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ROLE OF THE NEURONAL SPECIFIC TRANSCRIPTION COREGULATOR NPDC-1 IN RETINOID AND THYROID RECEPTOR SIGNALING IN HUMAN AND THE AXOLOTL AMBYSTOMA MEXICANUM

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION
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A dissertation submitted in partial fulfillment of the
Requirements for the degree of Doctor of Philosophy in the
Graduate Center for Toxicology at the University of Kentucky

By
Maria Theodosiou

Lexington, Kentucky

Director: Dr. Daniel J. Noonan, Professor of Biochemistry

Lexington, Kentucky

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ABSTRACT OF DISSERTATION

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AMBYSTOMA MEXICANUM

 Section I: This section is an introduction to the field of nuclear receptors. A
general overview of nuclear receptor-mediated transcriptional regulation is followed by
a review of literature on retinoid and thyroid receptor-mediated signaling.
 Section II: An introduction to NPDC-1 (neural proliferation, differentiation, and
control), its discovery and characterization with regards to developmental expression
and cellular localization. In addition NPDC-1 has been found to associate with a
number of cell cycle regulatory proteins. NPDC-1 is characterized as a regulator of
nuclear receptor-mediated transcriptional regulation. NPDC-1 was also demonstrated
to be regulated post-transcriptionally through the ubiquitin/proteosome degradation
pathway.
 Section III: Axolotl NPDC-1 (aNPDC-1) was cloned from axolotl brain and
analyzed for homology to NPDC-1 from higher vertebrates. The tissue distribution and
developmental expression of axolotl NPDC-1 were also examined.
 Section IV: The axolotl homolog for RARγ (aRARγ) was isolated from axolotl
brain. Axolotl NPDC-1 and aRARγ were then examined in a series of assays for
interactions. Axolotl NPDC-1 was found to repress transcription mediated by aRARγ to
a smaller extent than the repression observed in higher vertebrates. The DNA binding
of aRARγ-RXRα was increased in the presence of aNPDC-1 and complex mobility was
also observed. The domain of interaction
between aNPDC-1, aRARγ and hRXRα was localized in the amino terminus of aNPDC-
1. Axolotl NPDC-1 was also demonstrated to repress proliferation as measured by
reduced [^{3}H] thymidine incorporation.
 Section V: The axolotl homologs of TRα and TRβ (aTR) genes were isolated and
utilized in a series of experiments to demonstrate an interaction between aTRs and
aNPDC-1. As observed for βRE, aNPDC-1 increases the binding of aTR-RXR
heterodimer to xDR4, but no change in the mobility of the complex was observed.
Interaction between aNPDC-1, aTRα and aTRβ was localized to the amino terminus of
aNPDC-1. In contrast to previous observations for other nuclear receptors, aNPDC-1
was found to stimulate transcription mediated by axolotl TRs, suggesting a role for
aNPDC-1 in axolotl metamorphosis.

Section VI: A summary of data presented in the previous sections as well as a presentation of future directions and a proposed model for NPDC-1 actions in retinoid and thyroid-receptor mediated signaling in axolotl.

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Date

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To my family

ACKNOWLEDGEMENTS

The following dissertation would not have been possible without the love and support of a number of people. First and foremost, I would like to thank my Dissertation Chair and mentor, Dr. Dan Noonan, who exemplifies the high quality scholarship to which I aspire. A simple Thank you is not enough to express how thankful I am to have studied with Dr. Noonan and how much I have learned during my stay in his laboratory. It was a great experience; I truly learned a lot and will always cherish my time as a graduate student in his laboratory. In addition, I would like to thank Dr. Tom Vanaman, for helpful insights, comments and evaluations of data provided during lab meetings that allowed me to complete this project. Next, I wish to thank my full dissertation committee: Dr. Noonan, Dr. Nick Koszewski, Dr. Haining Zhu and Dr. Natasha Kyprianou for all their help and guidance. I want to thank my outside examiner, Dr. Michael Kilgore for accepting the task. A very special thank you goes to the later Dr. Zimmer who passed away recently. Dr. Zimmer was not only a valuable member of my committee but also a supporter during my graduate studies and a friend. He will be greatly missed but his legacy will continue. I feel truly blessed to have known him. And of course my graduate school tenure would not have been possible without the encouragement and support of Dr. Tomas Prolla at the University of Wisconsin-Madison. Under the guidance of Dr. Prolla, I learned a great deal and he encouraged me to follow my dream and go to graduate school.

This body of work, was made possible, with the help and guidance of a number of people here at the University of Kentucky. I wish to thank Dr. S. Randal Voss and his laboratory and especially James Mohaghan, for cloning the axolotl homolog of NPDC-1 and launching the studies in this dissertation and for all their help and guidance. Studies utilizing axolotl tissues would not have been possible without them. I would also like to acknowledge past and present members of the Noonan lab for their friendship and scientific input, especially Dr. Mike Spencer, and Brian York.

In addition to the technical and instrumental assistance above, I received equally important assistance from family and friends. Most importantly, I have to acknowledge my parents for encouraging me to follow my dreams and happiness. Obtaining a PhD was something I always wanted to do, and they supported me fully in pursuing it, from thousands of miles away. My mother, with whom I have had a number of emotional conversations over the course of the years, my father who was

always there to let me know that I always have choices, and that the world does not end with a bad grade or an unsuccessful experiment, thank you from the bottom of my heart. Thank you for everything. My brother for providing laughter and encouragement when it was most needed and my grandmother, who is a truly exceptional individual, thank you both for your love and your support. I could not have done this if I did not have your support even if you are thousands of miles away. My friends from the Panagia Pantovasilissa Greek Orthodox Church, Father George and Presbytera Harriet, Philip and Sonia Campbell, Peter and Kallie Sawaya thank you for being my substitute family and for making Lexington feel like a home away from home.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FILES

Maria Dissertation

ABBREVIATIONS

9cRA	9-cis Retinoic Acid
atRA	all-trans Retinoic Acid
ACTH	adrenocorticotropin
AF-2	Activation Function 2
β-gal	β-galactosidase
βRE	RARβ gene response element
CDK	Cyclin Dependent Kinases
CMV	Cytomegalovirus
CNS	Central Nervous System
CRABP	Cellular Retinoic Acid Binding Protein
CRBP	Cellular Retinol Binding Protein
DBD	DNA binding domain
DRn	Direct Repeat
ERAP	Estrogen Receptor-Associated Protein
GST	Glutathione-S-Transferase
HA	Haemaglutinin
HAT	Histone Acetyltransferase
HDAC	Histone DeAcetylase Complex
HLH	Helix-loop-helix
HRE	Hormone Response Element
HSP	Heat Shock Protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
LBD	Ligand Binding Domain
MAPK	Mitogen Activated Protein Kinase
NCoR	Nuclear Receptor Corepressor
NPDC	Neural Proliferation, Differentiation Control
NR	Nuclear Receptor
PCR	Polymerase Chain Reaction
RA	Retinoic Acid
RAE	Retinoic Acid Embryopathy
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Element
Rb	Retinoblastoma
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RXR	Retinoid X Receptor
RXRE	Retinoid X Response Element
SMRT	Silencing Mediator for retinoid and thyroid receptors
SRC-1	Steroid Receptor Coactivator-1
T3	3, 5, 3'-triiodothyronine
T4	3, 5, 3', 5'-tetraiodothyronine (thyroxine)
TH	Thyroid Hormone
TR	Thyroid receptor
TRE	Thyroid Response Element
TRH	Thyrotropin releasing hormone
TSH	Thyroid Stimulating Hormone
TRE	Thyroid Response Element
VAD	Vitamin A Deficiency
ZPA	Zone of Polarizing Activity

SECTION I: NUCLEAR RECEPTOR SIGNALING

Chapter 1: General Overview of Nuclear receptors

The proper organization and maintenance of the functions of a complex body plan by multicellular organisms requires specific intracellular signaling (1). The activities of intracellular kinase cascades and/or signaling pathways are induced by growth factors, neurotransmitters and peptide hormones binding to membrane receptors (1). Steroid hormones and other small hydrophobic signaling molecules, such as vitamins and metabolic intermediates, either enter the cell or are generated within the target cells and bind to cognate members of the nuclear receptor family (1). Nuclear receptors are important integrators of intracellular and extracellular signals and regulate gene expression in multicellular organisms.

The nuclear receptor superfamily is the largest group of eukaryotic transcription factors (2) and consists of structurally related proteins that are ligand inducible and regulate the transcription of target genes by binding to *cis*-acting DNA sequences (3). NRs regulate transcription by direct physical association with a variety of hormonal or other metabolic signals, by integrating diverse signaling pathways (as they are themselves targets of post-translational modification) and by signal transduction crosstalk (regulating the activities of other signaling cascades) (1). Through their ability to control gene expression, NRs regulate complex genetic and physiological events that trigger key steps in embryogenesis, development, metabolism, control/maintenance of homeostasis, reproduction, cellular proliferation, differentiation and death (1, 4, 5). The NR family can be separated into three

subgroups based on the chemical similarity of their ligands and on phylogenetic analysis of their DNA binding domain (3). The first subgroup are the steroid receptors and includes the androgen receptor (AR), mineralocorticoid receptor (MR), estrogen receptor (ER), glucocorticoid receptor (GR) and progesterone receptor (PR). The second subgroup is comprised of non-steroidal, RXR heterodimeric receptors such as the thyroid receptor (TR), vitamin D receptor (VDR), peroxisome proliferators activated receptor (PPAR), farnesoid X receptor (FXR) and retinoic acid (RAR) receptor, as well as the insect ecdysone receptor (EcR). The third subgroup is composed of the orphan receptors for which there is no known physiological ligand. For some time a distinction was made between classical nuclear receptors and orphan receptors. Recently, some orphan receptors have been matched with potential physiologic ligands, but there might be some orphan receptors that do not require a ligand to function (6). The classification of nuclear receptors into 6-7 phylogenetic families containing both orphan and non-orphan receptors further dismisses such discrimination (7). Many nuclear receptors also have been found to have different subtypes and isoforms (8-10). Thyroid receptor for example has two subtypes, $TR\alpha$ and $TR\beta$, with two isoforms that are created by alternative RNA splicing ($TR\alpha_1$, $TR\alpha_2$, $TR\beta_1$, and $TR\beta_2$). With the discovery that different receptor subtypes can respond differently to the same ligand (11) as well as the discovery of subtype selective ligands, the existence of receptor variants (12-15) increases the potential complexity and subtlety of the receptors' regulatory properties. The classification of nuclear receptors is made by virtue of evolutionarily conserved DNA-binding and ligand-binding regions. Comparison of the DNA-binding region reveals that they are derived from a common ancestral gene and that evolution of this family was completed before the arthropod/vertebrate split (3). It is speculated that there was a rapid evolution of

these genes as they are implicated in cell-cell communication, a crucial need for metazoans (the rapid divergence of early nuclear receptor genes coincides with the metazoan evolution burst), and when these genes were fixed in their present function only minor rearrangements occurred leading to an overall strong gene conservation (3).

Nuclear receptor proteins are single polypeptide chains with 5-6 major modular domains. The domain structures are variable in length among the receptors. Furthermore, some functions, such as activator binding, do not always correspond to a simple structural domain. To date, about 70 nuclear receptors have been identified, and with the exception of some unusual NRs which appear to contain only regions homologous to the DNA- or ligand-binding domains, all members display an identical structural organization. It is possible that a number of orphan receptors function exclusively as constitutive activators or repressors of transcription. Rev-Erb is such an example of a constitutive repressor that recruits corepressors but lacks a functional transcription activation function AF-2 (1). Traditionally the letter names A/B, C, D, E and F have been given to the different domains of a nuclear receptor (Figure 1). The amino terminal A/B domain is followed by a DNA-binding domain consisting of two zinc fingers (domain C), a hinge region D, and the ligand-binding domain (E) (16). Some nuclear receptors also contain a carboxyl-terminal region F that has no known function so far. The N terminal domain (A/B) is the least conserved domain across the superfamily. The A/B domain varies in length considerably between receptors. In the VDR the domain consists of 23 amino acids whereas in mineralocorticoid receptor it is 550 amino acids long. This domain contains one or more autonomous transcriptional activation functions (AF-1) which can activate transcription in a constitutive manner. Comparison of nuclear receptors from different subfamilies shows that the A and B domain cannot always be
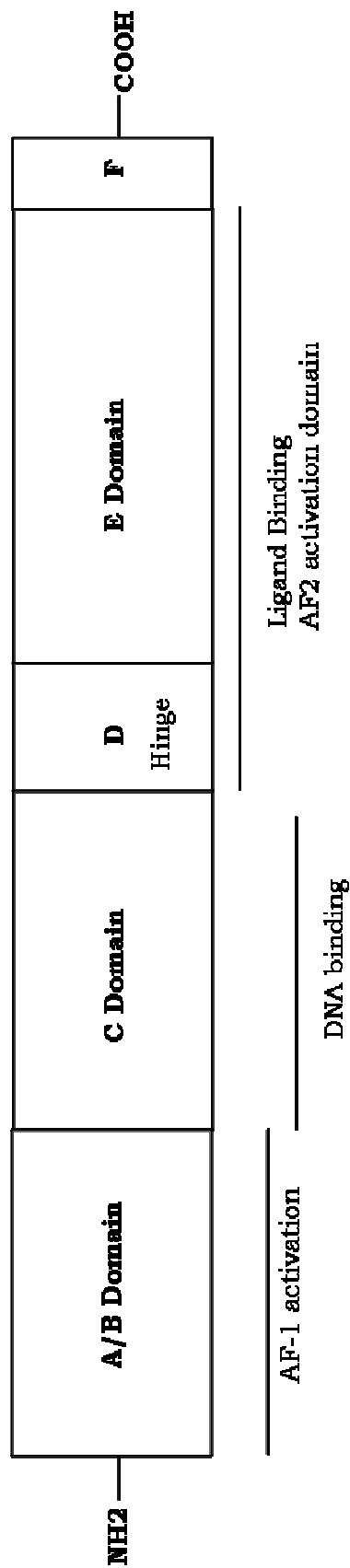
Figure 1: Schematic Representation of Nuclear Receptors. Nuclear Receptors all share a common modular structure

distinguished from each other. Efforts to determine the three-dimensional structure of this region have proven to be fruitless. Through alternative splicing and differential promoter usage, the presence or absence of different activation functions can be regulated (17). The N-terminus has also been found to be subject to posttranslational modifications such as phosphorylation.

The DNA binding domain (DBD), or C domain, lies adjacent to the A/B domain. This region is highly conserved and confers specificity for recognizing and binding to hormone response elements (HREs) on DNA (18). The DBD contains approximately 68 amino acids although it varies (e.g. DBD of PPAR consists of 67 amino acids while that of TR consists of 70 amino acids) (3). It is hypothesized that the C domain of the ancestral NR gene also contained 68 amino acids (3). The 68 amino acids in the DBD compose two zinc finger motifs, the N-terminal motif Cys-X2-Cys-X13-Cys-X2-Cys (CI) and the C-terminal motif Cys-X5-Cys-X9-Cys-X2-Cys (CII). The four cysteines in each motif chelate one Zn^{2+} ion. The two zinc finger structures play complimentary roles in the DNA binding process (19). The first zinc finger interacts with DNA in the major groove and is responsible for the sequence specificity of binding (20, 21). This allows for the specific recognition of short and imperfect inverted repeats of DNA (for steroid receptors) or direct repeats (for RXR heterodimeric receptors). The second finger and the region adjacent to it may be responsible for the definition of optimal spacing and alignment of half-sites as well as the direction of subunit interactions (20, 21). The region adjacent to the zinc finger may also be responsible for stabilizing the binding of protein monomers to single half-sites for some orphan receptors (20, 21). Several sequence elements within the C domain have been identified that contribute or define response element specificity, dimerization interface and contacts with the DNA backbone and residues flanking the DNA core recognition sequence. These sequences

have been termed P-, D-, A- and T- boxes. The P-box contributes to binding motif specificity, the D-box provides a dimerization interface, the T-box region forms a helix that corresponds to a dimerization surface in RXR homodimers and the A-box is the two zinc finger motifs (1).

The ligand binding domain (LBD) or E region is the hallmark of a nuclear receptor. The E domain is highly structured and encodes a number of distinct functions. The LBD confers specificity for ligand binding and performs a number of functions related to ligand binding such as receptor release from the heat shock complex, translocation to the nucleus, homodimerization, heterodimerization and transcriptional activation (19, 20, 22, 23). Hormone agonists promote these functions whereas antagonists can selectively block them (22). Evidence has been presented that ligand binding is an ability acquired during evolution and that the ancestral NR was an orphan (5, 24).

In addition to the three structural domains, nuclear receptors also have a flexible hinge region, D, that is found between the DBD and LBD. This region may allow the DBD and LBD to adopt different conformations without steric hindrance problems. A nuclear localization signal sequence can also be found in region D [reviewed in (17)]. The hinge region may also contain recognition sites for certain activator and/or repressor molecules (25).

Nuclear receptor-specific actions are derived from a combination of different parameters such as availability of ligand, receptor and non-receptor factors, structure of target site, interactions with other proteins such as general transcription factors and influences of other signaling pathways. These interactions result in ligand-regulated and ligand-independent effects on initiation of transcription of target genes (26). The genetic activities of NRs result from both direct modulation of the activity of

cognate gene programs and mutual interference with the activity of other signaling pathways (5). Ligands for nuclear receptors are able to circulate in the body bound to plasma proteins. Dissociation from these proteins allows for cell entry and association with their respective receptors. Steroids and Vitamin D probably enter cells through passive diffusion while thyroid hormone and retinoic acid entry require specific transport processes (26) (Figure 2). Non-liganded steroid receptors are bound to heat shock proteins (26) and are found in the cytoplasm. Upon binding of hormone, the steroid receptors dissociate from the heat shock proteins and are able to translocate to the nucleus, homodimerize and bind to HREs in the promoters of target genes (23). In contrast, TR subfamily members (such as TR, RAR and VDR) are predominantly nuclear localized and bound to their response element (usually as heterodimers with RXR) free of hsp proteins (26).

All nuclear receptors recognize derivatives of the same hexameric DNA core motif, 5'-PuGGTCA (Pu=A or G) (1). Mutation, extension, duplication and distinct orientations of repeats of this motif give rise to response elements that are selective for a given class of receptors. A spacer rule has been proposed to describe the preference of the various direct repeat (DR)-recognizing receptors. For example TR-RXR heterodimers show a preference for DR4, and RAR-RXR heterodimers have a preference for DR5 response elements. This rule is known as the DRn rule where n is the number of nucleotides between the two half-site motifs. These response elements can also be promiscuous, with more than one receptor dimer capable of binding to them (2). The half-site motifs also exhibit degeneration with some receptors having a preference for individual half-site motifs. For example, the preference for the half-site motif 5'-PuGGTCA over 5'-PuGTTCA follows the order TR>RXR>RAR (1). In addition to nucleotide preference in the half-site motif, certain receptors exhibit a preference for
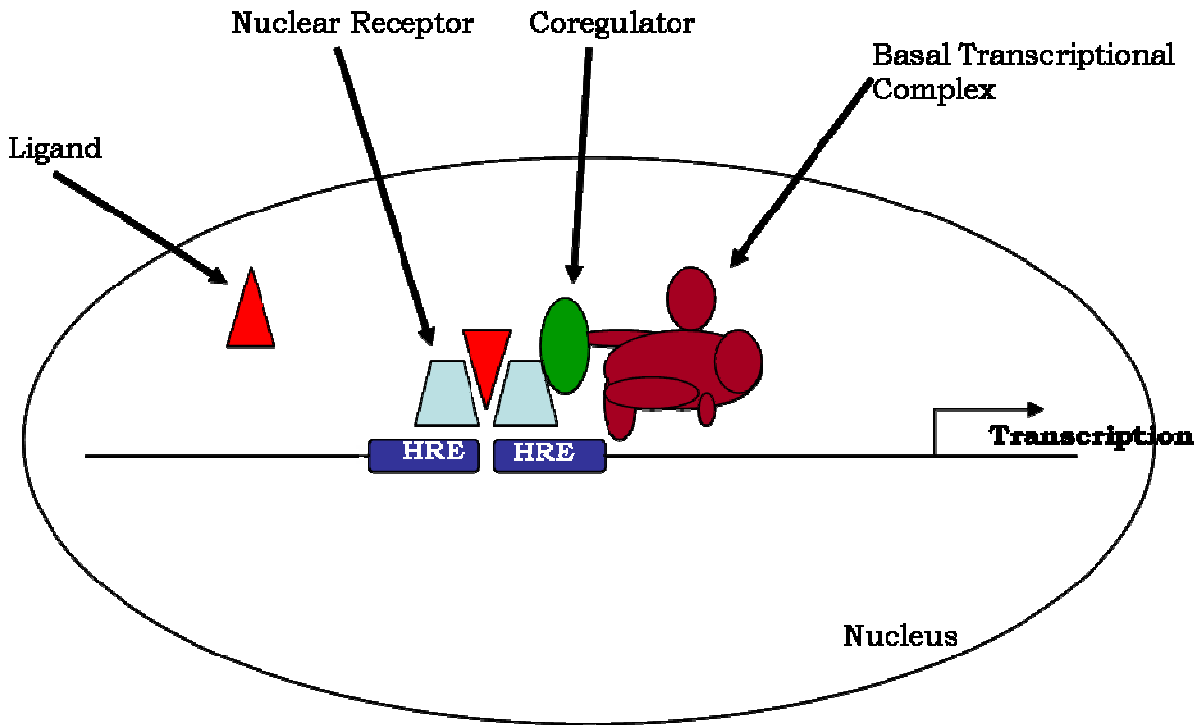
Figure 2: **Receptor Interactions in the Nucleus.** Ligand enters the nucleus and associates with its respective receptor. In the case of steroid receptors, this causes dissociation from heat-shock factors and translocation to the nucleus and dimerization. In the case of TR subfamily members the receptor is already in the nucleus bound to its respective response element. Binding of ligand induces interactions with coregulators and the basal transcriptional machinery and transcription occurs.

certain nucleotides in the DR spacer (1). Steroid hormones bind to response elements containing two half-sites (AGAACA) oriented as palindromic repeats separated by 3bp (IR3 elements) with each half-site binding a receptor monomer. Some orphan receptors recognize response elements composed of single half-sites and are believed to bind as monomers to their cognate response elements. In this case, additional residues 3' of the half-site are also recognized (27). A summary of NR DNA binding is presented in Tables 1 and 2. In the end the specificity of response element, DNA binding is due to both the recognition of a half-site motif and how or whether the receptor is dimerized.

Nuclear receptors can bind their response element as homodimers or heterodimers. Steroid receptors classically bind to their response elements as homodimers while RAR, TR, RXR and VDR have the potential to heterodimerize as well as homodimerize. The ability of RXR to promiscuously form heterodimers with RAR, TR, VDR and orphan receptors gives this receptor the ability to play an important role in various diverse signal transduction pathways. Heterodimerization is beneficial in that it increases DNA binding efficiency relative to the corresponding homodimers, leads to novel response element repertoire and allows for two signaling inputs, that of ligands of RXR and its partner (1). The signaling pathway identity for receptors like RAR, TR and VDR is maintained by the "RXR subordination" phenomenon, in that in heterodimers there is no response to RXR ligand and transcription is "controlled" by the RXR partner receptor. This phenomenon solves a potential problem that could arise from the ability of RXR to heterodimerize with a number of receptors and activate a number of signaling cascades in response to its ligand. It is unclear whether some RXR complexes will allow for RXR signaling in absence of a ligand for the partner of RXR (1).

Receptor subfamily	Bound to DNA?	Response element	Consequence of DNA binding
Steroid	Associated with heat shock factors	Palindromes AGAACA	Binding of hormone leads to conformational change, dissociation from HSF and binding to DNA as homodimers.
TR/RAR	Bound to DNA in absence of ligand	Direct repeats (A/G)GGTCA (A/G)GTTCA	Binding of hormone causes conformational change in binding domain and transcriptional activation as heterodimers.
Orphan	Bound as monomers or dimers to direct repeats		

Table 1: Nuclear Receptor Overview. Table 1 is a general overview of nuclear receptor subfamilies and their characteristics.

Receptor	Dimerization potential	Ligand	Response Element
ER	Homodimers	Estrogen	Palindromes
PR	Homodimers	Progesterone	IR3
GR	Homodimers	Glucocorticoids	Palindromes
AR	Homodimers	Androgen	
MR	Homodimers	Aldosterone, glucocorticoids	
TR	Homo or heterodimerization	Thyroid hormone	DR4
RAR	Homo or heterodimerization	All-trans-retinoic acid	DR5/DR2
RXR	Homo or heterodimerization	9-cis retinoic acid	DR1
PPAR	Heterodimers	Peroxisome proliferators	DR1/DR2
FXR		Bile Acids, Farnesol	DR5/IR1
VDR	Homo or heterodimerization	Vitamin D3	DR3
LXR		oxysterols	DR4
PXR			DR3
CAR			DR5

Table 2: Nuclear Receptors and their binding characteristics. This table is an overview of the general dimerization, DNA binding and ligand characteristics of nuclear receptor family members

The LBD of nuclear receptors has been a subject of much research and the first crystal structures of certain nuclear receptor LBDs were reported in 1995 (1). More recently the LBDs of heterodimeric receptors also have been solved (1). All the NR LBDs display a similar structure with 12 α-helices (H1-H12) and one β-turn arranged as an antiparallel α-helical sandwich in a three-layered structure (1, 5). Insight into the mechanism by which AF-2 becomes transcriptionally active was gained by the study of apo (non-liganded) and holo (liganded) LDB structures (Figure 3). Upon ligand binding H11 gets aligned with H10, leading to the swinging of H12 to unleash a loop that flips under H6, carrying along the N-terminal part of H3. In its final position H12 seals the ligand-binding cavity and further stabilizes ligand binding by contributing additional ligand-protein interactions (1). This is known as the mousetrap model. Coactivators are allowed to bind on the novel surfaces generated by agonist binding, and then these are able to recruit additional transcription factors. Simultaneously with agonist (but not necessarily antagonist) binding, corepressors bound to the apo-LBD dissociate. Certain antagonists force H12 into a third position distinct from the holo position whereby it impairs coactivator binding (1). The recent crystallization of heterodimer LBDs allowed for the comparison to homodimer LBDs. Overall, the structure is very similar; however within the heterodimer there is not equal contribution to the heterodimerization interface (1).

Observations of ligand-dependent transcriptional interference led to the conclusion that coactivators exist. The first evidence for the existence of coregulators was the observation that ERAP-140 and ERAP-160 (estrogen receptor associated proteins) interacted with GST-ER in the presence, but not the absence of, estradiol or diethylstilbestrol (28). An estrogen antagonist and anti-estrogen compounds were unable to promote the association of ER with ERAP. Mutational analysis showed that
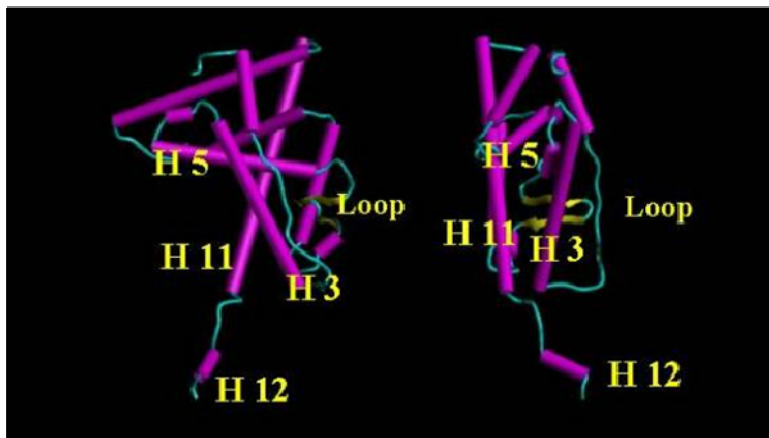
Figure 3A
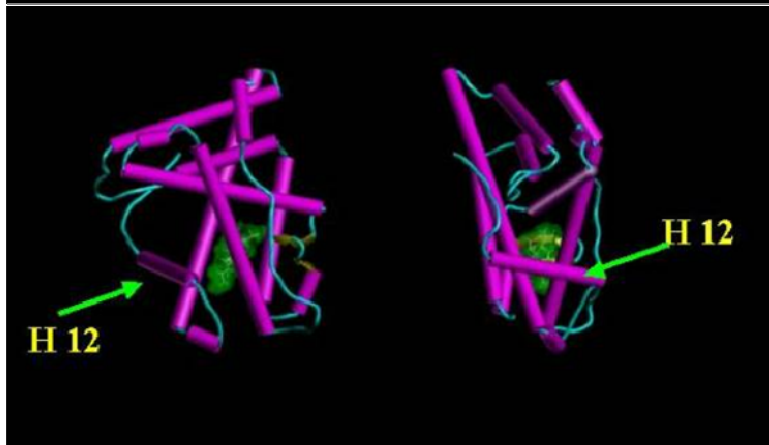
Figure 3B

Figure 3: Orthographic view of nuclear receptors. In figure 3A, unliganded receptor is shown. Helix 12 is located below and the volume is defined by H12, H11, H5 and H3 provides an opening in the LBD. Polar regions on H5 and H3 near the loop assist in docking the polar regions of the ligand. Figure 3B is an orthographic view of nuclear receptor after ligand binding. The result is a lipophilic ligand surrounded by the hydrophobic interior of the receptor. The resting place of H12 with respect to H11 and H3 is sequence and ligand dependent.

Figures taken from: http://www-personal.umich.edu/~lpt/domain.htm

this ligand-dependent interaction correlates with the ability to activate transcription. The first coactivator cloned and characterized, SRC-1, was shown to enhance the transcriptional activity of nuclear receptor family members in the presence of ligand (28). There are two intrinsic transferable activation domains in SRC-1 (AD1 and AD2), which are needed to achieve maximum steroid receptor activity (28, 29). SRC-1 appears to function in both ligand-dependent AF2 and ligand-independent AF-1 transactivation functions of nuclear receptors, as it is able to interact with both the carboxyl and amino terminal domains of nuclear receptors (28, 29). The carboxyl-terminus of SRC-1 possesses intrinsic HAT activity and also interacts with other HATs such as p300/pCAF (28). pCAF differs from other coactivators in its ability to bind the DBD of nuclear receptors in the absence of ligand. However, *in vivo* there appears to be a ligand dependency for pCAF, because in the absence of ligand nuclear receptors normally are associated with corepressors which inhibit pCAF recruitment.

Almost all coactivators identified to date have an LXXLL motif (leu-XX-leu-leu; X is any amino acid). This motif is referred to as an NR box and is necessary and sufficient to mediate interaction with nuclear receptors in the presence of ligand. The specificity of NR box usage is determined by the carboxyl-terminal sequences to the LXXLL motif (28). The presence of multiple NR boxes in the coactivator protein provides the specificity and flexibility for the assembly of nuclear-receptor coactivator complexes that serve diverse biological functions.

Upon ligand binding the receptor undergoes a conformational change and functions as a transcriptional activator. In the unliganded form, the AF-1 domain of the NR assumes a conformation that promotes interaction with corepressors. Several models have been postulated for NR transcriptional activation that attempt to incorporate the roles of ligand binding with coactivator recruitment (figure 4) (30).

This corepressor complex has histone deacetylase activity, which is associated with a compact "turned off" chromatin conformation, and transcription is repressed. Binding of ligand induces a conformational change that makes it impossible for the receptor to be bound to the corepressor complex, but able to complex now with activator proteins. Associated with coactivator complexes are HATs and ATP-dependent chromatin remodeling proteins. These proteins cumulatively relax chromatin structure permitting access to this structure by the basal transcriptional machinery.
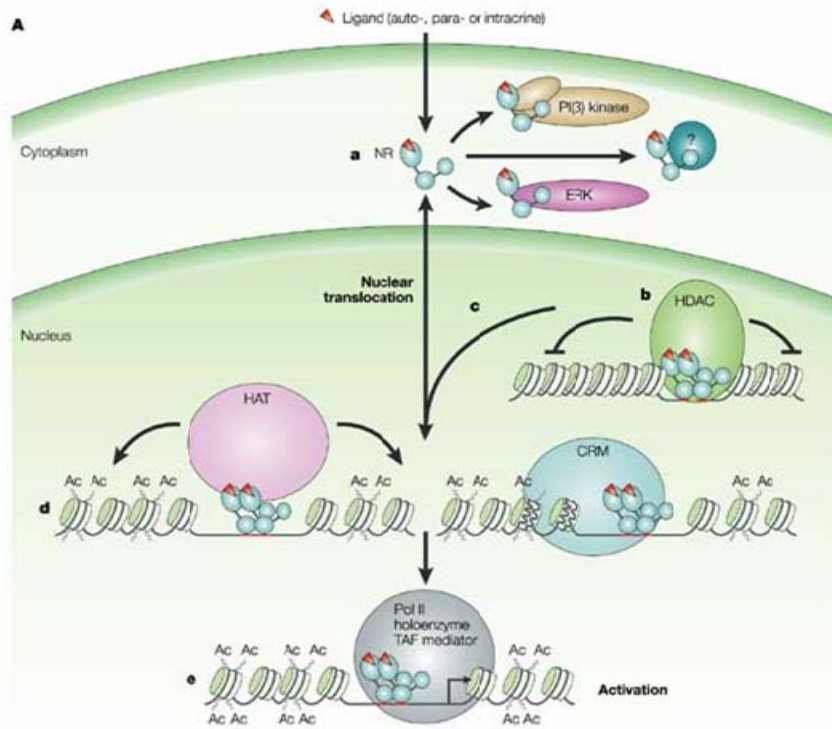
Gronemeyer *et al* Nature reviews 2004 3:950-964

Figure 4: Nuclear Receptors in Action. A | Mode of action of nuclear receptors (NRs). After diffusion through the cytoplasmic membrane, the ligand can interact with its cognate receptor where it can exert a 'non-genomic effect' by interacting directly, for example, with kinases (a). The ratio between cytoplasmic and nuclear location can vary between different receptors and is affected by the nature of a ligand. Ligand binding modulates the interaction of the receptor with a plethora of factors. In the absence of ligand, several nuclear receptors are believed to be bound to the regulatory regions of target genes as a corepressor or histone deacetylase (HDAC) complex (b). Histone deacetylation is responsible for the chromatin condensation that accounts for the gene-silencing effect of apo receptors. Ligand binding releases the HDAC complex (c) and results in the recruitment of histone acetyltransferase (HAT) and chromatin-remodelling (CRM) complexes (d). The temporal order and requirement of these complexes can occur in a receptor-, target-gene- and cell-specific manner. In the last step (e), the polymerase II holoenzyme, which comprises the pol II enzyme, TAF (TATA-binding protein-associated factor) and mediator complexes, is recruited and increases the frequency of transcription initiation.

Figure taken from: Gronemeyer et al Nature reviews 2004 3:950-964

Chapter 2: Thyroid Receptors

Thyroid hormone (TH) is produced by the thyroid gland and is a very important factor in development, differentiation and metabolism (10). In the late 19th century it was realized that loss of thyroid function led to cretinism and myxederma and this led to the discovery of thyroid hormones (10). Thyroid hormones are the derivatives of the amino acid tyrosine covalently bound to iodine (Figure 5). The two principal thyroid hormones are thyroxine (T4 or 3,5,3',5'-tetraiodo-L-thyronine) and triiodothyronine (T3 or L-3,5,3' tri-iodo-L-thyronine). The basic structure of thyroid hormones is two tyrosines linked together with iodine at three (T3) or four (T4) positions on the aromatic rings. The majority of thyroid hormone secreted by the thyroid gland is T4, which is deiodinated in peripheral tissues such as liver and kidney to yield T3, a more active form of the hormone. Deiodination of T4 can also yield reverse-T3 which has no metabolic activity. In early development, lack of thyroid hormone leads to cretinism (growth disturbances and severe mental retardation) whereas later in life the thyroid hormone plays an important role in metabolism. Thyroid hormones are hydrophobic and more than 99% of circulating THs are bound to carrier proteins such as thyroxine-binding globulin, a glycoprotein synthesized in the liver. Carrier proteins allow a steady pool of THs from which active, free hormone is released for uptake into target cells.

Insight into the mechanism of action of thyroid hormone came about with the cloning of their intracellular receptors. In 1986, two groups cloned the cellular homolog of the oncoprotein c-*erb*A. Characterization of the protein product of this gene revealed that c-*erb*A was really a high affinity TR, in that it contained a DBD

OH
I   I
| |

OH
|
I

OH
I   I
| |

O
I   I
| |

O
I   I
| |

O
|
I

CH₂
HOOC   NH₂

CH₂
HOOC   NH₂

CH₂
HOOC   NH₂

Thyroxine (T4)

Triiodothyronine (T3)

"Reverse T3"
(inactive)
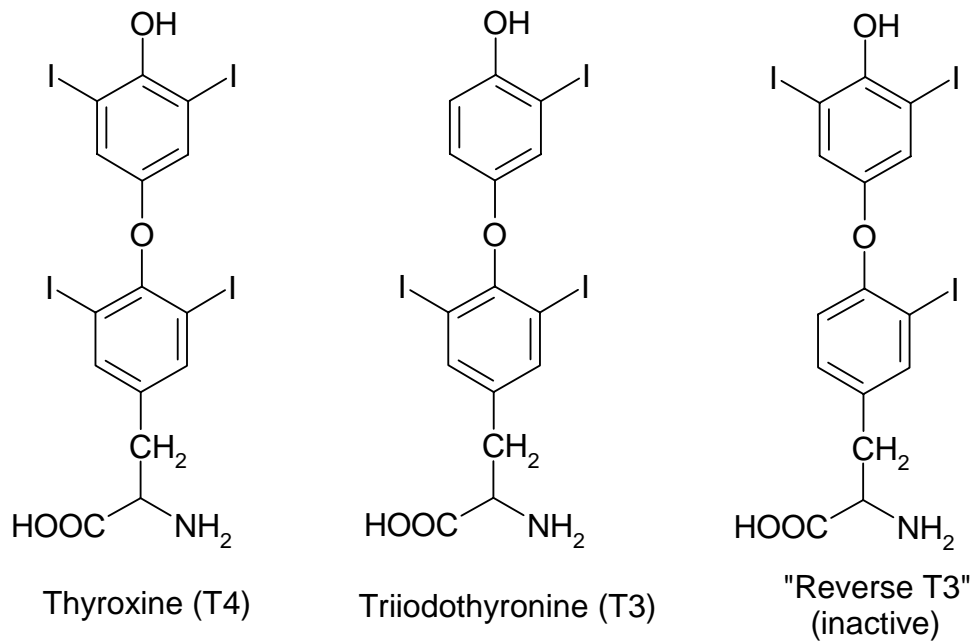
Figure 5: Structure of Thyroid Hormones. Thyroid hormones are derivatives of the amino acid tyrosine covalently bound to iodine. The two principal thyroid hormones are thyroxine (T4 or 3,5,3',5'-tetraiodo-L-thyronine) and triiodothyronine (T3 or L-3,5,3' triiodo-L-thyronine). Thyroxine is the principal hormone secreted by the thyroid gland, which is deiodinated to yield T3, the more active form of the hormone.

characteristic of nuclear receptors and a unique thyroid hormone binding domain (31, 32).

In vertebrates TRs are encoded by two separate genes, TRα and TRβ, which are located on different chromosomes. In humans TRα is located on chromosome 17 whereas TRβ is located on chromosome 3 (10). Alternative splicing gives rise to multiple TRs; TRα1, TRα2, TRβ1 and TRβ2. The last exon of TRα overlaps with the last exon of the orphan receptor Rev-erbα (1). Interestingly, this last exon is specific to the isoform TRα2, a dominant negative regulator of TRα1 isoform (1). The TRα gene is split into 10 exons spanning 27kb of genomic DNA (1). The positions of the exons are conserved across species (1).

The expression of TR isoforms is regulated both at the transcriptional and posttranscriptional level, and each isoform displays a characteristic pattern of regulation (10). The most highly regulated TR isoform is TRβ2 which is almost exclusively expressed in the hypothalamus and anterior pituitary. All tissues express TRα1, TRα2 and TRβ1, but each isoform has a characteristic pattern of distribution. The isoform TRα1 is highly expressed in brown fat and skeletal muscle, TRα2 is most prominent in the brain and TRβ1 is more homogeneously distributed with high concentrations in brain, liver and kidney (10). The first TR expressed in the fetus is TRα1 while TRβ expression in the brain increases dramatically after birth. Thyroid hormones have established effects on fetal and neonatal brain development with upregulation of TRβ receptor being critical for the expression of genes that are important in brain development.

Thyroid receptors share the same modular structure as the other nuclear receptor family members. Thyroid receptors contain an amino terminal transactivation domain (AF-1) that interacts with other transcription factors to repress

or activate transcription, a central DNA binding domain and a carboxyl terminus ligand binding/AF2 domain. The AF-1 domain varies considerably between the TRα and TRβ and within the two beta isoforms. The TRα2 isoform has a unique carboxyl terminus and does not bind triiodothyronine (T3) (10, 28). In this respect it acts similarly to a dominant negative mutant of TR, but it is able to be phosphorylated, which ultimately affects its DNA binding and dimerization (28, 33).

