TGGTGTTCTGTTGGTCTCCC

***NR6A1*, 9kb**: 5' – TAATTGGATGGCATTTTTGGG & 5' – TGGCATTTCATGACTTAGGG

***Prickle1*, 5kb**: 5' – CACCCCTGCTCTTGACTGCG & 5' – ACGTACAGTACACACACAGC

***Prickle1*, 9kb**: 5' – TCTTTTGTGCTCAGTGTAGCC & 5' – ACCCTGTGCCCAACCTTGCC

***Ror2*, 1kb**: 5' – AGGCTGTTGGTGAACAAAGG & 5' – GCCACCCACCATCAGAAGCC

***Ror2*, 7kb**: 5' – TCACAGTAAACACTTTCTGG & 5' – CCTGGCAGATCCTCTCTGGG

***Ror2*, 9kb**: 5' – AGGTGAAGGTCACGGCAACC & 5' – CCTGTGACCCATCCAATAGC

2.7 Western Blot Analysis

2.7.1 Protein Extraction from Cells

COS7 or P19 cells were grown to 95% confluency on 10cm plates, washed twice with ice-cold PBS then scraped into eppendorf tubes in 1mL PBS/1mM EDTA. Cells were recovered by centrifugation, lysed in 300µL Whole Cell Lysis buffer (WCLB) (20mM Tris [pH 8.0], 25mM NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 1% Trition, 10% glycerol, 1mM DTT, 1X protein inhibitor cocktail) and sonicated for 30 seconds (10% output duty cycle 10) on ice. The lysate was centrifuged to pellet cell debris and supernatant stored at -80°C prior to use.

2.7.2 Protein Extraction from Skin Samples

Protein was harvested from frozen dorsal skin from E18.5 mice from RAR$\alpha^{+/-}\gamma^{+/-}$ intercrosses using Whole Cell Extraction buffer (62.5mM Tris [pH 6.8], 25% glycerol, 2% SDS, 2% β-Mercaptoethanol, 1X protein inhibitor cocktail), and disrupted using a Polytron homogenizer. Samples were centrifuged to pellet debris, and protein-containing supernatant transferred to new tubes. Long-term storage was at -80°C.

2.7.3 Western Blotting

Proteins were resolved on 10% SDS-PAGE gels then transferred to Immobilon-P (Millipore) membranes by standard techniques. Membranes were blocked with 5% non-

fat milk powder in PBS:0.1% Tween 20 (PBST), then incubated overnight with appropriate primary antibody at 4°C (antibodies were generally diluted 1:1000 in PBST/milk solution). Blots were rinsed and then incubated with appropriate secondary antibody (HRP-conjugated, diluted 1:20000) for an hour at room temperature. Following a final rinse, immunodetection was carried out using Luminata Forte ECL substrate (Millipore) and the signal revealed on Bioflex® scientific imaging film (Clonex).

2.8 Immunoprecipitation Studies

COS7 cells were transfected with FLAG-tagged RARγ and GFP expression vector (to monitor transfection efficiency) as above. Media was changed after 24 hours, and protein isolated at 48 hours, as described above. 5% of cell supernatant was removed for an input control and the remaining lysate was pre-cleared with 5% protein A/G beads (SantaCruz) at 4°C for 2hrs. Separate A/G beads (50µL) were pre-bound with 5µg of antibody (or IgG control) for 2 hours at RT, and the cleared lysate incubated with the beads and rocked overnight at 4°C. The next morning a 5% depletion control was taken, beads were washed 3 X 2 minutes with WCLB at 4°C and used for western blot analysis.

CHAPTER 3

RESULTS

As described in the introduction, the microarray analysis identified several candidate genes that, after preliminary tests by RT-PCR and *in situ* hybridization studies, were confirmed to be induced by RA *in vivo* (data not shown). Of these, *NR6A1, Pk1* and *Ror2* were chosen for further study due to known or potential links to the planar cell polarity pathway.

3.1 EFFECT OF RETINOIC ACID ON GENE EXPRESSION

To assess further whether *NR6A1*, *Pk1* and/or *Ror2* are putative direct RA targets, embryo culture experiments utilizing cycloheximide were performed. Cycloheximide is a potent inhibitor of translational elongation, therefore if increases in transcription were observed following RA administration in the presence of cycloheximide it would suggest no intermediate proteins are necessary for a response, consistent with direct regulation.

Following embryo culture, transcript levels were assessed by reverse-transcriptase polymerase chain reaction (RT-PCR). As expected based on the microarray data, *Ror2* showed an increase in transcript levels following RA treatment [Fig 3.1; *Ror2,* lane 1 compared to lane 3]. Notably, cycloheximide alone also produced an increase in expression [Fig 3.1; *Ror2,* lane 1 compared to lane 2]; however this is consistent with other immediate-early target genes, and has been proposed to be due to message stabilization facilitated by cycloheximide blockage of mRNA degradation (Cosgaya *et al.,* 1998; Edwards and Mahadevan, 1992). Treatment by both cycloheximide and RA concurrently mediated a clear increase from control levels [Fig 3.1; *Ror2,* lane 1 compared to lane 4], although whether this is above cycloheximide-only treatment is difficult to determine. *Pk1* displayed a very similar pattern to *Ror2* whereby RA induced transcripts above baseline levels, but a much stronger induction was seen with

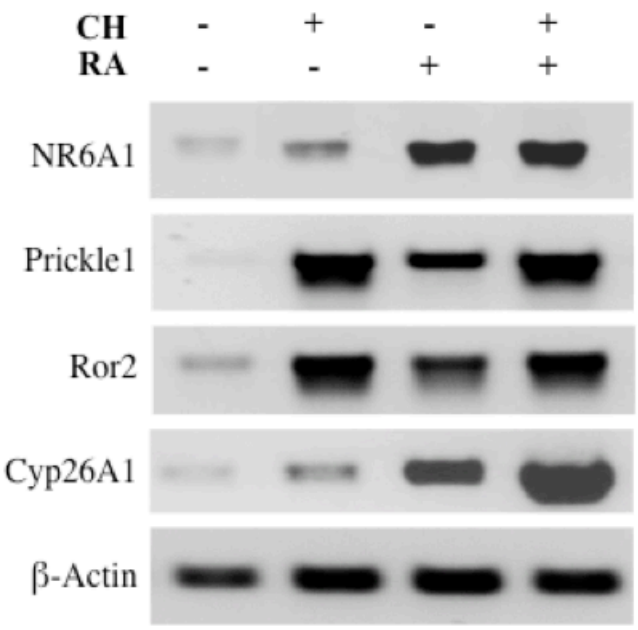

Figure 3.1 RT-PCR analysis of expression changes in target genes following cycloheximide/RA embryo culture.

Changes in gene expression were assessed by RT-PCR using cDNA generated from CD-1 embryos harvested at E8.5 which had been cultured in cycloheximide (0.1%), followed by RA (10^{-6}M) treated media (n=6). All three putative targets (*NR6A1, Pk1* and *Ror2)* show a clear induction of transcripts upon exposure to RA. *Cyp26A1* is a known RA-regulated gene and was used as a positive control, while *β-actin* was used as a loading control.
See text for details.

cycloheximide alone. Again, although an induction was seen when both treatments were combined, the high level of induction from cycloheximide made it difficult to determine any additional effects mediated by RA [Fig 3.1; *Pk1*]. Therefore from these studies it cannot be clearly concluded if RA induction of *Ror2* and *Pk1* occurs independently of *de novo* protein biosynthesis.

NR6A1 displayed a modest increase in transcript levels following treatment with cycloheximide alone [Fig 3.1; *NR6A1,* lane 1 compared to lane 2], and a more robust increase in expression levels following RA treatment [Fig 3.1; *NR6A1,* lane 1 to lane 3]. Importantly, treatment with both cycloheximide and RA yielded an increase in expression which was clearly above that of either treatment alone [Fig 3.1; *NR6A1,* compare lane 4 to 2 and 3]. This is similar to the superinduction effect exemplified by RA induction of direct targets such as *Cyp26A1* [Fig 3.1; *Cyp26A1*], where transcripts are induced by RA and further protected from degradation by cycloheximide. This finding is therefore consistent with *NR6A1* as a candidate for a direct RA target.

