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Todd HODGSON

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

M.Sc. (Biochemistry spec. HMG)

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Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

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Alpha-Thalaseemia Mental Retardation (ATR-X) Syndrome : Elucidating Cellular Functions of the ATR-X Gene

D. Picketts

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

CO-DIRECTEUR DE LA THÈSE - THESIS CO-SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

D. Gray

M. Holcik

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

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Alpha-Thalassemia Mental Retardation (ATR-X) Syndrome: Elucidating Cellular Functions of the ATRX Gene

By

Todd R. Hodgson

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Abstract

Mutations in the ATRX gene are responsible for the alpha-thalassemia mental retardation (ATR-X) syndrome. ATRX is a putative global transcription regulator and chromatin remodelling protein. The goal of this research is to characterize interactions ATRX has with other proteins involved in transcription regulation, and identify domains in ATRX that may be responsible for these interactions. Several stable NIH 3T3 tet-off cell lines have been established that contain a human ATRX transgene. In addition, ATRX, PML, and Daxx appear to co-localize in nuclear bundles, suggesting they may act together transiently, or in a complex, in a regulatory role. A domain has been identified on ATRX that appears to target the protein to nuclear bundles, and interact with PML and Daxx. ATRX patient mutations appear to alter these interactions. This work attempts to elucidate cellular functions of ATRX, in hopes of establishing a better understanding of the neuropathology of this complex disease.

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List Of Abbreviations

A...adenine (when referring to nucleic acids)
A...alanine (when referring to proteins)
aCoA...acetyl coenzyme A
APL...acute promyeolytic leukemia
Asn...asparagine
Asp...aspartic acid
ATP...adenosine-5'-triphosphate
ATPase...adenosine-5'-triphosphatase
ATR-X...Alpha-Thalassemia Mental Retardation Syndrome
ATRX...Alpha-Thalassemia Mental Retardation Syndrome gene or gene product (also called XH2 or XNP)
ATRXHA...HA-tagged ATRX
bp...nucleotide base pairs
BSA...bovine serum albumin
C...cytidine (when referring to nucleic acids)
C...cysteine (when referring to proteins)
cAMP...cyclic adenosine-5'-monophosphate
CAT...computerized axial tomography
CBP...chromatin binding protein, or
CBP...cAMP response element binding protein
cDNA...complementary deoxyribonucleic acid
CMV...cytomegalovirus
CNS...central nervous system
Co-IP...coimmunoprecipitation
C-Terminal...carboxylic acid end of protein
D...aspartic acid
DAPI...4',6-diamidino-2-phenylindole
DMEM...Dulbecco's Minimal Essential Medium
DMSO...dimethyl sulfoxide
DNA...deoxyribonucleic acid
DNMT...DNA methyl transferase
dNTP...deoxynucleotide-5'-triphosphate
ECL...enhanced chemoluminescence
E.coli...Escherichia coli
EDTA...ethylenediaminetetraacetic acid
EGFP...enhanced green fluorescent protein
FBS...fetal bovine serum
G...guanine (when referring to nucleic acids)
G...glycine (when referring to proteins)
GFP...green fluorescent protein
Glu...glutamic acid
Gly...glycine
GR...glucocorticoid receptor
H1...histone 1

H2A...histone 2A
H2B...histone 2B
H3...histone 3
H4...histone 4
H5...histone 5
HA...hemagglutinin nonapeptide epitope
HAT...histone acetyltransferase
HbH...hemoglobin β4-tetramer
HDAC...histone deacetylase
HIPK1...homeodomain interacting protein kinase 1
HP1...heterochromatin protein 1
HRP...horse radish peroxidase
HygB...hygromycin B
IF...immunofluorescence
IP...immunoprecipitation
IPTG...isopropyl β-D-thiogalactopyranoside
ISWI...imitation SWI
K...lysine
kDa...kiloDalton
kbp...kilobase pair
LB...Luria-Bertani
Leu...leucine
LN_2...liquid nitrogen
Lys...lysine
MBD...methyl binding domain
MCS...multicloning site
MDa...megaDalton
MeCP...methyl CpG-binding protein
MRI...magnetic resonance imaging
mRNA...messenger ribonucleic acid
MRX...primary/nonspecific X-linked mental retardation
MRXS...syndromic primary/nonspecific X-linked mental retardation
ND10...nuclear domain 10 (also PML body or POD)
NLS...nuclear localization signal
nm...nanometres
N-Terminal...amino end of protein
P...proline
PBS...phosphate-buffered saline
P_{CMV}...immediate early CMV promoter
PCR...polymerase chain reaction
PHD...plant homeodomain
PML...promyeolytic leukemia protein
P_{minCMV}...minimal CMV promoter
PMSF...phenylmethylsulfonyl fluoride
POD...promyeolytic oncogenic domain (also PML body)
R...arginine