Thyroid receptors differ from steroid receptors in that they are bound to DNA in the absence of ligand (10). TRs are able to bind to DNA as either homodimers or heterodimers with the major form of DNA-bound TR being the TR-RXR heterodimer. TRs recognize and bind to a DR4 element, two half-sites (AGGTCA) separated by 4 nucleotides, but are able to also recognize other forms of the repeats such as palindromic and inverted repeats (34, 35). The major functional form of the receptor is thought to be the TR-RXR heterodimer, as this has the highest DNA binding affinity. Apo-TR is also located in the nucleus and even in the absence of T3 it can bind to DNA (TREs) and act as a repressor of transcription (10, 28).

The heterodimerization of TR-RXR increases the ability of TR to bind to specific DNA sequences and contributes to the specificity of action of TR. The area of dimerization is, like in other RXR dimers, in the LBD and DBD domains. The interaction between the DBDs allows for specific interactions between monomers and the correct binding site recognition. The heterodimerization interface of RXR is in the second zinc finger in the D-box while the heterodimerization domain of TR is in the pre-finger region. The TR A-box is important in preventing binding on other response elements. TRs also interact with other transcription factors such as p53, AP-1 and Stat 5. TRs can also heterodimerize with RAR and chicken ovalbumin upstream promoter transcription factor (COUP-TF) although these interactions appear to be

weaker than that with RXR (10). Homodimers form at the expense of heterodimers

when there is a high enough concentration of TR (10), but it is not clear whether the

same domains are involved in TR homodimerization versus heterodimerization. The

heterodimerized receptors usually adopt a predefined polarity on direct repeats (26).

In the TR-RXR heterodimer, the 5' site is occupied by RXR and the 3' site by TR. In

the case of TR-VDR heterodimers, the 3' site is occupied by VDR in a DR3 and by TR

in a DR4 response element (26). TR-VDR heterodimers can activate transcription in

response to either ligand, but for the receptor at the 5' site a higher concentration of

hormone is required for activation (26).

Transcriptional activation of TRs commence upon ligand binding (Figure 6). In

the unliganded form, TR assumes a conformation that promotes interaction with

corepressors (28). Corepressor complexes associated with TRs have been shown to

contain histone deacetylase activity, which is associated with a compact "turned off"

chromatin conformation and repression of transcription. Binding of ligand induces a

conformational change that makes it impossible for the receptor to be bound to the

corepressor complex but able to complex now with activator proteins. Typical

coactivator complexes contain histone acetyltransferase (HAT) activity and ATP

chromatin remodeling proteins which are associated with "relaxed" chromatin and

transcription activation (Figure 6).

Transcriptional activity of TR is under the control of coregulators like all

nuclear receptor family members. SRC-1 is a coactivator of transcription in the

presence of ligand (28). SRC-1 is important in T3 action as mice without functional

SRC-1 are T3-resistant (28, 36). The carboxyl-terminus of SRC-1 possesses intrinsic

HAT activity and also interacts with other HATs such as p300/pCAF (28). In addition

to coactivators with HAT activity, TR uses a different complex called TRAP. TRAP does
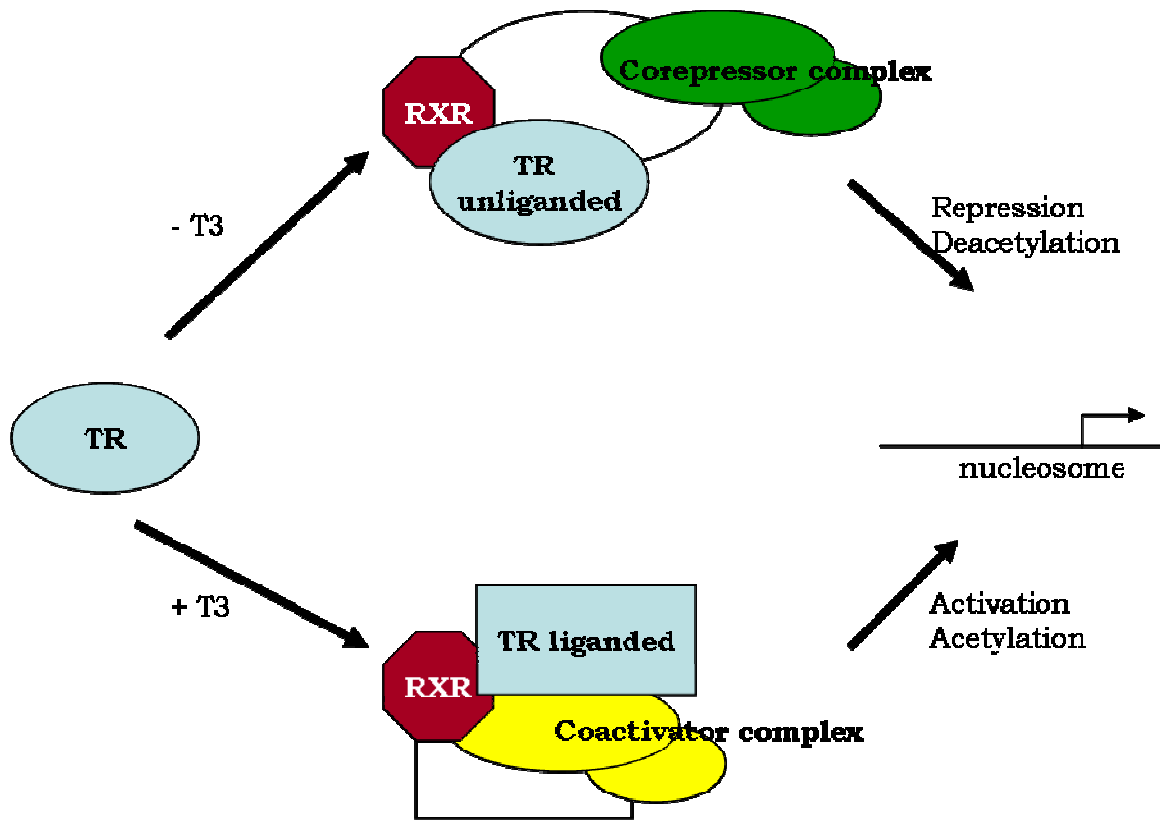
Figure 6: Model for thyroid receptor action. Model for activation and repression by thyroid hormone receptors. In the absence of T3, TR/RXR heterodimer recruits a corepressor complex that has histone deacetylase activity. In the presence of T3, TR/T3/RXR releases the corepressor complex and recruits a coactivator complex that has histone acetyltransferase activity. Enzymatic modification of nucleosomes by HDAC or HAT activity results in an open or closed chromatin structure that leads to transcriptional activation or repression.

Figure modified from Zhang et al, Annu Rev Physiol 2000 62:439-66

not possess HAT activity and is transcriptionally active even in the absence of chromatin. TRAP contains multiple polypeptides that are shared with a more general regulatory complex called the mediator complex SMCC (28).

Physiologically, aberrant coactivator recruitment is a feature of an autosomal inherited disease, thyroid hormone resistance (RTH). Thyroid hormone resistance disorder is caused by mutations in the TRβ gene and causes hypothyroidism characterized by goiter, elevated serum T3 and T4 and altered TSH levels. Children affected with this disorder have attention deficit disorder. This trait appears to be transmitted as a dominant negative mutation of the TRβ receptor.

Mice that are null for TRα1 show subnormal body temperature and mild abnormalities in cardiac function. TRα knockout mice (lacking both isoforms) are severely hypothyroid, exhibit growth arrest and have a very short lifespan. TRβ knockout mice have elevated TSH levels and are deaf whereas TRβ2 knockout mice have normal hearing and elevated TSH, suggesting that TRβ1 is responsible for the development of normal hearing and TRβ is responsible for normal serum concentrations of TSH. Finally, lack of TH and absence of TR do not have the same phenotype. Lack of TH has a more severe phenotype than receptor inactivation. The reason for this discrepancy is not fully understood, but it could be that in the case of receptor deficiency the receptor responsive genes are left in neutral, rather than being suppressed as would happen in the case of TH deficiency.

Chapter 3: Retinoid Receptors

Vitamin A is a fat soluble vitamin essential for the formation and maintenance of healthy teeth, skin, bones, mucus membranes, soft tissue and is also responsible for the promotion of good vision and immune function [web & (37)]. The active form of vitamin A is retinol, and is found naturally in animal liver, eggs and dairy products. Retinol can also be manufactured in the body from carotenoids such as β-carotene, found in green leafy, orange and yellow vegetables. Provitamin A compounds such as β-carotene have also been demonstrated to have anti-oxidant and anti-proliferative properties, and may suppress certain tumors. Retinol is converted to more active compounds called retinoids, such as retinoic acid (RA), by the zinc dependent enzyme retinol dehydrogenase (38). There are six known isomers of retinol; all-*trans*, 9-*cis*, 11-*cis*, 13-*cis*, 9,13-di-*cis* and 11,13-di-*cis* with the predominant physiological form being all-*trans*. Figure 7 shows the structure of β-carotene and several natural and synthetic retinoids.

Vitamin A deficiency (VAD) is associated with blindness, infectious diseases, diarrhea and malnutrition, the latter being a leading cause of death in children (39, 40). Less severe deficiencies in vitamin A retard growth and intensify iron deficiency anemia (41). Therapy with Vitamin A has been demonstrated to be effective in reducing the severity of VAD and therefore many immunization programs began including vitamin A (42, 43). Conversely hypervitaminosis A can result in toxicity to the liver, CNS, bone and skin (37, 44).

Vitamin A and retinoids play an important role in vertebrate development, and have been identified as potent teratogens. In 1933, Hale reported the first evidence of
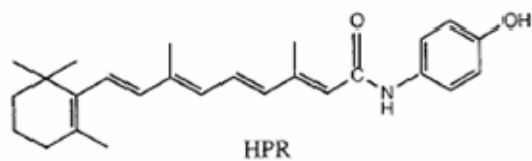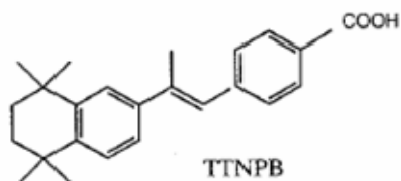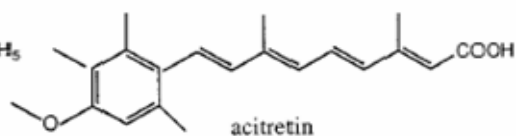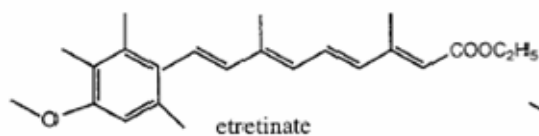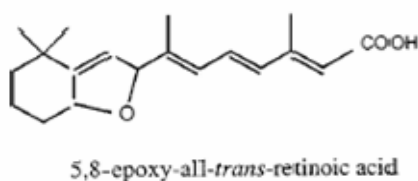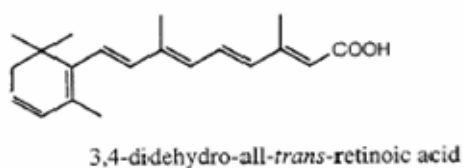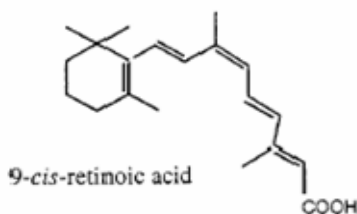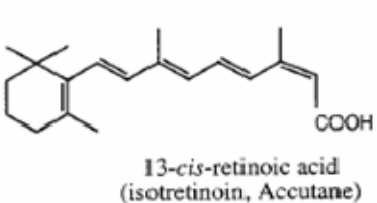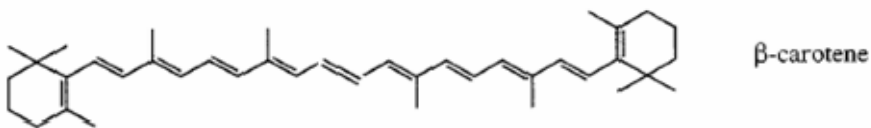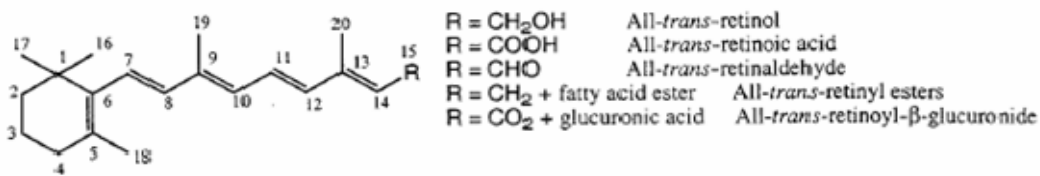
Figure 7: Structures of β-carotene and several natural and synthetic retinoids.

Figure from: Collins MD, Annu Rev Pharmacol Toxicol 1999 39:399-430

teratogenicity due to vitamin A deficiency; a deficient sow gave birth to piglets without eyeballs. In 1950, Wilson and Warkany reported that the offspring of rats fed vitamin A-deficient diets prior to and during gestation showed malformations in the eye, urogenital tract, heart and lung (37, 45). In 1954, Cochlan (46) was the first to report that excess vitamin A administered during pregnancy can be teratogenic. The malformations reported were exencephaly, hydrocephaly, spina bifida, eye defects, cleft palate and shortening of the mandible and maxilla (37). The teratogenicity of RA has been even more firmly established since 1954. At pharmacological concentrations, RA is able to induce malformations in all vertebrate species as well as certain invertebrates, with the extent of the malformations depending on the dose and developmental stage (37, 47, 48). Certain retinoids used for treatment of disease, such as Accutane® (13-*cis* RA) and etretinate, are reported human teratogens, and therefore prescribed with highly restrictive guidelines especially to women of childbearing age. As a toxicologist one can ask "How does RA fit with the six principles of teratology?" These would include:

a) Principle I of teratology is that "teratogenic susceptibility is determined by the genotype of the conceptus and the interaction of this genotype with environmental factors" (37, 49). Evidence thus far suggest that all species, to varying extents, are susceptible to the teratogenic effects of 13-*cis* RA (37).

b) Principle II states that "susceptibility to teratogenic agents depends on the developmental stage of the embryo or fetus at the time of exposure" (37, 49). Up until the first two weeks of human pregnancy, the embryo is relatively insensitive to teratogenesis. This changes though at the organogenesis stage at weeks 3-8 of human gestation when the embryo is highly sensitive to teratogens. Sensitivity gradually decreases after 8 weeks until parturition. A study of RA-induced

teratogenesis in hamsters suggests that small changes in gestational timing of exposure will result in major shifts in embryo lethality and malformations, and that for each malformation there is a "critical period" where exposure to an agent will cause malformation. Human epidemiological data describe a syndrome associated with 13-*cis* RA, called Retinoic Acid Embryopathy (RAE). Malformations associated with RAE, include craniofacial, cardiovascular, CNS and thymic abnormalities (37). The teratogenic effects of RA occur at stages where it is not normally present in the embryo. Most RA teratogenic effects in mice embryos occur at the stage of development prior to the onset of RA synthesis by the embryos. During this time the amount of RA required to induce malformations is 50-100 fold less than at other stages of development.

c) Principle III of teratology states that "teratologic agents work by specific mechanisms on developing cells and tissues to initiate pathogenesis" (37, 49). With respect to RA, embryonic pathogenesis occurs by an unknown specific mechanism early in development. Most human teratogens have unknown mechanisms of action, although there are a number of mechanisms that can be hypothesized with respect to RA-induced teratogenesis that are receptor-mediated or receptor independent.

d) Principle IV states that "perturbations of developmental processes can result in death, malformation, growth retardation, and/or functional disorder" (37, 49). Generation of transgenic knockout mice is a method often employed to study the function of a gene. Often though, when no gross anatomical phenotype is observed, functional redundancy is 'blamed', that is, the function of the gene in question can be performed by another gene. It is possible that deletion of a specific gene will produce a subtle phenotype that can be detected only through specific assays for postnatal functional and anatomic development (37). Mice null for RARβ, RXRβ and RXRγ were

observed to be deficient in forward locomotion compared to wildtype littermates. These mice had reduced expression of the dopamine receptors D1 and D2 which caused the phenotype. Expression of these dopamine receptors is induced by retinoid receptors [(37) and references therein]. In addition to physical malformations, RA is able to induce behavioral teratogenesis as observed in women who had prenatal exposure to 13-*cis* RA and gave birth to children with reduced intelligence [(37) and references therein].

e) Principle V states that "the nature of the influence (or agent) determines the extent of the interaction between the environmental agent and conceptus" (37, 49). This principle deals with the question of whether the agent acts on the embryo directly or indirectly through maternal toxicity. Transcriptionally active retinoids do not normally cross the placenta, but are synthesized within embryonic cells from maternal retinol. At elevated doses the normal biochemical pathways of retinoids change and there is placental transport.

f) Principle VI of teratology states that "there is an increase in the sequelae of abnormal development from the no effect level to the totally lethal level" (37, 49). This principle states that there should be a dose-response relationship associated with retinoid administration and this has been demonstrated in a number of animal experiments (37). Method of retinoid administration can also play a role; whereas oral administration may be teratogenic dermal administration often is not. Another factor of this principle is whether compounds have a threshold for teratogenesis. Retinoids are believed to have a threshold and generally follow a dose-response relationship [(37) and references therein]. Finally the teratogenic effects mediated by RA are receptor mediated, in that retinoids that are unable to activate RARs are nonteratogenic (50).

Retinoic acid plays a role in organogenesis as well as development. Retinoids have been shown to be important to the development of the heart and vasculature and in the absence of retinoids these structures do not form or are inoperatively lethal. In 1969, Thompson et al (51) showed that in the absence of retinoids the vasculature of domestic fowl does not develop and the embryo disintegrates (51). Furthermore, eggs from hens fed with a diet free of retinol, but adequate in RA, do not hatch, probably due to failure of RA to be transferred to the egg. In ovo administration of RA is also not as effective as retinol in permitting embryonal development (51, 52). The results first reported by Thompson were subsequently confirmed in the quail embryo (52, 53).

To study the effect of RAR and RXR in retinoid signaling, a number of studies were undertaken in mice. Transgenic knockout mice for RARα, RARβ and RARγ were found to be viable but displayed certain aspects of VAD syndrome and some congenital abnormalities in the tissues that normally express these receptors (54). The limited phenotype observed in these knockouts leads to the assumption that there is some functional redundancy between RARs. To study this further, double knockout mice were constructed and studied. Mice deficient in RARα/β, RARα/γ and RARβ/γ died from severe developmental defects in utero or shortly after birth (54-56). The severe malformations observed in RARα/γ null mice are similar to those of mice deficient in retinaldehyde dehydrogenase 2, which functions in RA synthesis, and highlight the importance of RA-liganded RARs early in development (57, 58). Some important early development RA functions that have been identified include hindbrain segmentation and closure, oocyte formation, axial rotation, development of forelimb buds and closure of the primitive gut (57). In addition to the above, RARs have been shown to be important for the ontogenesis of structures derived from mesenchymal cells, the development of ocular structures and the formation of the genital tract and ureters

[(57) and references therein]. Another function of RARs in development is the establishment of the anteroposterior axis of limbs, histogenesis of the retina, the regulation of lung branching morphogenesis and alveolar septation, as well as the morphogenesis of nasal cavities [(57) and references therein]. Data from RXRα null mice suggest that RXRα is the main RXR involved in embryogenesis (57, 59). RXRβ and RXRγ null mice do not have an obvious phenotype, even when one allele of RXRα is deleted.

The role of retinoids in carcinogenesis and disease processes has been extensively studied, as has their potential therapeutic value. As early as 1925, Wolbach and collaborators established that vitamin A deficiency causes squamous metaplasia and keratinization of epithelia (52). Because epithelial cancer is responsible for the death of many people retinoids became the focus of cancer research. RA is thought to inhibit tumor growth by inhibiting uncontrolled cell proliferation, inducing apoptosis of abnormal cells, and promoting normal cell proliferation (60). Pioneering efforts also showed retinoids to be effective in the inhibition of tumorigenesis of the skin and in the respiratory, mammary, buccal and stomach epithelia of rodents [(52) and references therein]. In preclinical studies, 9-*cis* RA has been shown to be effective in the chemoprevention of mammary and prostate cancer. Targretin (an RXR ligand; Ligand Pharmaceuticals, San Diego, CA) has been shown to be effective in the chemoprevention of mammary cancer, suggesting that RXR is important in epithelial cancer development. Retinoids (especially 13-*cis* RA) have been shown to be effective for the prevention of head and neck cancer, but have not yet been demonstrated to be effective in the prevention of lung cancer. Conversely, several large scale trials such as the Beta-Carotene and Retinol Efficacy Trial (CARET), the Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study and the Lung–

Study sponsored by the NCI, suggest retinoids may actually raise the risk of lung cancer in smokers. These studies also showed reduced tumor recurrence and mortality in nonsmokers and some small evidence of benefit to former smokers, suggesting that the response to retinoids differs between smokers and nonsmokers. A study by Kurie *et al* (61) examined the usefulness of 9-*cis* RA for the prevention of lung cancer in former smokers. Loss of RARβ expression in the bronchial epithelium is considered a biomarker of preneoplasia, and retinoids can restore expression of RARβ and presumably halt the progression of carcinogenesis. With this in mind, in a controlled study, 9-*cis* RA, 13-*cis* RA + alpha-tocopherol were used to evaluate squamous metaplasia, dysplasia and RARβ expression in former smokers. 9-*cis* RA was shown to be more effective than 13-*cis* RA in restoring RARβ expression. These findings suggested that ligands for both RARs and RXRs may be more effective chemopreventors than RAR-selective ligands. APL (acute promyelocytic leukemia) is caused by a translocation fusing the RARα gene to a gene called PML (for promyelocytes). Interestingly, the fusion protein is still RA responsive and RA treatment causes partial remissions in APL patients. RA therapy has been shown to improve the survival of children with neuroblastoma by suppressing residual disease after chemotherapy, and it now forms an important part of neuroblastoma treatment (62, 63). A study showed that in xeroderma pigmentosum, RA treatment results in a reduction of new and recurrent skin tumors (64). Finally, various skin conditions are treated with RA and its derivatives (65, 66). One of the better established targets of retinoids is the skin. Accutane (13-*cis* RA) is used for the treatment of cystic acne. Retin A is used as topical treatment for acne and etretinate is used for the treatment of psoriasis. Cumulatively these data demonstrate that retinoids and their receptors play critical roles in the development and homeostasis of a variety of tissues and that

perturbation of either the ligand or receptor function can have both disease and therapeutic implications.

Precursors for RA are derived from multiple food sources and converted to active forms of RA in the gut, liver and target tissues [reviewed in (37)]. Retinoids are absorbed in the duodenum where retinyl esters from animal products and dairy are hydrolyzed to retinol. Carotenoids, which are derived from plants, are metabolized to retinaldehyde in the intestine. Retinyl esters and retinaldehyde are then bound to CRBP II and this complex serves as a substrate for the lecithin-retinol acetyltransferase enzyme, which converts retinol to palmitate and other retinyl esters. Following their conversion, retinyl esters are packaged into chylomicrons and delivered to the liver where hydrolysis to retinol occurs. Retinol is then bound to lipocalin retinol-binding protein (RBP) and the complex is delivered to hepatic stellate cells where retinol is converted to retinyl esters, which are stored in lipid droplets in the liver. Prior to reentering the circulatory system retinyl esters must first form the retinol-RBP complex to mask its hydrophobicity and then complex with transthyretin to prevent kidney elimination. Once it is delivered to target tissues, retinol undergoes multiple enzymatic reactions to be converted to multiple products, including active retinoids. Like retinol, RA is not free but rather is bound to albumin upon entering the circulatory system. Figure 8 shows some of the major enzymatic pathways of retinol. Several cytochrome P450s have been implicated in the creation and destruction of RA [(37) and references therein]. There is a second family of retinol binding proteins, CRABP types I and II and CRBP I and II. These are found in specific anatomic locations and are highly conserved across species. These have higher affinity for RA than the RARs and are found in greater abundance in cells than their respective ligands (all-*trans*-retinol for CRBP and all-*trans*-RA for CRABP). Generation
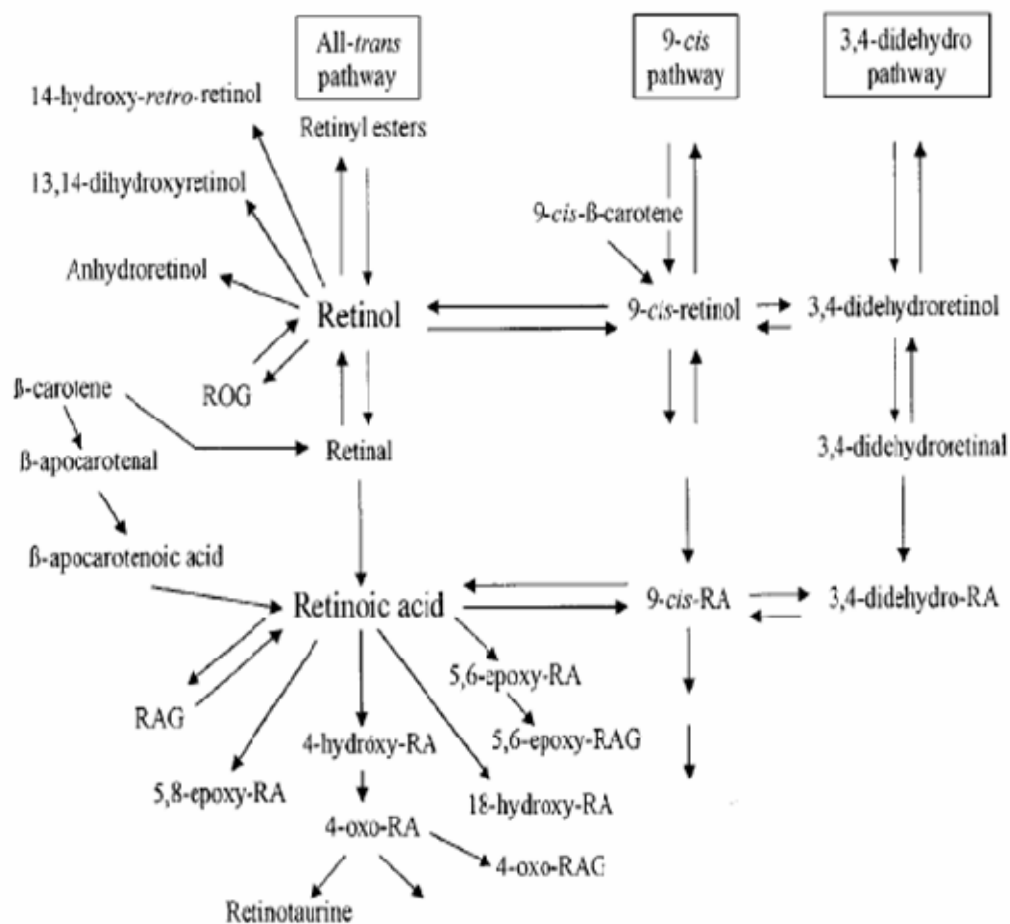
Figure 8: Principal retinoids in the metabolic pathway. The columns represent the various isomerization states, except for the 3,4-didehydro pathway; however, the pathway metabolites are only delineated for the all-*trans* forms. Shown here is the 9-*cis* pathway. In general the top has relatively reduced retinoids, whereas the bottom has relatively oxidized retinoids. ROG is all-*trans*-retinyl-β-glucuronide, RAG is all-*trans*-β–glucuronide, and RA is retinoic acid. The arrow from all-*trans*-oxo-RA with no product indicates that there are more oxidized products that are unknown.

Figure from: Collins Annu Rev Pharmacol. Toxicol. 1999 39:399-430

of transgenic knockout mice for these proteins has shed some light into their function. CRABP I null mice displayed no reported phenotype and CRABP II null mice had postaxial forelimb polydactyly. Deletion of both genes resulted in polydactyly, and did not display altered sensitivity to teratology following retinoid administration (37).

Retinoic acid exerts its effects by binding to retinoid receptors. Retinoid receptors can be divided into two subgroups, the retinoic acid receptors (RARs) and retinoid "X" receptors (RXRs). There are three isoforms of RAR, α, β and γ, and they bind to all-*trans* retinoic acid (atRA) and its isomer 9-*cis* retinoic acid (9cRA). Retinoid X receptors also have three isoforms α, β and γ and are able to bind to 9cRA with very high affinity but not to atRA. Sequence comparisons have shown that there is more conservation of a specific receptor isoform between species than is found between the three receptor isoforms within a species (8). This conservation leads to the conclusion that each receptor isoform must have a unique and specific function. RARs are phylogenetically more closely related to thyroid receptors but only distantly related to RXRs (1).

The first RAR (RARα) was independently cloned by three different groups in 1986-87 (67-69). In 1986, before the cloning of RARα or TRs, Anne Dejean characterized an integration site for HBV in a hepatocellular carcinoma, and cloned the corresponding cDNA which she named hap for hepatoma (69). In 1987, Pierre Chambon's group operating on the assumption that RA may, like steroid hormones, mediate its transcriptional effects via specific nuclear receptors, used a consensus oligonucleotide corresponding to a highly conserved sequence in the DBD of several members of the nuclear receptor family to probe two 'randomly primed' λgt cDNA libraries prepared using total poly(A)$^+$ RNA from the human breast cancer cell lines MCF-7 and T47D (68). The clone isolated was characterized and shown to bind to RA

with high selectivity and to retinol with lower affinity. At the same time, Ron Evans' group, using an oligonucleotide derived from a novel genomic sequence derived from hap (69), probed a λgt10 kidney cDNA library and isolated and characterized an RARα clone. Based on the high sequence homology hap had to RARα, it was subsequently determined by Anne Dejean's and Pierre Chambon's group to be a retinoic acid receptor isoform, and was named RARβ (70). Pierre Chambon's group was the first to identify a third isoform of RAR, RARγ, during their characterization of the murine RARα and RARβ (71).

The DBD of the three RARs is extremely conserved at 97-100% sequence identity and the LBD is also well conserved with 85-90% sequence identity (1). Of important note is that RARβ has been identified only in mammals and birds and not in amphibians and fish. The reason for this is not known. RARγ and has orthologs in amphibians, fish, mammals and birds (1).

The identification and characterization of a naturally occurring RARE resulted from the analysis of retinoic acid-inducible genes such as RARβ. The response element for retinoic acid receptors consists of two or more degenerate copies of the AGGTCA half-site motif organized in direct repeats or palindromes normally separated by 5 nucleotides, however there are exceptions to this. Specific proteins found in nuclear extracts were shown to increase the binding efficiency of RARs to these response elements (72, 73). Efficient binding of RAR to DNA occurs when partnered with RXR. When bound to DNA, the 5' half-site is occupied by RXR and the 3' half-site is occupied by RAR [(1) and references therein]. In addition to DR5, RAR-RXR heterodimers are able to activate transcription from DR1 and DR2 elements, such as those found in the promoters of cellular retinoic acid binding protein (CRABP) and cellular retinol binding protein (CRBP) (74, 75). When bound to DR1 elements the

RAR-RXR heterodimer is unresponsive to retinoic acid stimulation, most probably due to the inverted polarity of the heterodimer (5'-RAR-RXR-3') (76). When the heterodimer is in this inverted polarity, RAR ligands cannot induce dissociation of corepressors, and therefore transcriptional activation cannot occur. Dimerization occurs through interaction of the second zinc fingers of RAR and RXR. There are no homodimerization domains on RAR, making the RAR-RXR heterodimer solely responsible for the response to RA. Transcriptional activation by RAR is dependent on the pairing of RAR with RXR. Dimerization occurs on two interfaces, the DBD interface which is involved in the recognition of response elements on DNA and the LBD interface which is independent of the DNA binding activity of the complex.

RARs are transcription factors, and their target genes have been implicated in a large number of biological processes such as embryonic development and liver metabolism. Some of the first target genes reported for RARs include CRABP (74), CRBP (75) as well as RARβ itself. Another group of RAR regulated genes are the Hox genes, which play an important role in anteroposterior axis specification in development. Hox genes have also been shown to play an important role in transducing the morphogenic signal of RA, in that treatment of a developing embryo with exogenous RA leads to axial patterning and this is associated with repatterning of Hox gene expression domains (77). Somites are masked from left-right cues and are held in symmetrical register by an RA dependent signal (78). A symmetry-generating mechanism is necessary as proper somite development requires left-right refractoriness (78). To orchestrate differentiation and patterning a number of molecular signals such as Wnt, Notch, Hedgehogs and Fibroblast Growth Factors (FGFs) are employed in developmental settings often in conjunction with distinct cofactors (78). The anterior-posterior patterning of somites is defined by Notch, Wnt,

FGF8 and RA signaling (78, 79) while simultaneously, Notch, FGF8 and RA signaling are instrumental in left-right determination (78, 80). This signaling conflict makes it necessary to block the effect of side-specific signaling and to hold somitogenesis in synchronized and symmetrical register. This need is met by an RA-dependent signaling mechanism (78, 81-83). The mechanism by which RA masks left-right signals is unclear (78). FGF8, which is generated at the posterior end of the embryo during somitogenesis is antagonized by RA (78) and this is crucial for segmentation to proceed (78, 79). Blocking of endogenous RA production by antisense oligonucleotides against *raldh2* results in strongly biased LR asymmetric somite development (81). Progression of somitogenesis in zebrafish embryos depends on RA signaling and is tightly linked to the cascade of LR organ asymmetry information (81).

RARs have been shown to interact with a number of transcription factors and have been used as bait in various assays to identify nuclear receptor coregulators. RARs bind to p160 family members SRC-1, TIF2 and p/CIP in a ligand and AF-2 dependent manner (84). RARs also bind to p300 and CBP (85). RAR is able to interact with the TRAP220 protein, which belongs to the DRIP/TRAP/SMCC complex (86, 87). Apo-RAR is bound to NCoR and SMRT (84, 88); upon ligand binding this interaction persists when RAR is bound to a DR1 response element. This persistence in interaction could explain the repressive effect of RAR-RXR heterodimers bound to DR1 elements (1).

In summary, retinoid receptors propagate vitamin A induced events in cells. Vitamin A deficiency is associated with disease states such as blindness and malnutrition. Too much vitamin A is also detrimental to health leading to toxicity in tissues such as liver, CNS and skin. Vitamin A and its derivatives are also important signaling molecules for development where the amount and timing of expression of

retinoid receptors is critical. Mice deficient in more than one retinoid receptor display abnormalities and a shortened life span. Retinoids have also been proposed to act as antitumor agents and have been the subject of many clinical and chemopreventive trials with mixed results. For certain retinoid signaling is important for many biological systems and processes.

SUMMARY

Nuclear receptors are the largest family of transcription factors and are responsible for integrating multiple signaling pathways. They regulate complex physiological events and play a role in embryogenesis, development, metabolism and apoptosis. All nuclear receptors identified to date share the same modular structure. The DNA binding domain of nuclear receptors is the most conserved domain; the ligand binding domain is the least conserved. These receptors exert their function either as homodimers (steroid subfamily members) or as heterodimers (thyroid receptor subfamily members). Nuclear receptor dimers bind to their respective response elements. Hormone response elements are composed of two half-site motifs that are separated by 3, 4, or 5 nucleotides. The spacing between the two half-sites dictates the specificity with which the receptor will bind. The polarity of the heterodimers also dictates the response to ligand stimulation. Steroid family members are bound to heat-shock proteins in the absence of ligand while TR subfamily members are bound to their response element regardless of ligand presence. The determination of the crystal structure of these receptors showed that they share a common fold and that upon ligand binding, the receptor undergoes a conformational change. This model of conformational change is called the "mousetrap model".

An additional "layer" of transcriptional activation by nuclear receptors is the discovery of "coregulators". A number of transcriptional coregulators have been discovered to date. Corepressors have endogenous HDAC activity which keeps chromatin in a compact "turned off" conformation. Coactivators are associated with HAT activity which is associated with "relaxed" chromatin and allows for transcription to occur. A lot of these coregulators were discovered using retinoic acid receptors as

bait for the library screens. Using RXR as bait our lab identified a novel coregulator that had a high sequence homology to the published sequence for NPDC-1 (neural proliferation differentiation control) gene identified just three years earlier. The focus of this dissertation will be on studies that show NPDC-1 to be a coregulator of retinoid and thyroid receptor transcription.

SECTION II: NPDC-1 AS A MODULATOR OF TRANSCRIPTION EVENTS BY HUMAN RETINOID AND THYROID RECEPTORS

Chapter 4: NPDC-1

The discovery of transcription factors greatly enhanced our understanding of the mechanisms of development. In a search to define the mechanisms involved in neural cell commitment and differentiation, Pierre Rouget's group attempted to identify genes that may be involved in the control of neural cell proliferation and differentiation (89). Their criteria for selecting the sequences to be used as probes was both their preferential expression in neural precursor cells reaching confluence and their ability to encode amino acid sequences yielding helix-loop-helix motifs. Transcription factors in the basic helix-loop-helix family are key regulators in development, particularly cell type determination, terminal differentiation and sex determination (90). From their screen a unique gene was identified that was named NPDC-1 for Neural Proliferation, Differentiation and Control (89). Further analysis of the sequence showed some regions with homology to jun-fos leucine zippers, but the homology was too short to classify NPDC-1 as a member of the helix-loop-helix or leucine-zipper protein families (89). A stretch of potential phosphorylation sites was also identified between amino acids 234-244. This corresponds to the sequence Pro-Thr-Ser-Pro-Ser-Thr-Pro-Arg-Ile-Ser-Pro which represents three consensus sites recognized by mitogen-activated protein kinases (MAPK) (89). A Northern blot of RNA from proliferating, growth arrested or differentiating CLT.T.1.1 cells showed a 1.5kb mRNA that was preferentially expressed when the cells were growth arrested. In addition, tissue

analyses of NPDC-1 RNA expression suggested that, in mouse, NPDC-1 is almost exclusively expressed in brain (89). Finally, these initial studies also provided the first evidence suggesting NPDC-1 might function as a tumor suppressor, in that NPDC-1 was shown to both suppress proliferation and inhibit growth in soft agar (89).

The expression pattern of NPDC-1 during the development of the nervous system as well as the potential association of NPDC-1 with cell cycle regulatory proteins was examined by Dupont *et al* (91). Using total RNA from embryonic, postnatal and adult mouse brain they showed that NPDC-1 expression increased during development with the highest levels in adult brain. Using in-situ hybridization experiments, these investigators next examined the expression of NPDC-1 during development. In mouse brain NPDC-1 was observed to be expressed in different parts of the brain at different times of development, with the strongest expression observed in the adult hippocampus (Table 3). The expression of NPDC-1 was also studied in P19 and PC12 cells. P19 are a mouse embryonal carcinoma cell line that can be induced with RA to differentiate into neuronal cells or induced with DMSO to differentiate into muscle cells (92). Northern blot analysis of P19 mRNA showed that NPDC-1 expression was induced with RA treatment but not with DMSO treatment (91). PC12 cells showed endogenous expression of NPDC-1 mRNA could be substantially increased with NGF treatment (91). These results support the original claims of regulated expression of NPDC-1 in neuronal cells.

The interaction of NPDC-1 with cell cycle regulatory proteins was investigated by Dupont and Sansal (91, 93). Using *in vitro* pull-down assays these investigators were able to demonstrate that cyclin D1, Cdk2 and cyclin A are able to interact with NPDC-1 either directly or indirectly. The interaction between NPDC-1 and cyclin A

Newborn mouse CNS		Adult mouse CNS	
Neocortex		Neocortex	
Ventricular layer	++	Layer VI	+++
Intermediate layer	−	Layer V	+++
Inner cortical plate	++++	Layer IV	+
Bipolar cortical plate	++++	Layer III	−
Layer I	−	Layer II	+
		Layer I	−
Caudate putamen	+	Caudate putamen	+
Hippocampus		Hippocampus	
Subiculum	++++	Subiculum	++++
		CA4	++++
CA3	++++	CA3	++++
		CA2	++
CA1	++++	CA1	+++
Dentate gyrus		Dentate gyrus	+++
Primordium	+++		
Inner limb	++		
Colliculi		Colliculi	
Inferior	+++	Inferior	+++
Superior	+++	Superior	+++
Thalamus nuclei	+++	Thalamus nuclei	+++
White matter tracts		White matter tracts	
Corpus callosum	+	Corpus callosum	+
Cerebellum		Cerebellum	
External germinal layer	+	Molecular cell layer	−
Purkinje cell layer	++	Purkinje cell layer	+++
		Granular cell layer	+

*The intensity of the signals was evaluated by comparison to signals obtained with the control sense. NPDC-1 probe: − = signal less than or equal to sense control probe signal; + = low; ++ = moderate; +++ = high; and ++++ = very high.

Table 3: Expression of NPDC-1 mRNA in newborn and adult mouse CNS.

Highest levels of NPDC-1 are observed in the hippocampus, both in newborn and adult mouse. Expression of NPDC-1 not only varies between tissues but also within tissues.