3.2 BIOINFORMATIC ANALYSIS FOR CANONICAL RARES

As discussed in the introduction, a number of direct RA target genes have a canonical RA response element within their promoter regions. To examine if *Pk1, Ror2* or *NR6A1* contain such a motif, a region of 30kb upstream of the translational start sites was scanned for each candidate target gene using the Transcriptional Element Search System (TESS). Although no canonical RAREs were identified in this analysis, there were instances of single PuG(G/T)TCA half-sites, which may represent potential non-canonical response elements (data not shown). In this regard, it is well established that the majority of direct RA target genes are regulated through atypical RAREs (Balmer and

Blomhoff, 2002), necessitating a different approach to investigate the basis for retinoid regulation of these candidate targets.

3.3 CLONING OF NR6A1, Prickle1 AND Ror2 PROMOTER SEQUENCES

Several approaches were used to functionally analyze the promoter regions of the three candidate target genes for RA-responsive domains. The first was to use PCR to amplify regions of the upstream sequences of *Ror2, NR6A1* and *Pk1* loci and clone these sequences into a reporter vector containing a minimal basal promoter driving a luciferase reporter gene (see Materials and Methods for further reference). Unfortunately, the high GC content of these promoters negated this strategy. New sets of primers were then used to amplify shorter intervals of 2-3kb in length. Using this approach, two regions of the *NR6A1* promoter representing 6kb of sequence immediately upstream of the translational start site were successfully cloned into the reporter vector. These were transfected into COS7 [Fig 3.2A] or P19 [Fig 3.2B] cell lines, along with RXRα and RARγ expression vectors, and analyzed for RA response. While a significant induction was seen for the positive control vector, consisting of luciferase under the control of three tandem canonical RAREs, no response was observed from the *NR6A1* sequences in either cell line [Fig 3.2A & B]. While this suggests that there are no functional response elements in the 6kb of sequence located 5' to the *NR6A1* start site, it does not rule out regulatory elements elsewhere in the locus. Indeed, RAREs have been reported 10,000 bp upstream of transcriptional start sites, or up to 7000bp downstream (Balmer and Blomhoff, 2005). The lack of response could also be attributed to a lack of co-operative transcription factors necessary for RA induction of *NR6A1* in these cell lines.

3.4 CHROMATIN IMMUNOPRECIPITATION (ChIP) ANALYSIS

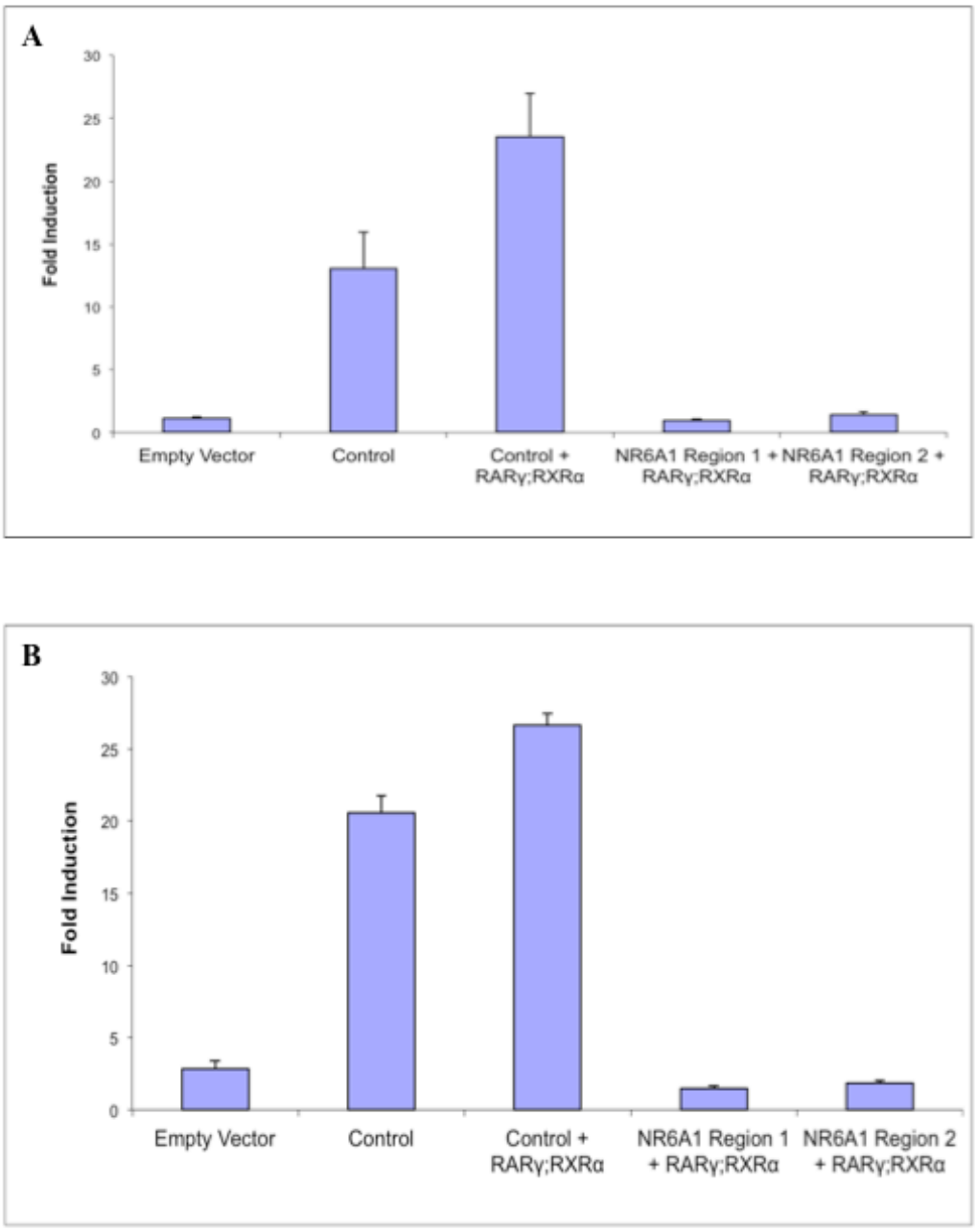

Figure 3.2 Luciferase reporter assay for investigation of RA-responsive regions in the *NR6A1* promoter.

Two 3kb regions of the *NR6A1* promoter were transfected into COS7 (A) and P19 embryonic carcinoma (B) cell lines along with RARγ and RXRα receptors, and assessed for luciferase reporter activity upon application of 10^{-6}M RA (n=3). A control vector consisting of three tandom RAREs upstream of the reporter cassette was used to verify RA induction.
See text for details.

3.4.1. Generation of a ChIP-Competent RAR Antibody.

Chromatin immunoprecipitation (ChIP) is a powerful means to examine target gene occupation by a transcription factor, such as the RARs. While this can be an extremely useful technique, it relies on an antibody-antigen interaction, and commercially available RAR antibodies are not suitable for such means. To circumvent this, I attempted to generate a highly specific antibody against RARγ which could be used for this purpose.

The F domain of the RARγ receptor presents a suitable antigen for several reasons. This particular domain shows the least conservation among nuclear receptors, including between RARs; it is therefore likely to be highly specific, and should minimize antibody cross-reactivity. It is also quite small, only 37 amino acids long, which should increase the likelihood of antibody specificity, and simplifies manipulation in order to generate appropriate fusion proteins for immunization and antibody purification. RARγ was chosen due to its expression in the caudal region of the embryo at tailbud stages and its known involvement in regulating critical genes, such as Cyp26A1, in this region (Abu-Abed *et al.,* 1998; 2003).

Briefly the RARγ F domain was PCR amplified then sub-cloned into both a glutathione S-transferase (GST) fusion protein expression vector and pMAL, a maltose binding protein (MBP) expression vector [Fig 3.3], and verified by sequencing. GST-RARγ was expressed and purified [Fig 3.4A] and the purified protein was then used for antigen immunization in two rabbits [Fig 3.5A]. Antibody purification was then performed using the MBP-RARγ protein coupled to CN-Br activated sepharose [Fig 3.4B]. The purified antibody was eluted from the beads and concentrated [Fig 3.5B].