RARα...retinoic acid receptor α
rDNA...ribosomal deoxyribonucleic acid
RhoGTPase...rhodamine guanine-5'-triphosphatase
RNA...ribonucleic acid
rtetR...reverse tetracycline repressor
RT-PCR...reverse transcriptase polymerase chain reaction
rtTA...reverse tetracycline transactivator
SAP...shrimp alkaline phosphatase
Ser...serine
SDS-PAGE...sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNF...sucrose non-fermenting
SWI...mating type switching
T...thymine
TAE...tris-acetate/EDTA buffer
TBST...tris-buffered saline with Tween 20
TD1...Targeting Domain 1
TD2...Targeting Domain 2
TEMED...N,N,N',N'- tetramethylethylenediamine
tetO...tetracycline resistance operator
tetR...tetracycline repressor
TF...transcription factor
TRD...transcription repressor domain
TRE...tetracycline response element
tTA...tetracycline transactivator
V...valine
VP16...virion protein 16
XLMR...X-linked mental retardation
Y...tyrosine

1 – Introduction

1.1 – X-Linked Mental Retardation.

Mental retardation affects approximately 2% of the population, with males being affected twice as often as females (Gibbons *et al.*, 1995a). Years of research have shown that many of the genes responsible for brain differentiation and function are X-linked (Ropers *et al.*, 2003). In fact, it is estimated that greater than 100 genes on the X-chromosome may be implicated in mental retardation (Stromme *et al.* 2002). As of the year 2000, over 200 X-linked mental retardation (XLMR) conditions had been described (Chiurazzi *et al.*, 2001). It is common in females who carry a defective X-linked mental retardation gene to have a highly skewed pattern of X-inactivation. This is probably because the mutation decreases viability of the affected cell and gives the cell that inactivates the mutant X-chromosome a growth advantage, thus a significant deviation from the normal 50:50 ratio of X-inactivation is observed (Raynaud *et al.*, 2000; Plenge *et al.*, 2002). Nonetheless, carrier females may exhibit mild effects of mental retardation, depending on the skewing pattern of X-inactivation in important tissues of neural development (Raynaud *et al.*, 2000).

Finding genes responsible for mental retardation is difficult, due mostly to mutations in different genes giving rise to heterogeneous phenotypes, or single mutations that lead to a variety of phenotypes (Gibbons *et al.*, 1995a). Researchers will often utilize phenotype splitting, whereby they will set out very specific diagnostic criteria, and use only those patients who fulfill it completely. Mental retardation that follows strict

diagnostic criteria of congenital abnormalities is generally defined as syndromic mental retardation (Gibbons *et al.*, 1995a).

The designation of syndromic XLMR (MRXS) versus non-syndromic XLMR (MRX) can be very confusing, as MRX conditions are sometimes redesignated as MRXS (Chiurazzi *et al.*, 2000). Generally, a syndrome is designated as such because of a clinically recognizable pattern of physical, neurological, and/or metabolic defects (Chiurazzi *et al.*, 2000; Ropers *et al.*, 2003). In pedigrees where the only constant symptom is XLMR, the MRX designation is assigned (Chiurazzi *et al.*, 2000; Ropers *et al.*, 2003). Also, it appears that genes responsible for syndromic XLMR generally code for multifunctional or wide target range proteins, whereas genes responsible for MRX generally code for proteins with less or more specific tasks (Chiurazzi *et al.*, 2000).

XLMR has numerous causative mechanisms. DNA fragile sites are implicated in Fragile X syndrome. Patients have large expansions of methylated CGG trinucleotide repeats (>200 repeats) in the promoter region of the FMR1 gene, which leads to methylation and transcriptional silencing of the gene (Verkerk *et al.*, 1991; Berry-Kravis *et al.*, 2003). This leads to a loss of expression of FMRP, an mRNA-binding protein that carries out a role in synaptic maturation and function (Bardoni and Mandel, 2002). Defects in the RhoGTPase cycle are identified as a cause of primary/nonspecific X-linked mental retardation (MRX). Over 11 genes have been implicated in MRX, one of which is Rho guanine nucleotide exchange factor 6 (Annunziata et al., 2003). The RhoGTPase cycle is important in cytoskeleton formation, cellular adhesion, intracellular signaling, gene expression, and cell cycle progression, whereby defects can lead to impaired polarization and impaired neural morphagenesis and connectivity (Toure *et al*, 2001;

Timpson *et al*, 2001; Rosenberger *et al.*, 2003; Speck *et al.*, 2003). The focus of this thesis is syndromal mental retardation caused by defects in the function of chromatin remodeling proteins. The modification of chromatin structure is important in properly regulating gene expression during neural development (Berube *et al.*, 2002). Numerous chromatin remodeling genes have been implicated in the development of XLMR, some examples of these are; MeCP2 (Rett Syndrome), RSK2 (Coffin-Lowry Syndrome), CBP (Rubinstein-Taybi Severe MR Syndrome), and ATRX (Alpha-Thalassemia Mental Retardation [ATR-X] Syndrome) (Gibbons *et al.*, 1995a; Chiurazzi *et al.*, 2000).