Figure from: Dupont et al, J of Neuroscience research 1998 51:257-267

was shown to be indirect, whereas the interaction between cyclin D, Cdk 2 and NPDC-1 was direct (91). These data cumulatively demonstrate that NPDC-1 can interact with a variety of cell cycle regulatory proteins and suggest that NPDC-1 plays a role in neuronal differentiation through its ability to regulate neuronal cell cycle progression.

NPDC-1 was also shown to complex with the cell cycle regulatory transcription factor E2F-1 (91, 93). Neuronal cell cycle progression and neuronal differentiation have been classically linked to the activities of the E2F family of transcription factors. The transcriptional activity of E2F-1 is due to its association with the retinoblastoma proteins pRb, p107 and p130 (94). NPDC-1 was shown to complex with E2F-1 suggesting that these two proteins interact to exert their function. Sansal *et al* (93) localized the domain of interaction between NPDC-1 and E2F-1 at the amino terminus of NPDC-1 although deletion of the carboxyl terminus reduced the interaction. The interaction between NPDC-1 and E2F-1 was also demonstrated *in vivo* in a two hybrid experiment (93). In gel shift experiments the investigators were able to demonstrate that NPDC-1 inhibited the binding of E2F-1 to DNA in a dose dependent manner (93). NPDC-1 was also shown to decrease the transcriptional efficiency of E2F-1 (93).

A widely accepted model is that association of E2F-1 with pRb represses the transcription of genes involved in cell cycle regulation and mediates different E2F activities depending on the cell type and proliferative or differentiated state of the cells (91). The observation that NPDC-1 can complex with E2F-1 suggests that NPDC-1 may play a role in the in terminal neural cell proliferation and differentiation through an ability to repress E2F-1 mediated transcription. A hypothetical model for NPDC-1 repression of E2F-1 mediated transcription is presented in figure 9. As seen in figure 9, activation of cyclin D1 leads to the phosphorylation of pRb which then dissociates
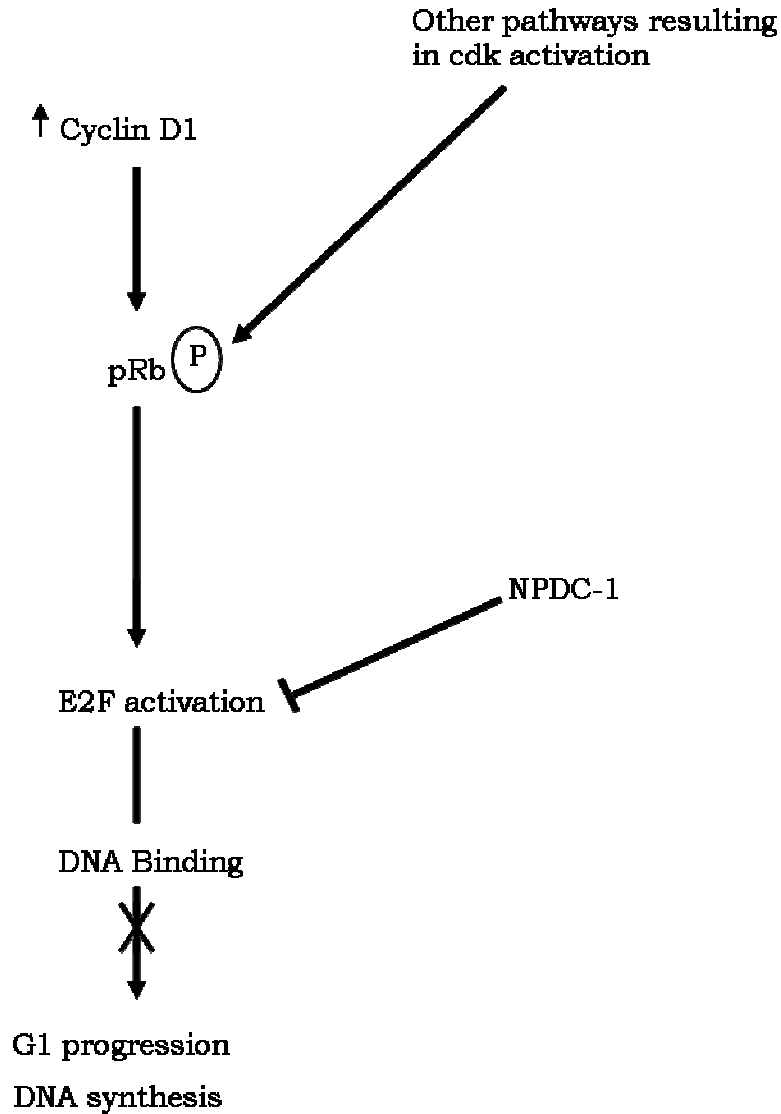
Figure 9: E2F-1 and cell cycle regulation. E2F-1 is normally associated with pRb and this leads to inhibition of gene transcription. Phosphorylation of pRb leads to dissociation and E2F-1 is able to stimulate cell cycle entry and progression. NPDC-1 has been shown to complex with E2F-1 and it can be hypothesized that these interactions repress E2F mediated transcription and therefore cell cycle progression.

from E2F-1 and allows E2F-1 to transactivate gene expression and promote cell cycle progression.

The human homolog for NPDC-1 was first published in 2001 when a Chinese group isolated a clone that shared a high homology to the mouse NPDC-1 gene by screening a human fetal liver cDNA library (90). Analysis of the composition of hNPDC-1 revealed a transmembrane segment at amino acids 182-202, and conserved HLH residues in the amino terminus. Prosite analysis revealed a cAMP and cGMP-dependent protein kinase phosphorylation site (aa 108-111), four protein kinase C phosphorylation sites (aa 9-11, 134-136, 216-218, 223-225), four casein kinase II phosphorylation sites (aa 171-174, 236-239, 275-279, 286-289), and eight N-myristoylation sites (aa 24-29, 33-38, 46-51, 61-66, 141-146, 162-167, 180-185, 294-299) (90). Add to these the three consensus sites for MAPK identified by Galiana *et al* (89), these observations would support the hypothesis that phosphorylation is important to NPDC-1 function.

The human genome sequencing project identified NPDC-1 localization at chromosome 9q34. Alignment of hNPDC-1 cDNA with its genomic sequence revealed that hNPDC-1 is composed of nine exons and eight introns (90). According to the NCBI website, (www.ncbi.nih.gov/IEB/Research/Acembly/av.cgi?db=human&l=NPDC1), NPDC-1 produces by alternative splicing 14 different transcripts all with introns, putatively encoding 14 different protein isoforms. The gene contains 17 introns and has 3 probable alternative promoters and four non-overlapping alternative last exons. The transcripts appear to differ by truncation of the 5' end, truncation of the 3' end, presence or absence of four cassette exons, and common exons with different boundaries. In addition, the gene has 3 shed variants, with exon contact but no

shared intron boundary. The mouse gene is similarly complicated (http://gbrowse.informatics.jax.org/cgibin/gbrowse/mouse_build_34?name=chr2:253 35246..25341657).

Northern blot analysis of human fetal tissues showed at least two transcripts of the gene (90). The smaller 1.55kb transcript appears to correspond to the full-length cDNA of NPDC-1, is the most abundant transcript and is most abundantly expressed in brain and heart (90). The bigger 4.6kb transcript is primarily expressed in the heart (90) (Figure 10). A further examination of hNPDC-1 mRNA expression patterns using dot blot technologies suggested NPDC-1 is expressed at low levels in a variety of tissues and fairly strongly expressed in brain, mammary gland, pituitary and prostate (90). There also appears to be a significant (1.82 fold) upregulation of NPDC-1 mRNA expression in adult brain when compared to fetal brain (90). Although both hNPDC-1 and mNPDC-1 are expressed highly in the brain, the published expression profiles of these two proteins differ substantially. mNPDC-1 was reported to be exclusively expressed in the brain and have only one transcript (89) whereas hNPDC-1 shows multiple transcripts and is expressed in other tissues besides brain (90). This could be explained by species differences or by the regions of NPDC-1 that were used as probes for the northern and dot blots. Our data and the available established EST sequence data strongly support the multiple transcript and more abundant expression of NPDC-1 (95).

Subcellular localization studies suggest NPDC-1 can localize to multiple locations within cells and this localization may be tissue or cell specific (96). NPDC-1 tagged with either FLAG or GFP was transfected into neurons and neuroendocrine cells (PC12 and GH3) to study its expression. In primary differentiated cortical neurons, NPDC-1 displayed a punctate staining along and at the distal part of neuritic
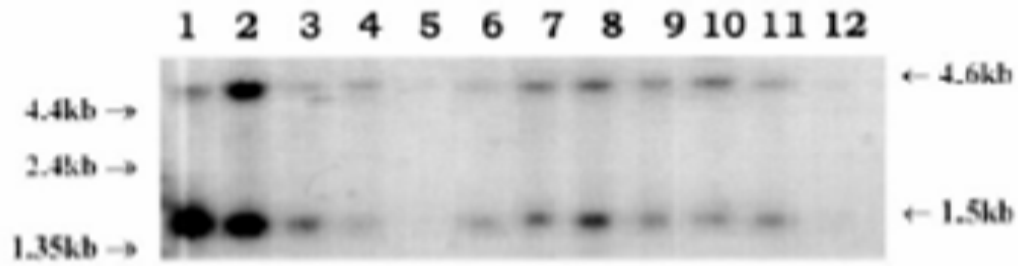
Figure 10: Multiple tissue Northern blot analysis of hNPDC-1 transcription in 12 human fetal tissues. Lanes 1-12: brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, peripheral blood leukocyte. The size markers 1.4, 2.4 and 4.4kb are indicated in the left.

Figure from: Qu *et al* Gene 2001 264:37-44

processes and accumulated in small varicosities (96). The fluorescence was primarily vesicular and a small fraction appeared diffuse in the cytoplasm. No NPDC-1 was observed in the nucleus of these cells. Immunocytochemical analysis of FLAG-NPDC-1 transfected PC12 and GH3 cells, revealed that the protein was inserted in the plasma membrane with the N-terminal region outside the cells (96). NPDC-1 was also detected in the endoplasmic reticulum and in the golgi apparatus (96). The presence of NPDC-1 in transferring positive punctate structures suggests that plasma membrane localized NPDC-1 may be internalized by endocytosis (96). At present it cannot be concluded that NPDC-1 is involved in synaptic vesicle trafficking (96), but appears to be associated with synaptic vesicles.

NPDC-1 knockout mice have also been generated and analyzed (97). No observable phenotype was detected in the null mice and histological analysis of adult and embryo brains showed no brain anomalies (97). This is surprising for a protein that appears to play a role in development and tissue differentiation. To complicate matters, studies in zebrafish show that NPDC-1 does have an observable phenotype. Zebrafish have been an invaluable tool for the study of developmental processes. In situ hybridization of zebrafish embryos at different stages of development show that diffuse expression of NPDC-1 mRNA occurs early in zebrafish development and is restricted to specific tissues as the embryo develops (98). NPDC-1 protein is observed to be expressed later in zebrafish development suggesting that either NPDC-1 is a maternally expressed mRNA and under tight translational control or it is rapidly degraded (99). Overexpression of NPDC-1 in zebrafish embryos led to abnormal development phenotypes. The most consistent phenotypes observed were no tail or duplicated head in place of the tail, and a milder phenotype where embryos lack much of their muscle layers and blood vessels (98). Of potential significance to NPDC-1's

mechanism of activity (and something further explored in this dissertation) is the observation that the teratogenic effects observed with overexpression of NPDC-1 in zebrafish parallel those observed with RA treatment of zebrafish embryos (100). Knocking down NPDC-1 in zebrafish embryos using NPDC-1 morpholinos disrupted normal organogenesis and resulted in a shortened lifespan. These data cumulatively would suggest that, at least in zebrafish, NPDC-1 plays a critical role in development. Possible explanations for these differences might include; the development of some compensation system in mice not present in zebrafish, an alternative function for NPDC-1 in zebrafish, or alternative expression of a variant of the NPDC-1 gene in mice.

In summary, NPDC-1 has been identified as a novel regulator of cell proliferation and differentiation in neuronal cells, but is also expressed in multiple tissues. NPDC-1 was found to associate, either directly or indirectly, with cell cycle regulatory proteins such as cyclin D, cdk2, cyclin A and E2F-1. Expression of NPDC-1 increases during development and the highest levels are observed in adult tissues. Knockout mice for NPDC-1 have no observable phenotype but in zebrafish NPDC-1 expression appears to be important for development.

Chapter 5: NPDC-1 as a regulator of Nuclear Receptor function

Retinoids have an established and profound effect on cellular differentiation and proliferation. Retinoids exert their effects by stimulating the RAR-RXR receptor complex and effect transcription of genes with RARE response elements. In mammalian cells, retinoid mediated transcription is influenced by the cell cycle regulator E2F-1. When E2F-1 is coexpressed with a βRE plasmid, transcription induced by atRA is inhibited (95), however the interaction between E2F-1 and RARs or βRE is not direct, suggesting an interaction with a common accessory factor or factors (95).

Using a novel yeast cDNA library screening protocol for identifying positive regulators of RXR mediated transcription, Henry *et al* identified the rat homolog of NPDC-1 (95). The rat NPDC-1 clone was subsequently used to clone a human NPDC-1 homolog and sequence analysis comparison of rat and human NPDC-1 with mouse NPDC-1 (figure 11) showed that the protein is well conserved between these species suggesting that the protein has a conserved function. Conserved motifs found within all three proteins include: a) an LXXLL motif (or NR box) which is a mediator of interaction between nuclear receptors and coregulators (28), b) a HLH motif, which provides the binding site for DNA. c) a MAPK kinase domain which is involved in phosphorylation, a way to regulate protein function, and d) a PEST domain which is involved in post-translational modification by targeting proteins for degradation by the ubiquitin-proteosome system. Tissue distribution comparison of rat and human NPDC-1, as analyzed by northern blotting, revealed rat brain to express a single mRNA species consistent with the published results for mouse, while rat lung, skeletal muscle, kidney and heart expressed multiple mRNA species. In human, multiple

```
mouse    1 MATPVPPPSPRHLRL LRLLL SGLILGAALNGATARRPDAT 40
rat      1 MATPVPPPSPRHLRL LRLLL SGLILGAALNGATARRPDAP 40
human    1 MATPLPPPSPRHLRL LRLLL SGLVLGAALRGAAAGHPDVA 40
           ****.********** ***** ***.***** **.* .**

mouse   41 TCPGSLDCALKRRAKCPPGAHACGPCLQSFQEDQRGFCVP   80
rat     41 TCPGSLDCALKRRAKCPPGAHACGPCLQSFQEDQRGVCVP   80
human   41 ACPGSLDCALKRRARCPPGAHACGPCLQPFQEDQQGLCVP   80
           .************* .************* *****.* ***

mouse   81 RKHLSSGEGLPQPRLEEEIDSLAQELALKEKEAGHSRLTA  120
rat     81 RKHQSSGEGLPQPRLEEEIDSLARELALKEKEAGHPRLTA  120
human   81 RMRRPPGGGRPQPRLEDEIDFLAQELARKE--SGHS---T  115
           * .    * * ******.*** **.*** **   .**    .

mouse  121 QPLLERAQKLLEPAATLGFSQWGQRLEPGLPSTHGTSSPI  160
rat    121 QPLPEASQKLLEPAATLGFSQWGQQLEPGLPSTHGTSSPT  160
human  116 PPLPKDRQRLPEP-ATLGFSARGQGLELGLPSTPGTPTPT  154
           **       *.* ** ******   ** ** ***** **  .*

mouse  161 PHTSLSSRASSGPVQMSPLEPQGRHGNGLTLVLILAFCLA  200
rat    161 PHTSLSARASSGPVQMSPLEPQGR-GNGLALVLILAFCLA  199
human  155 PHTSLGSPVSSDPVHMSPLEPRGGQGDGLALVLILAFCVA  194
           *****  .   ** **.******.*   * **.********.*

mouse  201 SSAALAVAALCWCRLQREIRLTQKADYAATAKGPTSPSTP  240
rat    200 STAALAVAALCWCRLQREIRLTQKADYTATAKGPTSPTTP  239
human  195 GAAALSVASLCWCRLQREIRLTQKADY-ATAKAPGSPAAP  233
           .***.**.****************** **** * **..*

mouse  241 RISPGDQRLAHSAEMYHYQHQRQQMLCLERHKEPPKELES  280
rat    240 RISPGDERLAHSAEMYHYQHQRQQMLCLERHKDPPKELES  279
human  234 RISPGDQRLAQSAEMYHYQHQRQQMLCLERHKEPPKELDT  273
           ******.***.*********************.*****..

mouse  281 ASSDEENEDGDFTVYECPGLAPTGEMEVRNPLFDHSTLSA  320
rat    280 ASSDEENEDGDFTVYECPGLAPTGEMEVRNPLFDHSTLSA  319
human  274 ASSDEENEDGDFTVYECPGLAPTGEMEVRNPLFDHAALSA  313
           ***********************************..***

mouse  321 PVPGPHSLPPLQ  332
rat    320 PVPGPHSSPPLQ  331
human  314 PLPAPSSPPALP  325
           *.* * * * *
```

Figure 11: Comparative alignment of rat and human NPDC-1 with the mouse sequence. Conserved amino acids are indicated with *.

Figure from: Henry *et al* Nuclear Receptor 2003 1

NPDC-1 mRNA species were detected in brain as well as in other tissues (figure 12). These data agree with previously published data by Qu *et al* (90).

To test whether NPDC-1 interacted with RXR, RAR and E2F-1, recombinant thioredoxin-tagged hNPDC-1 was used to pull-down protein from PC12 cell lysates that had been transfected with haemaglutinin (HA)-tagged E2F-1, human RARβ or human RXRα. Immunoblotting analysis showed that hNPDC-1 is able to bind to hRARβ, hRXRα and E2F-1, and binding to the retinoid receptors was observed to be ligand independent. Furthermore, these were the first studies to show a direct *in vivo* interaction between hNPDC-1 and E2F-1. In addition we were able to localize NPDC-1's RXR binding domain to the amino terminus, suggesting that the putative coiled-coil domain in this region may play a role in the interaction of NPDC-1 with RXRα. Finally, it has been shown that interactions between coregulators and NRs often occur at LXXLL motifs (1, 28). In the amino terminus of NPDC-1 is a conserved LXXLL motif and to evaluate the importance of this motif to RAR-mediated transcription events, a series of mutations were made to this motif and these mutants were analyzed for their impact on NPDC-1 repression of RAR-mediated transcription. The mutants had no impact on this event suggesting this motif was not essential for NPDC-1's RAR regulation.

To assess the functional significance of NPDC-1/NR interactions, the impact of NPDC-1 on nuclear receptor mediated transcription was measured in a mammalian transcription assay. Transcription mediated by RAR and RXR was strongly suppressed whereas VDR transcription was not affected. Transcription by PPAR and ER were also suppressed, but not as strongly as the retinoid receptors (Figure 13). Cumulatively, the above data suggest NPDC-1 functions as a repressor of NR-mediated transcription. This could either be through its ability to repress DNA binding or
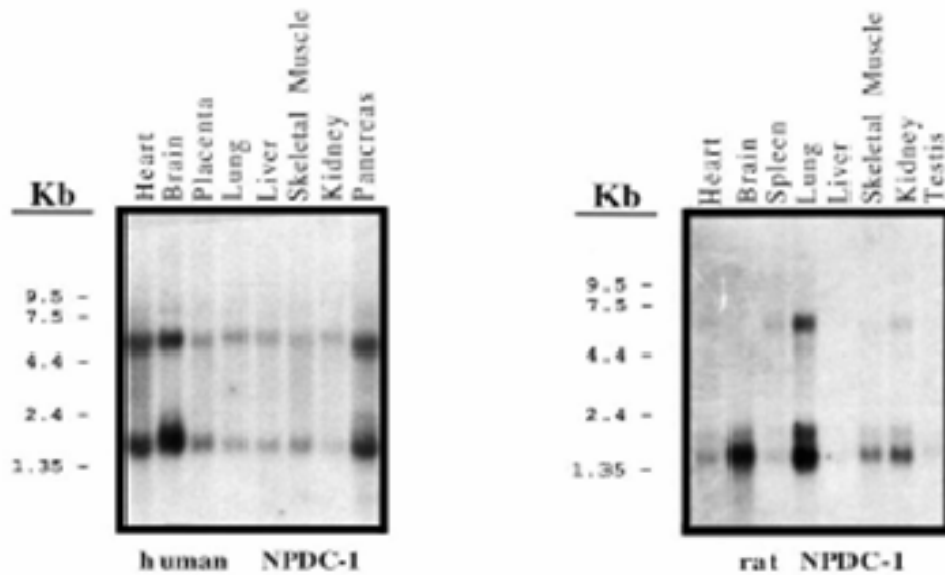
Figure 12: Northern Analysis of rat and human NPDC-1 distribution.

Approximately 2μg of poly A+ RNA from the indicated specific tissues was used to generate rat and human multiple tissue northern blots. Each blot was probed with a specific DNA derived radiolabelled DNA probes. The location of RNA size markers is indicated.

Figure from: Henry *et al* Nuclear Receptor 2003 1
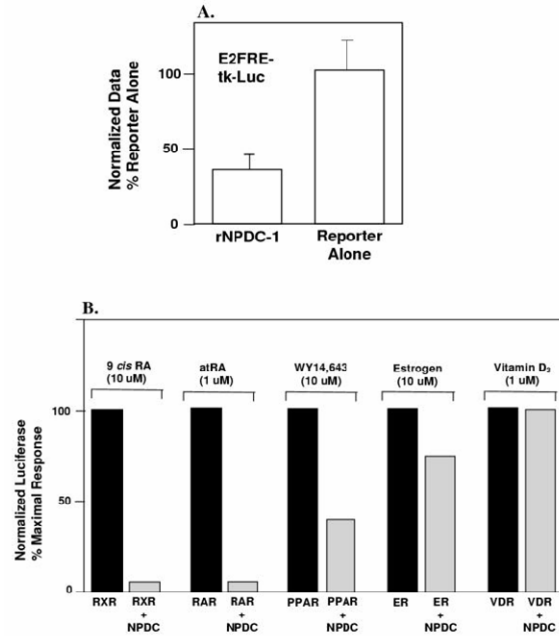
Figure 13: NPDC-1 selectively represses nuclear receptor mediated transcription. **A**. rNPDC-1 was analyzed for its ability to modulate the transcription mediated by endogenous E2F transcription factors. A pBL-E2FRE-tk-luc (E2FRE) reporter plasmid and a mammalian expression β-galactosidase construct were transfected into HepG2 cells with and without a mammalian expression construct for rNPDC-1. Luciferase data was normalized and data reported as a % of the constitutive activation of the E2F reporter plasmid. **B**. Mammalian expression plasmids for pRS-hRXRα, pRS-hRARβ, pRS-rPPARα, pRS-hERα and pRS-hVDR were transiently transfected into HEK 293 cells along with their respective luciferase reporter constructs and with or without rNPDC-1. Transfected cells were incubated with ligand at the indicated doses. A mammalian expression β–gal construct was included in all transfections to normalize for transfection efficiency. Data shown here is expressed in normalized values to β-gal.

Figure from: Henry et al Nuclear Receptor 2003 1

through its ability to recruit transcription repressor complexes (e.g. chromatin remodeling proteins). In a preliminary investigation aimed at distinguishing between these two possibilities, in these published studies I performed a series of experiments characterizing NPDC-1's impact on retinoid receptor DNA binding (figure 14). Using PC12 nuclear extract and recombinant NPDC-1, I studied the ability of NPDC-1 to modulate DNA binding to a DR5 βRARE (figure 14A). Two major complexes were formed in the presence of PC12 nuclear extracts. Addition of recombinant hNPDC-1 increased the intensity of the lower shift complex at the expense of the higher complex. NPDC-1 did not however form a measurable unique complex with the radiolabeled DNA, nor did it alter the mobility of the complexes, suggesting that NPDC-1 facilitates complex formation without remaining in strong association with either proteins or the DNA. In addition to the PC12 nuclear extracts, I also performed electromobility shift assays using pure recombinant protein in order to eliminate any effects from factors that may be present in the nuclear extract (figure 14B+C). Recombinant hRXRα and hNPDC-1 were tested for binding to a DR1 RXRE probe. RXRα was bound to DNA in the absence of ligand, forming a homodimer and a monomer complex. Addition of NPDC-1 increased both complexes without a shift in the complex mobility. Supershift experiments with α-RXRα and α-NPDC-1 demonstrated that NPDC-1 was not a part of the RXR/DR1 complex and that RXRα is a major component of both the upper and lower complex (95). Similar unpublished experiments were performed using nuclear extract from NT2 cells, which is a human neuronal cell line (Figure 15). Addition of NPDC-1 caused the formation of a higher complex, but did not diminish the intensity of the lower RAR/RXR complex on βRARE. When the NT2 cells were serum starved before making nuclear extract, addition of NPDC-1 caused a shift in the complex but with less intensity. Addition of atRA

A.

DR5 Probe	+	+	+	+
PC12 Extract			+	+
NPDC		+		+

B.

DR1 Probe	+	+	+	+
RXR			+	+
NPDC		+		+

C.

DR1	+	+	+	+	+	+
NPDC	+		+	+	+
RXR			+	+	+	+
α-RXR					+	
α-NPDC						+

←Supershift

Free Probe
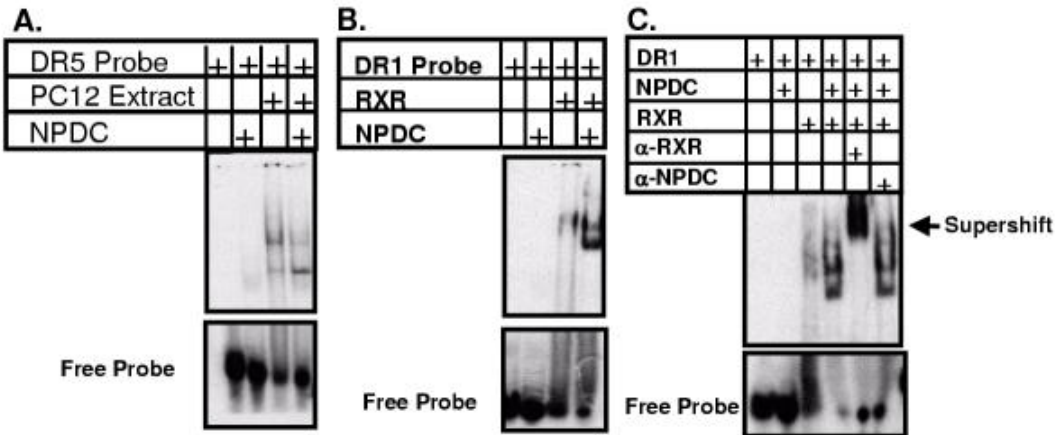
Figure 14: hNPDC-1 alters hRARβ and hRXRα DNA binding properties. Oligonucleotides for RARE (DR5, panel A) and RXRE (DR1, panels B and C) were ^{32}P labeled by T4 polynucleotide kinase. The resultant DNA binding probes were incubated with PC12 lysate (A), recombinant RXRα (B&C) and recombinant NPDC-1 (C) as indicated. DNA:protein complexes were resolved by non-denaturing PAGE and developed by autoradiography. In C, the presence of NPDC-1 within the RXR gel-shift complex was assayed by supershift. Antibodies specific for either NPDC-1 or RXR were used to identify proteins present in the complex.

Figure from: Henry et al Nuclear Receptor 2003 1

	1	2	3	4	5	6	7	8	9	10	11	12	13
Free Probe	x												
NT2 Nuc. X		x	x	x		x	x	x	x	x	x	x	x	x
hNPDC-1			x					x				x		
Cold competition						x					x			x
Anti RARβ				x					x				x	
Serum strarve							x	x	x	x				
atRA											x	x	x	x

→ NPDC-1 complex

→ RAR/RXR complex

Free Probe
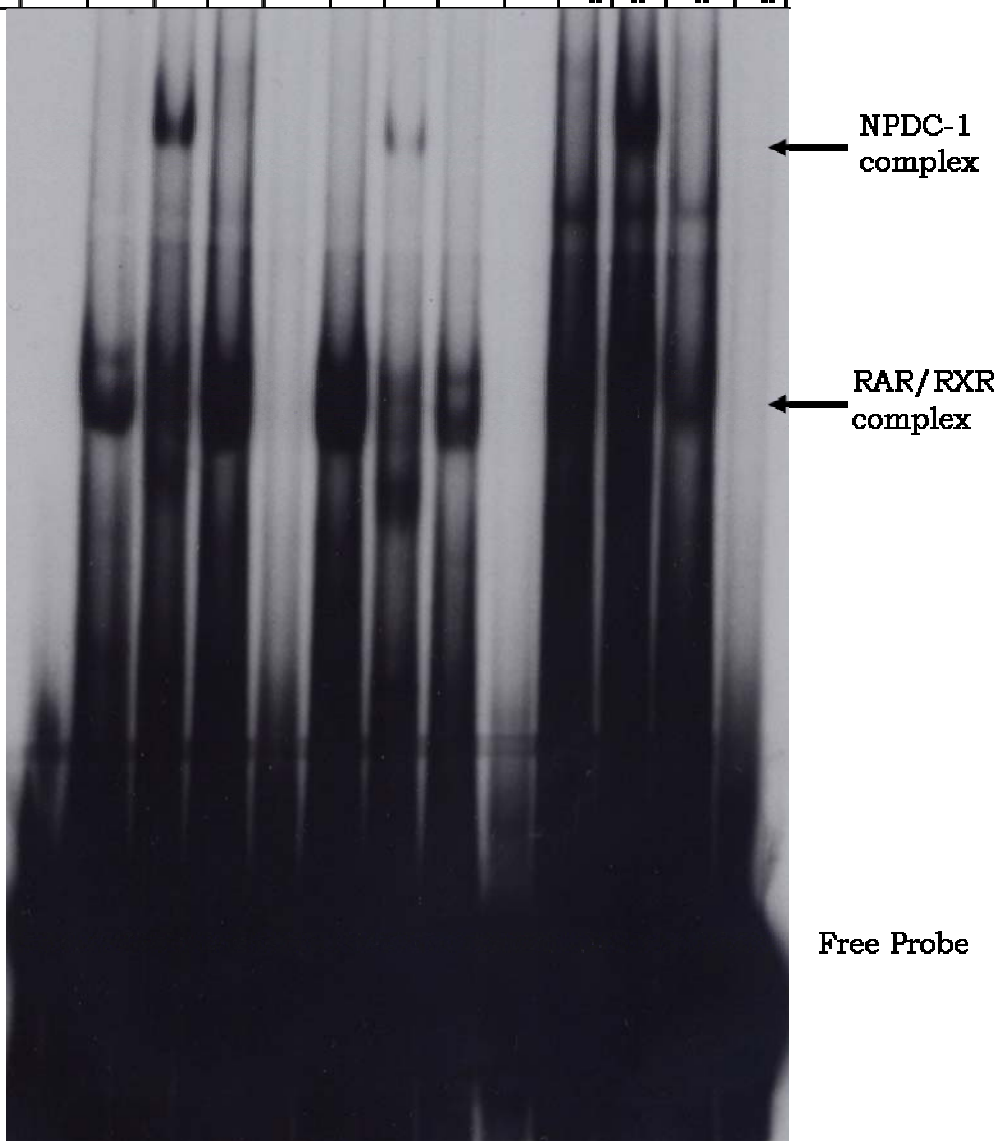
Figure 15: EMSA with NT2 nuclear extracts. Nuclear extracts from NT2 cells were assayed for binding to recombinant NPDC-1 and RARE. The βRE oligonucleotide was end-labeled with ^{32}P by T4 polynucleotide kinase according to the manufacturer's instructions. The resultant DNA binding probe was incubated with NT2 nuclear extract and recombinant hNPDC-1 protein. The resultant DNA:protein complexes were resolved by non-denaturing PAGE gels which were subsequently exposed to Kodak Xar-5 film. The RARβ antibody (lanes 4, 8, 12) was used to identify proteins in the complex and cold βRE oligonucleotide was used to show the specificity of the complex (lanes 5, 9, 13). Complex formation was also assayed in the presence of atRA (lanes 10-13). The RAR/RXR and RAR/RXR/NPDC-1 complexes are indicated by arrows.

*This is an accurate representation of experiment repeated at least three times.

resulted in a much stronger shifted complex with NPDC-1, similar to published results. Interestingly, addition of NPDC-1 caused a higher molecular weight complex to form without altering the intensity of the lower band, suggesting that in NT2 cells there is a direct association between RAR, RXR, NPDC-1 and DNA. I also tried to replicate these results *in vitro* using recombinant protein to determine the binding specificity of each protein to βRARE, and to eliminate any effect intrinsic to the nuclear extracts (Figure 16). NPDC-1, RARβ and RXRα alone did not bind to the βRARE and neither did RARβ/NPDC-1 or RXRα/NPDC-1 complexes. As expected RARβ and RXRα formed a complex, and the addition of NPDC-1 increased the intensity of the RARβ/RXRα complexes. As observed with PC12 extracts, no shift in complex size is observed with these recombinant proteins. The addition of RA to these reactions appears to slightly increase complex formation but has very little impact on NPDC-1's effects on the complex. Cumulatively these data suggest NPDC-1 can directly facilitate retinoid receptor DNA binding, but the mechanism is unclear and how this might be repressive to retinoid-mediated transcription is still a mystery.

In keeping with its hypothetical role as a regulator of cellular proliferation, NPDC-1 expression was also shown to be tightly regulated at the post-translational level (99). To study NPDC-1 function *in vivo*, we generated a green fluorescent protein (GFP)-tagged hNPDC-1 construct and transfected it into PC12 cells. Observation under a fluorescent microscope 48 hrs post transfection did not yield any green cells, although the native GFP expressed from vector alone was easily observed. Western blot analysis showed that NPDC-1 could be detected after prolonged exposure, but with a much lower intensity than GFP alone. This observation suggested that NPDC-1 is rapidly turned over in PC12 cells. Although a number of possible explanations could explain these results, one of the more prominent is that of targeted degradation

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
RAR			x		x		x	x			x		x	x
RXR				x		x	x	x				x	x	x
hNPDC-1		x			x	x		x		x	x	x		x
atRA 10uM										x	x	x	x	x

RAR/RXR complex

Free Probe
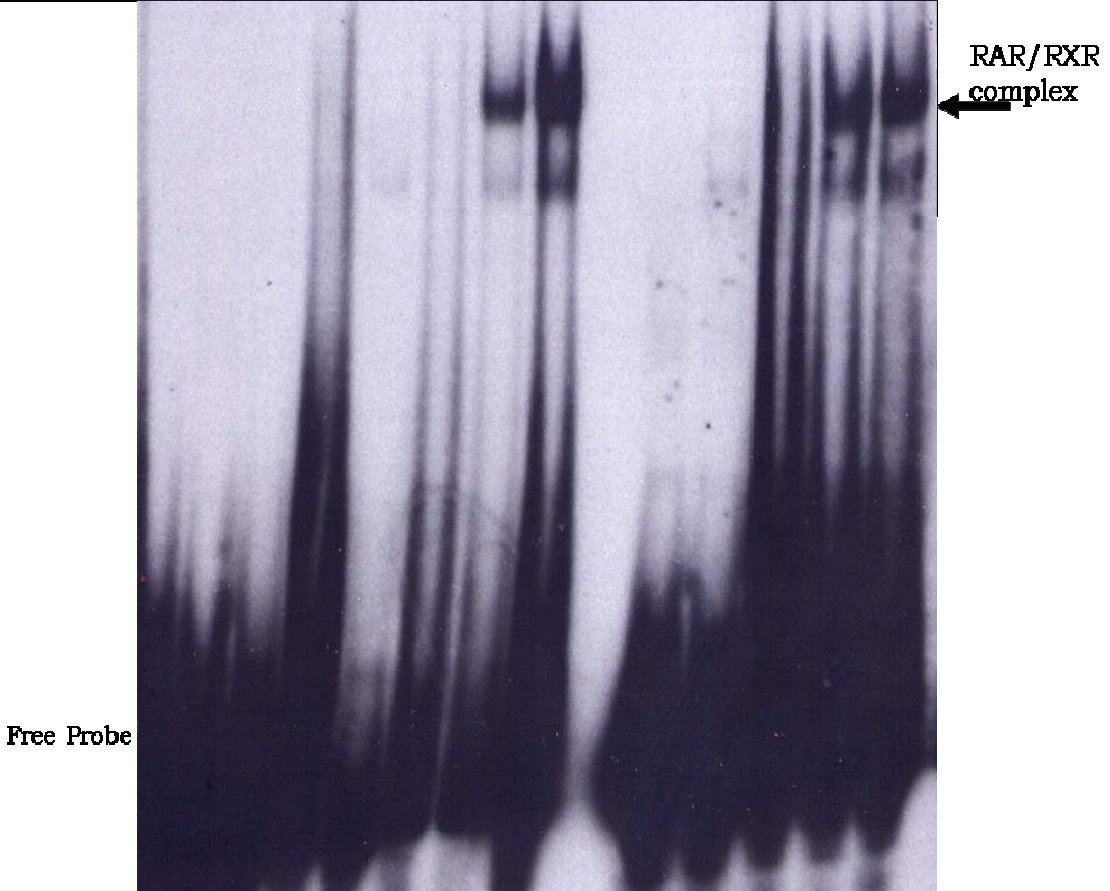
Figure 16: NPDC-1 makes the RAR/RXR complex stronger but does not complex with RAR or RXR. Oligonucleotide corresponding to the consensus RARβ-RARE was end labeled with ^{32}P by T4 polynucleotide kinase according to manufacturer's instructions. The resultant DNA binding probe was incubated with recombinant hRARβ, hRARα and hNPDC-1 proteins as indicated. The resultant DNA:protein complexes were resolved on non-denaturing PAGE gels which were subsequently exposed to Kodak Xar-5 film. Complex formation was also assayed in the presence of 10μM atRA (lanes 10-14).

* This is an accurate representation of an experiment repeated at least three times.

of NPDC-1 protein. The ubiquitin/proteosome system is an important mechanism for protein degradation (101). Proteins are targeted for destruction by the ubiquitin/proteosome system by the addition of multiple ubiquitin molecules. The ubiquitinated protein is targeted to the proteosome where it is cleaved into oligopeptides. There are a number of protease inhibitors that can be used to monitor proteosome function. One such inhibitor is MG-132, which is able to turn off the proteosome and allow targeted polyubiquitinated proteins to accumulate in the cell (102). To test whether the ubiquitin/proteosome system was the mechanism through which NPDC-1 is eliminated from the cell, we transfected PC12 cells with hNPDC-GFP or GFP and 24hrs post transfection treated the cells with 40μM MG-132 (figure 17). After an additional 24hrs of incubation cells were fixed and observed under a fluorescent microscope. Once again, GFP transfected cells were easily observable under the microscope whereas hNPDC-GFP transfected cells were not detectable. However, cells that were treated with MG-132 were detected under the microscope suggesting that NPDC-1 is indeed degraded by the ubiquitin/proteosome system (99). We went on in these published studies to further demonstrate that NPDC-1 expression is regulated post-translationally and NPDC-1 could be ubiquitinated both *in vitro* and *in vivo*. Taken together these observations provide conclusive evidence that NPDC-1 is targeted and degraded by the ubiquitin/proteosome pathway.

Ubiquitination complexes have been shown to bind target proteins at hydrophobic regions rich in proline, glutamate, serine and threonine (PEST). Sequence analysis of NPDC-1 showed a PEST degradation motif in the carboxyl terminus of hNPDC-1 (figure 18A+B) (99). For NPDC-1 this region was observed to be totally conserved between species suggesting its significance for NPDC-1 regulation. To study the importance of this PEST region further we generated a construct (termed
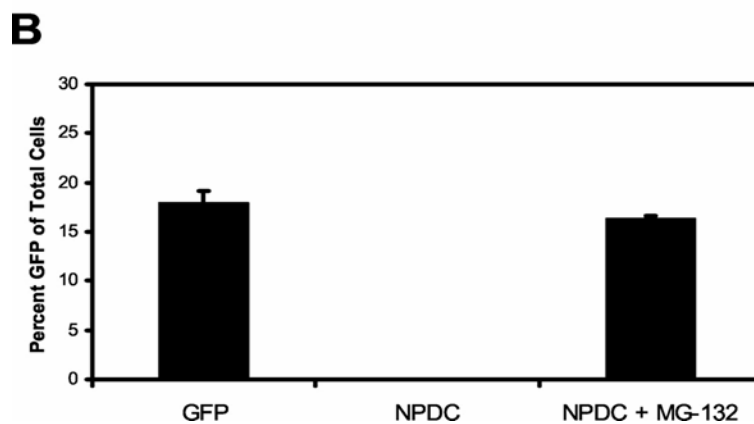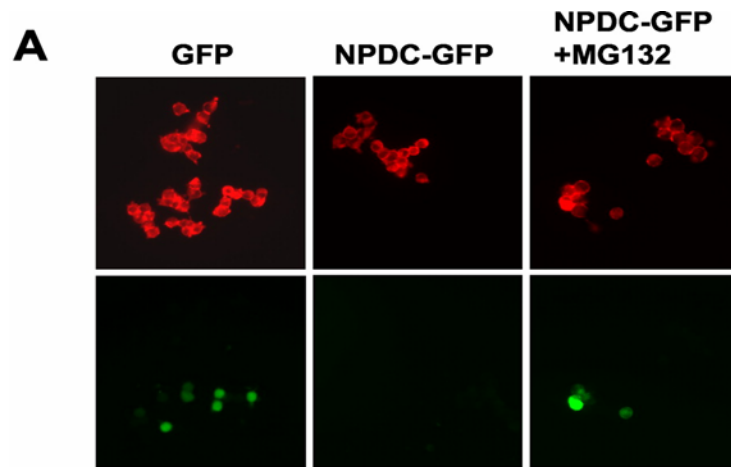
Figure 17: NPDC-1 degradation is mediated by the proteosome. PC12 cells were transfected with 1µg NPDC-GFP or GFP control vector. Cells were treated with 40mM MG-132 proteosome inhibitor where indicated. After 48hrs the cells were fixed and stained. In panel A cells were visualized by direct fluorescence (red all cells, green transfected cells). In panel B, cells expressing GFP or GFP-NPDC were counted and expressed as a percent of total cells.