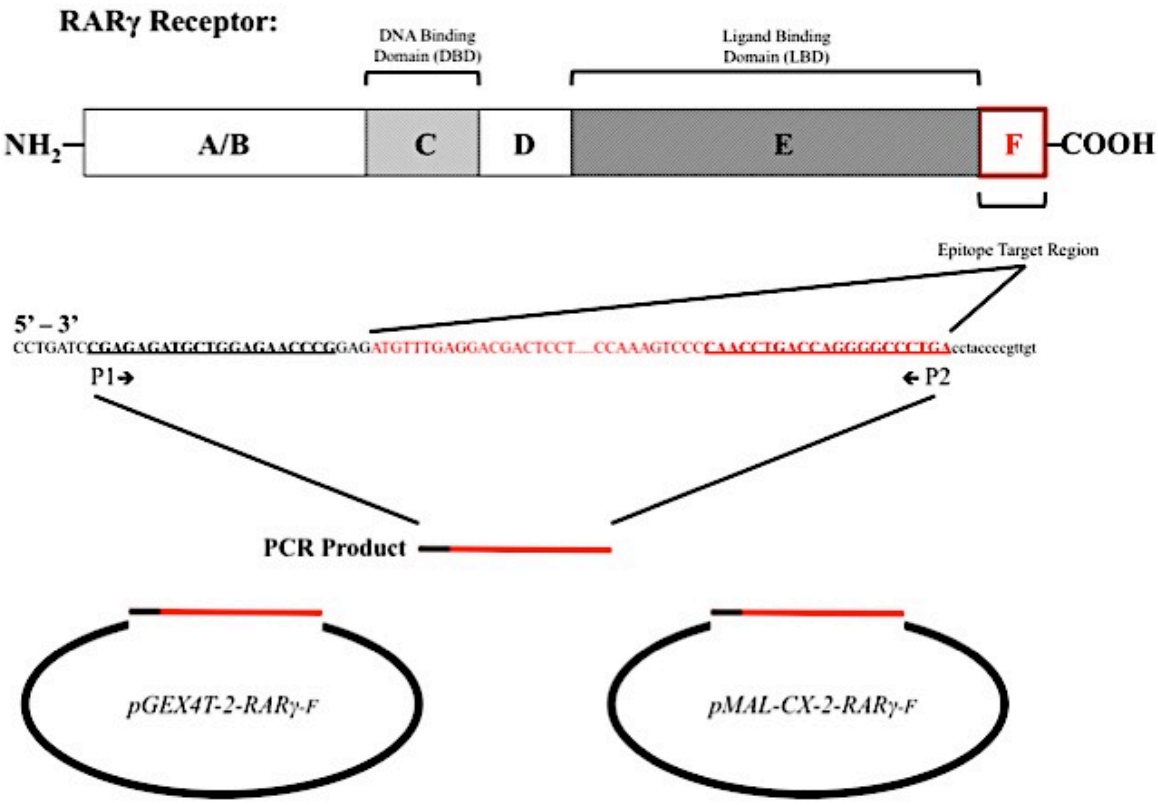

Figure 3.3 Schematic diagram of the epitope target region for a RARγ antibody.

A 136bp region representing the C-terminal F domain of RARγ was PCR amplified and cloned into GST and MBP expression vectors. RARγ was chosen for several reasons including its unique sequence and high abundance in the tailbud region of somitogenesis stage embryos.

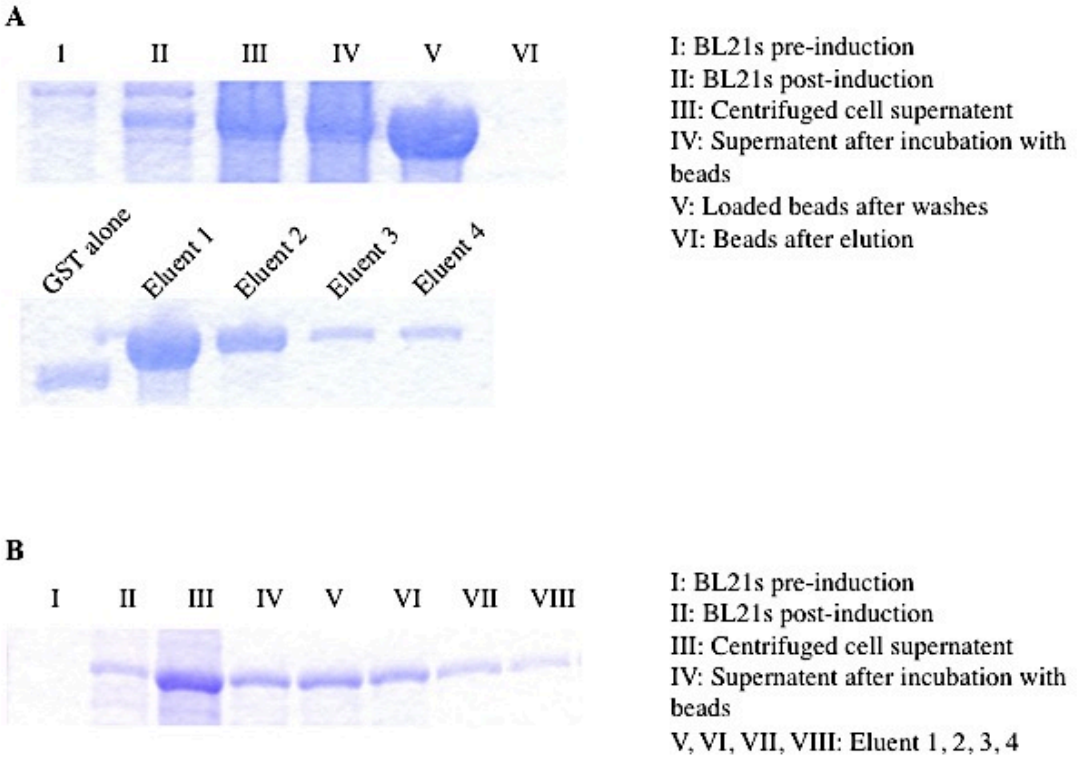

Figure 3.4 Coomassie blue staining of SDS-PAGE protein gels during production of RARγ fusion proteins.

BL21 *E. coli* cells were transformed with (A) GST- or (B) MBP-RARγ expression vectors and induced with IPTG. Cells were lysed and centrifuged, then the supernatant was bound to glutathione (for GST-RARγ purification) or amylose (for MBP-RARγ purification) coated agarose resin. Elution from the beads was performed using either reduced glutathione or maltose, as appropriate.