1.2 - Alpha-Thalassemia X-linked Mental Retardation (ATR-X) Syndrome.

1.2.1 – Clinical and genetic manifestations of ATR-X Syndrome

Alpha-Thalassemia X-linked Mental Retardation (ATRX) Syndrome is characterized by severe mental retardation, a characteristic facial appearance, microcephaly, seizures, abnormalities of genital development (varying in severity from undescended testes to male pseudohermaphroditism), and alpha-thalassemia (with varying severity) (see Figure 1) (Gibbons *et al.*, 1995a). Additionally, MRI and CAT scans in some ATR-X patients revealed no noticeable defects in brain morphology, but they were shown to have cerebral atrophy and enlarged ventricles (Gibbons *et al.*, 1995b; Berube *et al.*, 2002). ATR-X patients also show no signs of cytogenetic abnormalities (Gibbons *et al.*, 1995a).

Figure 1. ATR-X Syndrome.

Photographs of boys with ATR-X syndrome show the characteristic facial features; widely spaced eyes, small upturned nose, triangular-shaped mouth with full bottom lip, generally small head, and flat looking in the nasal bridge area (Gibbons *et al.*, 1995a). Source of photographs: http://www.imm.ox.ac.uk/mhu/home_pages/Gibbons/facial.html

http://www.imm.ox.ac.uk/mhu/home_pages/Gibbons/facial.html

Alpha-thalassemia is a rare form of anemia, whereby patients have the presence of hemoglobin β_4-tetramers (HbH) in their peripheral red blood cells, as detected by the redox dye, brilliant cresyl blue (Gibbons *et al.*, 1992). It is a result of drastic down-regulation of the α-globin gene, whereas the β-globin gene is unaffected, resulting in the abnormal HbH inclusions that consist of four β-globin chains, rather than the normal two α–globin chains and two β-globin chains (Gibbons *et al.*, 1995c).

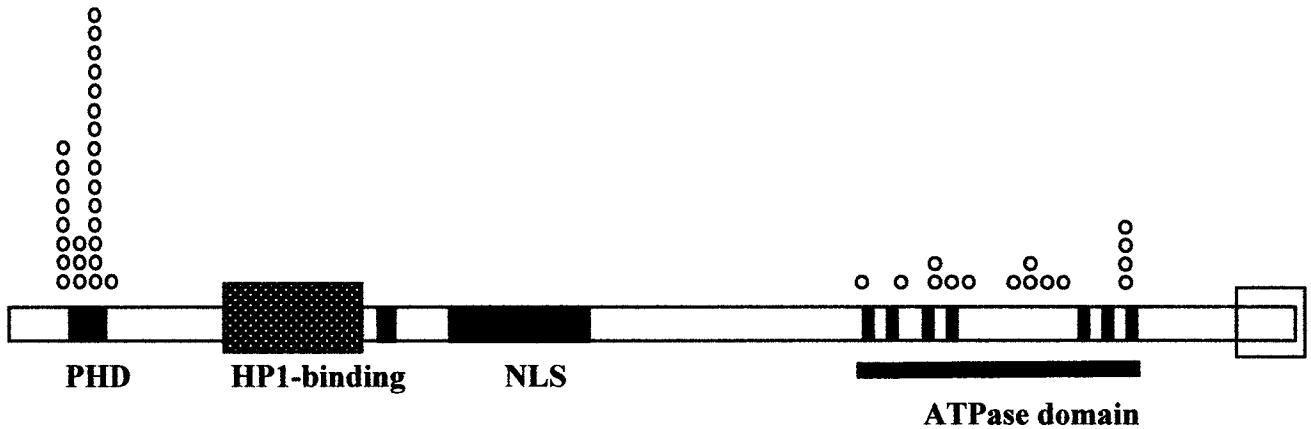
ATR-X syndrome is caused by mutations in the ATRX gene (also called XH2 and XNP), which is located at Xq13.3, spans over 300 kb of genomic DNA, has 35 exons, and codes for a protein product of approximately 280 kDa (Picketts *et al.*, 1996; Wada *et al.*, 2000). ATRX was found to be the causative gene of ATR-X syndrome in 1995 using a positional cloning approach (Gibbons *et al.*, 1995a). ATRX belongs to the SWI/SNF family of DNA-dependant ATPases and putative helicases (to be discussed in greater detail later). There are two domains (which are common to SWI/SNF family proteins) in the ATRX gene product that are significant with respect to ATR-X syndrome. These include a plant homeodomain (PHD)-type zinc-finger domain towards the N-terminal of the protein, and an ATPase/helicase domain towards the C-terminal (Picketts *et al.*, 1996; Gibbons *et al.*, 1997). Most mutations in ATR-X patients are found in these domains, with the most common occurrences being 60% in the PHD domain, and 25% in the ATPase domain (Picketts *et al.*, 1996; Gibbons *et al.*, 1997; Villard *et al.*, 1997; Berube *et al.*, 2000). There is no genotype/phenotype correlation in ATR-X patients. Regardless of where mutations lie, the inactivation of the ATRX gene generally results in a constant phenotype, suggesting that the two domains share a functional significance (Gibbons *et al.*, 1997). Of the mutations found in the ATRX gene, missense mutations are the most