Figure from: Spencer, M. L. et al. J. Biol. Chem. 2004;279:37069-37078
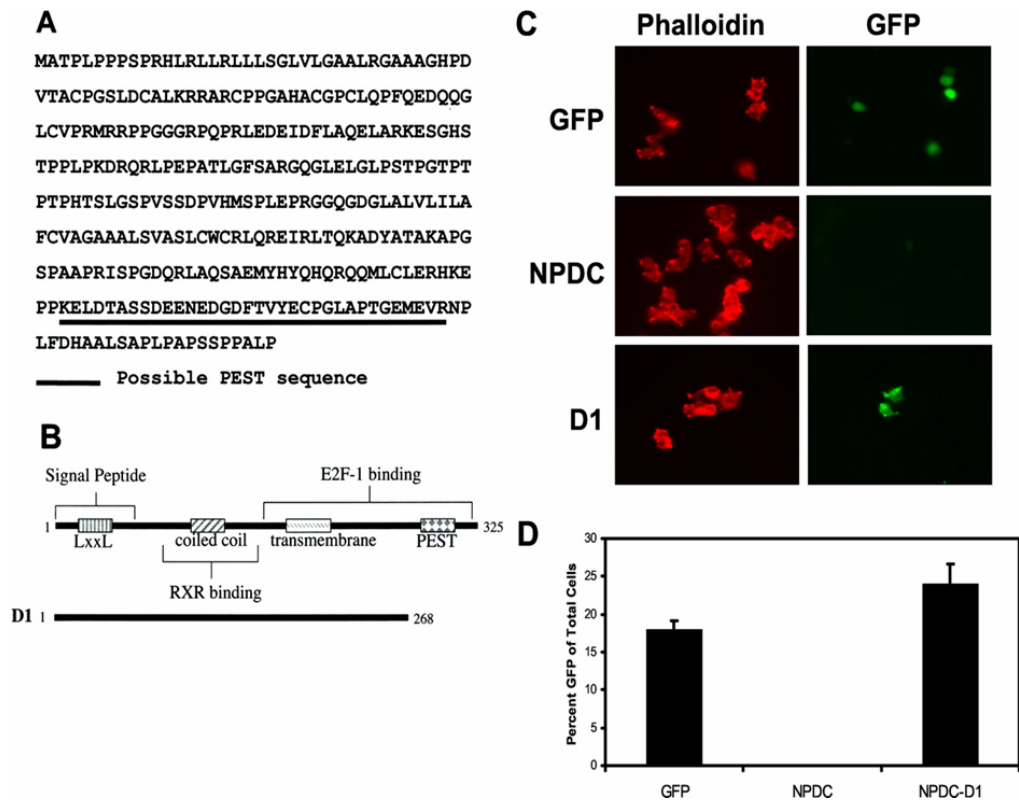
Figure 18: NPDC-1 contains the PEST degradation sequence. A. use of the website www.PESTfind to analyze hNPDC-1 for the presence of a PEST proteosomal degradation sequence identified a potential PEST sequence in the carboxyl terminus of hNPDC-1. B. GFP-tagged constructs were generated for NPDC-1 and a mutant hNPDC-1 gene truncated at amino acid 286 and missing the putative PEST sequence (D1). C. NPDC-1 and D1 constructs and GFP control protein were transfected into PC12 cells. After 48h the cells were fixed, stained and analyzed for fluorescence. D. Cells expressing GFP, NPDC-GFP and NPDC-D1-GFP were scored and expressed as a percent of all cells present.

Figure from: Spencer, M. L. et al. J. Biol. Chem. 2004;279:37069-37078

D1) where hNPDC-1 was truncated just before the PEST sequence. PC12 cells were transfected with GFP, hNPDC-GFP or hNPDC-D1-GFP and visualized under a fluorescent microscope (Figure 18C+D). As before, we were not able to detect the full length protein fusion, but we were able to detect the truncated construct which expressed as well as the GFP control. Subsequent *in vitro* ubiquitination experiments further demonstrated that hNPDC-D1 is not ubiquitinated, suggesting that the PEST sequence is functional and hNPDC-1 degradation can be inhibited by removal of the PEST sequence (99).

To marry our earlier studies looking at NPDC-1 effects on retinoid signaling, with these new data on NPDC-1 targeted degradation, we analyzed the impact the hNPDC-D1 mutant had on retinoid mediated transcription. In transcription assays using the βRE-tk-luc reporter we observed that hNPDC-D1 functioned as a significantly better inhibitor of retinoid transcription than full length hNPDC-1. This result suggests that proteosomal degradation of NPDC-1 could serve as a regulatory mechanism for retinoic-acid induced transcription (99).

In the papers first describing NPDC-1, a number of potential phosphorylation sites were reported (89, 90). Phosphorylation is one of the main mechanisms for regulating proteins that function in cellular proliferation and transcriptional regulation. We therefore initiated metabolic studies to evaluate the phosphorylation state of hNPDC-1 *in vivo* (Figure 19). hNPDC-1 was shown to be a phosphoprotein and that it could be phosphorylated by a number of kinases. These included ERK, glycogen synthase 3, casein kinase I and casein kinase II (99). Although phosphorylation of NPDC-1 by ERK and by CKII was previously demonstrated, GSK3 and CKI had not been reported or predicted.
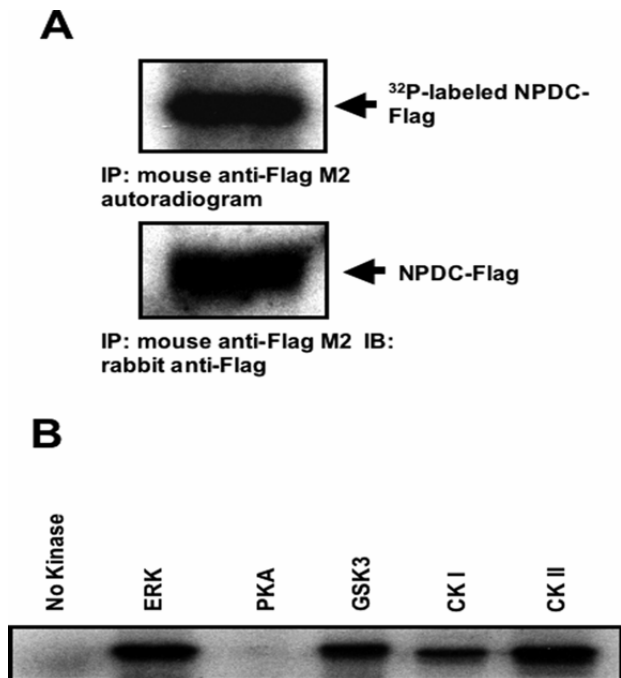
Figure 19: hNPDC-1 is a phosphoprotein. (A) PC12 cells were transfected with hNPDC-FLAG mammalian expression construct. After 24h, cells were rinsed and incubated in serum and phosphate free media. After 12h cells were labeled with 0.5mCi of ^{32}P orthophosphate for 4h. Cells were harvested, and FLAG antisera was used to immunoprecipitate hNPDC-FLAG. The precipitated material was divided evenly and subjected to SDS-PAGE. One gel was dried and developed by autoradiography and the other half was immunoblotted with anti-FLAG M2 antibody. (B) A series of protein kinases were tested for their ability to phosphorylate bacterially expressed hNPDC-1. 1μg of recombinant protein was incubated with 500U of kinase in a reaction mixture containing 5mCi of γ-ATP. Proteins were separated by SDS-PAGE and developed by autoradiography.

Figure from: Spencer, M. L. et al. J. Biol. Chem. 2004; 279:37069-37078

Phosphorylation is a major mechanism for the degradation of many PEST-containing proteins such as the cyclins (103). In this paper my major contribution was to evaluate the impact ERK phosphorylation of hNPDC-1 had on its ubiquitination kinetics *in vitro* (Figure 20). Recombinant thioredoxin-tagged NPDC-1 protein was phosphorylated for 1hr by ERK and then was subjected to ubiquitination time course assays containing rabbit reticulocyte and recombinant ubiquitin protein. Phosphorylation of hNPDC-1 by ERK increased the ubiquitination kinetics, suggesting the phosphorylation state of NPDC-1 is important for its targeted degradation. To examine this further, HEK293 cells were transfected with a hNPDC-FLAG expression construct and assayed for ubiquitination in the presence and absence of PD98059, a MAPK inhibitor, and in the presence and absence of the proteosomal inhibitor MG-132 (Figure 20B). The amount of ubiquitinated NPDC-1 was observed to decrease with increased PD98059 concentration. In addition to testing ERK for modified ubiquitination kinetics, I also tested GSK3 and CKII in a series of unpublished studies (Figure 21). These two kinases, previously shown to be able to phosphorylate NPDC-1, modified NPDC-1 ubiquitination kinetics in a similar manner to that observed with ERK, in that, in the presence of kinase, ubiquitination occurs earlier leading to an increased degradation rate.

Previously published data introduced NPDC-1 as an inhibitor of cellular proliferation (89, 93) and as an inhibitor of growth in soft agar. NPDC-1 has also been shown to associate with cell cycle components such as E2F-1 and various cyclins and cdks (91, 93). NPDC-1 levels also appear elevated in cells that have exited the cell cycle (89, 91). All of these observations taken together suggest that NPDC-1 can regulate cell proliferation and perhaps function as a tumor suppressor. Data from our lab (99) further support this hypothesis in that they show that NPDC-1 is regulated
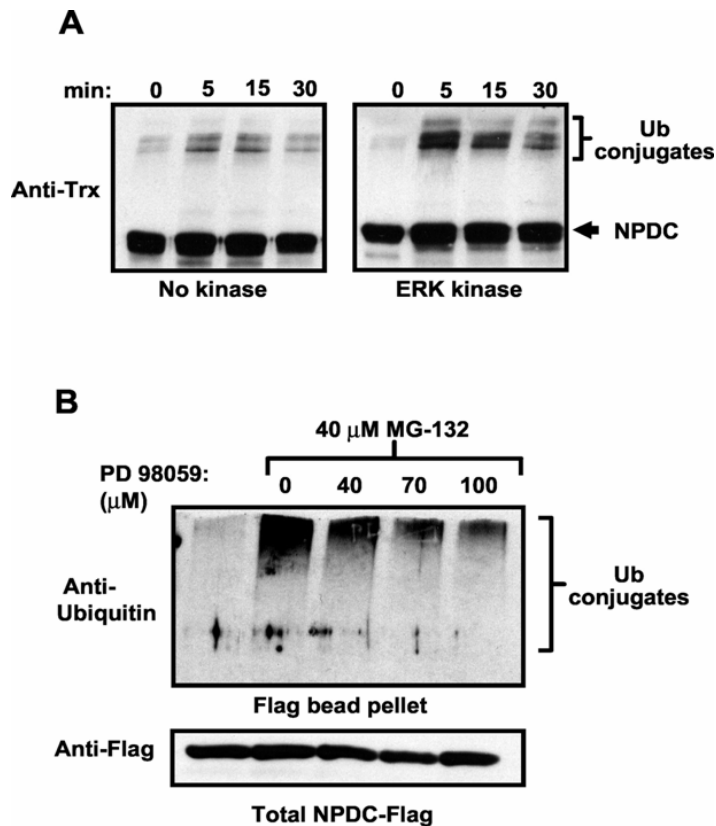
Figure 20: Phosphorylation increases the degradation kinetics of NPDC-1. (A) Recombinant S-thioredoxin-tagged hNPDC-1 was phosphorylated for 1h with 500U of ERK kinase in a reaction containing cold ATP. The reaction was added to an equal volume of ubiquitination buffer containing 10% rabbit reticulocyte and recombinant ubiquitin. Reactions were stopped at the indicated time points with 2X SDS-PAGE buffer, electrophoresed on SDS-PAGE and immunoblotted with anti-thioredoxin antibody for NPDC-1. (B) HEK293 cells were lysed in the presence of MG-132 and NEM to preserve ubiquitin chains. FLAG-NPDC-1 was pulled down with a-FLAG M2 beads. The pellet was divided and one half was blotted for ubiquitin and the other half was analyzed for total NPDC-1 with anti-FLAG-M2 antibody.

Figure from: Spencer, M. L. et al. J. Biol. Chem. 2004;279:37069-37078
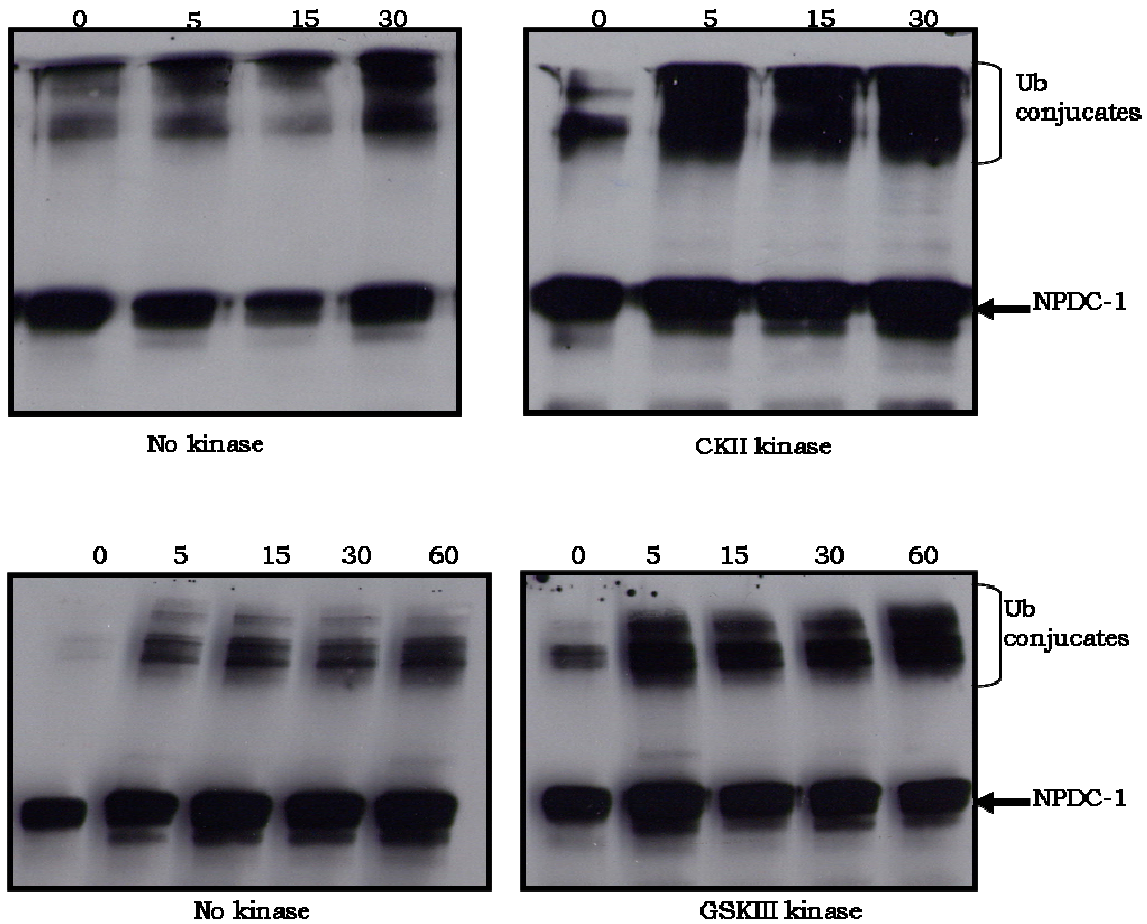
Figure 21: CKII and GSK3 increase the degradation kinetics of NPDC-1.
Recombinant S-thioredoxin-tagged hNPDC-1 was phosphorylated for 1h with 500U of either CKII (top) or GSK3 (bottom) in a reaction containing cold ATP. The reaction was added to an equal volume of ubiquitination buffer containing 10% rabbit reticulocyte and recombinant ubiquitin. Reactions were stopped at the indicated time points with 2X SDS-PAGE buffer and electrophoresed on SDS-PAGE gels, and immunoblotted for NPDC-1 with anti-thioredoxin antibody.

* This is an accurate representation of an experiment repeated at least three times

through phosphorylation and the ubiquitin/proteosome degradation pathway, not unlike other cell cycle associated proteins such as cyclins and cdks (103). Spencer *et al* (99) demonstrated that ERK is able to phosphorylate NPDC-1 and alter its ubiquitination kinetics. In the classic signaling MAPK cascade, MEK phosphorylates and activates ERK which then in turn phosphorylates and activates targets involved in cellular proliferation (Figure 22). This is accomplished by translocation of ERK to the nucleus. Furthermore, in unpublished data, I have been able to demonstrate that recombinant hNPDC-1 is able to directly bind and interact with GST-tagged MEK, ERK and Elk-1 (Figure 23A). The interaction between NPDC-1 and ERK was also shown *in vivo* (Figure 23B). Another potential link between the MAPK cascade and NPDC-1 is the observation that the MAPK signaling pathway can also be linked to cellular differentiation. An example of this is involvement of MAPK in PC12 neurite outgrowth (104). Treatment of PC12 cells with EGF activates the MAPK cascade transiently and results in proliferation while NGF stimulation results in a more sustained MAPK cascade and results in differentiation (105). Cumulatively these data suggest a role for NPDC-1 in MAPK-driven cellular proliferation and differentiation.

In summary, hNPDC-1 has been shown to act as a coregulator of retinoid receptors by suppressing transcription in the presence of retinoic acid. In addition, NPDC-1 is able to exert these effects by directly binding to hRARβ and hRXRα and alter the DNA binding of RAR/RXR heterodimers and RXR/RXR homodimers. The carboxyl terminus of NPDC-1 contains a PEST sequence which is conserved between species suggesting a function for this region. The level of NPDC-1 in cells is regulated by targeted degradation by the ubiquitin/proteosome system. Deletion of the PEST region leads to accumulation of NPDC-1 in cells and an even stronger repression of retinoid receptor mediated transcription.

Figure 22: MAPK/ERK in growth and differentiation.

Figure from: Cell signaling technology www.cellsignal.com
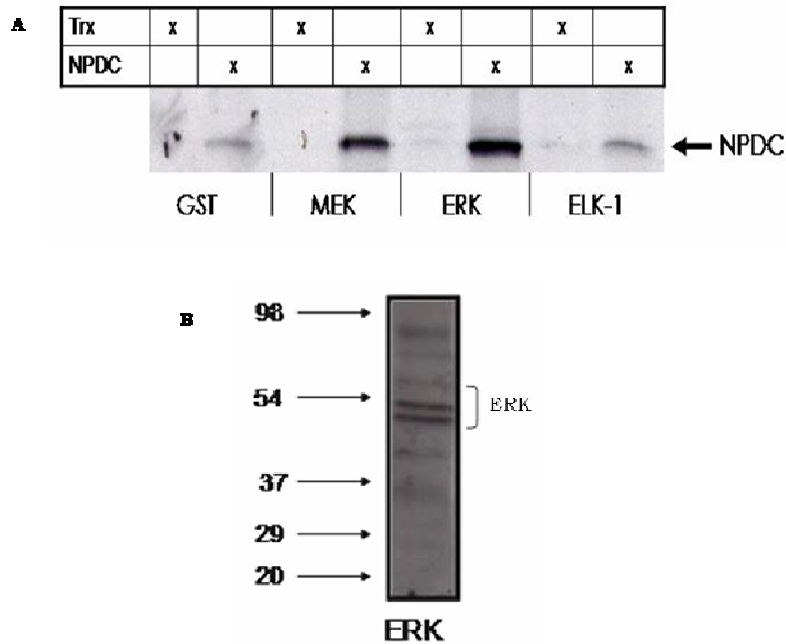
Figure 23: NPDC-1 is capable of interacting with MAPK proteins *in vivo* and *in vitro*. In panel A, recombinant GST-tagged MEK, ERK and ELK-1 were bound to glutathione beads, and used in pull-down for S-thioredoxin tagged NPDC-1 recombinant protein and using thioredoxin (Trx) as a control. Bound proteins were eluted from beads, separated by SDS-PAGE, immunoblotted onto nitrocellulose membranes and membranes were probed with α-thioredoxin antisera. The NPDC-1 specific band is indicated. In panel B, recombinant NPDC-1 is used in pull-down from HEK 293 cell extract. HEK 293 cell lysate (1mg) was incubated with recombinant hNPDC-1 bound to S-protein beads. The bound proteins were eluted from beads, separated by SDS-PAGE, immunoblotted onto nitrocellulose membranes and membranes were probed for ERK. The ERK specific band (42,44kDa) is indicated.

* This is an accurate representation of an experiment repeated at least three times

SUMMARY

Some inroads have been made in deciphering NPDC-1 and its role in cellular proliferation and differentiation, but a lot of questions remain. NPDC-1 has been shown to interact with cell cycle regulatory proteins such as E2F-1, cyclins and cdks. It has also been shown to impact retinoid signaling, an established player in development. NPDC-1 is also under heavy regulation in the cell and its levels never build up in proliferating cells, consistent with its higher expression in differentiated tissues and cells.

To examine the role of NPDC-1 in development and differentiation we sought a model system to carry out some *in vivo* studies. Axolotl is a well established, well studied model for development and differentiation. Thyroid hormone is used to induce metamorphosis of juvenile salamanders to adult. Retinoic acid is able to affect patterning in developing and regenerating limbs, and therefore impacts cellular differentiation. We therefore established a collaboration with Dr. Randal Voss in the Biology department to characterize NPDC-1 in axolotl and to examine its role in axolotl retinoic acid and thyroid hormone induced differentiation and development.

My hypothesis for the studies presented in this dissertation is that NPDC-1, through its ability to regulate the transcription events mediated by the intracellular receptors for retinoic acid and thyroid hormone, is able to impact the developmental and morphogenic pathways in axolotl. In studies presented in this dissertation I assemble the axolotl signaling components for retinoid and thyroid hormones and I investigate the impact that an axolotl NPDC-1 homolog has on gene expression events induced by these hormones.

SECTION III: ONGOING COLLABORATIONS FOR THE STUDY OF NPDC-1 IN THE AXOLOTL *AMBYSTOMA MEXICANUM*

Chapter 6: Introduction

In our hands the NPDC-1 protein has proven to be both interesting and challenging. NPDC-1 is a novel gene identified for its involvement in the control of neural cell proliferation and differentiation (89), and its high degree of conservation suggests it plays some universally critical function in the cells in which it is expressed. This is further supported by the accumulating data that demonstrate NPDC-1 activities are linked to cellular proliferation and differentiation. Our studies with NPDC-1 further link it to retinoid signaling, an event that has been linked to embryonic development, cellular differentiation and a variety of cancers. In spite of these data, none of the investigators working in the NPDC-1 field have been able to define its biochemical mechanism of activity nor its direct association with disease pathogenesis.

In an effort to biochemically define the *in vivo* role of NPDC-1 in development and differentiation, we instituted a collaboration with Dr. Randal Voss' laboratory here at the University of Kentucky. Dr Voss' lab studies the molecular evolution of the axolotl. The axolotl system, due to their unique regenerative capabilities, has been classically used to study differentiation and development. Dr. Voss' lab breeds various species of axolotl and studies the molecular evolution of gene families in this organism. The availability of the organism, its unique tools for studying differentiation

and the Voss' lab expertise in cloning and characterizing axolotl genes fit well with our NPDC-1 needs.

The axolotl is an interesting model system for the study of developmental and differentiation processes, and these processes in axolotl can be directly linked to and mediated by members of the NR family. The early development of axolotl is three times slower than that of *Xenopus* at the same temperature (106, 107), allowing for the study of developmental processes. Dr Voss has been working with the axolotl, *Ambystoma mexicanum*, a juvenile salamander that does not undergo metamorphosis without exogenous thyroid hormone treatment and activation of the thyroid receptor. Natural populations of axolotl can be purely neotenic, transforming or polymorphic (i.e. some remain as larvae and some undergo metamorphosis (108)). Phylogenetical analyses have shown that metamorphic failure evolved several times during *Ambystoma* evolution (108). Salamanders, like all urodeles, have the ability to regenerate limbs after amputation, and retinoic acid respecifies positional memory, this event resulting in the formation of extra limb elements (109-112).

The axolotl embryo development has been divided into 44 stages based on external morphology and on features visible in dissected embryos (113). The staging series originally published by Bordzilovskaya and Dettlaff (114) is also based on external morphology with stage timing beginning with the first appearance of the initial cleavage furrow on the animal pole (113). The early blastula stage is at stage 8, approximately 16h after first cleavage and early gastrula forms at stage 10 when the first signs of the dorsal blastopore are visible which occurs at 26h (113, 114). Early neurula stage where the boundaries of the neural plate are outlined, occurs at stage 13 at roughly 55-56.5h (113, 114). By stage 21 (72h), the neural folds are completely fused and at stage 22 (73h) the gill region and the pronephros are more distinct with

the tailbud being slightly outlined and five to six pairs of somites present (113, 114). The ventral side of the embryo becomes more concave as the downward curvature of the head increases (113, 114). At stage 23, approximately 74h post first cleavage, the primordium of the ear is outlined and the boundary between the hyoid arch and the first branchial arch is outlined (113, 114). By stage 27, 86h post first cleavage, the second branchial furrow appears and 12 somites are now present (113, 114). The body of the embryo begins to straighten, the tailbud enlarges and sixteen pairs of somites are present by stage 29, after 97h (113, 114). At stage 38, 178h post first cleavage, filament sprouts appear as two nodules on each gill and the limb buds are slightly outlined (113, 114). By stage 41, (265h) the gills continue to elongate, the number and length of filaments increases and the mouth is outlined (113, 114). Hatching begins at stage 41 as well (113, 114). By stage 43, which occurs 342h post first cleavage, the breaking through the mouth occurs or the mouth is already open (113, 114). A figure with pictures from the different stages of axolotl embryos is presented in figure 24 with pictures taken from Bordzilovskaya *et al* (114). About 4 weeks post fertilization, a 25mm axolotl has developed forelimbs and the hindlimb buds are detectable as bean-shaped structures on either side of the posterior intestine (115). It takes and additional three weeks for the hindlimbs to elongate posteriorly, splay laterally, develop digits and differentiate (115).

Cumulatively these data suggested the axolotl might be a useful animal model system for looking at the *in vivo* role of NPDC-1 in development and differentiation. Unfortunately the molecular genetics of the salamander system are not as developed as organisms like zebrafish and mice. Therefore prerequisite to establishing an axolotl model system for studying NPDC-1's role in NR-mediated development and
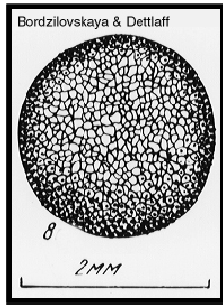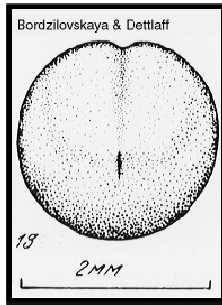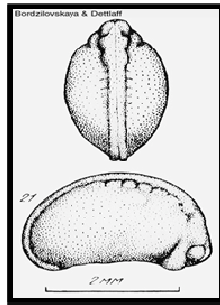
Stage 8   Stage 13   Stage 21   Stage 22   Stage 23
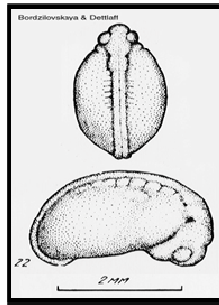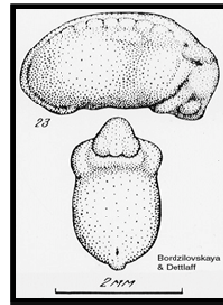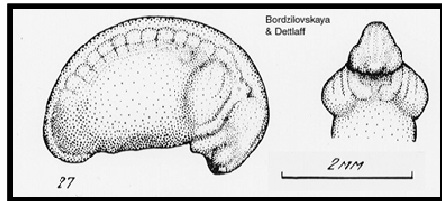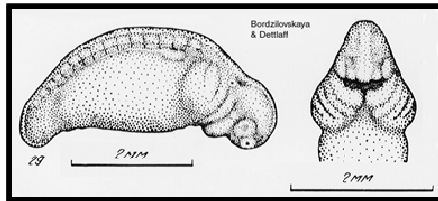
Stage 27   Stage 29
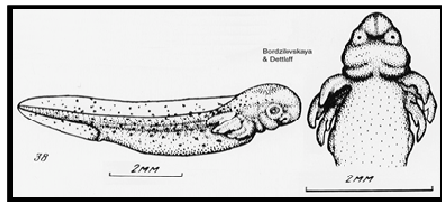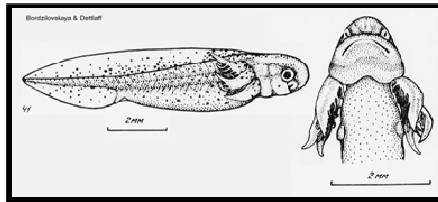
Stage 38   Stage 41
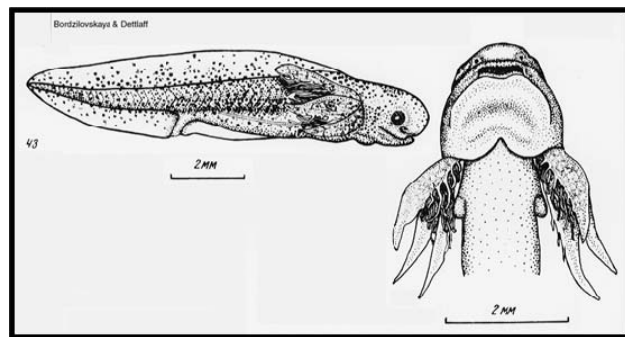
Stage 43

Figure 24: Developmental staging in axolotl embryos. Timing begins with the first appearance of the initial cleavage furrow on the animal pole. Stage 8 is the early blastula stage. Stage 13 is the early neurula stage I, where the blastopore is is narrow vertical slit, the boundaries of the neural plate are outlined, but neural folds are not yet raised above the surface of the embryo. By stage 21, the neural folds are completely fused and the posterior boundary of the gill region is more distinct. By stage 22, the gill region and pronephros are more distinct and the taolbud is very slightly outlined and 5-6 somites are present. The ventral side of the embryo becomes more concave. The primordium of the ear is outlined as a shallow depression in the region above the future hyoid arch at stage 23. In the dorsal region of the gill swelling, the hyobranchial furrow appears, outlining the boundary between the hyoid arch and the first branchial arch. At stage 27, the second branchial furrow appears faintly in the dorsal part of the gill swelling and 12 pairs of somites are present. At stage 29 the body of the embryo begins to straingthen, the tailbud enlarges and 16 pairs of somites are present. Flament sprouts appear as two nodules on each gill at stage 38. At stage 41, the gills continue to elongate, the number of filaments increases and they become longer. The forelimb buds are still small and hatching begins. At stage 43, the breaking through of the mouth occurs or the mouth is already open.

Figures from: Bordzilovskaya and Dettlaff, Developmental Biology of the axolotl, Oxford University Press, NY

differentiation, was the arduous task of both cloning and characterizing the axolotl NPDC-1 and nuclear receptor homologs. Dr Voss' lab was extremely vital to the initial cloning and characterization of these components, and in chapter 7 I present these early collaborative studies that were undertaken to identify the axolotl NPDC-1. In section IV of this dissertation I describe the molecular characterization of axolotl RARγ while in section V, I describe similar characterization information for axolotl TRα and TRβ. Throughout these sections I also investigate the relationships between axolotl NPDC-1 and signaling events mediated through these axolotl nuclear receptors.

Chapter 7: Results/Discussion

NPDC-1 was initially cloned as a mouse gene and characterized as a protein involved in neuronal cell proliferation and differentiation control (89). Later the human (90) and rat (95) homologs were identified and cloned, demonstrating NPDC-1 is conserved across species. To determine whether NPDC-1 is conserved in axolotl James Monaghan in Dr Randal Voss' lab identified an axolotl EST cDNA clone. This clone demonstrated high sequence homology to the mouse NPDC-1 gene, and using 5' RACE technology on total RNA isolated from adult axolotl brain, he identified a full length coding sequence for axolotl NPDC-1 (aNPDC-1). The aNPDC-1 amino acid sequence demonstrated 39.3% identity and 49.1% similarity with that of the human sequence (Figure 25). When the amino acid sequence for aNPDC-1 was aligned with human and rodent sequences, several motifs were observed to be highly conserved (Figure 25). These included the LXXLL motif, the PEST sequence as well as the six cysteines of the helix-loop-helix motif.

Examination of aNPDC-1 mRNA expression in various adult axolotl tissues was done by semi-quantitative RT-PCR using elongation factor 1 alpha (EF-1α) as a control. Axolotl NPDC-1 mRNA was found to be expressed in heart, brain, eye, skin, and gill and to a lesser extent in spinal cord, lung, muscle and liver (Figure 26). Minimal aNPDC-1 mRNA was detected in the spleen. Multiple tissue distribution of aNPDC-1 mRNA in axolotl agrees with previous published results for rat and human NPDC-1 mRNA (90, 95).

Previous data showed NPDC-1 expression in mouse to vary with development and highest levels to be detected in adulthood (91). To examine this in axolotl a semi-quantitative RT-PCR analysis was performed to look at aNPDC-1 expression at

```
human     MATPLPPPSPRHLRLLRLLLSGLVLGAALRGAAAGHPDVAACPGSLDCALKRRARCPPGA
mouse     MATPVPPPSPRHLRLLRLLLSGLILGAALNGATARRPDATTCPGSLDCALKRRAKCPPGA
rat       MATPVPPPSPRHLRLLRLLLSGLILGAALNGATARRPDAPTCPGSLDCALKRRAKCPPGA
axolotl   ---MEPPRATVGARLLLLLCGMLMLSSAAQTGDR------CPRKLDCTLLKRHYCPPGS
               ** :.    *** **  . *:*.:*  . .         **  .***:*  :*  ****:

human     HACGPCLQPFQEDQQGLCVPRMRRPPGGGRPQPRLEDEIDFLAQELARKE--SGHSTPPL
mouse     HACGPCLQSFQEDQRGFCVPRKHLSSGEGLPQPRLEEEIDSLAQELALKEKEAGHSRLTA
rat       HACGPCLQSFQEDQRGVCVPRKHQSSGEGLPQPRLEEEIDSLARELALKEKEAGHPRLTA
axolotl   DACGPCLPNHVENNDGKCVESATSQKGK-TSDRLPDEDIDFYASLLSPGK---AHQLQAV
          .****** . *:: * ** *  .:  ::  ::*** * *:  :  .*  .

human     PKD--RQR--LPEPATLGFSARGQGLELGLPSTPGTPTPTPHTSMGSPVSSDPVHMSPLE
mouse     QPLLEAAQKLLEPAATLGFSQWGQRLEPGLPSTHGTSSPIPHTSLSSRASSGPVQMSPLE
rat       QPLPEASQKLLEPAATLGFSQWGQQLEPGLPSTHGTSSPTPHTSLSARASSGPVQMSPLE
axolotl   MEST---------AAPE---------ESSEKNRAGNVTPKPLPPTKGGVKSSPVPK---E
               .*.             *  .   *. :* *  ..  ...*.**      *

human     PRGGQGDGLALVLILAFCVAGAAALSVASLCWCRLHREIRLTQKADY-ATAKAPGSPAAP
mouse     PQGRHGNGLTLVLILAFCLASSAALAVAALCWCRLQREIRLTQKADYAATAKGPTSPSTP
rat       PQGRG-NGLALVLILAFCLASTAALAVAALCWCRLQREIRLTQKADYTATAKGPTSPTTP
axolotl   ---KMSDALLLGLVVTCTAAGLTALIVATVCWCRLQKEMKLAEKADY-SSNKGASPPPYD
                :.* * *:::   *. :** **::*****::*::*::**** :: *.. .*.

human     RISPGDQRLAQSAEMYHYQHQRQQMLCLERHKEPPKELDTASSDEENEDGDFTVYECPGL
mouse     RISPGDQRLAHSAEMYHYQHQRQQMLCLERHKEPPKELESASSDEENEDGDFTVYECPGL
rat       RISPGDERLAHSAEMYHYQHQRQQMLCLERHKDPPKELESASSDEENEDGDFTVYECPGL
axolotl   RSSPVDKKLAQSAQMYHYQHQKQQMLSMENKDEPKLPDSNTTSDEENEDGDFTVYECPGL
          * ** *::**:**:*********:****.:*.:.:*    ..::******************

human     APTGEMEVRNPLFDHAALSAPLPAPSSPPALP
mouse     APTGEMEVRNPLFDHSTLSAPVPGPHSLPPLQ
rat       APTGEMEVRNPLFDHSTLSAPVPGPHSSPPLQ
axolotl   APTGEMEVKNPLFDDSSLHHPNSKPQ------
          ********:*****.::*   *  .  *
```

Figure 25: NPDC-1 protein alignment. Shown here is a sequence alignment between human, mouse, rat and axolotl NPDC-1. Conserved amino acids are denoted by *. Conserved across species are the LXXLL motif shown in pink, the HLH motif (the conserved cysteines comprising the HLH motif are shown in red), the PEST region which is shown in blue and the MAPK domain is shown in green.
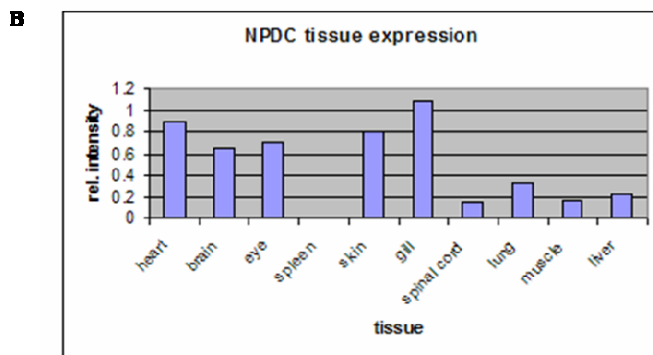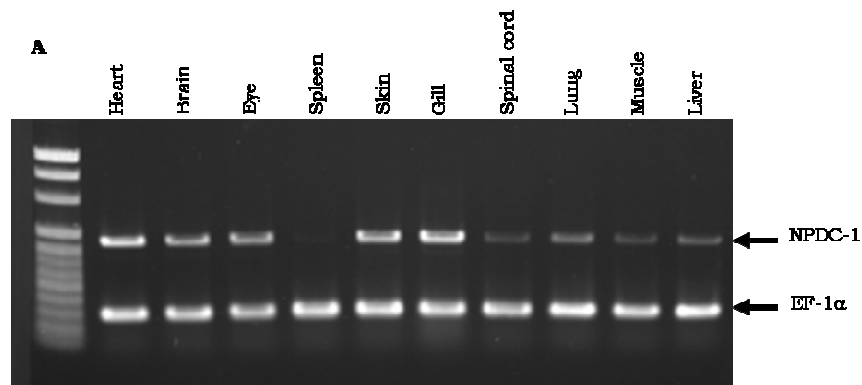
Figure 26: aNPDC-1 tissue distribution in axolotl. **A.** Semi-quantitative RT-PCR to examine tissue distribution of aNPDC-1 in non-metamorphosed axolotl tissues. mRNA from the listed tissues was isolated, reverse transcribed into cDNA and analysed by PCR using aNPDC-1 specific primers (NPDC-1). As a control, RT-PCR analysis of axolotl elongation factor 1α (EF-1α) was assayed using the same cDNAs. PCR products were run on 1.2% agarose gels and analysed on a Kodak Gel logic gel imaging system. **B**. Using Gel Logic software (Kodak), the relative intensity of the aNPDC-1 specific PCR bands from A were normalized to the EF-1α bands for the various tissues.

different stages of axolotl development. As shown in Figure 27 expression of aNPDC-1 mRNA peaks at hatching and remains at constant levels post-hatching.