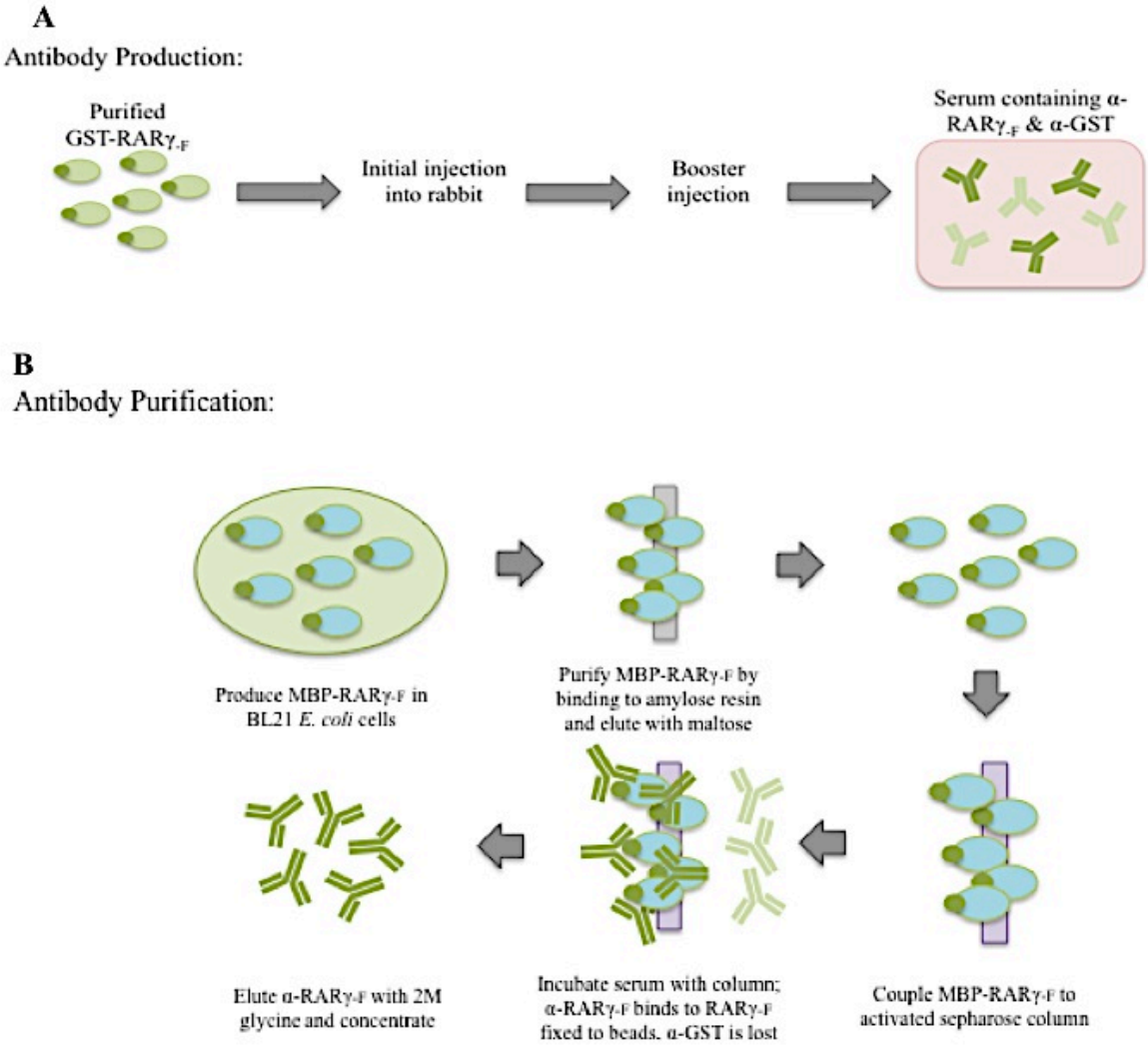

Figure 3.5 Model for the production and purification of RARγ antibody.

GST-RARγ was purified from *E. coli* cells by exploiting its high affinity for glutathione, using appropriately coupled agarose beads. The purified protein was then used for antigen immunization in two rabbits (A). α-RARγ was recovered from test sera by coupling purified MBP-RARγ to a CN-Br activated sepharose column, which was then incubated with the serum for antigen binding. Elution with 2M glycine from the column allowed collection of purified α-RARγ (B).

Western blot analysis revealed recognition of RARγ from transfected COS7 cell lysate [Fig 3.6A; lane 3], as well as protein harvested from bacteria transformed with the parental RARγ expression vector [Fig 3.6A; lanes 6 & 7], compared to negative controls. The lack of background and robust signal seen in these initial tests were encouraging as to the antibody's potential, especially since commercial antibodies lack robust avidity and specificity.

Further western blot analysis used protein samples from the skin of wild-type, RARγ$^{+/-}$ and RARγ$^{-/-}$ mouse embryos. Skin was chosen as it expresses abundant levels of RARγ. A clear signal was observed at the anticipated molecular weight in the wild-type sample, while the lane containing sample from null embryos was completely devoid of signal [Fig 3.6B; lane 3 compared to WT in lane 1]. Interestingly, the heterozygous tissue appeared to display a gene dosage effect with approximately half the intensity seen in wild-type samples [Fig 3.6B; lane 2]. Note also the lack of cross reactivity with other protein species, confirming that this antibody displays high specificity for RARγ, and is capable of detecting the endogenous receptor. These results are in marked contrast to those typical of a commercial antibody, exemplified by a western blot that was run in parallel using such a reagent [Fig 3.6C]. Note that the commercial antibody showed no discernable difference in signal intensity between genotypes, and exhibited a high degree of non-specific reactivity [Fig 3.6C; compare lane 1 to 2 or 3].

3.4.2 Immunoprecipitation Studies with α-RARγ

To test whether the antibody possessed immunoprecipitation (IP) capabilities, a necessary feature for a ChIP-competent antibody, COS7 cells were transfected with a FLAG-tagged RARγ expression vector, and lysates then immunoprecipitated with α-

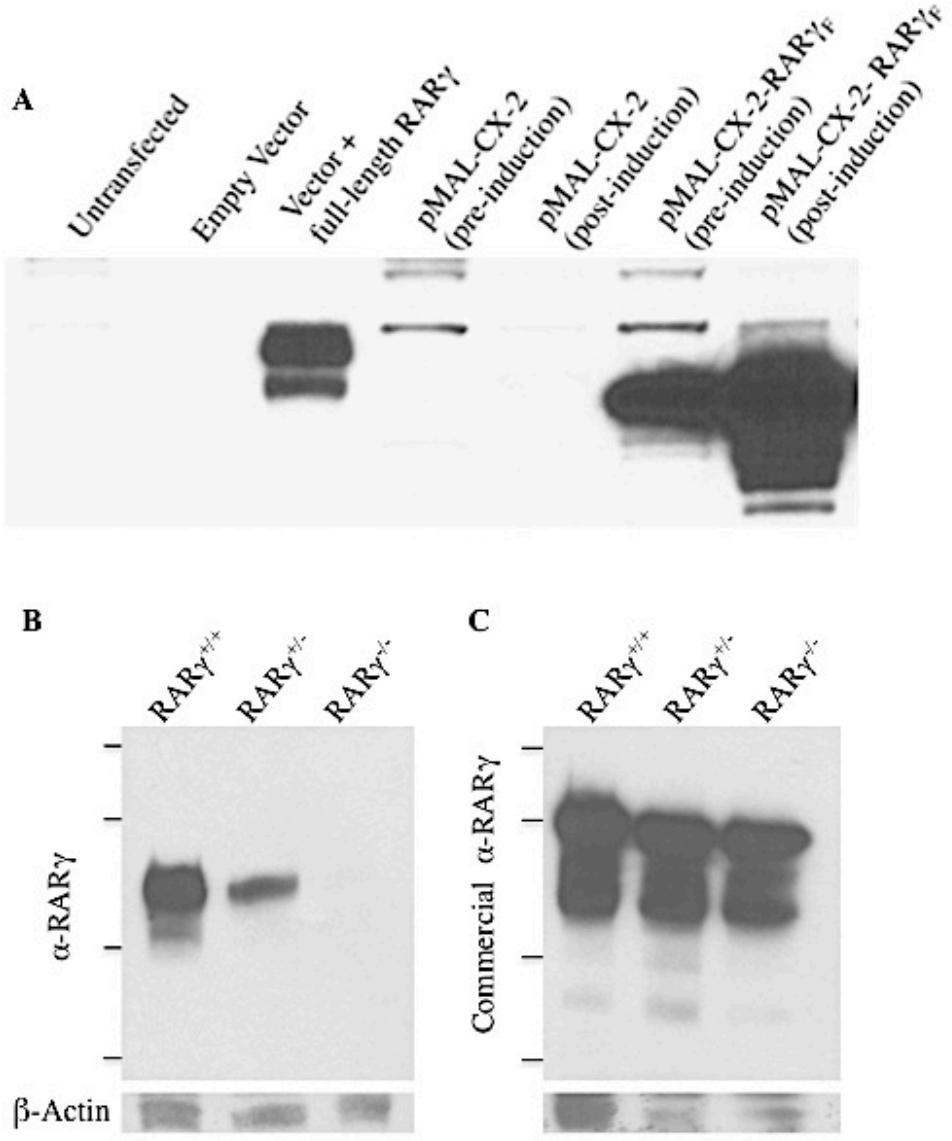

Figure 3.6 Western blot analysis using the new RARγ antibody on cell lysate and skin samples.