In summary, NPDC-1 appears to be a conserved protein across species suggesting its function is also conserved and important for physiological processes. The expression of aNPDC-1 was observed to occur in multiple tissues and to fluctuate during axolotl embryogenesis and early larval development. The cloning of the axolotl homolog of NPDC-1 was an essential component for the subsequent chapters presented in this dissertation and I wish to thank the Voss lab for helping me with these preliminary data and for tissues and other assistance provided in the dissertation work presented in Sections IV and V.
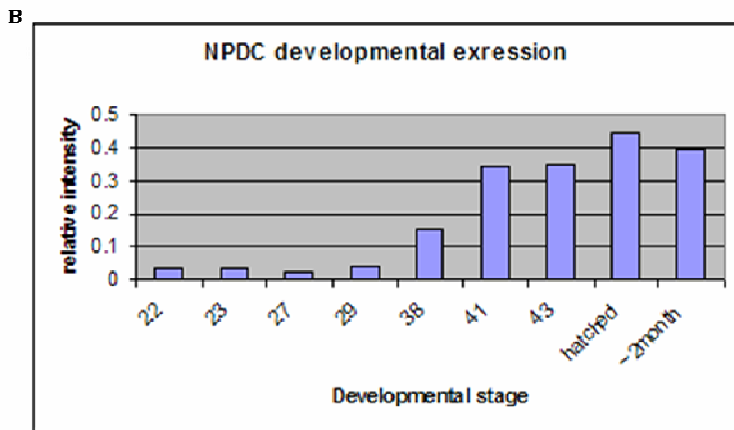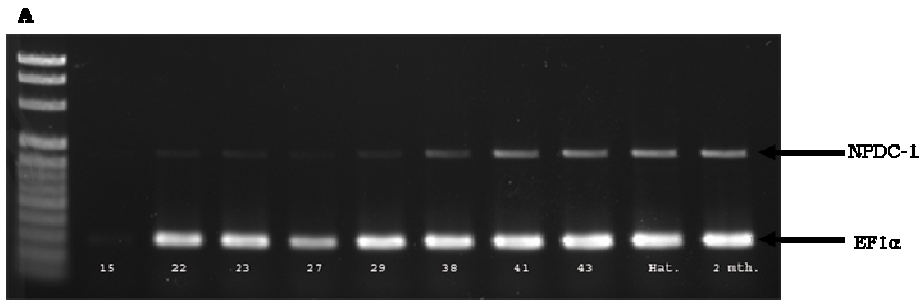
Figure 27: Developmental Expression of aNPDC-1. A. Semi-quantitative RT-PCR to examine the expression of aNPDC-1 during embryogenesis in axolotl. mRNA from the listed embryo stages was isolated, reverse transcribed into cDNA and analyzed by PCR using aNPDC-1 specific primers (NPDC-1). As a control, RT-PCR analysis of axolotl elongation factor 1α (EF-1α) was assayed using the same cDNAs. PCR products were run on 1.2% agarose gels and analyzed on a Kodak Gel logic gel imaging system. **B**. Using Gel Logic software (Kodak), the relative intensity of the aNPDC-1 specific PCR bands from A were normalized to the EF-1α bands for the various embryo stages.

SECTION IV: CONVERGENCE OF RETINOIC ACID AND NPDC-1 SIGNALING PATHWAYS IN AXOLOTL

Chapter 8: Introduction

Retinoids are important signaling molecules for a variety of biological phenomena such as differentiation, proliferation and cell communication (106). The effects of RA are mediated through the retinoid receptors (RARs and RXRs). Retinoic acid is important for the morphogenesis of the nervous system and appears to re-specify positional values within the neural plate (106). RA is a morphogen for the CNS, heart, respiratory system, limbs, eyes, urogenital system and skin (106).

Retinoic-acid induced embryonic patterning has been studied in many organisms including *Xenopus*, axolotl and chicken. Treatment of *Xenopus laevis* embryos with retinoic acid at gastrula and early neurula stages results in the transformation of anterior neural tissue to a more posterior fate (106, 116). In the regenerating amphibian limb, retinoids induce proximodistal duplications giving rise to additional skeletal elements that are already present in the stump (117, 118). Maden (110) described the effects of retinoids on limb regeneration in the axolotl *Ambystoma Mexicanum.* In this study, it was shown that retinoids cause specific alterations in the proximo-distal pattern of regenerating axolotl limbs and suggested that retinoids are responsible for changing the positional information of limb cells. Finally, in chickens, retinoids induced the formation of two sets of digits in the developing chick wing bud (117, 119, 120).

In *Xenopus*, the intracellular receptors for retinoids, RARα and RARγ, are expressed during embryogenesis (106, 121). RARα1 is expressed in the oocyte and in the embryo until gastrulation, whereas RARα2 is expressed at the end of gastrulation in the neuroectoderm (106). RARγ2 expression, on the other hand, begins in the oocyte and peaks at the developmental period during which the anteroposterior axis of the embryo is determined, which is between early gastrulation and mid-neurulation (106).

RAR expression in axolotl embryogenesis has been limitedly studied. Using an axolotl neurula cDNA library, Wirtanen *et al* (106) isolated the axolotl RARγ2 and identified a splicing variant RARγ3. Examination of aRARγ2 expression in axolotl embryos using semi-quantitative RT-PCR showed that the expression of aRARγ2 was monophasic. The levels of aRARγ2 were low during segmentation before the mid-blastula transition (MBT) (106), increased rapidly during gastrulation and reached a maximum during neurulation (stage 16.5). The levels at neurulation were 20-fold higher than at segmentation. The expression diminished between stages 16.5 and 28, then again between stages 36 and 38 and RNA levels remained low until stage 42 (hatched larvae) (106). It appears that in amphibians (X. *laevis* and axolotl) the main RAR isoform expressed during early development is RARγ2.

Retinoids are able to impact patterning in both developing and regenerating limbs (122-125). Retinoid administration has the ability to modify positional information along the three cardinal axes: the proximodistal (PD), anteroposterior (AP) and the dorsoventral (DV) (125). Positional memory of blastema cells along these axes is modified in one direction, positional memory is proximalized in the PD axis (111, 124, 125), posteriorized in the AP axis (119, 120, 125) and ventralized in the DV axis

(125-127). Metabolic inhibitors that block RA synthesis such as disulfuram and citral, interfere with limb regeneration (125, 128).

During chick limb development RA may act by modifying pattern directly and/or inducing an additional zone of polarizing activity (ZPA), which in turn may provide the pattern modification stimulus (117). The ZPA is a region at the distal-posterior margin of the developing limb bud capable of inducing mirror-image digit duplications when grafted to the anterior margin of a host limb (129). Regenerating amphibian limbs lack a ZPA or anything corresponding to it, therefore a ZPA is not necessary for the formation of an RA gradient in regenerating or developing limbs (117). In axolotls, an RA gradient is observed in regenerating limbs but not in *Xenopus* (117). While *Xenopus* is capable of limb regeneration it is not able to pattern the regenerate appropriately (117). The establishment of an RA gradient seems to be essential for pattern formation in regenerating and developing limbs (117).

The expression of sonic hedgehog (shh), a gene first associated with segmentation patterning in Drosophila melanogaster (130), also is expressed within the ZPA. Shh is a mediator of anterior-posterior (AP) patterning within the developing limb (129, 130). In chick limbs shh expression correlates with the mapped domain of maximal ZPA activity at most developmental stages (129-131) and shh peptides alone can induce the same mirror-image duplications observed after ZPA grafting (129). Retinoic acid is capable of inducing digit duplications (120, 129, 132) and upregulates expression of shh on the developing chick wing and zebrafish fin (129, 130, 133). Expression of shh is responsible for the activity of the ZPA and for establishing the anterior-posterior axis of the developing vertebrate limbs (129, 134).

Urodele limbs are homologous to those of other vertebrates and share conserved mechanisms of development (129), but have certain unique features that set

them apart from other tetrapods. These unique features include a reversed sequence of digit formation (anterior to posterior rather than the reverse as in other tetrapods), absence of a region equivalent to ZPA, ability to regenerate limbs as adults and their multiple responses to treatment with RA causing patterning changes in all three limb axes (129). Torok *et al* (129) studied shh expression in development and regeneration in axolotl. Shh expression occurs later, and is downstream of, Hox-d11 expression both in development and regeneration. Shh expression is detectable by stage 37 in axolotl embryos in mesenchymal cells on the posterior side of the limb bud and its expression is downregulated by the three digit stage (129). In regenerating limbs, shh is not expressed until a medium bud blastema has formed (129). In regeneration, as in limb development, shh is not necessary for the initial establishment of the AP axis (129). Initially, the shh-expressing domain is detected at the stump-blastema junction and later more distally in association with the base of the forming autopod (129). Expression of shh domain in regenerating limbs is small compared to developing limbs, and is very transient (129). Expression of shh is downregulated when RA levels in the blastema are high and is re-expressed as the levels decline (129). A unifying hypothesis is that RA acts to establish a ZPA (129, 135, 136) through its ability to downregulate expression of shh.

In summary, retinoids are important for embryonic patterning, limb development and limb regeneration. Administration of exogenous retinoids leads to skeletal deformities in regenerating limbs of tetrapods. The urodele axolotl *Ambystoma mexicanum* is an ideal model system for the study of developmental patterns. Studies in axolotl have shown the establishment of an RA gradient in regenerating limbs that are directly linked to this regeneration process. In this section of the dissertation, I investigate a potential molecular mechanism for regulation of

axolotl retinoic acid signaling. These studies include cloning of the axolotl RARγ gene and the analysis of axolotl NPDC-1 expression on aRARγ DNA binding and aRARγ-mediated gene expression events.

Chapter 9: Experimental Procedures

<u>Chemicals, Enzymes and Antibodies</u>

Standard chemicals used throughout these studies, unless otherwise stated, were purchased from Sigma chemical or Fisher Scientific. Restriction enzymes, polymerases, ligases and kinases used, unless otherwise stated, were purchased from New England Biolabs. Benzonase nuclease was purchased from Novagen. Anti-thioredoxin antibody was purchased from Santa Cruz.

<u>NPDC-1 antibody</u>

The peptide sequence NH_2-EENEDGDFTVYECPGLAPT-COOH that is found in the PEST regions of NPDC-1 and observed to be conserved from axolotl to humans, was used as an immunogen to generate a rabbit polyclonal antibody directed against NPDC-1. The synthesis of the peptide, development of the antibody and affinity purification of the antibody was performed by Washington Biotechnology (Baltimore, MD).

<u>Plasmids and Constructs</u>

<u>aRARγ</u>: Using axolotl lung RNA as template, an RT-PCR was performed using primers Forward: 5'-AA<u>G CGG CCG C</u>GT ACG ACT GCA TGG AGG CC-3' and Reverse: 5'-AA<u>G GAT CCC</u> TAG AGC TCT TTG AAC CT-3' to amplify the aRARγ2 cDNA. To generate a FLAG-tagged mammalian expression construct, the amplified fragment was then subcloned into the *NotI/BamHI* sites of p3XFLAG-CMV-10 vector (Sigma). This construct was used in subsequent PCR reactions to obtain aRARγ2 with the appropriate overhangs for further subcloning. Using the primers Forward: 5'-AA<u>G</u>

GAT CCA TGT ACG ACT GCA TGG AGG CC-3' and Reverse: 5'-AAC TCG AGC TAG AGC TCT TTG GAA CT-3', aRARγ was subloned into the *BamHI/XhoI* sites of the mammalian expression vectors pcDNA3.1/Zeo/3HA (a gift from Dr. Andres at the University of Kentucky) and pcDNA3.1/Zeo (Invitrogen). Amplification with Forward: 5'-AAG GAT CCT ACG ACT GCA TGG AGG CC-3' and Reverse: 5'-AAC TCG AGC TAG AGC TCT TTG GAA CT-3' allowed the subcloning of aRARγ into the *BamHI/XhoI* sites of the bacterial protein expression vector pGEX-KG (Amersham), which contains a Glutathione tag. Amplification with Forward: 5'-AAG GAT CCA TGT ACG ACT GCA TGG AGG CC-3' and Reverse: 5'-AAC TCG AGC ACT AGA GCT CTT TGG AAC T-3' was performed for cloning into the *BamHI/XhoI* sites of the bacterial protein expression vector pET32a (Novagen) which expresses a His-thioredoxin tag fusion protein.

aNPDC-1: To create a bacterial aNPDC-1 expression construct, full length aNPDC-1 (pGEM aNPDC-1 a gift from R. Voss lab) was digested with *AvrII*, filled in with Klenow and then digested with *NotI*. This fragment was then subcloned into the *EcoRV/Not I* sites of the bacterial protein expression vector pET32c (Novagen) (pET32c-aNPDC-CDS). A bacterial expression helix-loop-helix deletion construct (pET32-aNPDC-ΔHLH) was constructed by digesting the full length pET-aNPDC with *Sma/NotI* and inserting the fragment into the bacterial protein expression vector pET32a digested with *EcoRV/NotI*. This deletes amino acids 1-97 and eliminates the transmembrane and helix loop helix domain of aNPDC-1 on the amino terminus. To generate the PEST deletion construct (deletion of amino acids 209-288), PCR amplification was used. Using primers Forward: 5'-AAA GGA TCC GAG CCT CCC AGG GCG ACC-3' and Reverse: 5'-AAG GAC TCG AGT TAT TAT TAT TGT GCC AAC TTC TTG TC-3' the ΔPEST fragment was amplified, isolated and cloned into the *BamHI/XhoI* sites of the bacterial protein expression vector pET32a. To generate pcDNA3.1-aNPDC-CDS, full length

aNPDC-1 was digested with *SacII* restriction enzyme, filled in with Klenow and digested with *NotI* and inserted into the *EcoRV/NotI* sites of the mammalian expression vector pcDNA3.1/Zeo. To generate pcDNA-aNPDCΔPEST, pET32a-aNPDC-CDS was digested with *BglII/EcoRI* and inserted in to the *BamHI/EcoRI* sites of pcDNA3.1/Zeo.

Other plasmids and constructs

In these experiments the following plasmids were used that were generated previously. The βRE-tk-luc plasmid was generated previously (in our lab) by subcloning a single copy of annealed synthetic oligonucleotides for βRE 5'-AGC TTA AGG GTT CAC CGA AAG TTC ACT CGC AT-3' and 5'-AGC TAT TGC GAG TGA ACT TTC GGT GAA CCC TTA-3' into the *HindIII/BamHI* sites of pBL-tk-luc vector (137). This plasmid contains an imperfect DR5 and was used for both the mammalian transcription assays and for generating a DR5 response element for electromobility gel shift assays. The β-galactosidase plasmid used in the mammalian transcription assay contains the β-galactosidase gene driven by a CMV promoter was purchased from Clontech.

RT-PCR

Axolotl tissue cDNA derived from multiple mRNA populations (a gift from Dr. Voss' lab), was subjected to PCR using the following aRARγ primers: forward: 5' CCC AGA CAA ATA TAC AGA AAC TGC-3' and reverse: 5' CCA TGC ACC ACA GGC CAT TTG AGA-3' as published by Wirtanen *et al* (106). cDNA template corresponding to 400ng total RNA was used per PCR reaction. PCRs were performed on a T-Gradient thermocycler PCR machine as described by the manufacturer (Biometra) using the following cycling conditions $94^0Cx3min$, 94^0Cx45s, 50^0Cx45s, 72^0Cx45s for 32 cycles, $72^0Cx\ 5min$. PCR products were resolved on a 1.2% agarose gel and detected using the Gel logic gel imaging system (Kodak).

Recombinant Protein Expression

Glutathione: To develop GST and GST-aRARγ fusion proteins, BL21-RP *Escherichia coli* cells were transformed with bacterial expression constructs in the pGEX-KG plasmid system. Transformants were plated on double selection media containing carbanecillin (50μg/ml) and chloramphenicol (34μg/μl) overnight at 37^0C. Isolated colonies were picked and used to grow a 2.0mL starter culture which was then used to inoculate 500mL of 2X-YT (16mg/ml Tryptone, 10mg/ml NaCl, 10mg/mL Yeast extract, pH 7.2-7.4) supplemented with carbanecillin and chloramphenicol. Cultures were grown to log phase at room temperature (OD_{600} of 0.6). Protein expression was induced for 6h by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (Gold BioTechnology, Inc) to a final concentration of 1mM. Bacterial cells were pelleted by centrifugation for 15 minutes at 6000rpm and resuspended in 5mL of 10mM Tris-HCl pH 7.5 containing 100mg of lysozyme (Sigma), followed by the addition of 20mL of Triple Detergent Lysis buffer [50mM Tris-HCl pH 8.0, 150mM NaCl, 0.02% sodium azide (v/v), 1% NP-40 (v/v), 0.5% sodium deoxycholate (v/v), 1μM PMSF and 1X protease inhibitor cocktail (Roche Applied Science) and Benzonase Nuclease (1μl/ml)] and incubated at room temperature with agitation (150rpm) for 30 minutes on an orbital floor shaker. The soluble fraction was clarified by ultracentrifugation for 1h at 50000rpm in a Ti70 rotor. GST fusion proteins were purified by Fast Performance Liquid Chromatography (FPLC) on a GSTrap Fast Flow column (GE Life Sciences). Eluates were desalted by FPLC on a G-50 desalting column into TBS-P (10mM Tris-HCl pH 8.0, 150mM NaCl, 10% glycerol (v/v), 1mM DTT) and the resulting proteins were aliquoted and stored at -70^0C until use. The protein concentration was determined using Bradford reagent (Advance, Cytoskeleton Inc).

<u>Thioredoxin:</u> To generate Thioredoxin and thioredoxin-tagged aNPDC-1, aNPDCΔPEST, aNPDCΔHLH and aRARγ, bacterial expression constructs in the pET32 (Novagen) system were transformed into BL21-RP *E. coli* cells. Transformants were plated on double selection media containing carbanecillin (50μg/ml) and chloramphenicol (34μg/μl) and incubated overnight at 37^0C. Isolated colonies were picked and used to grow a 2.0mL starter culture which was then used to inoculate 500mL of 2X-YT supplemented with carbanecillin and chloramphenicol. Cultures were grown at room temperature, to an OD_{600} of 0.6. Protein expression was induced for 6h by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1mM. The transformed bacterial cells were pelleted by centrifugation at 6000rpm for 15 minutes and resuspended in 5mL of 10mM Tris-HCl pH 7.5 containing 100mg of lysozyme (Sigma) followed by the addition of 20ml of Triple Detergent Lysis buffer and incubated at room temperature with agitation (150rpm) for 30 minutes on an orbital floor shaker. The soluble fraction was clarified by ultracentrifugation for 1h at 50000rpm in a Ti70 rotor. The clarified lysate was purified by FPLC on a HiTrap chelating HP column (Amersham Biosciences) preloaded with Nickel. Bound protein was washed with 10 column volumes of His wash (50mM NaH_2PO_4, 300mM NaCl, 20mM Imidazole pH 8.0) and eluted in His elution buffer (50mM NaH_2PO_4, 300mM NaCl, 500mM Imidazole pH8.0). Eluates were desalted by FPLC on a G-50 desalting column into TBS-P (10mM Tris-HCl pH 8.0, 150mM NaCl, 10% glycerol (v/v), 1mM DTT), and the resulting proteins were aliquoted and stored at -70^0C until use. The protein concentration was determined using Bradford reagent (Advance, Cytoskeleton Inc).

<u>Pulldown Assays</u>

Recombinant GST-aRARγ and GST-hRXRα (10μg/reaction) were prebound to glutathione sepharose beads (50% slurry in APB buffer) [APB: 50mM Tris-HCl ph 7.5, 150mM NaCl, 10% glycerol (v/v), 1% NP-40 (v/v)] by rotating for 1h at 4⁰C. To the GST prebound proteins, equimolar amounts of thioredoxin or thioredoxin-tagged aNPDC-1 proteins were added as described in results and figure legends. The proteins were allowed to interact by rotating for at least 1h at 4⁰C. The beads were recovered by centrifugation in a microcentrifuge and washed 5 times with 1ml APB. The bound proteins were analyzed by western blot analyses probing with antisera for thio-probe (Santa Cruz).

Western Blot analysis

Western blot analyses were performed as previously described (99). Briefly, proteins were separated by SDS-PAGE electrophoresis and electroblotted onto nitrocellulose membranes (Stratagene Inc). Blocking was performed in PC buffer (1% casein in 1X PBS) [PBS: 0.30g/L NaH_2PO_4 anhydrous, 1.15g/L Na_2HPO_4 anhydrous, 9g/L NaCl, pH 7.2-7.4] overnight. Primary antibody was diluted in PCT buffer (1% casein in 1X PBS, 0.01% Tween-20) and incubated for 1h. Blots were subsequently washed with TBST buffer (20mM Tris pH7.5, 137mM NaCl, 0.1% Tween-20), then incubated with an appropriate secondary antibody coupled to horseradish peroxidase, reacted with ECL reagents (Pierce) as described by the manufacturer and detected on X-ray film by autoradiography.

Electromobility Shift Assays

EMSAs were performed as described previously (95). Briefly, βRE-tk-luc construct, generated previously as described in plasmids and constructs section, was digested

with *HindIII/BamHI* to obtain a double stranded RARE response element (βRE) containing 5' overhangs. The overhangs were radioactively labeled with [α-^{32}P]-dCTP using a standard Klenow fill-in reaction (138). The radioactive response element was purified by centrifugation through a microspin G-25 column (Amersham). The radiolabeled response element was incubated in binding buffer [for 1X binding buffer: 5% glycerol, 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM DTT, 50mM NaCl, 10mM Tris pH 7.5, 0.25mg/mL poly (dIdC)-poly (dIdC)] with recombinant aRARγ, hRXRα, aNPDC-1, aNPDCΔPEST or aNPDCΔHLH in the presence of 1μM atRA as indicated. The response element/protein complexes were separated by electrophoresis on 5% non-denaturing polyacrylamide gels. The gels were dried and the results visualized by autoradiography on X-ray film.

Mammalian Transcription Assays

Cos7 cells were transfected using SuperFect reagent (Quiagen) using a modification of the manufacturer's protocol. Briefly, 5μg total DNA (1.25μg βRE-tk-luc, 0.25μg pcDNA, 0.5μg pcDNA-aNPDC-1, 0.25μg 3HA-pcDNA-aRARγ, 0.5 μg cmv-βgal and 2.5μg pUC) were combined with 150μl OptiMEM (Gibco) and vortexed briefly. To the OptiMEM-DNA mixture 20μL of SuperFect reagent was added and incubated at room temperature for 20 minutes. During this time, Cos7 cells were washed with 1XPBS once, and then 3ml of OptiMEM was added. To the OptiMEM-DNA mixture, 2mL of OptiMEM was added and after mixing by pipetting up and down, the mixture was added to the 10cm plates dropwise. After 4h incubation at 37 degrees, the media was removed and the cells were washed with 1XPBS 3 times. After trypsinizing, the cells were resuspended in 10mL DMEM containing 10% CAFBS (charcoal absorbed fetal calf serum) and 20μl cells/well were plated into a 96 well plate with and without 1μM

atRA. Cells were assayed in 6 wells per treatment. The cells were incubated for 24h in the presence of retinoic acid, and then luciferase and normalizing β-gal activities were determined as previously described (139). The highest and lowest values for each treatment were dropped. Activity was expressed as the average of luciferase response normalized to the average β-gal response/minute.

Proliferation Assay

HEK293 cells were transfected using a standard calcium phosphate precipitation protocol as previously described (140). Briefly, HEK293 cells at 70% confluence were transfected with 3HApcDNA-aRARγ, pcDNA-aNPDC-CDS or pcDNA-aNPDCΔPEST and βRE-tk-luc luciferase reporter plasmid. The total amount of plasmid per 10cm^2 plate transient transfection was brought up to 20μg by the addition of the appropriate amount of pUC-19 plasmid. The calcium phosphate/DNA mix was allowed to incubate with the cells for 6h at 37^{0}C and then cells were washed three times with 1X PBS, and fresh media (DMEM with 10% FBS) was added. Transfections were incubated overnight and the next morning (~12h later) the cells were trypsinized and counted. 50,000 cells were plated per well in a 24 well plate; six wells per transfection with three wells being treated with 1uM atRA and three wells serving as controls. Each well received 2μl of [^{3}H] thymidine as well at 120Ci/mmol. At 6h, 24h and 48h following treatment the cells were harvested and lysed. DNA was precipitated using 10% TCA and radioactivity was trapped by filtration through a vacuum filter apparatus (Hoefer Scientific Instruments) on glass fiber filter circles (Fisherbrand). Total radioactivity was measured using a scintillation counter.

Bradford Assay

Proteins were diluted 1:200 in 1X Bradford reagent (Advance, Cytoskeleton Inc) and the OD_{595} was measured using a BioRad microplate reader. The OD reading of each protein was adjusted to a blank and the concentration of each protein was calculated based on the dilution factor and the manufacturers' instructions.

Chapter 10: Results

Cloning of axolotl RARγ

Further studies into the biochemical mechanism of the putative retinoid receptor coregulatory protein NPDC-1 necessitated the identification of an animal model system in which the discrimination between differentiation and developmental effects was possible. The axolotl system appears to fit these needs and the availability of both breeding colonies and the laboratory of one of the leaders in molecular evolution of axolotls, made this system accessible. The axolotl is an interesting model system for the study of development and regeneration in view of the fact that urodeles are capable of regenerating perfect limbs following amputation (141-145). Furthermore, its early development is three times slower than that of *Xenopus* (106, 107) at the same temperature, thus favoring the identification and analysis of intermediate rather than terminal differentiation products (106). Finally, retinoids have been implicated in both axolotl embryogenesis and limb regeneration (141-145). The axolotl was therefore chosen as an animal model for studying NPDC-1 modulation of retinoid receptor signaling.

Having identified and cloned an axolotl NPDC-1 homolog, it next became vital that we generate clones for the axolotl RAR homolog. We were fortunate in this regard in that the axolotl RARγ gene had been previously identified and partially characterized by Wirtanen *et al* in 2000 (106). Using the published sequence, oligonucleotide primers were designed and used in an RT-PCR approach to isolate an aRARγ cDNA from axolotl lung mRNA. This cDNA was subcloned into the pcDNA and 3HApcDNA vectors for mammalian expression of the protein, and into pGEX-KG and pET32 for bacterial expression of the protein. Sequence analysis showed this aRARγ

to be identical to the previously published axolotl sequence, and comparison of the aRARγ cDNA with other RARγ sequences shows it to be well conserved between humans and rodents, with differences mostly confined in the amino terminus (Figure 28). As expected the DBD is the most conserved (98.5% conservation), but with the LBD also being fairly well conserved (89% conservation) (Figure 28). Now with the availability of an axolotl homolog of RAR it was possible to initiate molecular investigations into the mechanisms governing RA action in urodeles.

Tissue expression of aRARγ

While the embryogenic expression pattern of aRARγ has been published (106) the localization of RARγ in various tissues in axolotl has not been determined. To determine the pattern of expression of RARγ in axolotl, RT-PCR was used. Using previously published aRARγ-specific primers, PCR was performed on cDNA from various axolotl tissues (Figure 29). RARγ was observed to be expressed very highly in axolotl heart, highly in skin and eye and at low levels in lung, spinal cord, gill and liver. Very little if any expression was observed in muscle, spleen and brain.

Expression of aRARγ during late embryogenesis and early larva stage

Axolotl aRARγ has been previously shown to express early in development (106), to peak at neurulation (stage 16.5) and to diminish by stage 28. In my studies, the first embryonic stage examined for aRARγ expression was stage 27, but as opposed to these earlier studies that were terminated at hatching, my analyses continued through 2 months post-hatching. As shown in Figure 30, aRARγ mRNA levels decreased between stage 43 and hatching. The aRARγ mRNA levels are observed to increase 2 months post-hatching back to levels similar to pre-hatching.

```
human      MATNKERLFAAGALGPGSGYPGAGFPFAFPGALRGSPPFEMLSPSFRGLGQPDLPKEMAS
mouse      MATNKERLFAPGALGPGSGYPGAGFPFAFPGALRGSPPFEMLSPSFRGLGQPDLPKEMAS
xenopus    ----MYDCMEAFPLMPRPLYDMSPQGPCMLRKAG---------CFGGLDPFGWMQSHSM
axolotl    ----MYDCMEAFMLAPHPLYDVTNPGACMLRKARLSP-------CFGGLDPFGWPQPASL
               :.  * *. * :    .:               .* **.  .  :  :

human      LSVETQSTSSEEMVPSSPSPPPPPRVYKPCFVCNDKSSGYHYGVSSCEGCKGFFRRSIQK
mouse      LSVETQSTSSEEMVPSSPSPPPPPRVYKPCFVCNDKSSGYHYGVSSCEGCKGFFRRSIQK
xenopus    QSVETQSTSSEEMVPSSPSPPPPPRVYKPCFVCNDKSSGYHYGVSSCEGCKGFFRRSIQK
axolotl    QSVETQSTSSEEMVPSSPSPPPPPRVYKPCFVCNDKSSGYHYGVSSCEGCKGFFRRSIQK
            ************************************************************

human      NMVYTCHRDKNCIINKVTRNRCQYCRLQKCFEVGMSKEAVRNDRNKKKKEVKEEGSP-DS
mouse      NMVYTCHRDKNCIINKVTRNRCQYCRLQKCFEVGMSKEAVRNDRNKKKKEVKEEGSP-DS
xenopus    NMVYTCHRDKNCQINKVTRNRCQFCRLQKCFQVGMSKEAVRNDRNKKKKEIKEEVVLPDS
axolotl    NMVYTCHRDKNCQINKVTRNRCQYCRLQKCFEVGMSKEAVRNDRNKKKKEIKEEVVT-DS
            ************ **********:*******:******************:***    **

human      YELSPQLEELITKVSKAHQETFPSLCQLGKYTTNSSADHRVQLDLGLWDKFSELATKCII
mouse      YELSPQLEELITKVSKAHQETFPSLCQLGKYTTNSSADHRVQLDLGLWDKFSELATKCII
xenopus    YEMPPEMEELIQKVSKAHQETFPSLCQLGKYTTNSSADQRVQLDLGLWDKFSELSTKCII
axolotl    YEMPPEMEALIAKVSKAHQETFPSLCQLGKYTTNSSADHRVQLDLGLWDKFSELATKCII
            **:.*::* **  ****************************:***************:*****

human      KIVEFAKRLPGFTGLSIADQITLLKAACLDILMLRICTRYTPEQDTMTFSDGLTLNRTQM
mouse      KIVEFAKRLPGFTGLSIADQITLLKAACLDILMLRICTRYTPEQDTMTFSDGLTLNRTQM
xenopus    KIVEFAKRLPGFTTLTIADQITLLKSACLDILMLRICTRYTPEQDTMTFSDGLTLNRTQM
axolotl    KIVEFAKRLPGFATLTIADQITLLKAACLDILMLRICTRYTPEQDTMTFSDGLTLNRTQM
            ************: *:***********:*********************************

human      HNAGFGPLTDLVFAFAGQLLPLEMDDTETGLLSAICLICGDRMDLEEPEKVDKLQEPLLE
mouse      HNAGFGPLTDLVFAFAGQLLPLEMDDTETGLLSAICLICGDRMDLEEPEKVDKLQEPLLE
xenopus    HNAGFGPLTDLVFSFADQLLPLEMDDTETGLLSAICLICGDRMDLEEPEKVEKLQEPLLE
axolotl    HNAGFGPLTDLVFAFAEQLLPLEMDDTETGLLSAICLICGDRMDLEEPEKVDKLQEPLLE
            *************:** ********************************:*********

human      ALRLYARRRPSQPYMFPRMLMKITDLRGISTKGAERAITLKMEIPGPMPPLIREMLENP
mouse      ALRLYARRRDPAKPYMFPRMLMKITDLRGISTKGAERAITLKMEIPGPMPPLIREMLENP
xenopus    GLKFYARRRPDKPYMFPRMLMKITDLRGISTKGAERAITLKLEIPGPMPPLIREMLENP
axolotl    ALKIYARRRPNKPYMFPRMLMKITDLRGISTKGAERAITLKMEIPGPMPPLIREMLENP
            .*::***** *  :*****************************:*****************

human      EMFEDDSSQPGPHPNASSEDEVPGGQGKGGLKSPA----
mouse      EMFEDDSSKPGPHPKASSEDEAPGGQGKRGQSPQPDQGP
xenopus    EAFEDGAATPKPSERSSSESSNGSPTGEDSSGSKTP---
axolotl    EAFEDDALSSPKAEEKPIKVEEIPAEKPSSKEL------
            * ***.:  .   . . :  .        .
```


Conservation of RARγ domains between
human and salamander

Figure 28: Sequence alignment of RARγ.

RARγ appears to be largely conserved

between higher and lower vertebrates.

Conserved amino acids are deonoted by *.

Figure 29: aRARγ tissue distribution in axolotl. A. Semi-quantitative RT-PCR to examine tissue distribution of aRARγ in non-metamorphosed axolotl tissues. mRNA from the listed tissues was isolated, reverse transcribed into cDNA and analysed by PCR using aRARγ specific primers (aRARγ). As a control, RT-PCR analysis of axolotl elongation factor 1α (EF-1α) was assayed using the same cDNAs. PCR products were run on 1.2% agarose gels and analysed on a Kodak Gel logic gel imaging system. **B**. Using Gel Logic software (Kodak), the relative intensity of the aRARγ specific PCR bands from A were normalized to the EF-1α bands for the various tissues.

Figure 30: Developmental Expression of aRARγ. A. Semi-quantitative RT-PCR to examine the expression of aRARγ during embryogenesis in axolotl. mRNA from the listed embryo stages was isolated, reverse transcribed into cDNA and analyzed by PCR using aRARγ specific primers (aRARγ). As a control, RT-PCR analysis of axolotl elongation factor 1α (EF-1α) was assayed using the same cDNAs. PCR products were run on 1.2% agarose gels and analyzed on a Kodak Gel logic gel imaging system. **B**. Using Gel Logic software (Kodak), the relative intensity of the aRARγ specific PCR bands from A were normalized to the EF-1α bands for the various embryo stages.

Localization of domain of interaction between aNPDC-1 and aRARγ

Previous studies had shown that hNPDC-1 complexes with hRARβ and hRXRα (95). The first question addressed in my studies was whether this interaction between NPDC-1 and RAR also occurs in the axolotl, and if so, if the region of interaction could be identified. To investigate this interaction 10μg of GST-aRARγ and/or GST-hRXRα was bound to glutathione sepharose beads and allowed to interact with equimolar amounts of thioredoxin or thioredoxin-tagged aNPDC-1 proteins. Bead bound proteins were resolved by SDS-PAGE and immunoblotted using a Thio-probe polyclonal antibody (Santa Cruz). As shown in Figure 31B, full length aNPDC-1 (aNPDC-CDS) and aNPDC-1 with its PEST domain deleted (aNPDCΔPEST) were able to complex with aRARγ, but not the aNPDC-1 protein with its helix-loop-helix domain deleted (aNPDCΔHLH). These data suggest that the domain of interaction between aRARγ and aNPDC-1 is at the amino terminus within amino acids 1-97. The domain of interaction between aNPDC and hRXRα also lies within the amino terminus (Figure 31C). These findings are consistent with what was observed with hNPDC-1 and hRXRα (95). As shown in Figure 31D, the differences in binding observed between the different aNPDC-1 constructs, and aRARγ, and hRXRα are not due to unequal loading, but rather are due to the region deleted being critical for interaction between the retinoid receptor and aNPDC-1. These data would suggest that the amino terminus of aNPDC-1 is important for interaction with nuclear receptors. Within this region are found the LXXLL motif, the signal peptide, the HLH motif and a coiled-coil domain (95). Further studies are necessary to more definitely establish which of these domains might be responsible for NR binding.

A.

Recombinant aRARγ ➡️

pGEX aRARγ pET aRARγ

GST Trx

B.

Trx aNPDC-CDS aNPDCΔPEST aNPDCΔHLH

C.

Trxn aNPDC-CDS aNPDC-ΔPEST aNPDC-ΔHLH

D.

Thioredoxin aNPDC-CDS aNPDC-ΔPEST aNPDC-ΔHLH
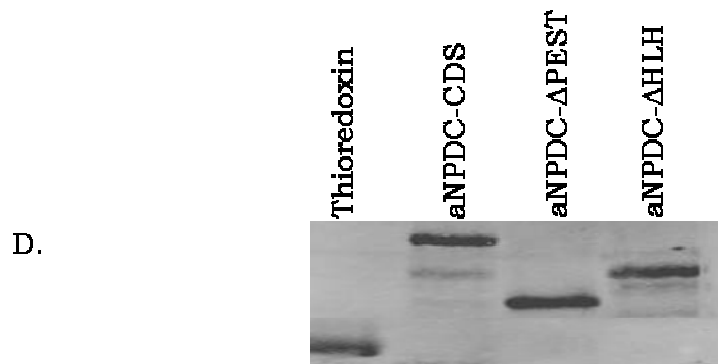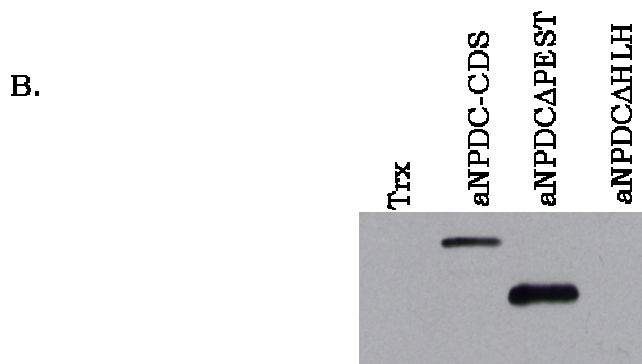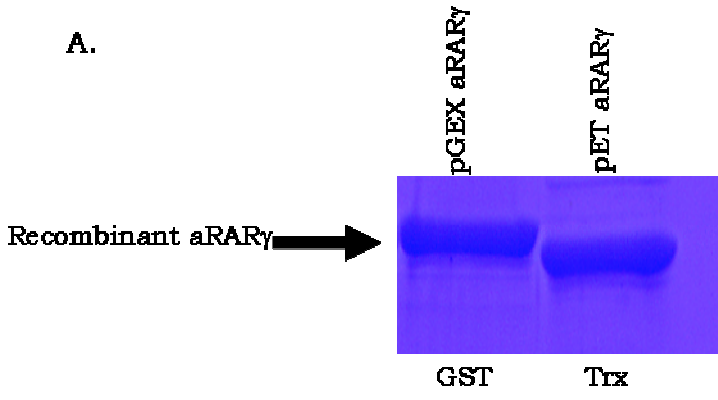
Figure 31: Direct *in vitro* interaction between aRARγ and aNPDC-1. In panel A, recombinant aRARγ protein is shown. Protein tagged with either Glutathione (GST) or Thioredoxin (Trx) was generated as described in materials and methods. 10μg of purified recombinant protein was resolved on a 10% SDS-PAGE gel and stained with Coomassie Blue. In panels B and C, 10μg of GST-aRARγ (B) or 10μg of hRXRα (C) were bound to glutathione beads and allowed to react with equimolar amounts of thioredoxin (Trx), aNPDC-1, aNPDCΔPEST and aNPDCΔHLH. Bead bound proteins were resolved on a 10% SDS-PAGE gel, and immunoblottled on nitrocellulose membranes. The membranes were then blotted with antisera for Thio probe. In panel D, equimolar amounts of thioredoxin and thioredoxin-tagged proteins were bound to S-protein beads and resolved on a 10% SDS-PAGE gel and immunoblotted for thioredoxin using anti-thioredoxin antisera.

Analysis of the impact aNPDC-1 has on aRARγ DNA binding

Initial observations demonstrated that hNPDC-1 impacted RAR/RXR heterodimer DNA binding, but did not shift the retinoid receptor complex (95). In the studies presented here, I investigated whether a similar result is observed with the axolotl RARγ and NPDC-1. A βRE oligonucleotide was obtained through the digestion of the βRE-tk-luc plasmid with *HindIII/BamHI* enzymes. The oligonucleotide was radioactively labeled with ^{32}P using a Klenow fill-in reaction. The radiolabeled βRE was then allowed to complex with recombinant proteins and the DNA/protein complexes were resolved on a non-denaturing polyacrylamide gel. Shown in Figure 32 is a representative EMSA of the observations made. Neither aRARγ, aNPDC-1 nor any of the aNPDC-1 deletions able to independently complex with the βRE DNA response element (lanes 2-5). The combination of hRXRα and aRARγ recombinant proteins was observed to form a shift complex (lane 6). Specificity of the complex was shown by competing it away with cold oligonucleotide (lane 7). Addition of aNPDC-1 made the aRARγ/hRXRα complex stronger in intensity and generated a second higher band (lane 8) which most likely represents an aNPDC-1 shifted complex. This is supported by the observation that anti-NPDC-1 antibody (raised against the conserved PEST region) eliminates the upper complex (lane 9) and diminishes the lower complex. It is worth noting that the commercial anti-thio probe antibody used in these studies is fairly dilute and not advertised as useful in supershift assays. Therefore the mild response observed here (lane 10) could be a reflection of this caveat. Although the above data support the hypothesis that axolotl NPDC-1, like rodent and human NPDC-1, can modulate retinoid receptor DNA binding, this generation of an additional aNPDC-1-specific higher mobility shift complex appears to be unique to axolotls. s. mRNA from the listed tissues waation that anti-NPDC-1 antibody also reduces the

	1	2	3	4	5	6	7	8	9	10
aRARγ (GST)					x	x	x	x	x	x
hRXRα (GST)						x	x	x	x	x
aNPDC (Trx)		x						x	x	x
aNPDCΔPEST (Trx)			x							
aNPDCΔHLH (Trx)				x						
Cold Competition							x			
α-NPDC antibody									x	
α-Thio probe antibody										x
1μm atRA	x	x	x	x	x	x	x	x	x	x

RAR/RXR/NPDC complex

RAR/RXR complex

Free Probe
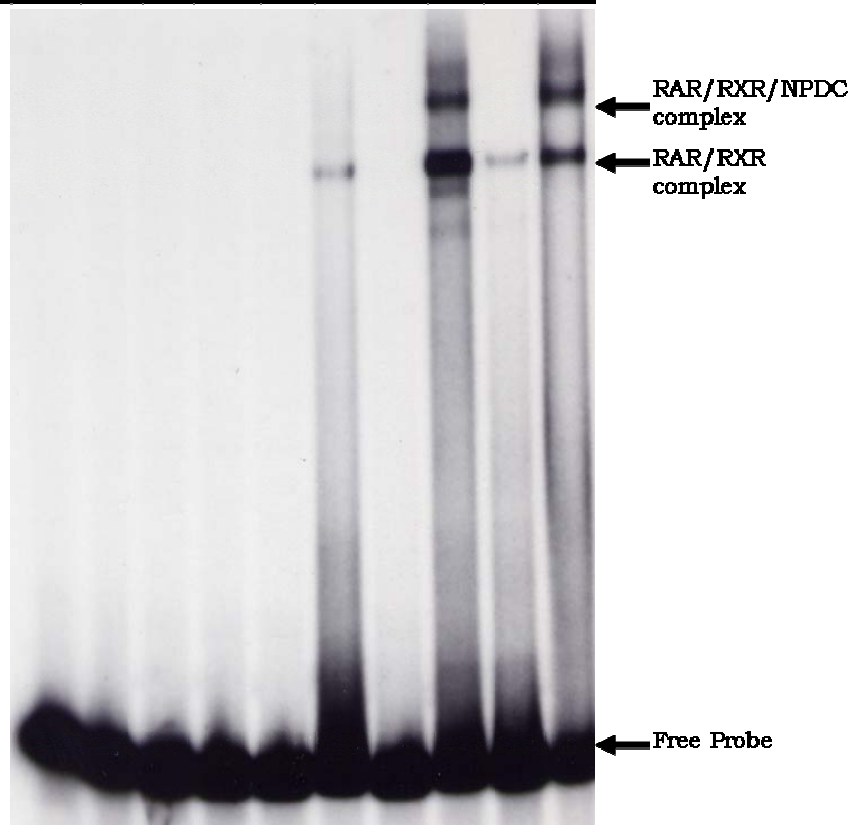
Figure 32: Electromobility Shift Assay with axolotl NPDC-1 and RARγ.

An oligonucleotide corresponding to βRE was radiolabelled with ^{32}P-dCTP with Klenow enzyme. The resultant DNA binding probe was incubated with recombinant hRXRα, aRARγ, aNPDC-1, aNPDCΔPEST and aNPDCΔHLH. Unlebelled (cold) oligo was used to show specificity of binding and antisera specific for NPDC-1 and thioredoxin were used to assay supershifts. In all reactions 1μM atRA was included. DNA:protein complexes were resolved on non-denaturing PAGE gels which were subsequently exposed to Kodak Xar-5 film.

*This is an accurate representation of an experiment performed at least three times.

intensity of the lower band in these complexes warrants further study and suggests that aNPDC-1 can both facilitate aRARγ/hRXRα DNA binding as well as form novel aRARγ/hRXRα/aNPDC-1 complexes.

Analysis of the impact aNPDC-1 has on transcription events mediated by aRARγ

The ability of rat and human NPDC-1 to impact retinoid receptor mediated transcription has been previously reported (95). To investigate whether aNPDC-1 can likewise impact aRARγ mediated transcription, *in vitro* transcription analyses were performed. Using a lipid based transfection method, Cos7 cells were transfected with mammalian expression constructs for aRARγ and aNPDC-1, a βRE luciferase reporter plasmid and a β-galactosidase expression plasmid (for normalizing transfection efficiency). Transfected cells were stimulated with 1μM atRA for 24h. After 24h the cells were lysed and assayed for luciferase and β-galactosidase activity. In Figure 33, the average luciferase values normalized to their respective β-gal activities are shown for the various transfection scenarios. As seen here, and as previously observed with hNPDC-1, aNPDC-1 repressed aRARγ-mediated transcription. Transcription repression was observed to be approximately 30%. This value is somewhat lower than that reported for rat and human receptors (90%), but it must also be noted that these comparisons are difficult to make due to the use of the different host cell systems and the different transfection technologies employed (95).

Proliferation Assay

A hallmark of rodent NPDC-1 was its observed ability to inhibit cell proliferation (89). To evaluate this with respect to aNPDC-1, I investigated the effects over-expression of aNPDC-1 and aNPDC-1ΔPEST had on cell proliferation (Figure 34). Using

Impact of axolotl NPDC-1 on aRARγ-mediated transcription
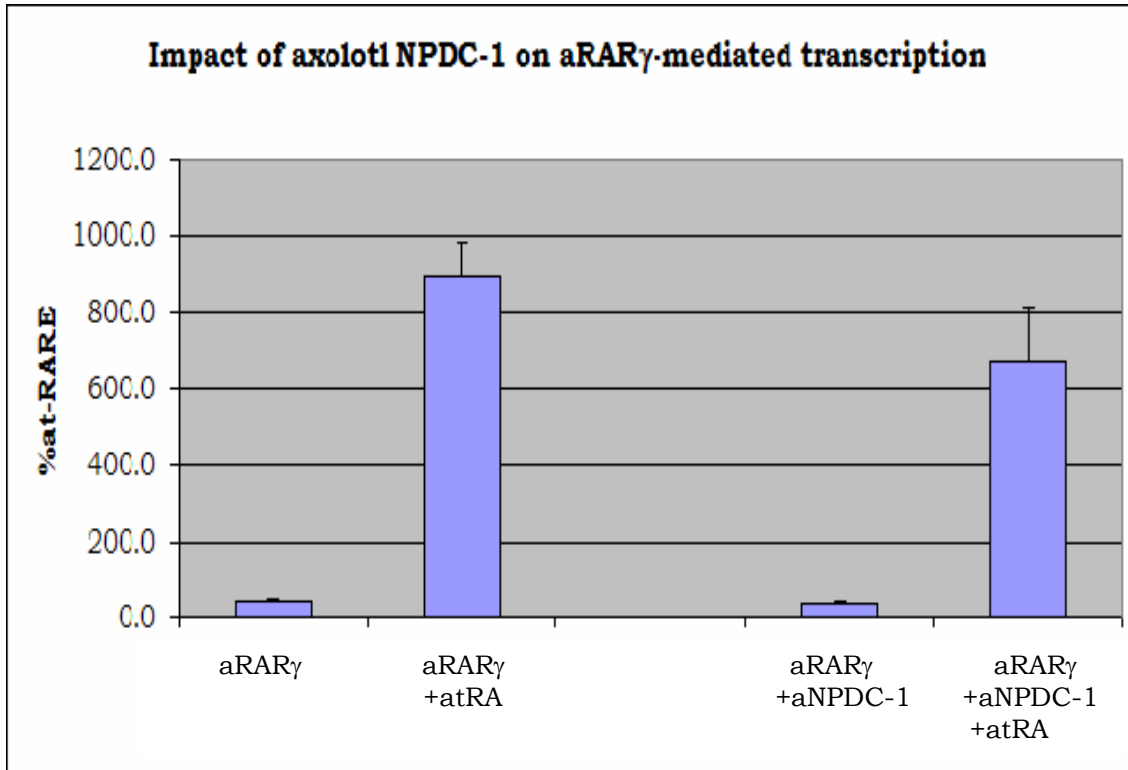
Figure 33: Impact of aNPDC-1 on aRARγ-mediated transcription. Cos7 cells were transiently transfected with mammalian expression plasmids for aNPDC-1, aRARγ, βRE-tk-luc, β-galactodisase and treated with 1μM atRA. Luciferase data was normalized to β-galactosidase and data was reported as a % of the response of aRARγ and aRARγ+aNPDC-1 in the presence of βRE.

* This result presents the average from experiments repeated at least four times.
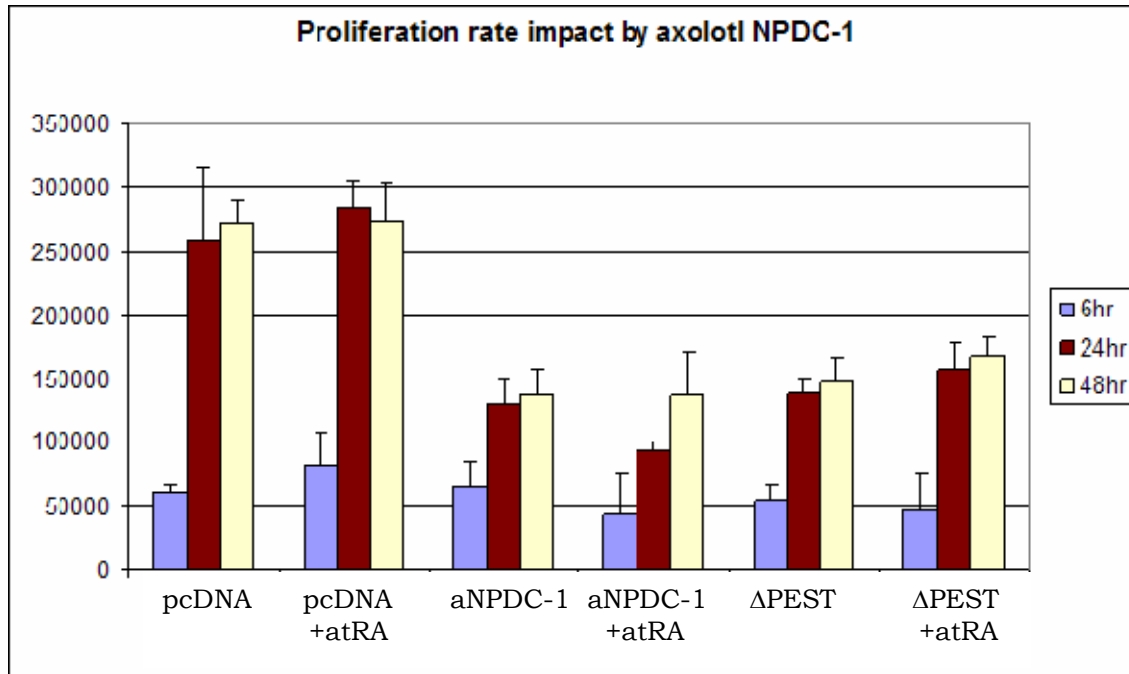
Figure 34: aNPDC-1 negatively impacts proliferation of HEK293 cells. HEK 293 cells were transfected with mammalian expression plasmids for aNPDC-1 and aNPDCΔPEST using calcium phosphate technologies. Cells were allowed to recover overnight before being plated at a density of 50,000 cells/well in a 24-well plate. Cells were incubated with 2μCi [3H]-thymidine/well and either treated with 1μM atRA or untreated for 6h, 24h, 48h. Cells were lysed and DNA was precipitated with 10% TCA and radiation was trapped by filtration through a vacuum filter apparatus. The incorporation of [3H]-thymidine was measured by TCA precipitation.

calcium phosphate precipitation technologies, HEK 293 cells were transfected with mammalian expression plasmids for aNPDC-1 or aNPDCΔPEST and a βRE-tk-luc reporter plasmid. Transfections were allowed to recover overnight and the next morning the cells were trypsinized, counted and plated at a density of 50,000 cells/well in a 24 well plate. Cells were either treated with 1μM atRA or untreated, and incubated in the presence of 2μCi [^{3}H]-thymidine/well for 6h, 24h and 48h. The incorporation of [^{3}H] thymidine was measured by TCA precipitation and the average incorporation for 3 wells was plotted. The over-expression of full-length aNPDC-1 was observed to significantly reduce proliferation at all three timepoints. Although the aNPDC-1 with the PEST deletion also significantly reduced proliferation, its impact was not statistically different from that observed for full-length aNPDC-1. In addition, the presence of retinoic acid appears to significantly inhibit the 24h aNPDC-1 proliferation results whereas it had no effect on the aNPDCΔPEST transfections. Cumulatively, these data support a role for NPDC-1 in suppression of cellular proliferation.

Chapter 11: Discussion

The NPDC-1 protein has been shown to associate with cell cycle regulators and regulate cellular proliferation events, and we have previously shown that NPDC-1 can regulate retinoic acid signaling events, a classical pathway for defining early embryonic differentiation events in both higher and lower eukaryotes. In spite of these advances in NPDC-1 cellular biochemistry, the precise biochemical mechanism by which NPDC-1 functions has not been defined by any of the investigators working with it, nor has NPDC-1 been directly associated with any disease pathogenesis. In an effort to define the biochemical role of NPDC-1 *in vivo*, we established a collaboration with Dr Randal Voss at the University of Kentucky. Dr. Voss is a leader in the field of axolotl development and evolution, and his laboratory houses one of the few established facilities for breeding and studying axolotl development. Axolotl represents a unique and well-defined system for the study of proliferation and differentiation, both functions impacted by NPDC-1 and retinoic acid (RA) signaling. Metabolic inhibitors of RA, such as citral, inhibit limb regeneration (125, 128) in axolotls, suggesting retinoic acid impacts patterning in the developing axolotl limb (122-125) and is able to modify positional information in the regenerating limb (111, 119, 120, 124-127). Given the phenotype of RA signaling in axolotl and its similarity to NPDC-1 signaling, we investigated the impact of NPDC-1 on the intracellular mediator of axolotl retinoic acid signaling, the axolotl retinoic acid receptor (aRAR).

To initiate our studies, it was necessary to first isolate and clone the axolotl homolog for NPDC-1 (aNPDC-1). As described in Chapter 6, aNPDC-1 was isolated from axolotl brain using 5' RACE. Sequence comparison of the axolotl homolog with the sequence of NPDC-1 from higher vertebrates showed conservation of certain key

regions of the protein such as the LXXLL motif, MAPK domain, PEST sequence and the helix-loop-helix motif. Overall the protein is conserved between higher vertebrates and axolotl with a consensus of 97.3% and identity of 36.3%, compared to 97.9% consensus and 73.3% identity between human, rat and mouse sequences. The biggest deviation in sequence conservation is observed between human and axolotl with only 49.1% consensus conservation and 39.3% identity conservation.

The tissue distribution of NPDC-1 is as varied in axolotl tissues as it is in human and mouse. Although NPDC-1 was isolated initially as a neuronal protein it has been demonstrated by a number of groups (90, 95) to have a more diverse distribution in higher vertebrates. In studies presented here it is demonstrated that in lower vertebrates such as the axolotl, the pattern of expression of NPDC-1 is just as widespread. In axolotl, NPDC-1 is highly expressed in heart, brain, skin, gill and eye. High expression of NPDC-1 was also observed to occur in human heart and brain tissues (95). The high level of NPDC-1 expression in juvenile axolotl gill would also appear to agree with previously reported data showing high level of expression of NPDC-1 in both human and rat lung (95). In metamorphosized axolotls, it is reasonable to hypothesize that NPDC-1 expression in lung would be higher than in juvenile animals. Cumulatively these tissue distribution data would circumstantially suggest that NPDC-1 serves a similar role in axolotl as it does in higher vertebrates.

Axolotl NPDC-1 also appears to functionally share with higher vertebrate NPDC-1 its ability to suppress cellular proliferation. In the original studies characterizing NPDC-1, cells transfected with NPDC-1 were observed to have lower proliferation rates. The doubling times of cells stably transfected with NPDC-1 were higher compared to controls, and the addition of NPDC-1 antisense oligonucleotides restored the generation times to control levels, demonstrating that NPDC-1 was responsible for

the observed slower proliferation rates (89). In studies presented here it was observed that transfection of aNPDC-1 into HEK293 cells resulted in decreased [3H] thymidine incorporation signifying a lower proliferation rate, in agreement with these previously reported results for mouse NPDC-1 (89). In the studies presented here it was also observed that treatment of aNPDC-1 transfected HEK cells with 1µM atRA resulted in a lower proliferation rate compared to untreated cells. The potential mechanism for NPDC-1's actions in inhibiting cellular proliferation is not fully understood, but NPDC-1 has been reported to associate either directly or indirectly with cell cycle regulatory proteins such as E2F-1, cyclin A, cyclin D1 and cdk2 (91, 93). Further examination is necessary to determine whether these associations are responsible for NPDC-1's properties as an anti-proliferative protein.

Similar to NPDC-1, my studies in axolotl also mandated that I clone an axolotl homolog for retinoic acid receptor. This was accomplished by utilizing RT-PCR to clone the RARγ from axolotl lung. Sequence comparison between human and axolotl RARγ showed a 98.5% conservation of the DBD and 89% conservation of the LBD. Although three distinct isoforms of RARs have been identified in human (68), mouse (146), and chicken (147), the amphibians *Notophtalmus viridescens* (148-150), *Xenopus laevis* (151, 152), and zebrafish (153) have been reported to express only two isoforms, RARα and RARγ. In the study by Wirtanen *et al* (106) looking at RAR expression in axolotl, cDNAs coding for a full-length aRARγ and partial sequence for aRARα were isolated by screening an axolotl neurula cDNA library. In the same study, these investigators isolated three splice variants of aRARγ; aRARγ1, aRARγ2 and aRARγ3. The axolotl homologs for RXR have not been cloned so far, but all RXR homologs have been identified in other amphibians such as *Xenopus* (151, 154, 155) and in zebrafish (153). Although the studies presented here are restricted to aRARγ2,

the overlap in tissue distribution with aNPDC-1 would suggest these data are biologically relevant.

Amphibian RARγ tissue distribution appeared to also mirror that observed for higher vertebrate RARγ. In humans RARγ mRNA is the predominant RAR subtype expressed in both fetal and adult skin (1, 156). A low level of RARγ mRNA expression can also be found in lung (1). The size of murine RARγ mRNA transcript is the same size as in human and is found in the skin and at lower levels in lung and spleen (1, 71). In studies presented here I demonstrate axolotl RARγ localizes to the heart, eye, skin, and to a lesser extent gill, spinal cord, liver and lung. These findings are in line with previously published data on axolotl and other amphibians where it was shown that RARγ is expressed, at both the mRNA and protein levels, in mesenchymal and epidermis tissues of normal and regenerating limbs of the newt (1, 150, 157). In *Xenopus*, RARγ is expressed during early development in the anterior and posterior ends of the neurula, and later in the head mesenchyme and tail bud (1). This localization of RARγ is consistent with the spatial and temporal pattern of retinoic acid sensitivity of *Xenopus* (1, 152).

Axolotl RARγ also appeared to function as a transcription factor in a similar manner to that observed for the higher vertebrate form of the receptor. RARs have been shown to heterodimerize with members of the RXR family of receptors and bind DNA response elements for retinoid receptors (RAREs) composed of two half-site motifs separated by 5 bases (DR5). In the studies presented here I demonstrate that aRARγ can bind to, and initiate transcription from, a DR5 RARE. This is consistent with published studies for higher vertebrates (95). As with higher vertebrate RARs, aRARγ utilizes RXR as a heterodimer partner. Although human RXRα was used for the studies presented here, as mentioned above, previously published data support the

existence of RXR isoforms in axolotls (158), but axolotl RXRs have yet to be cloned and sequenced. Cumulatively these data would suggest that aRARγ signaling functions through a similar mechanism to that of higher vertebrate RARs and most probably regulates the expression of similar sets of genes.

Expression of aNPDC-1 and aRARγ were observed to overlap during axolotl development, but peak at different times. NPDC-1 was initially isolated in terminally differentiated neuronal cells and shown to be inhibitory to cellular proliferation (89). In addition, NPDC-1 expression in mouse brain was found to increase during development with peak levels occurring at adulthood (91). A similar developmental pattern of NPDC-1 expression was observed in our studies in axolotl, where aNPDC-1 expression was strongly detected beginning at stage 38 and peaked at hatching. Although the pattern of RARγ expression during axolotl development appeared to partially overlap with that seen for NPDC-1, aRARγ expression was observed to occur earlier in development and peak prior to that seen for aNPDC-1 expression. In axolotl development, Wirtanen *et al* observed RARγ2 levels begin to rise by stage 11, peak by stage 16 and decline as the embryo approaches hatching (106). In studies presented here we have also detected aRARγ levels to decrease at hatching but extending our analyses we observed aRARγ to begin to increase 2 months post-hatching. In essence, RARγ expression in axolotl increases during the gastrula and neurula stages, through the fusion of the neural fold, gill region and ear pit development and extension of the body to 20 somites, whereas NPDC-1 expression peaks substantially later during the development and differentiation of gills, gill filaments and mouth (113). From a purely developmental perspective, it appears that NPDC-1 and RARγ expression are antagonistic in that aRARγ expression is reduced to background levels as aNPDC-1 is beginning to increase its expression.

Previous studies with higher vertebrate NPDC-1 demonstrated it could impact RAR signaling through direct binding of RXR and RAR-RXR heterodimers (95). In the research presented here I demonstrate that axolotl NPDC-1 can also bind aRARγ, and that this binding event can be localized to the amino terminus within amino acids 1-97. Two highly conserved domains occur within this amino terminus region. The first is an LXXLL motif. This motif has been previously reported to be an important nuclear receptor binding site for a variety of transcription coregulatory proteins (1), although Henry *et al* (95) demonstrated that, at least with respect to human NPDC-1 and RAR signaling, this motif appears not to be critical for transcriptional regulatory events modulated by NPDC-1. The second motif is a helix-loop-helix (HLH) domain that occurs downstream of the LXXLL motif. This domain has been shown to function as a binding site for DNA, and proteins belonging to the HLH family have been shown to be key regulators of development, particularly cell type determination, terminal differentiation and sex determination (90). Further studies will be required to establish what, if any, role the HLH domain might be playing in aNPDC-1-aRARγ binding events.

Most coregulatory proteins mediate their regulation of transcription through modulation of the protein complexes that associate with specific DNA regulatory sequences in the promoters of the genes they regulate (18). In the studies presented here I demonstrate that the mechanism for aNPDC-1 regulation of aRARγ-mediated transcription appears complex and may involve both direct associations with aRARγ as well as facilitating complex formation on RAR response elements. It was observed that the presence of aNPDC-1 increased the amount of response element DNA bound of the RAR-RXR heterodimer complex, suggesting that aNPDC-1 is capable of altering the DNA binding capacity or affinity of the RAR-RXR heterodimers. These results parallel

what we have published for higher vertebrates, in that it was shown that in PC12 extracts, NPDC-1 increases the intensity of the complex formed on a DR5 response element without altering the mobility of the complexes formed (95). In the same published study, human NPDC-1 was similarly shown to enhance the binding of recombinant human RXRα complexes to a DR1 response element (95). These data cumulatively suggest that NPDC-1 is able to alter the DNA binding capacity of the RAR-RXR complex without altering the mobility of the complex. The mechanism for this observation is unclear but one possibility is that NPDC-1 facilitates complex formation on the response element, but its loose associations are lost in the subsequent electrophoretic procedures. There have been previous reports in literature where addition of nuclear extracts increased the binding efficiency of RAR to DNA (72, 73), but these reports focused on the DNA binding activity of RARα and not the RAR-RXR heterodimer. Yang *et al* and Glass *et al* (72, 73), demonstrated that addition of nuclear extracts from various cell lines increased the DNA binding affinity of RARα, and this was dependent on the origin of the nuclear extract. It is possible that merely the presence of RXR from the nuclear extract would change the complex mobility and intensity, although the study by Glass *et al* (73) also suggests that specific combination of retinoic acid receptors and cell-type specific coregulator proteins may function to integrate the effects of retinoic acid on patterns of gene expression within specific cell types during development. Based on the size of the various proteins observed to cross-link with RARα in the study by Glass et al (73), NPDC-1 appears not to be one of them.

Interestingly, in contrast with what we have published for high vertebrate NPDC-1-RAR DNA binding events, using recombinant axolotl NPDC-1 and RARγ proteins, a unique shift in the RAR-RXR complex was also observed with the addition

of aNPDC-1. This more logical increase in complex size due to binding of aNPDC-1 is supported by the observation that NPDC-1 antibody effectively eliminated the complex. It should be noted that NPDC-1 antibody also eliminated the enhanced complex described above, suggesting that these multiple effects axolotl NPDC-1 is having on aRARγ-RXR DNA binding complexes are both NPDC-1-specific. These data would also support the hypothesis presented above that aNPDC-1 can transiently or loosely associate with aRARγ-RXR complexes and facilitate their DNA binding. Cumulatively, these data demonstrate that aNPDC-1 shares many of the features higher vertebrate NPDC-1 proteins possess with respect to their impact on RAR-RXR DNA binding, but also suggests aNPDC-1 may complex with aRAR-RXR heterodimers in a somewhat different capacity.

The potential functional consequences of aNPDC-1 binding of aRARγ and its impact on aRARγ DNA binding, appears to be a repression of aRARγ-mediated transcription events. In studies presented here I demonstrate that DR5-driven reporter gene transcription can be moderately repressed by over-expression of aNPDC-1 in *in vitro* transcription analyses. This is similar to what has been reported for human NPDC-1 (95), although the repression observed for aNPDC-1 is not as great as the repression observed for hNPDC-1 (95). This could be due to a number of factors, not the least of which is the expression of axolotl proteins in mammalian cells. It is difficult to predict if the axolotl proteins are expressed and processed correctly in the mammalian cells. Ideally, these studies should be performed in an axolotl cell line, but due to the lack of any established axolotl cell line and the culturing and transfection limitations of axolotl primary cells, I was relegated to using the Cos7 mammalian cell line for the studies presented here. The Cos7 cell line was chosen primarily because of its low endogenous level of RAR and its transfectability.

Cumulatively these studies present a potential paradox in that, although NPDC-1 can enhance RAR-RXR DNA binding to a DR-5 response element, the end result is repression of gene expression events from this response element. Several possible explanations for these observations exist and might include aNPDC-1 enhancing RAR/RXR heterodimer binding but blocking ligand binding and subsequent recruitment of transcription co-activator complexes, aNPDC-1 enhancing RAR/RXR heterodimer binding that results in the recruitment of transcription co-repressor complexes, or even the possibility that aNPDC-1 facilitates the formation of a homodimer complex (RAR or RXR) that can compete for binding to the DR5 element and in that capacity repress RAR/RXR heterodimer-mediated transcription. Further studies will be required to establish the biochemical mechanism for repression of RAR-mediated transcription events by NPDC-1.

In summary, this study presents the first evidence for interaction between NPDC-1 and RARγ in axolotl. The axolotl is an established animal model system for studying development and differentiation, functions that are impacted by both RAR and NPDC-1. In studies presented here, it is demonstrated that aRARγ is able to bind to and activate transcription from a DR5 element, and that aNPDC-1 is able to repress this transcription. The axolotl NPDC-1 was also shown to decrease cell proliferation, confirming previously published results for mouse NPDC-1 (89). Finally, it was shown that aNPDC-1 could alter aRARγ-RXR DNA binding complexes, suggesting that aNPDC-1 can directly modulate gene expression events mediated through retinoic acid signaling. Future directions for these studies include the utilization of the axolotl as a model organism to study the interactions between aNPDC-1 and aRAR *in vivo* and their impact on differentiation. Overall, these studies are the first step towards

dissecting the impact of NPDC-1 on RAR mediated signaling in development and differentiation.

SECTION V: CONVERGENCE OF THYROID AND NPDC-1 SIGNALING PATHWAYS IN AXOLOTL

Chapter 12: Introduction

Amphibian metamorphosis is a postembryonic developmental process governed by thyroid hormone (TH) during which larval tissues are being replaced by adult tissues. The dramatic changes that occur during metamorphosis, which are easily observed due to the accessibility of the animals compared to amniotes, include organogenesis and tissue remodeling (159). At the biochemical level, metamorphosis involves a coordinated process of cell proliferation, differentiation and death (159). During premetamorphosis, the circulating levels of TH are minimal (160), increasing slowly (161) and peaking at metamorphosis (108). This peak is caused by an increased secretion of circulating TSH and is accompanied by dramatic and rapid morphological and biochemical changes (161). The circulating levels of the TH isoforms, T3 and T4, return to premetamorphic levels several days after metamorphosis is completed (161). TH is responsible for metamorphosis, although other hormones such as prolactin, glucocortocoids and estrogen are thought also to play a role in this process (160-165). Furthermore, it has been clearly demonstrated that the T4 isoform is responsible for induction of metamorphosis (159, 166, 167). During metamorphosis, enzymatic processes convert T4 to the more biologically active and potent T3 isoform, and T3 levels rise and reach a peak at climax (159, 165, 168). In addition to T3, corticosteroid levels also rise and have been demonstrated to synergize with T3 to promote metamorphosis (159, 165, 168). Administration of TH to

anuran tadpoles, even before the period of metamorphosis, triggers the process (108), and blockage of endogenous TH production by goitrogens blocks metamorphic induction and produces giant tadpoles (108).

Thyroid gland secretions are under strict hormonal control. Thyrotropin (TRH) secreted by the pituitary gland controls T3 production and adrenocorticotropin (ACTH) controls the production of corticosteroids (159, 165, 167). The biosynthesis and secretion of both TRH and ACTH are under the control of neurohormones produced by the hypothalamus, thus placing the neuroendocrine system at the center of the metamorphological processes (159). The primary regulator of TSH secretion is thyrotropin-releasing hormone (TRH). However, TRH does not appear to control thyroid secretions during metamorphosis (159, 167, 169), but corticotrophin-releasing hormone (CRH) may function in this capacity instead, controlling the release of both TSH and ACTH during metamorphosis (159, 165, 167, 170-174).

In nature, the importance and extent of metamorphosis across amphibian species varies (108). Alternative mechanisms such as neoteny (sexual maturity in the larval form) and direct development can be found in both urodeles and anurans [(108) and references therein]. In *Ambystoma*, neoteny is facultative, whereas in *Necterus*, *Proteus* or *Siren* species it is obligatory (108). Populations of *Ambystoma* found in nature can be purely neotenic, transforming or polymorphic (108). In the laboratory, most *Ambystoma* species undergo metamorphosis when treated with TH, whereas the concentration of TH necessary to trigger metamorphosis varies (108). In salamander the term metamorphosis is used to describe changes that occur naturally or can be induced by TH in sexually mature adults, and therefore are not necessary to complete the life cycle (115, 175). Spontaneous metamorphosis has been described for some salamander species where they resorb their gills and tail fins, change their head shape

and undergo skin thickening (115). Phylogenetic analyses have shown that metamorphic failure evolved independently several times during *Ambystoma* evolution, suggesting that there is an increased ability to select this life history trait in all species of this genus (108).

The reasons some *Ambystoma* do not undergo metamorphosis are unclear and seem dependent on environmental conditions (161). The cause of this metamorphic failure has been a subject of study at the molecular level. Axolotl tissues respond to TH changes and this suggests that failure resides with TH production in these animals and abnormally low TSH secretion (108, 161, 176). With the cloning of the axolotl thyroid hormone receptors in 2004 it was observed that both TRα and TRβ are expressed in larval and adult axolotl, and that their target genes could be regulated by TH (108, 177). Furthermore, these receptors were also observed to be upregulated in axolotl tissues following TH administration (108).

Structurally, axolotl TR homologs show a strong sequence identity to known TRα and TRβ sequences, with the strongest conservation in the DBD and LBD. Within the DBD the helix responsible for contact with the HRE is fully conserved. Within the LBD there is strong conservation in the 12 α-helices that form the core of the 3-D structure (178), suggesting that axolotl TRs have the capability to bind TH and transactivate target genes in response to hormone (108). Further experiments presented by Safi *et al* (108), show that axolotl TRs are functional, able to bind DNA and T3, and able to activate transcription off a xDR4 luciferase reporter in a T3 dependent manner (108).

During metamorphosis of axolotls the levels of TRs are modified. At premetamorphosis the levels of TRα mRNA are higher compared to TRβ mRNA. At the beginning of metamorphosis, TH binds to TRα, which then induces early genes such

as TRβ, which upregulates its own transcription and mediates the response to TH by inducing different cascades of gene activation (108). The levels of TRβ never exceed those of TRα (161), and after the climax of metamorphosis the levels of both isoforms return to normal (179-181).

The role of thyroid hormone in axolotls is not confined to metamorphosis, but rather extends to the developmental changes that take place in the nervous system prior to metamorphosis, such as the remodeling of CNS regions necessary for the transition from larval to adult life. The importance of TH for the maturation of neurosecretory centers was demonstrated by Norris and Gern (182) who showed that neotenic tiger salamanders could be induced to metamorphose when injected intrahypothalamically with T4 but not intraperitoneally (170). During this time of premetamorphosis CNS remodeling, certain larval structures are eliminated and adult-specific neural structures develop.

The axolotl is an excellent model for the study of postembryonic development, in that their life cycle has distinct boundaries and these changes from juvenile to adult can be regulated *in vitro* by TH (108). This process of metamorphosis is highly regulated and is mediated through the modulation of gene expression events via TH interaction with its nuclear receptor family members. Because of its importance and level of commitment, it stands to reason that the process of thyroid receptor-mediated gene transcription is itself under close regulation during metamorphosis. In the previous chapters I defined a role for the axolotl nuclear receptor coregulatory protein aNPDC-1 in regulation of axolotl retinoid signaling, an event closely aligned with axolotl embryonic development. In this section of the dissertation I investigate the possibility that this same coregulatory protein might be involved also in the regulation of thyroid hormone signaling. Here I demonstrate that axolotl NPDC-1 can alter

transcription mediated by thyroid receptors, can complex with both aTRα and aTRβ, and can modify TR/RXR DNA binding. These preliminary studies into the regulation of thyroid hormone signaling provide the foundation for dissecting the molecular mechanisms regulating amphibian morphogenesis, which, due to the conservation seen in these protein structures, could prove applicable to our understanding of thyroid signaling in high vertebrates.

Chapter 13: Experimental Procedures

Chemicals, Enzymes and Antibodies

Standard chemicals used throughout these studies, unless otherwise stated, were purchased from Sigma chemical or Fisher Scientific. Restriction enzymes, polymerases, ligases and kinases used in these studies, unless otherwise stated, were purchased from New England Biolabs. Benzonase nuclease was purchased from Novagen. The antibodies used for these studies were purchased from Santa Cruz unless otherwise stated.

Plasmids and Constructs

aTRα: To clone axolotl TRα into the bacterial expression vector pGEX-KG, a PCR reaction was performed on pGEM-aTRα (a gift from Dr. Voss' lab) using the following primers: Forward 5'-GGA A<u>GA ATT C</u>TA GAC CAG AAC CCC AGC GAT-3' and Reverse: 5'-GCA A<u>AA GCT T</u>TC AAA CTT CCT GGT CTT CGA A-3'. The amplified receptor was then subcloned into the *EcoRI/HindIII* sites of the bacterial expression vector pGEX-KG. To clone aTRα into the mammalian expression vector pcDNA3.1/Zeo, full length aTRα was digested with *SacII*, filled-in with Klenow and subsequently digested with *Not I* and inserted into the *EcoRV/NotI* sites of pcDNA 3.1/Zeo. The primers forward: 5'-TT<u>G AAT TC</u>A TGG ACC AGA ACC CCA G-3' and reverse: 5'-TAT <u>AAG CTT </u>TCA AAC TTC CTG GTC TTC-3' were used to amplify aTRα and clone it into the *EcoRI/HindIII* sites of the bacterial expression vector pET32.

aTRβ: To clone axolotl TRβ into the bacterial expression vector pGEX-KG, a PCR reaction was performed on pGEM-aTRβ (a gift from Dr. Voss' lab) using the following primers: forward: 5'-GGC C<u>GA ATT C</u>TA CCC ATC AGT ATG TCA GGG-3' and reverse:

5'-GGC C<u>AA GCT T</u>TT AGT AGT TGG AGA CGA TCA G-3'. The amplified receptor was then subcloned into the *EcoRI/HindIII* sites of the bacterial expression vector pGEX-KG. The reverse primer was modified using the redundancy of the genetic code to increase the T_m of the primer. To clone aTRβ into the mammalian expression vector pcDNA3.1/Zeo, full length aTRβ was digested with *SacII*, filled-in with Klenow and subsequently digested with *Not I* and inserted into the *EcoRV/NotI* sites of pcDNA 3.1/Zeo. The primers forward: 5'-TT<u>G AAT TC</u>A TGC CCA TCA GTA TG-3' and reverse: 5'-TAT <u>AAG CTT </u>CTA ATC CTC AAA GAC C-3' were used to amplify aTRβ and clone it into the *EcoRI/HindIII* sites of the bacterial expression vector pET32. To clone aTRβ into the *EcoRI/HindIII* sites of pTriex (Novagen) (can be used for both mammalian and bacterial expression), the primers used were: forward: 5'-TT<u>G AAT TC</u>T ATG CCC ATC AGT ATG-3' and reverse: 5'-TAT A<u>AA GCT T</u>CT ACT GTG GCT TAG AG-3'.

<u>aNPDC-1:</u> The generation of aNPDC-1 clones was described in section IV materials and methods.

<u>RPA probe plasmids:</u> RT-PCR was performed on axolotl lung RNA to obtain aTRα, aTRβ, aNPDC-1 and RPL13A (a control gene) for use as RPA probes. The primers used for aTRα were: forward 5'-GCT T<u>GA ATT C</u>GC ATC GCT GTG GGC ATG GC-3' and reverse: 5'-CAC G<u>AA GCT T</u>GG CCG GTG CTG CAT GGA CT-3'. For aTRβ the primers used for the RT reaction were forward: 5'-GTC T<u>GA ATT C</u>GC ACC TAC GTT GGC ATG GC-3' and reverse: 5'-CAC G<u>AA GCT T</u>CG TTG GTG GCC ATG TGT GC-3'. For aNPDC-1, the primers used were forward: 5'-GTC T<u>GA ATT C</u>GA CAG ATC CTC GCC AGT AG-3' and reverse: 5'-CAC G<u>AA GCT T</u>CG TGA AAT CGC CAT CCT C-3' whereas for RPL13A the following primers were used: forward: 5'-GTC T<u>GA ATT C</u>GG CAG GCT GCC CTG GAA AG-3' and reverse: 5'-CAC G<u>AA GCT T</u>CA ACC GCA CAA TCT TGA GC-

3'. The amplified fragments were then subcloned into the *EcoRI/HindIII* sites of pGEM4.

Other plasmids and constructs: For these experiments, in addition to the plasmids described above, the previously described TRE-tk-luc plasmid (183) and RSV-βgal plasmid (137) were utilized.

RT-PCR Assays

Axolotl tissue cDNA (a gift from Dr. Voss' lab), was subjected to PCR using the following primers: For aTRα forward: 5'-CTG GAT AAA GAT GAA CCG TGC GTT-3' and reverse: 5'-ACT TCA GCA TTT CCT CTT TGC GCC-3' and for aTRβ forward: 5'-ATG GAT TTC TAA CTC AGA CGA CAC CG-3' and reverse: 5'-CCA AAA TGT TTC TTT TTT TAC ACT CGA CT-3'. cDNA template corresponding to 400ng total RNA was used per PCR reaction. PCR were performed on a T-Gradient thermocyler as described by the manufacturer (Biometra) using the following cycling conditions: 94^0Cx3min, 94^0Cx45s, 55^0Cx45s, 72^0Cx45s for 32 cycles, 72^0Cx 5min. PCR products were resolved on a 1.2% agarose gel and detected using the Gel logic gel imaging system (Kodak).

Recombinant Protein expression

Glutathione: To develop GST and GST-aTRα and GST-aTRβ fusion proteins, BL21-RP *Escherichia coli* cells were transformed with bacterial expression constructs in the pGEX-KG plasmid system. Transformants were plated on double selection media containing carbanecillin (50μg/ml) and chloramphenicol (34μg/μl) overnight at 37^0C. Isolated colonies were picked and used to grow a 2.0mL starter culture which was then used to inoculate 500mL of 2X-YT (16mg/ml Tryptone, 10mg/ml NaCl, 10mg/mL Yeast extract, pH 7.2-7.4) supplemented with carbanecillin and chloramphenicol.

Cultures were grown to log phase at room temperature (OD_{600} of 0.6). Protein expression was induced for 6h by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (Gold BioTechnology, Inc) to a final concentration of 1mM. Bacterial cells were pelleted by centrifugation for 15 minutes at 6000rpm and resuspended in 5mL of 10mM Tris-HCl pH 7.5 containing 100mg of lysozyme (Sigma), followed by the addition of 20mL of Triple Detergent Lysis buffer [50mM Tris-HCl pH 8.0, 150mM NaCl, 0.02% sodium azide (v/v), 1% NP-40 (v/v), 0.5% sodium deoxycholate (v/v), 1μM PMSF and 1X protease inhibitor cocktail (Roche Applied Science) and Benzonase Nuclease (1μl/ml)] and incubated at room temperature with agitation (150rpm) for 30 minutes on an orbital floor shaker. The soluble fraction was clarified by ultracentrifugation for 1h at 50,000rpm in a Ti70 rotor. GST fusion proteins were purified by Fast Performance Liquid Chromatography (FPLC) on a GSTrap Fast Flow column (GE Life Sciences). Eluates were desalted by FPLC on a G-50 desalting column into TBS-P (10mM Tris-HCl pH 8.0, 150mM NaCl, 10% glycerol (v/v), 1mM DTT) and the resulting proteins were aliquoted and stored at -70°C until use. The protein concentration was determined using Bradford reagent (Advance, Cytoskeleton Inc).