Protein harvested from transfected COS7 cells (lanes 1-3) and the parental MBP alone or MBP-RARγ expression vectors were expressed in bacteria both before and after IPTG induction (lanes 4-7) and used to assess antibody specificity. The full-length RARγ protein runs at a predicted size of 54kDa, while the pMAL protein is 43kDa and pMAL-RARγ approximately 47kDa (A). Skin samples from wild-type, $RAR\gamma^{+/-}$ and $RAR\gamma^{-/-}$ embryos were also used for western blot analysis (B), which were run in parallel with a commercially available antibody (C) to compare specificity.

RARγ. Samples were resolved on an SDS-PAGE gel and probed in a western blot with anti-FLAG to detect the immunoprecipitated receptor. Untransfected COS7 lysate controls were used to control for specifity, and, as evidenced by the lack of any significant signal on the blot [Fig. 3.7], confirmed that the antibody has low cross-reactivity. In contrast, a robust signal was detected after IP from FLAG-tagged RARγ COS7 lysate, with concomitant depletion in the supernatant [Fig. 3.7]. This result shows that this new RARγ antibody displays a considerable IP capacity, a necessary quality for ChIP studies.

3.4.3 Chromatin Immunoprecipitation with α-RARγ

To determine whether the antibody was suitable for *in vivo* ChIP analysis, immunoprecipitated DNA was used to scan for known RAREs. To do this, primers were designed to amplify regions corresponding to well-characterized RAREs on established direct RA targets (Houle *et al.,* 2000; Loudig *et al.,* 2000) [Fig. 3.8A]. *Cyp26A1* is regulated by RARγ in the caudal embryo (Abu-Abed *et al.,* 1998), therefore it served as a persuasive positive control for RARγ occupation, while *Cdx1* contains a RARE (Houle *et al.,* 2000) and a CDRE (Cdx Response Element) in its proximal promoter (our own unpublished data), allowing it to double as both a positive control and to evaluate immunopreciptation efficiency. On the *Cyp26A1* promoter, robust binding was observed by α-RARγ [Fig 3.8B; *Cyp26A1,* lane 6], and showed no non-specific binding. On the *Cdx1* promoter, occupation of the region was shown by both α-RARγ and α-Cdx2, as expected, while also showing little non-specific binding [Fig. 3.8*; Cdx1*, lane 6 and 7]. An upstream region of *Cdx2* was used as an off-target control to confirm that the

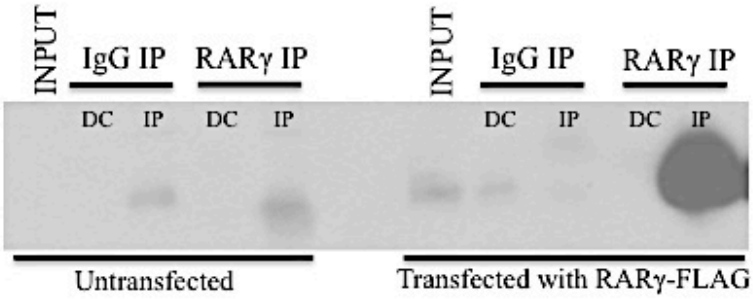

Figure 3.7 Immunoprecipitation of RARγ-FLAG by α-RARγ from COS7 cells.

COS7 cells were transfected with a RARγ-FLAG expression vector, then immunoprecipitated using either α-IgG (control) or α-RARγ. Depletion controls (DC) were taken of the cell lysate following immunoprecipitation (IP). Protein detection was performed by western blot using an α-FLAG antibody.

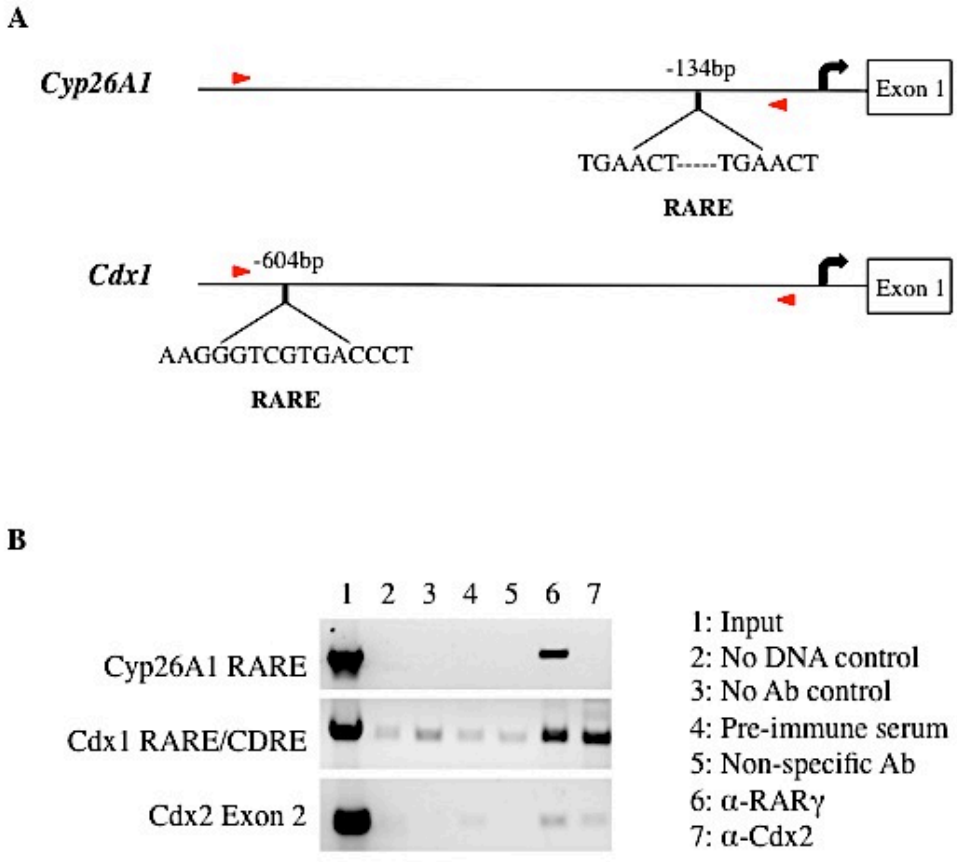

Figure 3.8 ChIP utilizing the new RARγ antibody to assess for RAREs in the promoters of known RA-regulated genes.

E8.5 embryos were used to prepare chromatin for immunoprecipitation using α-RARγ and α-Cdx2, along with controls. PCR primers (red arrow heads) were designed against the proximal promoters of Cyp26A1 and Cdx1, which both contain established RAREs (A), to verify antibody specificity. ChIP pull-downs confirmed antibody binding in these regions, along with Cdx2 binding on the Cdx1 promoter, and displayed minimal signal on a Cdx2 off-target control pull-down (B) (n=3).

antibodies were binding specific target regions [Fig. 3.8; *Cdx2* Exon 2], and as gauged by the lack of binding, appears to be sufficiently specific.

Once this was demonstrated, further ChIP analysis was used to identify regions on the candidate genes potentially harbouring RAREs. PCR analysis for the target genes was designed as a 'promoter walk' using primers to scan sections over 10Kb (at 2Kb intervals) of the target promoters. As the nature of chromatin shearing would create a variety of fragment sizes that would overlap between the regions not directly targeted by PCR, any antibody binding within the 10Kb proximal promoter should be identified [Fig. 3.9A].

ChIP analysis of the *Nr6A1* promoter showed robust binding around the -5Kb region [Fig. 3.9B, lane 6] as well as binding near the -3Kb and -9Kb regions [Fig. 3.9B, lane 6]. Weaker binding at the -7Kb region is also observed; this could be attributed to incomplete shearing of the chromatin, which would produce larger fragments and subsequent overlap into other regions. RAR occupation was also observed on *Ror2* and *Pk1*. The Ror2 promoter displayed the strongest binding near the 1Kb region [Fig. 3.9C, lane 6], but interestingly also showed moderate binding with α-Cdx2 in the same region [Fig. 3.9C, lane 7]. On the *Pk1* promoter, strong binding was seen around the -5Kb and -9Kb regions [Fig. 3.9D, lane 6]. While the investigation of the putative targets showed several regions of binding on some targets, this could potentially be attributed to incomplete shearing, or due to multiple RAR occupancy.

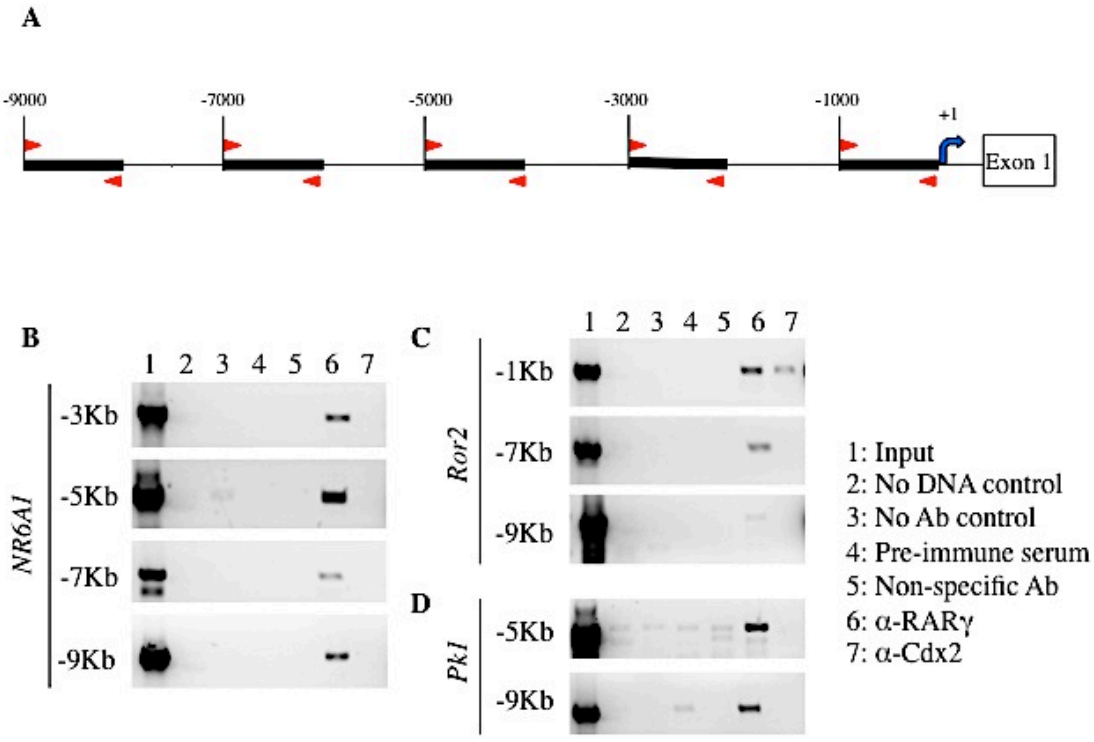

Figure 3.9 ChIP promoter walk utilizing the new RARγ antibody to assess for RAREs in the promoters of *NR6A1*, *Pk1* and *Ror2*.

E8.5 embryos were used to prepare chromatin for immunoprecipitation. RARγ occupation of the *NR6A1, Pk1* and *Ror2* promoters was assessed using the α-RARγ antibody. RT-PCR analysis was designed as a promoter walk with primer sets spanning alternate 1Kb segments of each promoter (A). *NR6A1, Pk1* and *Ror2* were then analyzed using a selection of controls including pre-immune serum and non-specific antibody pull-downs, alongside experimental IPs (n=3).

CHAPTER 4

DISCUSSION

4.1 RA REGULATION OF NR6A1, Prickle1 AND Ror2

Almost a century of research has revealed many of the dynamic roles retinoids play throughout life. Not only important for embryogenesis, vitamin A metabolites are also involved in the maintenance of vision, reproduction and homeostasis in the adult. Although a metabolic pathway for the generation of RA, as well as a mechanism for its regulation of target gene expression has been characterized, several key areas, including the nature of retinoid target genes, continue to pose challenges. Because of this, a global picture of RA signaling and its interactions with other pathways in the developing embryo have been difficult to elucidate. Part of this stems from the diverse nature of RA regulation of direct retinoid target genes; some contain canonical response elements while many others do not. Similarly, although new potential retinoid targets are constantly being discovered, clear evidence of direct regulation is often lacking. For example, Balmer and Blomhoff (2002) reported that, of the hundreds of RA-responsive genes, only 27 have been substantiated to be direct targets, with an additional 105 that appear likely to be directly regulated but require further evidence.

This project aimed to explore *NR6A1*, *Prickle1* and *Ror2* as novel retinoid target genes in the developing embryo. Confirmation of RA-mediated regulation was established through embryo culture studies, where induction of target gene expression was shown following the addition of RA. Evidence for a potential direct regulatory mechanism of *NR6A1* was observed by virtue of cycloheximide-insensitive induction by RA in embryo culture which prompted further investigation into its regulation. To this end, *NR6A1* promoter sequences were cloned into reporter vectors, which were then surveyed for RA-dependent activity in COS7 and P19 cell lines. Although no regulatory

regions could be identified by this approach, this work warrants further examination and will be addressed in future studies, as discussed below. In parallel, a novel α-RARγ antibody was generated and characterized. This reagent was subsequently validated for ChIP studies on known retinoid targets, including *Cdx1* and *Cyp26A1*, demonstrating occupation by RARγ in E8.5 embryos. Further analysis suggested association of RARγ with several regions of the *NR6A1*, *Pk1* and *Ror2* loci, supporting a direct role for retinoid signaling in directing their expression in the developing embryo.

4.1.1 RA Signaling in the Caudal Embryo

A major aspect of this work was to validate novel candidate RA target genes, the nature of which suggest potential interactions between RA and other signaling networks. Of the pathways that have been characterized as RA-dependent, the opposing RA and Wnt/FGF activities in the tailbud/trunk region of the early embryo are of particular interest (Aulehla and Pourquié, 2010). The caudal embryo is a highly dynamic region that experiences rapid changes in gene expression and precisely coordinated cell movements. The tailbud region of embryos contains a population of undifferentiated progenitor "stem" cells, necessary for embryo elongation; this population has yet to be exposed to any differentiating factors. As cells exit the caudal stem cell region, they leave the FGF field of expression and are subsequently exposed to RA emanating from more anterior regions of the embryo. We hypothesized that this prompts induction of retinoid target genes necessary for normal embryogenesis, and therefore we hypothesized that the naïve tailbud tissue may be a useful population to identify novel RA targets.

A microarray analysis examining RA-induced genes in this region of E8.5 embryos provided a number of putative targets for subsequent investigation. From the

analysis, three strong candidates were chosen, based on their relationship to the PCP pathway. Two of the genes, *Prickle1* and *Ror2* encode proteins for known members of the planar cell polarity pathway. A third, *NR6A1,* codes for an orphan receptor with a knockout phenotype reminiscent of PCP disruption. These genes were verified for RA-mediated induction by embryo culture followed by RT-PCR, which confirmed regulation of all three, while induction in the presence of cycloheximide strongly suggested direct regulation of *NR6A1*. Although *in vitro* attempts to study functional response elements in the genes will require further work, other experiments showed convincing evidence of RARγ-dependent regulation.

4.1.2 RA Signaling and Planar Cell Polarity

Planar cell polarity (PCP) is now well documented to be one of the major driving forces in a number of morphogenic events, including neural tube closure (Simons and Mlodzik, 2008; Wallingford and Harland, 2001). Mice with disruptions in a number of core PCP members, including *Loop-tail* (*Vangl2*), *Celsr1* (mouse ortholog of fly *flamingo*/*starry night*), and *Scrib1* among others all display severe neural tube defects indicative of PCP's central role in neurulation (Curtin *et al.,* 2003; Kibar *et al.,* 2001; Murdoch *et al.,* 2003).

Prior to neural tube closure, somitogenesis operates in the trunk region of the embryo, forming transient somites that will eventually give rise to the dermatome, myotome and sclerotome (see Dequeant and Pourquie, 2008). This process relies on a determination front whereby cells from the caudal tailbud region are maintained in an undifferentiated state and are displaced anteriorly as the embryo elongates until they reach a region conducive to somitogenesis; RA emanating posteriorly from somites is

believed to play a central role in this event, driving gene expression necessary for this differentiation process (Dubrulle and Pourquie, 2004). Importantly, processes involved in somitogenesis are also impacted by lesions in PCP players, as evidenced by the phenotype of Wnt5a null mutants (Yamaguchi *et al.,* 1999).