Thioredoxin: To generate Thioredoxin and thioredoxin-tagged aNPDC, aNPDCΔPEST, aNPDCΔHLH, aTRα and aTRβ, bacterial expression constructs in the pET32 (Novagen) vector system were transformed into BL21-RP *E. coli* cells. Transformants were plated on double selection media containing carbanecillin (50μg/ml) and chloramphenicol (34μg/μl) and incubated overnight at 37°C. Isolated colonies were picked and used to grow a 2.0mL starter culture which was then used to inoculate 500mL of 2X-YT supplemented with carbanecillin and chloramphenicol. Cultures were grown at room temperature, to an OD_{600} of 0.6. Protein expression was induced for 6h by the

addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (Gold Biotechnology Inc) to a final concentration of 1mM. The transformed bacterial cells were pelleted by centrifugation at 6000rpm for 15 minutes and resuspended in 5mL of 10mM Tris-HCl pH 7.5 containing 100mg of lysozyme (Sigma), followed by the addition of 20ml of Triple Detergent Lysis buffer, and incubated at room temperature with agitation (150rpm) for 30 minutes on an orbital floor shaker. The soluble fraction was clarified by ultracentrifugation for 1h at 50,000rpm in a Ti70 rotor. The clarified lysate was purified by FPLC on a HiTrap chelating HP column (Amersham Biosciences) preloaded with Nickel. Bound protein was washed with 10 column volumes of His wash (50mM NaH_2PO_4, 300mM NaCl, 20mM Imidazole pH 8.0) and eluted in His elution buffer (50mM NaH_2PO_4, 300mM NaCl, 500mM Imidazole pH8.0). Eluates were desalted by FPLC on a G-50 desalting column into TBS-P (10mM Tris-HCl pH 8.0, 150mM NaCl, 10% glycerol (v/v), 1mM DTT), and the resulting proteins were aliquoted and stored at -70^0C until use. The protein concentration was determined using Bradford reagent (Advance, Cytoskeleton Inc).

Pulldown Assays

Recombinant GST-aTRα and GST-aTRβ (10μg/reaction) were prebound to glutathione sepharose beads (50% slurry in APB buffer) [APB: 50mM Tris-HCl ph 7.5, 150mM NaCl, 10% glycerol (v/v), 1% NP-40 (v/v)] by rotating for 1h at 4^0C. To the GST prebound proteins, equimolar amounts of thioredoxin or thioredoxin-tagged aNPDC-1 proteins were added as described in results and figure legends. The proteins were allowed to interact by rotating for at least 1h at 4^0C. The beads were recovered by centrifugation in a microcentrifuge and washed 5 times with 1ml APB. The bound

proteins were analyzed by western blot analyses probing with antisera for thio-probe (Santa Cruz).

For pulldown studies investigating aNPDC-1 and aTR interaction in HEK cells, HEK cells transfected with tagged constructs for aNPDC-1 and aTRs were lysed and purified as described below and processed as described above.

TR Cell Extract Preparation

HEK cells were transiently transfected with either the expression vector pTriex or pTriex-aTRβ using the calcium phosphate transfection method as previously described (140). Following 48h incubation at 37^{0}C, the cells were washed with 1X PBS [PBS: 0.30g/L NaH$_2$PO$_4$ anhydrous, 1.15g/L Na$_2$HPO$_4$ anhydrous, 9g/L NaCl, pH 7.2-7.4] and lysed in 1ml APB [APB: 50mM Tris-HCl ph 7.5, 150mM NaCl, 10% glycerol (v/v), 1% NP-40 (v/v)]. The cell lysate was then spun briefly to precipitate cellular debris. The supernatant was collected, the concentration was determined using the Bradford method, and was stored at -80^{0}C until needed.

Cross-linking aNPDC-1 to Cyanogen bromide sepharose

Recombinant axolotl NPDC-1 protein was cross-linked to Cyanogen Bromide-activated Sepharose 4 Fast Flow agarose (Amersham) according to manufacturers' instructions. Briefly, the matrix was washed in low pH (pH 2-3) to remove additives added by the manufacturer. The washed medium was then mixed with coupling solution [0.1M NaHCO$_3$ pH 8.3 containing 0.5M NaCl] and ligand (aNPDC-1) and coupling was allowed to occur at 4^{0}C overnight. The excess ligand was then washed with 5 volumes coupling buffer and non-reacted groups on the medium were blocked by incubation in 0.1M Tris-HCl pH 8.0 for 2 hours. The coupled medium was then washed using

alternate low and high pH solutions. For low pH washes 0.1M acetate buffer pH 3-4 containing 0.5M NaCl was used and for high pH washes 0.1M Tris-HCl buffer pH 8-9 containing 0.5M NaCl was used. The washes were repeated a total of 5 times. The coupled medium was stored at 4^0C in 20% ethanol to prevent microbial growth.

Western Blot Analysis

Western blot analyses were performed as previously described (99). Briefly, proteins were separated by SDS-PAGE electrophoresis and electroblotted onto nitrocellulose membranes (Stratagene Inc). Blocking was performed in PC buffer (1% casein in 1X PBS) [PBS: 0.30g/L NaH_2PO_4 anhydrous, 1.15g/L Na_2HPO_4 anhydrous, 9g/L NaCl, pH 7.2-7.4] overnight. Primary antibody was diluted in PCT buffer (1% casein in 1X PBS, 0.01% Tween-20) and incubated for 1h. Blots were subsequently washed with TBST buffer (20mM Tris pH7.5, 137mM NaCl, 0.1% Tween-20), then incubated with an appropriate secondary antibody coupled to horseradish peroxidase, reacted with ECL reagents (Pierce) as described by the manufacturer and detected on X-ray film by autoradiography.

Electromobility Shift Assays

To generate nuclear extracts for EMSAs, cells were washed with non-sterile PBS twice and scraped into 1mL PBS and transferred to microcentrifuge tubes on ice. The cells were then spun for 2 minutes at 2500 rpm. The resulting pellet was resuspended into buffer A [10mM Hepes pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.2mM PMSF] and incubated on ice for 10 minutes. After vortexing, the lysate was spun at top speed for 3 minutes. The pellet was then resuspended into 100μl buffer C [20mM Hepes pH 7.9, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, and

0.2mM PMSF]. Following vortexing, the resuspended pellet was incubated on ice for 1hr. After a 3 minute spin at top speed, the supernatant was aliquoted into 10uL aliquots and stored at -80^{0}C until needed.

EMSAs were performed as described previously (95). Briefly, to generate the DR4 response element the following oligos were annealed, Forward: 5'-GG<u>G TCG AC</u>C TAG GCA GGT CAT TTC AGG ACA GCC CAG CG-3' and Reverse: 5'-GGG TCG ACG CTG GGC TGT CCT GAA ATG ACC TGC CTA GG-3'. The sequence for *Xenopus* DR4 used here was first reported by Safi *et al* to be responsive in axolotl (108) and in the sequence shown here the *SalI* site was added. The annealed oligos have 5' overhangs. The overhangs were radioactively labeled with [α-^{32}P]-dCTP using a standard Klenow fill-in reaction (138). The radioactive response element was purified by centrifugation through a microspin G-25 column (Amersham). The radiolabeled response element was incubated in binding buffer [for 1X binding buffer: 5% glycerol, 1mM MgCl$_2$, 0.5mM EDTA, 0.5mM DTT, 50mM NaCl, 10mM Tris pH 7.5, 0.25mg/mL poly (dIdC)-poly (dIdC)] with recombinant aTRα, aTRβ, hRXRα, aNPDC, aNPDCΔPEST or aNPDCΔHLH in the presence of 1μM T3 as indicated. The response element/protein complexes were separated by electrophoresis on 5% non-denaturing polyacrylamide gels. The gels were dried and the results visualized by autoradiography on X-ray film.

Mammalian Transcription Assays

Cos7 cells were transiently transfected by the method of Calcium Phosphate as previously described (140). Briefly, 20μg total DNA (5μg TRE-tk-luc, 1μg pcDNA, 2μg pcDNA-aNPDC, 1μg pcDNA-aTRα or 1μg pcDNA-aTRβ, 5 μg rsv-βgal and 7μg pUC) were dissolved in 420ul of water and combined with 60μl 2M CaCl$_2$ and vortexed briefly. The DNA-CaCl$_2$ mixture was added dropwise, with light vortexing, to 500μl 2X HBS

[10g/L Hepes, 16g/L NaCl pH 6.95] that was mixed with 10ul 100X Phosphate [70mM Na$_2$HPO$_4$, 70mM NaH$_2$PO$_4$] and incubated at room temperature for 30 minutes. After the 30 minute incubation the DNA precipitate was mixed by pipetting up and down and then the mixture was added to the 10cm plates dropwise. After 6h incubation at 37 degrees, the media was removed and the cells were washed with 1XPBS 3 times. After trypsinizing, the cells were resuspended in 10mL DMEM containing 10% CAFBS (charcoal absorbed fetal calf serum) and 20ul cells/well were plated into a 96 well plate with and without 1μM T3. Cells were assayed in 6 wells per treatment. The cells were incubated for 24h in the presence of thyroid hormone (T3), and then luciferase and normalizing β-gal activities were determined as previously described (139). The highest and lowest values for each treatment were dropped. Activity was expressed as the average of luciferase response normalized to the average β-gal response/minute. The data presented here is an average of at least four independent experiments.

Nucleic Acid isolation

Axolotl tissues were a generous gift from Dr. Voss' lab. Tissues were snap frozen in liquid nitrogen and kept at -80^{0}C until needed. Frozen tissue was ground to a powder in liquid nitrogen using mortar and pestle. The powderized tissue was then transferred to a Dounce homogenizer containing 1mL TRIzol reagent (Sigma). RNA was obtained following the manufacturers protocol. In short, tissue was homogenized and allowed to stand for 5 minutes at RT. Then 0.2mL chloroform was added per 1mL of TRIzol reagent and after shaking the sample was allowed to stand at RT for 5 minutes and then centrifuged at 12,000g for 15 minutes at 4^{0}C. The aqueous phase was then transferred to a new tube and the RNA was precipitated with 0.5mL isopropanol per 1mL TRIzol reagent. The RNA pellet was rinsed with 75% ethanol, air-

dried and resuspended in autoclaved distilled water. The $O.D_{260}$ was measured using a spectrophotometer and the concentration determined using the formula: O.D260*dilution factor*40=mg/mL.

RNAse Protection Assays

RNAse protection assays were performed as previously described (184). The RPA probe plasmids, generated as described above, were linearized with *EcoRI* and purified from agarose gels using DEAE paper purification. Briefly, the band to be purified was run onto DEAE-cellulose paper which was previously soaked in 2.5M NaCl, washed with DI water and stored at 4^0C in 1mM EDTA. The DEAE paper with DNA was then placed in a 1.5mL tube and washed with water to eliminate possible agarose and other contaminants. After all water was removed, the paper is saturated with elution buffer [0.01M Tris pH 7.5, 1.5M NaCl, 0.001M EDTA]. The elution buffer was separated from the DEAE paper following vigorous vortexing (2-5minutes) and a centrifugation for 2-5 minutes. The solution was transferred to a 1.0 mL syringe and passed through a column (Promega) to eliminate contamination from the paper. To the eluate, an equal volume of isopropanol was added and the DNA was precipitated at -20^0C for at least 30 minutes.

The linearized plasmids were used with the Promega riboprobe kit to synthesize a labeled cDNA probe using T7 RNA polymerase and [^{32}P] UTP. Following measurement of the cpm, the probe was adjusted to 500cpm x #UTP in probe sequence, and incubated overnight with RNA sample (10μg/reaction) and allowed to form complexes. The next day, the RNA/probe complexes were subjected to RNase digestion, precipitated and separated on a 5% sequencing gel. Gels were analyzed by autoradiography.

<u>Bradford Assay</u>

Proteins were diluted 1:200 in 1X Bradford reagent (Advance) and the OD_{595} was measured using a BioRad microplate reader. The OD reading of each protein was adjusted to a blank and the concentration of each protein was calculated based on the dilution factor and the manufacturers' instructions.

Chapter 14: Results

Developing axolotl thyroid receptor plasmid tools for analyzing receptor-mediated gene expression events

Amphibian metamorphosis results in extensive organogenesis and tissue remodeling, each involving coordinated processes of cell proliferation, differentiation and death (159). TH, as a controller of metamorphosis, initiates the many gene expression events required for morphogenesis through its ability to bind and activate a pair of nuclear receptor transcription factors. It has been observed in higher vertebrates that the expression and activation of these thyroid hormone receptors are also under complex biochemical regulatory mechanisms including; the physical expression of the receptors, the DNA binding of the receptors, the ligand binding of the receptors and the ability of the receptors to interact with a variety of coregulatory proteins. Our preliminary studies with the nuclear receptor coregulatory protein NPDC-1 suggest it may play a central role in coordinating nuclear receptor-mediated gene expression during embryogenesis in axolotls. We therefore hypothesized that NPDC-1 might also be serving a similar function with respect to thyroid receptor-mediated events during morphogenesis. To preliminarily investigate this hypothesis we developed a set of axolotl thyroid receptor (aTRα and aTRβ) recombinant DNA tools. For the identification of the axolotl TR homologs, it was fortunate that they had been previously identified and partially characterized by Safi *et al* in 2004 (108). Using the published sequence, the axolotl homologs were cloned by PCR into the pGEM-Easy T vector using axolotl brain cDNA as template. Integrity of the constructs was confirmed by sequencing using vector specific primers. PCR primers were designed also to clone aTRα and aTRβ into the mammalian expression vector pcDNA 3.1/Zeo and the

bacterial expression vector pGEX-KG. Sequence comparison of the cloned aTRα and

aTRβ revealed them to be identical to the previously published sequence (108), and, as

defined by these investigators, to have strong sequence identities with the human and

rat TR sequences. With the availability of the axolotl homologs for TRα, TRβ and

NPDC-1, it was possible to investigate aNPDC-1's ability to regulate gene expression

events mediated by thyroid receptors in axolotl.

Tissue distribution of aTRα and aTRβ in axolotl

To characterize the tissue distribution of the aTRα, aTRβ and aNPDC-1, an

RNAse protection assay was performed as previously described (184). Axolotl tissues

(a generous gift from Dr. Voss' lab) were harvested and snap frozen in liquid nitrogen

and kept at -80⁰C. Frozen tissue was ground and total RNA was isolated using TRIzol

reagent (Sigma). Total RNA was allowed to hybridize with ^{32}-P-labeled complementary

RNAs (cRNAs) of unique fragments for aTRα, aTRβ, aNPDC-1 and axolotl ribosomal

protein L13A (aRPL13A).   Single-stranded RNA was digested away with RNAse,

hybridized fragments were separated on denaturing urea-polyacrylamide sequencing

gels, and gels were analyzed by autoradiography. As observed in Figure 35, all three

axolotl proteins (aTRα, aTRβ, and aNPDC-1) are highly expressed in brain and gill,

whereas lung is the only tissue tested that expressed all three proteins lowly. The

axolotl homolog of NPDC-1 was not detected in the spleen, and the liver had an

intermediate level of expression. On the other hand, aTRβ was poorly expressed in

both liver and spleen in addition to lung, and aTRα was lowly expressed in liver and

intermediately expressed in spleen. A possible explanation for the low levels of protein

expression in the lung is that the animals used to obtain the tissues were juvenile and
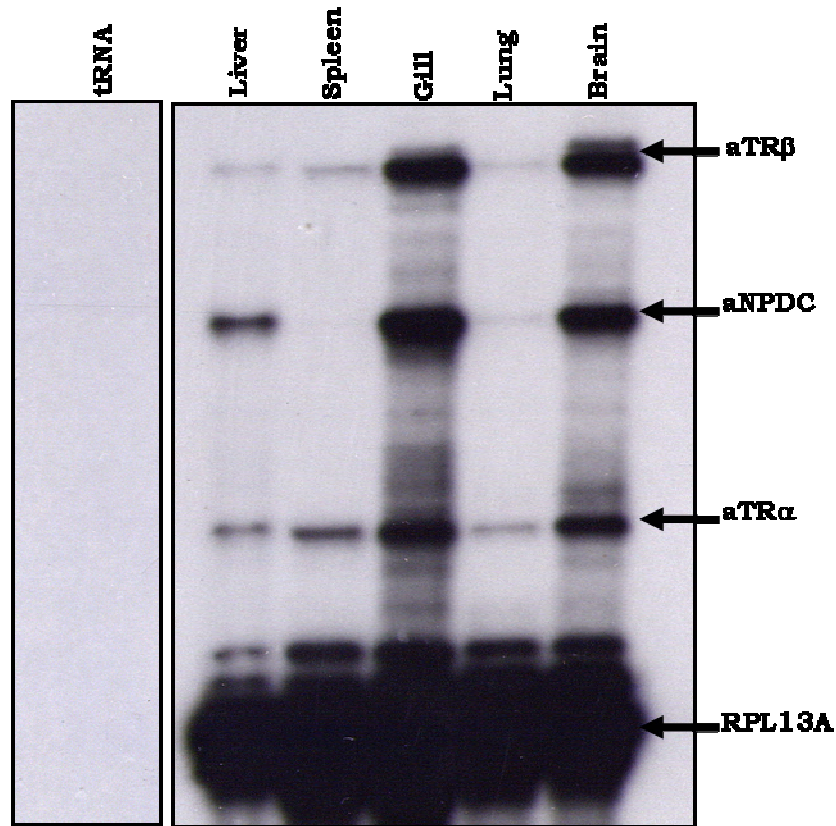
Figure 35: Tissue distribution of aTRα, aTRβ and aNPDC-1 in axolotl tissues. Tissues from adult axolotl were harvested and snap frozen. Frozen tissue was ground to powder under liquid Nitrogen. RNA was extracted using TRIzol reagent and the RNA was allowed to hybridise with ^{32}P-labelled complimentary RNAs (cRNAs) of unique fragments of aTRα, aTRβ, aNPDC-1 and aRPL13A (ribosomal protein L13A). Single straned RNA was digested away with RNAse and hybridized fragments were separated on denaturing urea-polyacrylamide sequencing gels. Gels were analysed by autoradiography.

therefore the lung was not fully developed. A different pattern of expression may be expected in metamorphosed axolotl.

A complimentary experiment to examine the tissue distribution of TRα and TRβ in axolotl tissue was performed by RT-PCR. Axolotl tissue cDNA was used in a PCR assay to examine tissue distribution. As shown in Figure 36, aTRα was observed to be expressed at high levels in the heart, and at lower levels in all other tissues examined, namely, brain, eye, spleen, skin, gill, spinal cord, lung, liver and muscle. The tissue distribution of aTRβ was examined and is shown in Figure 37. As shown in Figure 37, aTRβ was expressed highly in heart, eye, skin, gill, brain and spinal cord and at lower levels in spleen, lung, liver and muscle.

Developmental expression of aTRα and aTRβ

A wealth of information exists on the pattern of TRα and TRβ expression during amphibian metamorphosis. However, the embryonic developmental pattern of TRs in axolotl has not been previously reported. Using cDNA from different stages of axolotl embryonic development, PCR analysis was performed to determine the pattern of expression of aTRα and aTRβ. As shown in figure 38, aTRα is expressed in late embryogenesis at stage 27, and its levels drop in later stages and remain fairly constant at hatching and larva stage. In figure 39, the expression pattern of aTRβ in embryogenesis is shown. The levels of aTRβ appear to peak at stage 36 and drop gradually until hatching and remain constant at the larval stage 2 months post-hatching. At all stages though, aTRβ levels of expression are lower than aTRα levels, consistent with previously published results for other amphibians before metamorphosis.

Figure 36: aTRα tissue Expression in axolotl. **A.** Semi-quantitative RT-PCR to examine tissue distribution of aTRα in axolotl tissues. mRNA from the listed tissues was isolated, reverse transcribed into cDNA and analysed by PCR using aTRα specific primers (aTRα). As a control, RT-PCR analysis of axolotl elongation factor 1α (EF-1α) was assayed using the same cDNAs. PCR products were run on 1.2% agarose gels and analysed on a Kodak Gel logic gel imaging system. **B**. Using Gel Logic software (Kodak), the relative intensity of the aTRα specific PCR bands from A were normalized to the EF-1α bands for the various tissues.

Figure 37: aTRβ tissue Expression in axolotl. **A.** Semi-quantitative RT-PCR to examine tissue distribution of aTRβ in axolotl tissues. mRNA from the listed tissues was isolated, reverse transcribed into cDNA and analysed by PCR using aTRβ specific primers (aTRβ). As a control, RT-PCR analysis of axolotl elongation factor 1α (EF-1α) was assayed using the same cDNAs. PCR products were run on 1.2% agarose gels and analysed on a Kodak Gel logic gel imaging system. **B**. Using Gel Logic software (Kodak), the relative intensity of the aTRα specific PCR bands from A were normalized to the EF-1α bands for the various tissues.
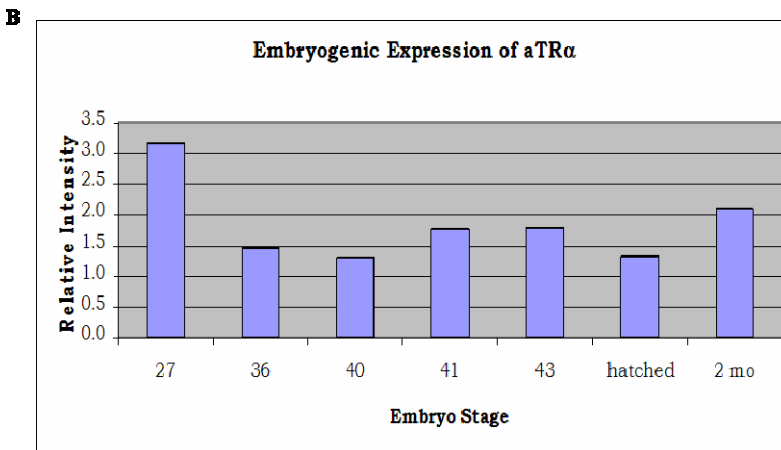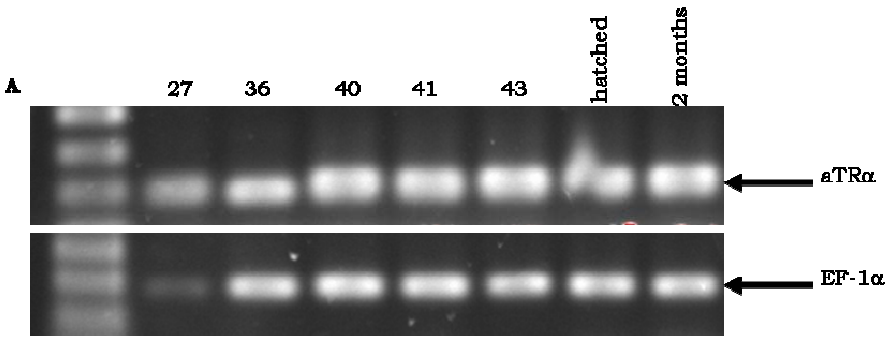
Figure 38: Developmental Expression of aTRα. A. Semi-quantitative RT-PCR to examine the expression of aTRα during embryogenesis in axolotl. mRNA from the listed embryo stages was isolated, reverse transcribed into cDNA and analyzed by PCR using aTRα specific primers (aTRα). As a control, RT-PCR analysis of axolotl elongation factor 1α (EF-1α) was assayed using the same cDNAs. PCR products were run on 1.2% agarose gels and analyzed on a Kodak Gel logic gel imaging system. **B**. Using Gel Logic software (Kodak), the relative intensity of the aTRα specific PCR bands from A were normalized to the EF-1α bands for the various embryo stages.

Figure 39: Developmental Expression of aTRβ. A. Semi-quantitative RT-PCR to examine the expression of aTRβ during embryogenesis in axolotl. mRNA from the listed embryo stages was isolated, reverse transcribed into cDNA and analyzed by PCR using aTRβ specific primers (aTRβ). As a control, RT-PCR analysis of axolotl elongation factor 1α (EF-1α) was assayed using the same cDNAs. PCR products were run on 1.2% agarose gels and analyzed on a Kodak Gel logic gel imaging system. **B**. Using Gel Logic software (Kodak), the relative intensity of the aTRα specific PCR bands from A were normalized to the EF-1α bands for the various embryo stages.

Expression of aTRα, aTRβ and aNPDC-1 during metamorphosis

To compare the pattern of expression of the axolotl TRs with that of aNPDC-1 during metamorphosis, tissues (a generous gift from Dr. Voss' lab) were obtained from juvenile axolotls treated with 150nM T4 for up to 28 days. Total RNA was isolated from the tissues and analyzed by RNAse protection assay for expression of aNPDC-1, aTRα, and aTRβ (Figure 40). Single-stranded RNA was digested away with RNAse, hybridized fragments were separated on denaturing urea-polyacrylamide sequencing gels and gels were analyzed by autoradiography. Overall, the levels of aNPDC-1 mRNA appear to decrease with T4 treatment in gill, skin and liver and increase with T4 treatment in lung. The levels of aTRβ mRNA were observed to decrease with T4 treatment in skin and increase with T4 treatment in gill. In lung and brain, the levels of aTRβ mRNA appear to peak at day 8 following T4 treatment and decrease at days 14 and 28. The levels of aTRα mRNA, generally expressed at lower levels than aTRβ and aNPDC-1 mRNA, were observed to decrease with T4 treatment in skin, appear unchanged in liver and gill, increase with T4 treatment in lung, and in brain appear to peak at day 8 and decrease after that. No clear pattern in expression emerges from this, although it must be noted that RNA levels do not always translate to protein levels. The changes observed here may not accurately correlate with changes in the protein levels for these mRNAs, because it is fairly well established that during metamorphosis TRβ levels increase (161), and that NPDC-1 can be post-translationally regulated through targeted protein degradation (99). In spite of these shortcomings, these data clearly demonstrate that TH can modulate the mRNA levels for these putative regulators of axolotl metamorphosis.

Day of T3 treatment

aTRβ aNPDC aTRα RPL13A

0 8 14 28 Skin
0 8 14 28 Liver
0 8 14 Gill
0 8 14 28 Lung
0 8 14 28 Brain
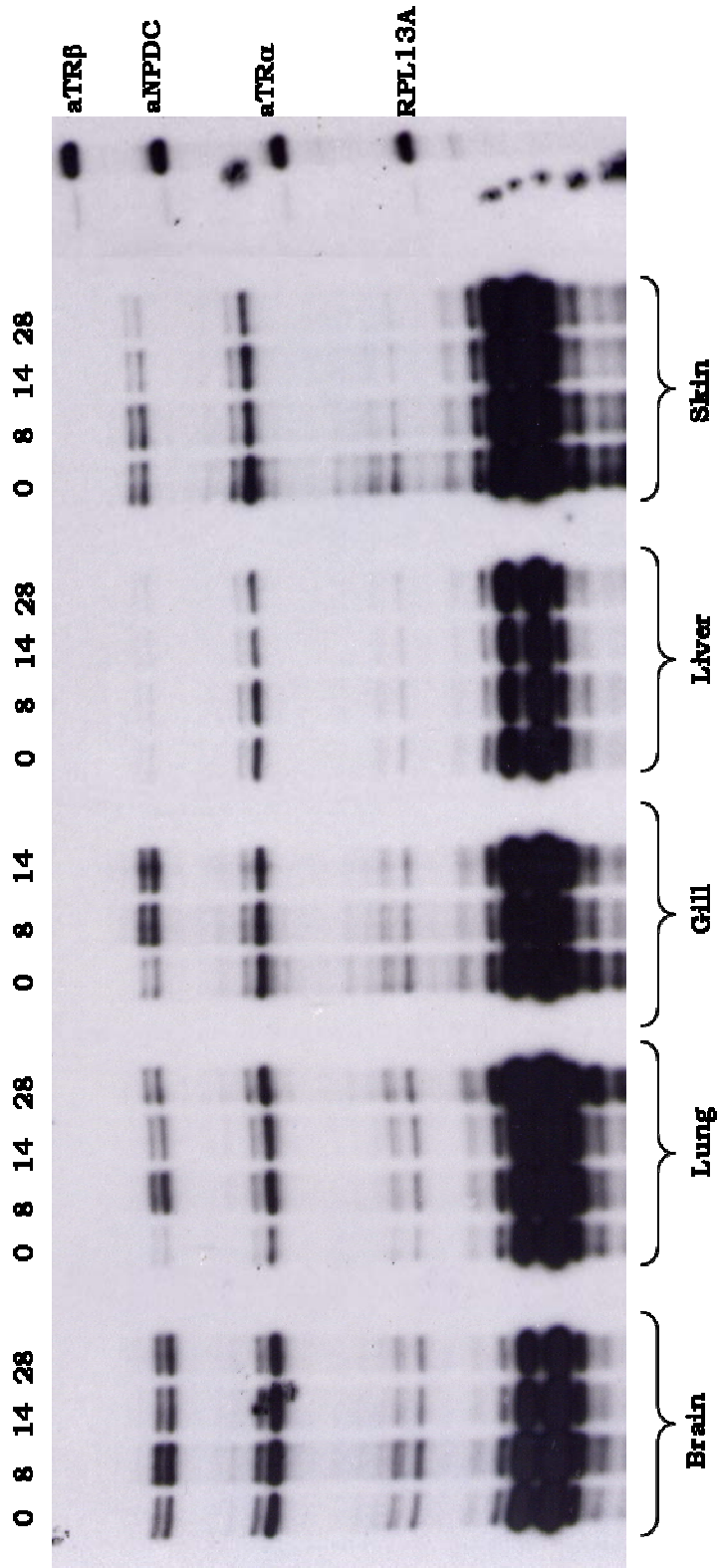
Figure 40: Expression of aTRα, aTRβ and aNPDC-1 during metamorphosis. Tissues from axolotl treated with 150nM T4 for up to 28 days were harvested at day 0, 8, 14 and 28 and snap frozen. Frozen tissue was ground to powder under liquid Nitrogen. RNA was extracted using TRIzol reagent and the RNA was allowed to hybridise with ^{32}P-labelled complimentary RNAs (cRNAs) of unique fragments of aTRα, aTRβ, aNPDC-1 and aRPL13A (ribosomal protein L13A). Single stranded RNA was digested away with RNAse and hybridized fragments were separated on denaturing urea-polyacrylamide sequencing gels. Gels were analysed by autoradiography.

Localization of the domain of interaction between aNPDC-1 and aTRs

Higher vertebrate NPDC-1 has been previously shown to directly bind retinoic acid nuclear receptors and this binding appears to directly impact gene expression events mediated by these receptors (95). In previous chapters of this dissertation I demonstrate that this is also the case with the axolotl RARγ. To investigate this same possibility for the thyroid receptor family members, recombinant protein pulldown experiments were performed. The first question investigated was whether axolotl NPDC-1 and axolotl TRs could interact. To address this question, we generated recombinant axolotl TR proteins that were tagged with GST at their amino terminus (Figure 41A). For each pull-down reaction, 10μg GST-aTRα or GST-aTRβ were bound to glutathione sepharose beads and allowed to interact with equimolar amounts of thioredoxin or thioredoxin-tagged aNPDC-1 proteins (figure 41C). Bead bound proteins were resolved by SDS-PAGE and immunoblotted. Blots were probed with an anti-Thio-probe polyclonal antibody (Santa Cruz). As shown in figure 41B, both full length aNPDC-1 and aNPDC-1 with its PEST domain deleted were able to complex with aTRα and aTRβ, but the aNPDC-1 protein with its helix-loop-helix domain deleted was not. These data suggest that the domain of interaction between aTRs and aNPDC-1 is at the amino terminus within amino acids 1-97, and are consistent with what was observed for binding of aNPDC-1 and aRARγ.

aNPDC-1 binding of aTRβ in cellular lysates

To further validate the above *in vitro* findings for aTR recombinant proteins, aNPDC-1 was examined for its ability to selectively bind aTRβ from cellular lysates. HEK293 cells were transfected with the expression vector pTriex or pTriex-aTRβ. The transfected cells were harvested 48h later, lysed in APB buffer in the presence of

A.

aTRα   aTRβ

B.

Thioredoxin
aNPDC-1
aNPDCΔPEST
aNPDCΔHLH
Thioredoxin
aNPDC-1
aNPDCΔPEST
aNPDCΔHLH

aTRα   aTRβ

C.

Thioredoxin
aNPDC
aNPDCΔPEST
aNPDCΔHLH
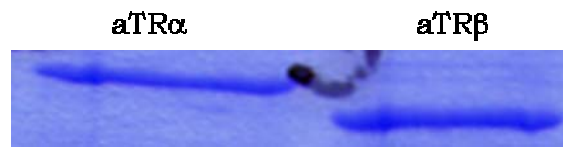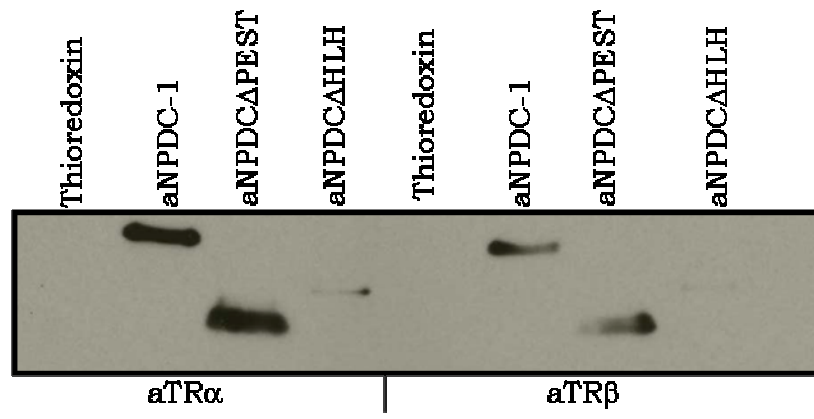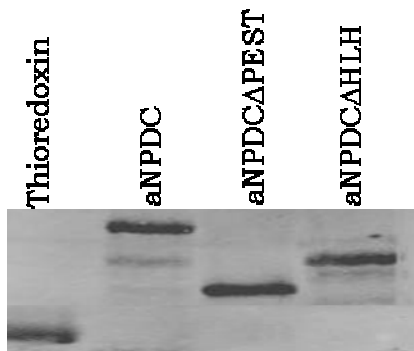
Figure 41: Direct *in vitro* interaction between aTRα, aTRβ and aNPDC-1. In panel A, recombinant glutathione-tagged axolotl TR protein is shown. 10μg of purified recombinant protein was resolved on a 10% SDS-PAGE gel and stained with Coomassie Blue. In panel B, 10μg of GST-TRα or 10μg of aTRβ were bound to glutathione beads and allowed to react with equimolar amounts of thioredoxin (Trx), aNPDC-1, aNPDCΔPEST and aNPDCΔHLH. Bead bound proteins were resolved on a 10% SDS-PAGE gel, and immunoblottled on nitrocellulose membranes. The membranes were then blotted with antisera for Thio probe. In panel C, equimolar amounts of thioredoxin and thioredoxin-tagged proteins were bound to S-protein beads and resolved on a 10% SDS-PAGE gel and immunoblotted for thioredoxin using anti-thioredoxin antisera.

nuclease and protease inhibitors and used in pulldown experiments. For the purposes of this experiment, full-length aNPDC-1 was cross-linked to cyanogen bromide activated sepharose beads. To bead-bound aNPDC-1, 1mg of extract was added and following incubation at 4^0C with rotation, the beads were washed with APB as described in materials and methods. The bead-bound proteins were resolved by SDS-PAGE and immunoblotted. The blots were probed with an anti-thio antibody (Santa Cruz). As observed in Figure 42 (top), aNPDC-1 is able to specifically complex with the aTRβ from the HEK293 lysates, whereas vector alone is not. Figure 42 (bottom), represents an equivalent amount of the two HEK lysates (from pTriex and pTriex-aTRβ cells) run in separate lanes and probed for cytoplasmic thiolase. These studies demonstrate that aNPDC-1 can specifically pull-down aTRβ from cellular lysates, suggesting that the interactions between aNPDC-1 and aTRβ are highly specific and most probably biologically relevant.

Analysis of the impact aNPDC-1 has on aTR DNA binding

My previous studies with aRARγ suggested that aNPDC-1 could directly impact aRARγ DNA binding. This potential was also investigated with respect to aTR DNA binding. The group that first identified and cloned the axolotl homologs for TRα and TRβ identified a *Xenopus* DR4 (xDR4) response element that could be bound specifically by axolotl TRs (108). The axolotl TRs were shown to bind to xDR4 only as TR-RXR heterodimers and the specificity of the binding was demonstrated by competition with cold and non-specific oligonucleotides (108). Due to the lack of any available cloned axolotl RXR, for experiments in this study I was relegated to using recombinant human RXRα. Although the sequence of axolotl RXRα is not known, it can be reasonably deduced that both the protein sequence and structure between
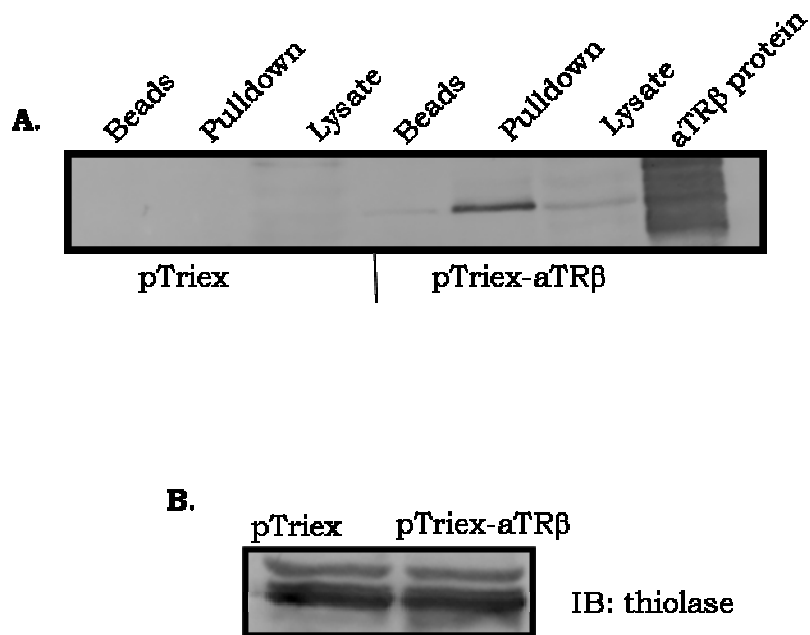
Figure 42: *In vivo* **interaction between aTRβ and aNPDC-1**. HEK293 cells were transfected with either pTriex or pTriex-aTRβ and the lysate was used in pulldown experiments. Full-length aNPDC-1, crosslinked to cyanogen bromide activated sepharose beads, was incubated with 1mg of cell extract. The bead-bound proteins were resolved by SDS-PAGE and immunoblotted onto nitrocellulose membrane and blotted with an anti-thioredoxin antibody. As observed in panel A, aNPDC-1 is able to specifically complex with aTRβ. Panel B, represents an equivalent amount of the 2 HEK lysates run in separate lanes and probed for cytoplasmic thiolase to demonstrate equal loading.

higher (such as human and rat) and lower vertebrates (such as *Xenopus* and axolotl) is highly conserved. This assumption is supported by the recent publication in which antisera developed to the human RXR isoforms were observed to specifically detect RXR protein isolated from the testis of the marbled newt *T. marmoratus marmoratus* (158).

In Figures 43 and 44, annealed oligonucleotides for *Xenopus* DR4 (108) were radiolabeled with ^{32}P using a Klenow fill-in reaction. The radiolabeled xDR4 was then allowed to complex with aTRα (43), aTRβ (44), hRXRα (43 and 44) and/or aNPDC-1 (43 and 44) recombinant proteins. The DNA/protein complexes were resolved on non-denaturing polyacrylamide gels and visualized by autoradiography. As shown in Figure 43, aTRα, while not able to complex with DNA effectively on its own (lane 2), forms a strong heterodimer complex with hRXRα (lane 3), which is competed effectively by the addition of excess cold xDR4 oligonucleotide (lane 4). Addition of recombinant aNPDC-1 (lane 5) results in the formation of a stronger upper complex than the one observed for TR/RXR alone. The recombinant aNPDC-1 mutants, aNPDC-1ΔPEST (lane 6) and aNPDC-1ΔHLH (lane 7), conversely do not appear to be able to impact aTRα/hRXRα heterodimer DNA binding.