My work has shown that *Ror2* and *Pk1*, both of which are central to PCP signaling in vertebrates, are induced by RA *in vivo.* Although a direct regulatory relationship was not immediately clear, a link between these genes and retinoid signaling is consistent with the hypothesis of a relationship between RA and PCP. These genes are both expressed throughout caudal tissues when PCP is active; this event is also dependent on RA signaling and exclusion of canonical Wnt signaling which PCP has been well documented to antagonize (see Veeman *et al.,* 2003). Regulation of these *Ror2* and *Pk1* genes by RA may therefore serve as a mechanism to begin the cytoskeletal rearrangements necessary for PCP-dependent neural tube closure.

In addition to neural tube closure and somitogenesis, retinoid signaling has also been found to be important for development of other tissues dependent in part on correct PCP signaling. Inner ear development has become one of the best-understood examples of PCP signaling in vertebrates. Proper positioning of PCP proteins is necessary for correct alignment of stereociliary bundles in the cochlea, which would otherwise become disordered and affect sensing abilities of the hair cells (Denman-Johnson and Forge, 1999). RA signaling has now become associated with inner ear development, as RALDH2-deficient mice show abnormalities in the formation of these structures (Romand *et al.,* 2006). Therefore, it is possible that some of the PCP defects seen in the

ear could also stem from RA regulatory events, and perhaps, some of the PCP events necessary for neurulation could be mediated by retinoic acid.

4.1.3 RA Regulation of *NR6A1*

NR6A1 also emerged from this project as a strong candidate for direct RA regulation. Studies by other groups had previously identified *NR6A1* as an RA-regulated transcript in neural cell lines, although the nature of this regulation, or regulation by RA *in vivo*, have not been established (Bauer *et al.,* 1997; Heinzer *et al.,* 1997). The present study presents evidence for direct RA-mediated induction of *NR6A1* in the developing embryo, and suggests potential promoter regions responsible for this effect.

NR6A1 showed a response to RA and cycloheximide in embryo culture comparable to *Cyp26A1*, a *bona fide* direct RA target. NR6A1 has established roles in neurulation, and knockout phenotypes mimic those associated with both abnormal PCP and RA signaling, including failure of complete neural tube closure (Chung *et al.,* 2001). It is possible that NR6A1 could play a yet undiscovered role as a player in PCP signaling, and/or could potentially mediate the RA signal to other transcription factors or PCP members, which could be examined through future studies to help elucidate a mechanistic interaction between them.

4.1.4 Future Studies for Classification of *NR6A1, Pk1* and *Ror2* as RA Targets

Further investigation will be necessary to identify the nature of the relationship between RA and these candidate genes, especially identification of the means of retinoid regulation. While the embryo culture studies highlighted an induction of these genes by RA treatment, the inability to clone promoter sections for analysis of reporter activity meant that promoter studies, to isolate and identify regulatory sequences, could not be

accomplished. BAC recombineering was attempted as another strategy to investigate gene promoter responsiveness (refer to Appendix 1), and despite the difficulties encountered, may prove to be an effective means for addressing these questions. Indeed, assuming the technology can be optimized, BACs containing promoter sequence of candidate RA targets could be generated then quickly and easily screened for retinoid response. With isolation of a functional RARE as the fundamental role, the same recombineering technology could be used to delete successive sections of promoters and ultimately reveal the responsive sequence, as confirmed by mutagenesis studies. The positive results from the ChIP promoter analysis also provide clear evidence for RARγ occupation of the promoters for all three of the candidate target genes, consistent with a direct regulatory mechanism. These findings support further analysis to both better define the basis for RA-dependent regulation of these genes, as well as to use the anti-RARγ antibody for broader experimentation, as described below. Confirmation of direct regulation of these genes by RA would support a mechanistic basis for a link between PCP and RA. This would be an important advance in our understanding of the relationship between these fundamental pathways.

4.2 DEVELOPMENT OF A SPECIFIC RARγ ANTIBODY

Comprehensive investigation into RA signaling has been hampered by several factors, including the lack of suitable antibodies to explore the *in vivo* occupation of RARs on target genes. Rather classification of direct RA targets has generally relied on promoter analysis using mutagenesis and other labor-intensive means to correlate regulatory sequences with functional response. Production of an appropriate antibody

would facilitate broader screening initiatives such as genome-wide screens using ChIP-seq.

To this end, one fundamental goal of my work was to produce an RARγ antibody that would be suitable for ChIP studies. An antibody against the F domain of RARγ was chosen for development because of the prominent role of this receptor in gene regulation in caudal embryonic tissue and because of the particularly unique sequence of this receptor domain. Although the RARs share a relatively high sequence similarity in other domains, especially their DNA-binding and ligand-binding domains, the F domain is poorly conserved among the RARs, representing an ideal epitope for RARγ-specific antibodies.

The RARγ antibody has proven to be a highly specific antibody, as evidenced by western blot and ChIP analyses. While other RAR antibodies are available, they are generally restricted to use in cell culture studies using overexpression approaches. The quality of the antibody generated in these studies is perhaps best evidenced using skin samples from wild-type and RARγ knockout mice. This analysis clearly showed the antibody was not only highly specific compared to a typical commercially available reagent, but was clearly able to recognize the endogenous receptor. In addition, as the results from the immunoprecipitation studies indicate the antibody can complex with, and precipitate, the receptor in solution. Finally, the ChIP analyses indicate that the antibody specifically recognizes RARγ on DNA *in vivo* on known RAREs. This particular finding, demonstrating RARγ occupation on the *Cyp26A1* promoter, and the lack of off-target interactions, provided the substantiating proof to confidently use the antibody on embryonic material for screening the target genes for RAREs. The choice to use

Cyp26A1 for validation was severalfold; firstly, it is a well known and well described direct target of retinoid signaling with characterized RAREs. It is also known to be expressed, and RA-regulated, in the caudal embryo – the region we were interested in examining through precocious differentiation for the microarray – allowing it to be model for our expected response from the candidate genes. Most importantly, *Cyp26A1* in the caudal embryo is specifically regulated by RARγ (Abu-Abed *et al.,* 1998), making it an ideal target with which to validate the antibody. The antibody was also successful in binding the RARE present on the *Cdx1* promoter, showing that it can recognize diverse retinoid targets genes, and displayed no binding with other controls, confirming its specificity. Once this was substantiated, and using the previous findings from the embryo culture studies suggestive of direct regulation, we were confident in using the antibody for ChIP studies to explore the target genes for RAREs.

The results from the ChIP promoter walk highlighted several key areas of the candidate target promoters that may harbor RAREs. While we observed multiple sites along the promoters that showed some degree of binding, these could be explained as non-functional motifs which still bind RARγ, or could represent cryptic RAREs that could not be identified using a software program such as TESS, Unfortunately, as the promoter cloning studies were inconclusive or unsuccessful, further experimentation will be needed to confirm these regions for their responsiveness. However, in the areas where strong binding was observed, if a functional RARE is identified, this will underscore the usefulness of the new antibody for identification of retinoid targets *in vivo*.

4.3 FUTURE INVESTIGATION INTO RA REGULATION

The microarray investigating RA-induced expression of genes provided a wealth of potential targets to investigate. Without the use of an RARγ antibody, confirmation of direct RA targets requires considerable time and effort to test promoter regions for response using tissue culture and other approaches. However, this new reagent will allow screening and identification by more contemporary approaches to assess retinoid regulation in the embryo, as well as in other settings.

Of techniques to explore, chromatin immunoprecipiation followed by sequencing (ChIP-seq) has become an increasingly powerful tool to investigate DNA-protein interactions on a genome-wide scale, making it a highly attractive means to investigate RA-mediated gene regulation. This technology expands on the traditional ChIP assay, using the precipitated DNA fragments as input for high throughput sequencing. ChIP-seq experiments have now surpassed ChIP-on-chip technology, where immunoprecipiated DNA fragments are identified by hybridization to a microarray plate, in terms of resolution, coverage of the genome, background noise, and requires less starting material thereby decreasing amplification needs (Mardis, 2007; Park, 2009). While there can be sequencing errors, and the technology is prone to a bias towards GC-rich fragments, it is now considered one of the premiere methods to investigate DNA-protein interactions. As such, the RARγ antibody opens many new avenues of exploration.

Additionally, the initial microarray used to identify candidate targets still remains a rich source of unexamined potential retinoid target genes. Ideally, a ChIP-seq analysis would further validate some of these genes as direct targest, which then could be further characterized using BAC recombineering studies to identify response elements. This

could potentially uncover a wealth of information regarding other gene pathways that are regulated by retinoids during development which have yet to be discovered.

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APPENDIX 1

To assess larger regions of the *NR6A1* promoter, and circumvent the persistent difficulties of PCR-amplifying promoter segments, a bacterial artificial chromosome (BAC) recombineering approach was used. This technology allows the precise modification of large sections of DNA for a wide variety of downstream applications (Copeland *et al.,* 2001; Muyrers *et al.,* 1999).

1. Direct Cloning and Colony Lift Hybridization

Bacterial artificial chromosome (BAC) vectors containing on average 30kb of upstream promoter sequence for each target gene were acquired and used for amplification by PCR. Various combinations of PCR conditions were thoroughly investigated, however no representative amplicon could be generated for further manipulation. A 'shot-gun' approach was designed which would allow for larger stretches of sequence to be ligated into a reporter vector containing a minimal TK basal promoter and 3' luciferase cassette, used in earlier cloning experiments. This method involved the use of colony lifts and radioactively labeled probes for unique promoter fragments to identify which section of the BACs had been cloned into the reporter vector.

Briefly, BACs were digested with a particular restriction enzyme (RE) that would cut the upstream region into approximately 10 sections. The digested BAC fragments were then ligated at random into the reporter vector upon digestion with the same enzyme. Ligation products were transformed into bacteria, and the identity of the fragment sub-cloned revealed by a colony lift. This technique is a modified southern blot, whereby bacterial colonies are lifted onto nitrocellulose filters, lysed, and treated as to fix

DNA to the filter. Probe hybridizations used radioactively labeled oligonucleotides unique to each digestion product, allowing subsequent detection by autoradiography.

After several attempts at the method above, it was modified to enrich for chances of recovering particular regions. The BAC sequence was analyzed for low frequency RE combinations that could produce distinctive band sizes. After digestion, the products were seperated electrophoretically on an agarose gel where the predicted band size was extracted and used for further ligation work. The fragment was then identified by colony lift hybridization, as previously.

The benefit of these two methods over standard PCR techniques was that larger sections of the BACs could be recovered for subsequent reporter assays. Unfortunately, as they were met with little success, another approach was designed. This next technique possessed the added benefit of allowing the entire BAC to be used for reporter assays instead of isolated sections.

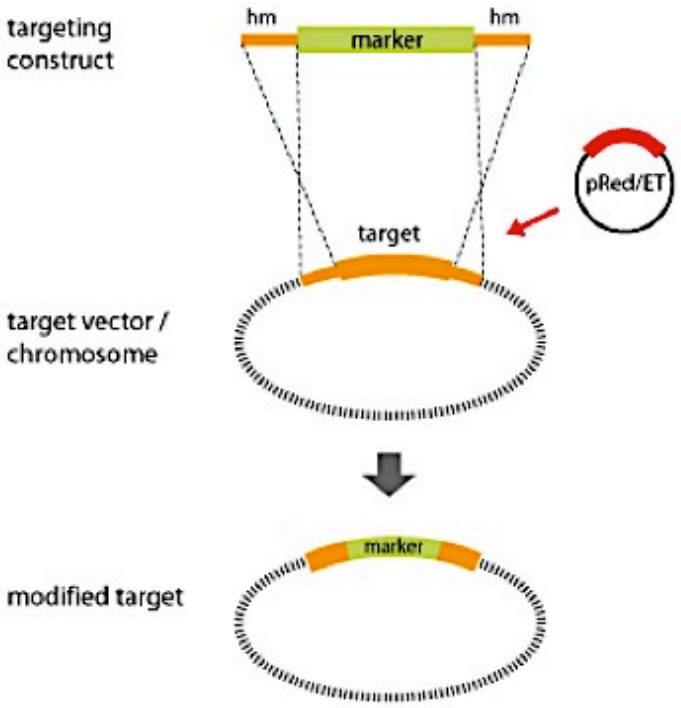
2. Homologous Recombination

Due to its increasing popularity for a variety of uses, homologous recombination, a means for modifying BAC sequences, was explored as a potential technique. This technology offers several advantages to conventional cloning in that BACs are generally large enough to sufficiently represent entire eukaryotic genes. As gene promoters are generally poorly described, inclusion of large areas of upstream sequence, as in BAC vectors, increases the likelihood of containing important sequences required for gene activation. Successful incorporation of a luciferase construct downstream of the start site would essentially turn the modified BAC into a reporting vector.

As mentioned previously, homologous recombination involves expression of a Red/ET expression plasmid that, under permissive conditions, allows expression of phage-derived exonucleases and annealing proteins responsible for recombination. Regions of sequence homology between the BAC and insert fragment define where recombination events are to occur, allowing precise placement of target sequences and avoiding the concerns of mutation inherent with PCR-mediated strategies [Fig 5.1]. This technique allows the user to insert, delete or modify specific regions of DNA facilitating a broad range of subsequent downstream applications.

Briefly, the Red/ET expression plasmid, containing a tetracycline resistance marker, was transformed into the *E. coli* BAC host. These cells were then grown in tetracycline and chloramphenicol (BAC-specific selectable marker) treated media. Following a sufficient growth and selection period, expression of the recombinase proteins was induced. In this case, induction was dependent on a temperature shift from 30°C to 37°C, and the addition of L-arabinose to the media. After a short growth period, the cells were prepared for electroporation of the linear insert fragment. Cells from this procedure were then plated and selected for according to the insert DNA.

For this project, homologous recombination was initially employed to insert a luciferase expression cassette downstream of target gene promoters. Upon completion, these modified BACs could be expressed in cell lines and assessed for induction responses after RA treatment. While a significant amount of troubleshooting was necessary to acquire any recombinant colonies, it became obvious that once optimized, a more streamlined screening approach was necessary. Hundreds of recombinant colonies were generated, but with no secondary selectable marker, each colony was laboriously



Supplementary Figure 5.1 Strategy for homologous recombination.

An insert is PCR-amplified which consists of the sequence of interest to be integrated, generally including a marker to aid selection, flanked by two regions of BAC homology (hm). Expression of a pRED/ET expression vector is induced which produces the proteins necessary for recombination to occur. This allows incorporation of the insert into the host BAC, which can then be transformed into a suitable *E. coli* host and screened for using the selectable marker.

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screened for proper insertion of the target sequence with no conclusive positive results. A major challenge to this PCR strategy was that identification was dependent on the presence of a specific amplicon spanning the BAC/insert junction site. Naturally, this was difficult to optimize with no positive control template, and as no definitive positive colonies were detected, this suggested that although conditions were now more favorable for colony growth, proper recombination was either at a low frequency, or conditions were still not optimized for correct target insertion. This meant that a new strategy would have to be employed to improve screening capabilities.

While optimization attempts using the luciferase cassette were still carried out, parallel experiments were conducted with a modified insert fragment. Instead of a luciferase sequence, an excisable NEO cassette containing an SV40 promoter flanked by loxP sites was used for recombination, which would allow more efficient colony screening owing to its antibiotic resistance. Once NEO-containing recombinants were successfully recovered, new obstacles were encountered stemming from the multiple loxP sites now present in the modified BACs. This caused persistent difficulties during cre-mediated excision attempts, whereby combinations of removals were impossible to predict. Unfortunately, after several attempts to transiently transfect NEO-containing recombinants into cell lines and perform the cre-mediated excision necessary to remove the SV40 promoter, it was ultimately determined that a new strategy might need to be employed to successfully generate the desired recombinants. As such, this aspect of the project will have to be considered at a future time.