In figure 44, the ability of aTRβ to complex with DNA was examined. Similar to the observations made for aTRα, aTRβ is not able to complex with DNA alone (lane 2), but is able to complex with DNA when heterodimerized with RXRα (lane 3). The complex can be competed away with cold oligonucleotide (lane 4). Also similar to the aTRα observations, addition of recombinant aNPDC-1 results in a stronger complex, but the mobility of this complex is not altered. The aNPDC-1 deletion mutants, aNPDC-1ΔPEST (lane 6) and aNPDC-1ΔHLH (lane 7), do not appear to impact the aTRβ/hRXRα heterodimer DNA binding. A similar effect to that observed with pure

	1	2	3	4	5	6	7
aTRα (pET)		x	x	x	x	x	x
hRXRα			x	x	x	x	x
aNPDC-1					x		
aNPDCΔPEST						x	
aNPDCΔHLH							x
cold oligo				x			

aTRα/hRXRα complex

aTRα/hRXRα complex

Free probe
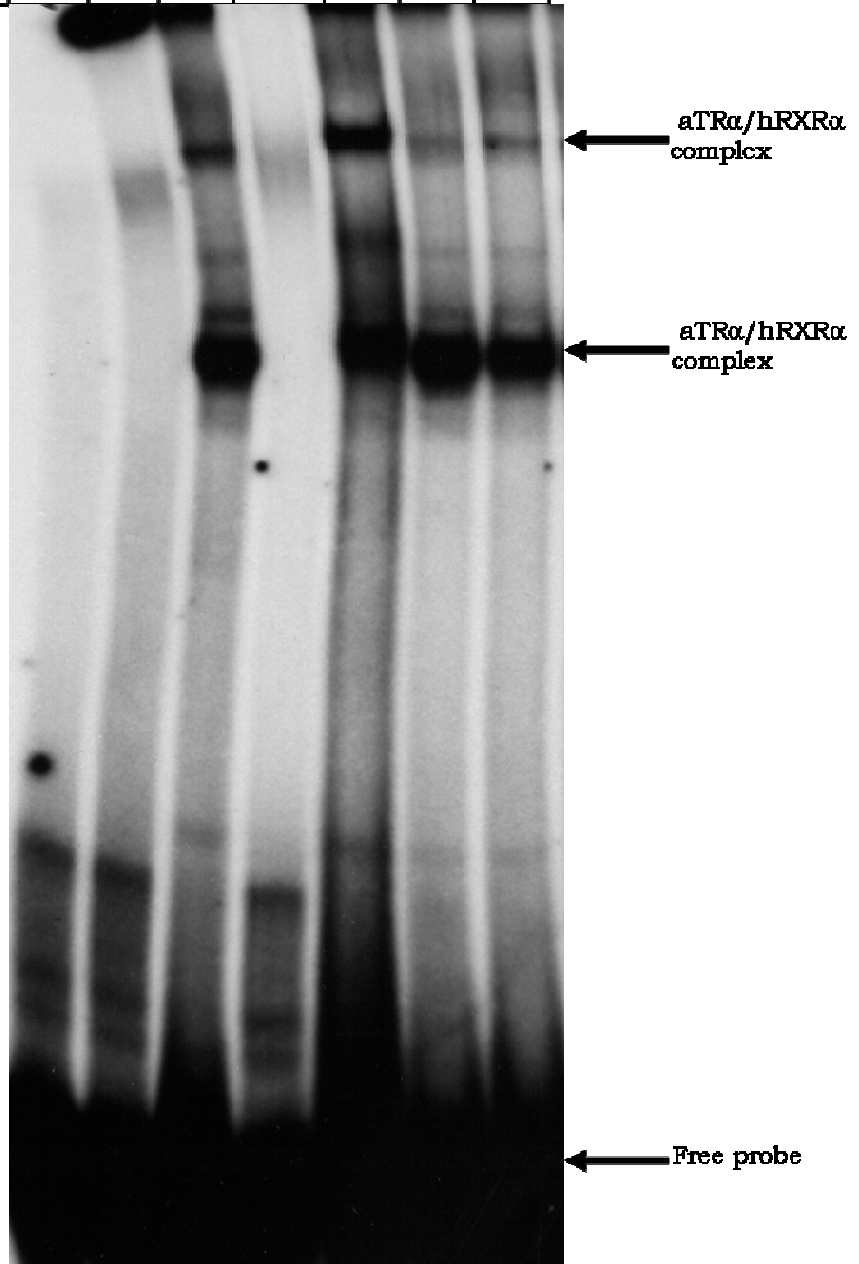
Figure 43: Electromobility Shift Assay with axolotl NPDC-1 and aTRα.

An oligonucleotide corresponding to xDR4 was radiolabelled with ^{32}P-dCTP with Klenow enzyme. The resultant DNA binding probe was incubated with recombinant hRXRα, aTRα, aNPDC-1, aNPDCΔPEST and aNPDCΔHLH. Unlebelled (cold) oligo was used to show specificity of binding. DNA:protein complexes were resolved on non-denaturing PAGE gels which were subsequently exposed to Kodak Xar-5 film.

*This is an accurate representation of an experiment performed at least three times.

	1	2	3	4	5	6	7	8	9	10	11	12
aTRβ (pET)		x	x	x	x	x	x	x	x			
hRXRα		x	x	x	x	x	x	x	x			
aNPDC-1					x				x			x
aNPDCΔPEST						x						
aNPDCΔHLH							x					
cold oligo				x							x	
Cos7 Nuc. X								x	x			
Axolotl Skin Nuc. X										x	x	x

TR/RXR complex

Free probe

- 160 -
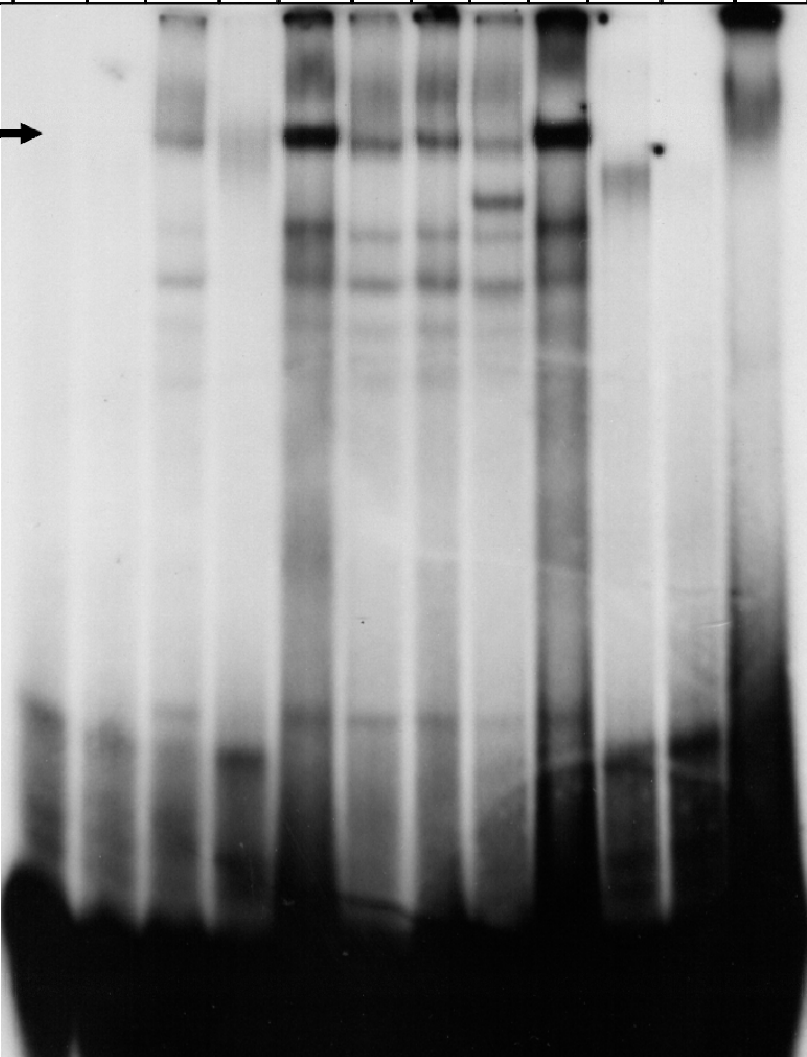
Figure 44: Electromobility Shift Assay with axolotl NPDC-1 and aTRβ.

An oligonucleotide corresponding to xDR4 was radiolabelled with ^{32}P-dCTP with Klenow enzyme. The resultant DNA binding probe was incubated with recombinant hRXRα, aTRβ, aNPDC-1, aNPDCΔPEST and aNPDCΔHLH or nuclear extract from either Cos7 cells or axolotl skin. Unlabelled (cold) oligo was used to show specificity of binding. DNA:protein complexes were resolved on non-denaturing PAGE gels which were subsequently exposed to Kodak Xar-5 film.

*This is an accurate representation of an experiment performed at least three times.

recombinant protein are observed in the presence of Cos7 nuclear extracts supplemented with recombinant protein. When aTRβ and hRXRα are added to Cos7 nuclear extracts (lane 8), no difference is observed compared to recombinant protein alone (lane 2). However, addition of aNPDC-1 (lane 9) results in a stronger upper complex than that of recombinant proteins alone. This observation is in agreement with recombinant protein data and with observations made for other response elements, such as the RARE (95).

I also examined axolotl skin nuclear extract for its ability to form shift complexes with the labeled xDR4 element in the absence of supplemented recombinant proteins (Figure 44, lane 10). Addition of lysates forms what appears to be a single shift complex, and cold oligonucleotide is able to compete this band away (lane 11). Interestingly, addition of aNPDC-1 recombinant protein results in a single more intense complex with altered mobility from that observed with extract alone (lane 12). Therefore, similar to the study with recombinant proteins, complex formation is enhanced by the addition of aNPDC-1, but unique to this study is the increased mobility of the complex and the increase size of the complex relative to that seen with recombinant proteins. These data suggest that the composition of axolotl DR4 binding complexes found in skin nuclear extracts vary substantially from that of recombinant TR/RXR proteins, and that aNPDC-1 impacts these complexes in a different manner. The ramifications of heterogeneity in axolotl skin nuclear extracts as well as the caveat of alternative TR/RXRs make it difficult to predict what is happening here, although if one hypothesizes that this DR4 is binding axolotl TR/RXR heterodimers in these skin extracts, then aNPDC-1 drastically changes the recruitment of proteins to this complex. Further investigation into the mechanisms of aNPDC-1 impact on nuclear receptor DNA binding is necessary to establish the potential for tissue-specific

TR/RXR/DR4 binding complexes in axolotl.

Preliminary analysis of the impact aNPDC-1 has on aTR gene expression events

In an attempt to assign a functional significance to the observed direct binding of aNPDC-1 to TRs and the alteration of aTR DNA binding by aNPDC-1, the impact of aNPDC-1 on aTR-mediated transcription was investigated. To study the transcriptional impact of aNPDC-1 on aTR, calcium phosphate technologies were used to co-transfect Cos7 cells with mammalian expression constructs for aNPDC-1, aTRα or aTRβ, a β-galactosidase expression plasmid, and a TRE-tk-luc reporter plasmid. Following transfection, the cells were stimulated for 24h with 1μM T3. After 24h the cells were lysed and assayed for luciferase and β-galactosidase activity. In Figure 45, the standardized luciferase values normalized to β-galactosidase are shown. In contrast to previous observations for other nuclear receptor family members, aNPDC-1 appeared to stimulate transcription mediated by both aTRα and aTRβ. This observation may be significant in further dissecting NPDC-1 signaling and determining a biological function/significance for this protein. NPDC-1 and thyroid receptors would appear to overlap in function through their shared roles in regulation of proliferation and differentiation, and these data suggest that this overlap is more than coincidental. Cumulatively these data suggest that aNPDC-1 may play a role in amphibian metamorphosis through its ability modulate transcription events mediated by thyroid hormone signaling pathways.

Impact of axolotl NPDC-1 on axolotl thyroid receptor-mediated transcription
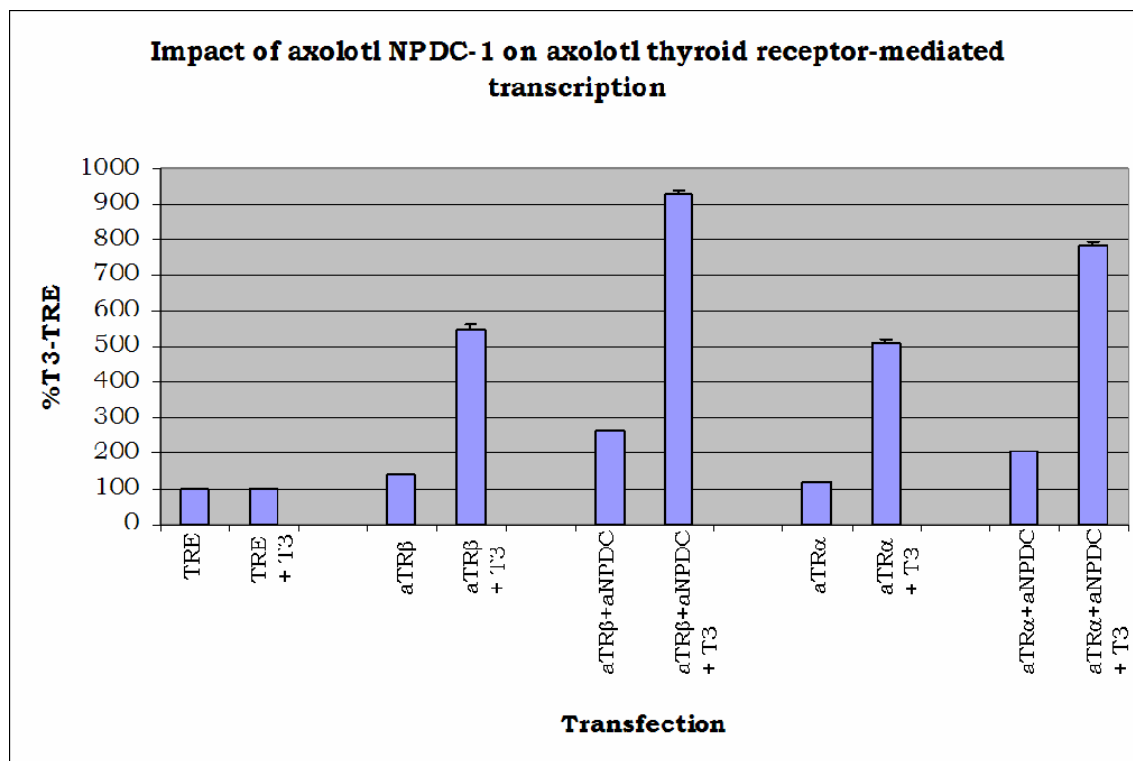
Figure 45: Impact of aNPDC-1 on aTR-mediated transcription. Cos7 cells were transiently transfected using calcium phosphate precipitation methods with mammalian expression plasmids for aNPDC-1, aTRα, aTRβ, TRE-tk-luc, β-galactosidase and treated with 1μM T3. Cells were lysed and assayed for luciferase and β-galactosidase activity. Luciferase data was normalized to β-galactosidase and data was reported as a % of the constitutive activation of the reporter plasmid (TRE).

* This result presents the average from experiments repeated at least four times.

Chapter 15: Discussion

The emphasis of my dissertation research was to investigate axolotl as a model system for studying the role of NPDC-1 in cellular and organismal differentiation events. Axolotl would appear to be an ideal system for doing this in that its life cycle transitions through 2 major differentiation events (embryogenesis and morphogenesis), these transition events can be readily reproduced in laboratory environments, and axolotls express a conserved homolog of NPDC-1. In the results from Chapter 14 I present strong circumstantial evidence that axolotl NPDC-1 can modulate the activities of the axolotl intracellular receptors for thyroid hormone. Amphibian metamorphosis is a developmental process governed by thyroid hormones. Prior to metamorphosis, the circulating levels of thyroid hormones are minimal (160) and increase slowly (161) peaking at metamorphosis (108), largely due to increased TSH levels (161). In nature, the importance and extent of metamorphosis across amphibian species varies (108) and alternative mechanisms such as neoteny and direct development can be found (108). The axolotl *Ambystoma Mexicanum* is a facultative neotenic species and is sexually mature as a larva, but the reason for this phenotype is not clear and seem dependent on environmental conditions (161). In the laboratory setting, most *Ambystoma* species undergo metamorphosis when treated with TH (108). The replacement of larval with adult tissues that occurs during metamorphosis results in organogenesis and tissue remodeling (159). Each of these processes involves a coordinated process of cell proliferation, differentiation and death (159). These functions of TH, echo similar functions by the NPDC-1 protein, which has been identified as a neuronal protein involved in cellular proliferation and

differentiation (89) and is described in previous chapters of this dissertation. Both proteins have also been shown to be expressed in CNS development (91, 170).

To investigate the impact of aNPDC-1 had on axolotl thyroid hormone signaling it was necessary that I first clone the axolotl homologs for the intracellular receptors for thyroid hormone. Conveniently the axolotl homologs for TRα and TRβ had recently been cloned and partially characterized, and the receptors we identified proved to be identical to those previously described receptors. While the presence of thyroid receptors in axolotl has been suspected for a while, it was not fully established until recently when Safi *et al* cloned the axolotl thyroid receptors and shown them to be functional (108). Axolotl TRs were shown to have strong homology to other known TRα and TRβ sequences, with the DBD and LBD being the most conserved. The sequence conservation between human and axolotl TR DBDs was observed to be 98% for aTRα and 92.5% for aTRβ. An overall sequence comparison between human, rat and axolotl TRα showed a consensus conservation of 83% and identity conservation of 66.5%, whereas axolotl TRβ does not appear to be as well conserved with consensus conservation of 68.4% and identity conservation of 49%. The sequence comparison for aTRβ is a little bit deceptive as most of the sequence deviation between human and axolotl TRβ occurs in the A/B domain due to the considerably larger size of hTRβ (108). Comparison of the sequence of axolotl TRα with axolotl TRβ shows that the protein is conserved between the two isoforms for the most part, with consensus conservation at 83% and identity conservation at 76.3%, deviating mostly at the amino terminus due to size differences between the two isoforms. The human isoforms are similarly conserved with consensus at 77.6% and identity at 76.6%, with the carboxyl terminus not being conserved between the two isoforms.

The tissue distribution of thyroid receptors is as varied in axolotl as it is in mammals. In mammals, TRα is expressed in brain, kidney and heart among other tissues (185, 186), and TRβ is expressed in most tissues (32, 187). In studies presented here, using RNAse protection and PCR technologies, I demonstrate that in axolotl, TRα mRNA is expressed in heart, and in lower levels in brain, eye gill, spleen, spinal cord, lung, liver, muscle and skin, whereas TRβ mRNA is expressed in high levels in heart, brain, eye, skin, gill and spinal cord and at lower levels in spleen, lung, liver and muscle. Due to the absence of an antibody that could recognize the axolotl form of TRs, it was not possible to examine TR expression at the protein level.

The expression of TR mRNA has been extensively studied in metamorphosis in amphibians. In the closely related species to axolotl, *Xenopus*, TRα mRNA levels appear to fluctuate from tissue to tissue within the tadpole during metamorphosis, but are always higher than TRβ mRNA levels, which appear to fluctuate mostly in the head portion and appear at a higher level in the tail at metamorphotic climax (181, 188-190). Data presented in this dissertation demonstrate that, in axolotl tissues, the level of expression of TRα mRNA appears to be lower than TRβ mRNA, and tissue-specific fluctuations were observed for both the TRα and TRβ mRNA expression. Tissue-specific correlations between *Xenopus* and axolotl TR mRNA expression during metamorphosis cannot be made, as the studies in *Xenopus* were performed with portions of tadpoles and not isolated tissues (181). However, if brain in axolotl is used to compare with the head portion of *Xenopus,* and axolotl liver with the middle portion, a comparison can be made. Brain TRα mRNA levels in axolotl showed temporal expression and were at lower levels than TRβ, whereas in *Xenopus,* head TRα mRNA was present at much higher levels than TRβ mRNA and remained unchanged during metamorphosis. In axolotl liver, both TRα and TRβ mRNA was virtually undetectable

whereas in *Xenopus* both TRα and TRβ mRNA displayed temporal expression. There are differences between these two amphibians and their TR expression, and this could simply be due to differences in their life cycle. Whereas *Xenopus* will undergo metamorphosis as part of its normal life cycle, axolotl is a neotenic amphibian and is sexually mature as a larva, and will not undergo metamorphosis unless exposed to exogenous TH.

Most coregulatory proteins mediate their regulation of transcription through modulation of the protein complexes that associate with specific DNA regulatory sequences in the promoters of genes they regulate (18). In the studies presented here, I demonstrate that the mechanism for aNPDC-1 regulation of aTR-mediated transcription appears complex and may involve both direct associations with aTR as well as facilitating complex formation on TR response elements. As observed previously for retinoid receptors [(95) and other sections of this dissertation], the presence of aNPDC-1 increased the intensity of the TR-RXR heterodimer complex binding to DNA, whereas the aNPDC-1 deletion mutants are not able to alter TR-RXR complex binding to DNA. The ability of aNPDC-1 to increase the intensity of TR-RXR complex binding to DNA was observed for both recombinant proteins and in the presence of Cos7 nuclear extract. Interestingly, when axolotl skin nuclear extract was used, addition of aNPDC-1 results in a mobility shift of the complex on xDR4. It appears that the effects of aNPDC-1 on DNA binding are variable depending on the origin of the protein (recombinant, cell or tissue). The mechanism for aNPDC-1 effects on DNA binding is unclear. One possibility is that NPDC-1 facilitates complex formation on the response element, but its loose associations are lost in the subsequent electrophoretic procedures. These observations with respect to aNPDC-1 and aTR DNA binding parallel what was observed for aNPDC-1 and aRARγ DNA

binding, in that complex formation is stronger in the presence of aNPDC-1. In contrast to what was seen with aRAR-aNPDC-1 binding, no unique TR-RXR-NPDC-1 complex was formed with purified proteins. Cumulatively these data demonstrate that aNPDC-1 alters the DNA binding capabilities of the TR-RXR heterodimer, although the mechanism for this is unknown.

The potential functional consequences of aNPDC-1 binding of aTRs and its impact on aTR DNA binding appear to be a stimulation of aTR-mediated transcription events. Here, I show that aTRs are able to stimulate transcription off of a human thyroid receptor response element, and this observation is in agreement with what has been reported elsewhere for axolotl TRs (108). Unique to these dissertation studies are the demonstration that TR-based DR4-driven reporter gene transcription can be stimulated by overexpression of aNPDC-1 in an *in vitro* transcription analysis. This observation is not only unique with respect to what has been reported for aNPDC-1 effects on aTRs, but also unique with respect to what has been reported for NPDC-1 effects on nuclear receptor-mediated transcription. All previous reports demonstrate NPDC-1 to be a repressor of retinoid receptor-mediated transcription (95), and studies in this dissertation demonstrate that aNPDC-1 can also repress axolotl RARγ-mediated transcription. Retinoid and thyroid receptors are both members of the same subfamily of nuclear receptors, are bound to DNA in the absence of ligand, and activate transcription as heterodimers. It is therefore surprising, that NPDC-1 has opposing effects on these receptors. These analyses were performed in the Cos7 mammalian cell line, expressing axolotl TR and NPDC-1 proteins. It is difficult to predict if the axolotl proteins were correctly expressed and processed correctly in the mammalian cells. Ideally, these studies would be repeated in an axolotl cell line, but due to the lack of established axolotl cell lines and transfection limitations, we were limited to the

Cos7 cell line. A more reasonable explanation for these contrasting responses would be the specific recruitment of alternative coregulatory complexes by the binding of NPDC-1. Thyroid and retinoid receptors have been shown to associate with a variety of coregulatory complexes (28-30, 84-87), and it can be hypothesized from the observations in this dissertation that, at least in the axolotl system, NPDC-1 functions as a molecular target for either co-repressor complexes in the case of retinoid receptors or co-activator complexes in the case of thyroid receptors. Further studies will be required to establish the validity of this hypothesis. Cumulatively, these studies suggest aNPDC-1 can modulate gene expression events mediated by aTRs and that this ability could have direct implications in the regulation of metamorphosis in axolotls.

In summary, this study presents the first evidence for interaction between NPDC-1 and TR in axolotl. The axolotl is an interesting system that allows the study of development and differentiation, functions that are impacted by both TR and NPDC-1. It is the first report of NPDC-1 acting as an activator of transcription and may help shed some light into the biological function of NPDC-1 and its biochemical mechanism. This study showed that axolotl TRs are able to activate transcription off a human DR4 element, and aNPDC-1 is able to bind to aTRs and stimulate transcription. Future directions for these studies include the utilization of the axolotl as a model organism for the study of aTR and aNPDC-1 interactions *in vivo* and their impact on differentiation and morphogenesis. Overall, these studies are the first step towards dissecting the impact of NPDC-1 on TR mediated signaling in development and differentiation.

SECTION VI: PLACING EVERYTHING INTO CONTEXT

The studies in this dissertation attempt to address the hypothesis that the nuclear receptor coregulatory protein NPDC-1, through its ability to modulate transcription events mediated by retinoid and thyroid families of nuclear receptors, can serve as a critical regulator of differentiation and development in the axolotl animal model system. Towards establishing the validity of this hypothesis I cloned and/or characterized axolotl homologs for; a) the nuclear receptor co-regulatory protein aNPDC-1, whose expression appears to be linked to the regulation of differentiation; b) a nuclear receptor critical for axolotl embryonic differentiation and development, aRARγ; and c) the nuclear receptors critical for axolotl morphogenesis, aTRα and aTRβ. These homologs were shown to be structurally homologous to those found in higher vertebrates and to contain all of the functional domains identified in these higher vertebrate forms.

Examination of the tissue-specific expression and distribution of these axolotl homologs for NPDC-1, RARγ, TRα and TRβ suggested that these various regulators of differentiation and morphogenesis might be functionally related. Axolotl NPDC-1 is expressed in brain, heart, eye, skin, gill, spinal cord, lung, liver and muscle whereas aRARγ is expressed in heart, eye, gill, skin and lung. The TRs add another level of complexity to analyzing the associations between these three proteins. Axolotl TRα was detected in heart, brain, eye, spleen, gill, spinal cord and lung whereas TRβ was found in all tissues aTRα was detected and in skin, liver and muscle. Overall, all axolotl tissues examined with the exception of spleen were observed to express all four proteins, but some seem better suited for further studies by strongly expressing all four proteins, and these are heart, eye and skin. Tissues expressing all four proteins

may be utilized in future experiments to study the impact of aNPDC-1 on RARγ and TR target genes during development.

The developmental expression of these proteins also varies. For example, aRARγ mRNA expression peaks early in development and then reduces at hatching and increases again at the larval stage while aNPDC-1 mRNA appears to peak slightly later in axolotl embryogenesis and maintain this level of expression through hatching and into larval development. Levels of Thyroid receptors were examined after stage 27 by which time aRARγ levels have decreased and aNPDC-1 levels are on the rise. Levels of aTRβ mRNA are detectable at stage 27 and peak at stage 36 and gradually decrease until hatching and remain at constant levels 2 months post-hatching at the larva stage, whereas aTRα levels are detected at higher levels than aTRβ, are first detected at stage 27, are observed to decrease by stage 36 and remain at low levels until hatching and remain relatively constant post-hatching. The developmental expression pattern of these mRNAs suggests that gene expression events regulated by RARγ occur early during embryogenesis and are down-regulated just prior to hatching when gene expression events regulated by NPDC-1 and TRs begin to peak. This, perhaps not coincidentally, also parallels the impact aNPDC-1 has on gene expression events mediated by aRARγ and the aTRs, wherein it was observed that aNPDC-1 repressed retinoic acid-mediated signaling events but facilitated thyroid hormone-mediated signaling events. Further studies will be required to establish any direct links between NPDC-1, these signaling pathways and axolotl development.

Having established the possibility that these various regulators of differentiation and development overlap in both the tissues and the times of development they are expressed, I next examined whether there might be any direct linkage between these proteins. Using pull-down and immunoprecipitation analyses I was able to

demonstrate that aNPDC-1 interacts directly with aRARγ, aTRα and aTRβ. The interaction between these nuclear receptors and aNPDC-1 was localized to the amino terminus of aNPDC-1 between amino acids 1 and 97. This region of the aNPDC-1 protein houses a conserved LXXLL motif, a HLH domain and a coiled coil domain. Further studies are necessary to determine the exact domain where aNPDC-1 and nuclear receptors interact.

Having established this potential for direct interactions between aNPDC-1 and these nuclear receptor mediators of differentiation and development, I then performed 2 sets of experiments to investigate the possible functional consequences of these direct interactions between aNPDC-1 and the axolotl nuclear receptors. The first of these was an analysis of the impact aNPDC-1 had on nuclear receptor DNA binding. It was observed that aNPDC-1 modified DNA binding by both retinoid and thyroid receptors. In agreement with previous studies, addition of aNPDC-1 recombinant protein resulted in stronger RAR-RXR DNA binding. Contrary to previous observations for human NPDC-1 and RARs where no altered complex mobility was observed (95), addition of aNPDC-1 also resulted in a complex with altered mobility, consistent with the formation of a RAR-RXR-NPDC-1 complex. With respect to aTRs aNPDC-1 resulted in stronger TR-RXR complex without altering complex mobility.

In a second set of experiments looking at function, I analyzed the impact aNPDC-1 had on transcription events mediated by the aTR and aRAR nuclear receptors. These studies revealed that the effects of aNPDC-1 on aRARγ and aTR mediated transcription was varied. Previous studies have shown hNPDC-1 to be a strong repressor of RAR and RXR mediated transcription (95). Axolotl NPDC-1 was also found to repress aRARγ-mediated transcription, but not as strongly as the human homologs. With human proteins a 90% repression was observed for RAR and RXR,

whereas with axolotl proteins only 30% repression was detected. This discrepancy may be due to a number of factors such as proper processing of axolotl proteins in mammalian cells and the different isotypes examined. On the other hand, aNPDC-1 was observed to stimulate axolotl thyroid receptor-mediated transcription for both TR homologs. Transcription mediated by aTRβ was stimulated 200%, whereas transcription mediated by aTRα was stimulated 163%. This observation is consistent with a proposed role for aNPDC-1 as a propagator of axolotl morphogenesis.

Therefore, the studies presented in this dissertation strongly implicate axolotl NPDC-1 in the regulation of gene expression events mediated by the axolotl retinoid and thyroid receptors. As extensively discussed in the introduction and throughout this dissertation, these 2 families of nuclear receptors play critical roles in both higher and lower vertebrate embryogenesis and development. The axolotl is an ideal animal model system for studying differentiation and development because its life cycle is conveniently segmented into embryogenesis, larval development, morphogenesis and adult development. Furthermore, the axolotl species used in these studies is neotenic, meaning it does not spontaneously go through morphogenesis, but will go through normal morphogenesis upon stimulation with thyroid hormone. This provides the selective experimental advantage of accurately staging the morphogenesis events in a laboratory setting. These reasons cumulatively were the impetus behind my research investigation into the potential utility of the axolotl for studying the molecular mechanism of NPDC-1. Marrying my preliminary studies of characterizations of NPDC-1, RAR and TR expression and function, with what has previously been reported for both axolotl and other amphibian embryogenic and morphogenic events, I have developed a potential model for aNPDC-1's role in these events (Figure 46). This model hypothesizes that NPDC-1 may serve as a critical moderator of retinoid and

thyroid signaling pathways during embryogenesis and morphogenesis. The 3 major molecular keys to this hypothesis are the data supporting NPDC-1 as a repressor of cellular proliferation events, data supporting NPDC-1 as a repressor of retinoid signaling events and data supporting NPDC-1 as a stimulator of thyroid hormone signaling events. Based upon the embryonic and developmental patterns of aNPDC-1 expression, aRARγ expression and aTR expression, one can hypothesize that during early embryogenesis of axolotls when cellular proliferation and patterning occurs, aRARs would be highly expressed and aNPDC-1 expression would be suppressed. This is reflective of what others have shown for aRARγ mRNA expression (106) and what I show in this dissertation for aNPDC-1 expression. At the molecular level, data presented in this dissertation coupled with our previously published (95) and unpublished data would suggest this expression pattern is more than coincidental, in that we are able to demonstrate that aNPDC-1 can directly bind and inhibit gene expression events mediated by aRARγ, and in unpublished studies looking at the human NPDC-1 gene promoter we have identified a highly specific RAR response element. These data support the possibility that aRARs may be involved in some coordinate upregulation of aNPDC-1 expression and that upregulation of aNPDC-1 may be involved in some coordinate down-regulation of aRAR-mediated events. This is represented in the model posed in Figure 46 wherein it is seen that during embryogenesis (stages 1-43) of the axolotl aRARγ expression is shown to initiate early, peak and then decline as the embryo nears hatching (stage 43). On the other hand, data for aNPDC-1 expression would suggest it begins to take off slightly later in embryogenesis, peak around hatching and remain at a moderately high level of expression.

aTRα
aTRβ
aRARγ
aNPDC-1

Larva

Stage 38
Stage 27
Stage 13
Stage 1
0

Metamorphosed axolotl

Day 0
Day 8
Day 14
Day 28
0
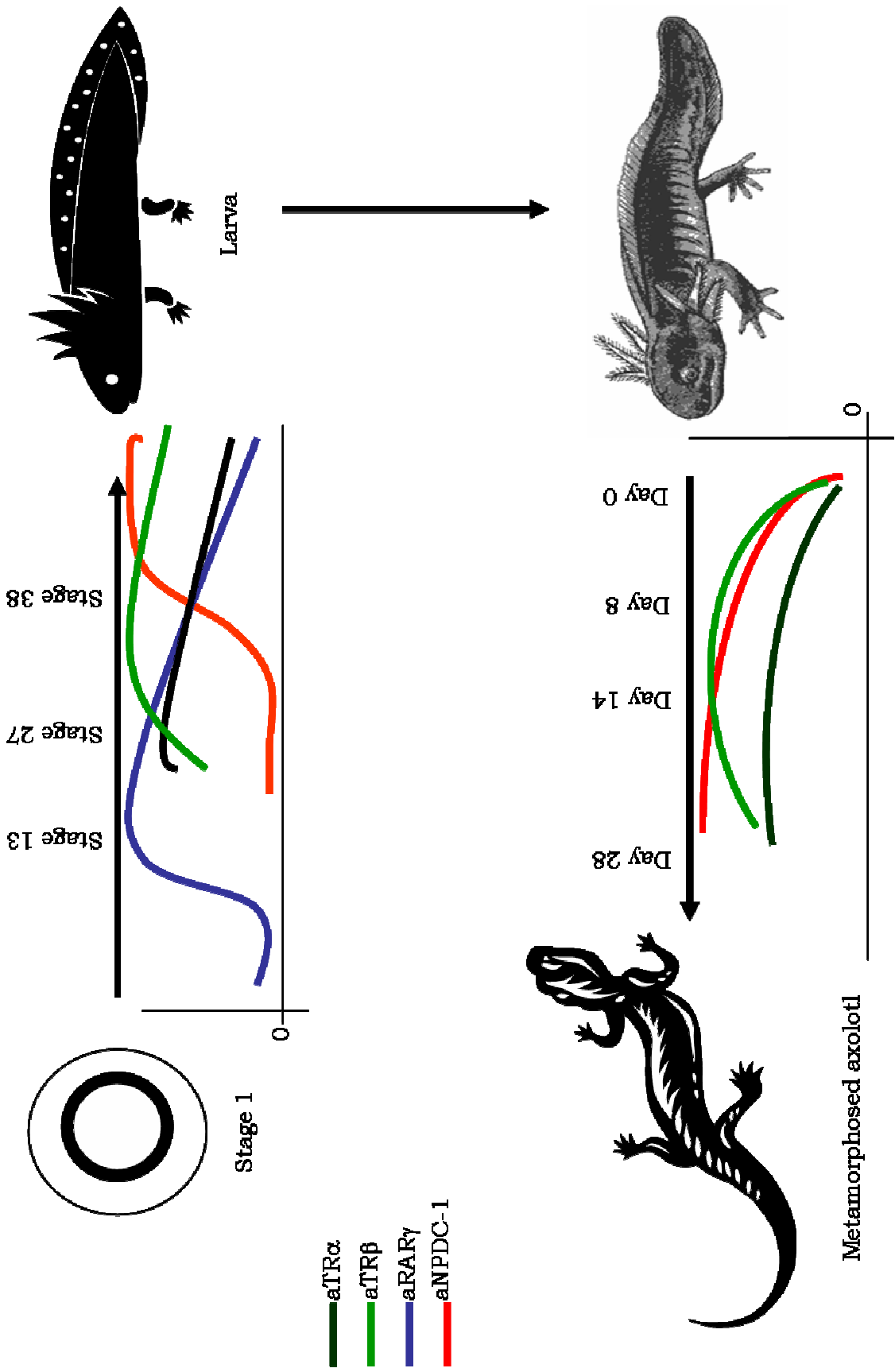
**Figure 46: Proposed model of the role of aNPDC-1 in the signaling of aRARγ and aTRs**. aNPDC-1 may act as a moderator of embryogenesis and morphogenesis in axolotl.

The story for thyroid signaling is less clear primarily due to the limited data available, but combining what I have been able to demonstrate in this dissertation along with extrapolations from other published reports (181, 188-190) one might hypothesize that molecular signaling by thyroid hormone at onset of morphogenesis is strongly facilitated by the presence of NPDC-1. This is depicted in Figure 40 where in axolotl lung, a tissue that undergoes extensive changes during morphogenesis, aNPDC-1 expression appears to increase while aTRβ levels display a temporal expression. At day 8 following T4 treatment, aNPDC-1 and aTRβ levels both increase in axolotl lung, but while aNPDC-1 levels continue to remain high during metamorphosis, aTRβ levels decrease. The levels of aTRα also appear to increase in axolotl lung by day 8 of metamorphosis and remain higher than at day 0 (before T4 treatment).

There are obviously a lot of gaps in this model of NPDC-1's role in regulation of differentiation and development in axolotl, and many other components in this process that need to be married into the model. Fortunately, with the tools developed through the studies in this dissertation we may now be able to more accurately define the molecular role of this highly conserved nuclear receptor coregulatory protein. With this in mind I have listed several future directions that expand on the studies and findings from this dissertation.

FUTURE STUDIES

The preliminary studies presented in this dissertation suggest the hypothesis that aNPDC-1 plays a critical role in aRAR- and aTR-mediated gene expression events. To establish this hypothesis it will be important that these preliminary data be expanded in a more comprehensive manner. The axolotl is a convenient system for

doing this in that tissues can be cultured *in vitro*. Therefore, the proposed studies would be to investigate the effects aNPDC-1 small interfering RNAs (siRNAs) have on global gene expression in various hormone-treated, *in vitro* cultured axolotl tissues or primary cell cultures. Micro array analyses could be tailored toward a variety of RAR-specific, TR-specific and other developmentally regulated genes. These studies would be expected to provide direct evidence for NPDC-1 regulation of molecular events associated with axolotl development, as well as potential tools for further dissecting the molecular mechanism by which NPDC-1 is regulating these events.

Pending the results of these *in vitro* analyses, this work could be expanded to look at the effects of conditionally knocking out the aNPDC-1 gene in axolotl. A variety of published data strongly support a role for NPDC-1 in brain development. Similarly, both retinoids and thyroid hormone have been implicated in the embryonic and premetamorphic patterning of axolotl brain. Therefore, the experiment would be to either conditionally knock out or overexpress the aNPDC-1 gene in a tissue-specific manner. A cre-loxP system could be used to conditionally knockout the gene, and if the cre recombinase was placed under an inducible and neuronal-specific promoter, the activity could be targeted to the brain. Alternatively, overexpression of NPDC-1 could be accomplished by creating transgenic axolotls that have a ligand-inducible (e.g. doxycycline) aNPDC-1 gene inserted into their genome. The developmental impact of knocking out and over-expressing NPDC-1 could be monitored, both organismally and molecularly (e.g. using similar gene array analyses mentioned above), in the presence and absence of hormone-stimulated events.

At the molecular level, perhaps the most surprising observation made in my dissertation studies is the intriguing possibility that axolotl NPDC-1 can function as both a nuclear receptor co-repressor or co-activator protein, dependent upon which

receptors transcriptional apparatus it is associating with. What makes this finding even more interesting is the observation that these antagonistic activities can occur in the same host cell, suggesting that it is either something innate to aNPDC-1's interaction with the specific nuclear receptor, aNPDC-1's interaction and recruiting of other coregulatory proteins, or something unique in the response elements used in these studies. To investigate these possibilities I would propose a set of experiments that specifically elucidate the protein complexes that associate with the 2-specific response elements (βRE and TRE) used in my dissertation studies. These experiments could be accomplished using matrix-associated (e.g. biotin labeled response elements and streptavidin agarose) pull-down purification technologies to isolate proteins associated with these response elements, and proteomic technologies to specifically identify these proteins.

In summary, these studies are the first step towards assigning a measurable, biological function to NPDC-1. The evolutionary conservation of NPDC-1 suggests an important role for this protein in development and differentiation of organisms, but so far the biochemical mechanism for this protein is yet to be fully determined. Hopefully, these studies shed some light on the biochemical function of NPDC-1 and provide a model system and template for establishing NPDC-1's molecular mechanism for regulation of differentiation.

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Publications:

Abstracts

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