common, and of these missense mutations, approximately 36% of them represent the R246C amino acid change (Villard and Fontes, 2002). Other identified mutations include three nonsense mutations, seven splicing defects, and eight deletions (Villard and Fontes, 2002). Also, a truncating mutation that deletes the last 100 amino acids from the ATRX protein, and mutations in the last two exons, have been shown to result in severe urogenital deformities, suggesting the importance of that portion of the protein in urogenital development (Picketts *et al.*, 1996; Villard and Fontes, 2002). However, a splice mutation caused by a point mutation in exon 11 creates three cryptic splice sites, two of which remove the C-terminal end of the protein and do not cause severe urogenital abnormalities in ATR-X patients, which suggests residual activity of the third splice variant, which is only missing one amino acid residue (Fichera *et al.*, 1998). It has also been suggested that the ATRX protein is required in a very specific amount for normal development. This is evident in a splicing mutation whereby there is about 30% normal ATRX mRNA, yet patients still develop ATR-X syndrome, and conversely, overexpression of ATRX protein in mice leads to early lethality (Picketts *et al.*, 1996) (see Figure 2 for a schematic diagram of ATRX showing locations of mutations).

Female carriers show a highly skewed level of X-inactivation, and occasionally have HbH inclusions in their blood, but other wise have no other manifestations of the disease (Gibbons *et al.*, 1992; Gibbons *et al.*, 1995a). Females in ATR-X families, who do not carry mutations, do not show a skewed pattern of X-inactivation, which shows that the skewing pattern is caused by the presence of ATRX mutations (Lossi *et al.*, 1999). Two female carriers have been identified who are mosaic for ATRX mutations; one has two affected sons, but the mutation was not detected in her somatic tissue so she is

Figure 2. Schematic diagram of the ATRX gene product.

The ATRX gene product is a 280 kDa protein. Shown in the diagram are a plant homeodomain (PHD)-type zinc-finger domain towards the N-terminal of the protein, and an ATPase/helicase domain towards the C-terminal. Most mutations (indicated by o) in ATR-X patients are found in these domains, with the occurrences amounting to about 60% in the PHD domain, and 25% in the ATPase domain. The box at the C-terminal end represents the deletion that results in severe urogenital abnormalities. Also shown are the heterochromatin protein 1 (HP1)-binding domain and the nuclear localization signal (NLS).


PHD
HP1-binding
NLS
ATPase domain

assumed to have germline mosaicism, whereas the second female has two daughters who share their affected brother's haplotype but do not carry the disease, and is thus assumed to be gonosomal mosaic (Bachoo and Gibbons, 1999). This provides evidence that post-zygotic mutation occurs, and is a consideration in the course of genetic counseling for ATR-X families (Bachoo and Gibbons, 1999).

ATRX gene mutations are likely the cause of many forms of XLMR without alpha-thalassemia, as they have been shown in Juberg-Marsidi syndrome, Carpenter-Warziri syndrome, XLMR with spastic paraplegia, Holmes-Gang syndrome, Smith-Fineman-Myers syndrome, and other syndromal forms of XLMR (Villard *et al.*, 1996; Gibbons and Higgs, 2000; Guerrini *et al.*, 2000; Stevenson *et al.*, 2000; Villard *et al.*, 2000; Berube *et al.*, 2002; Yntema *et al.*, 2002).

One more thing to note is, although ATRX belongs to the SWI/SNF family of proteins, patients with ATR-X syndrome do not show phenotypic effects consistent with other diseases associated with mutations in this protein family, such as increased ultraviolet sensitivity, increased incidents of malignancy, or abnormal chromosome breakage and/or segregation (Gibbons *et al.*, 1995a). For example, ATRX is closely related to RAD54, a DNA repair protein. Patients were tested for defects in DNA repair and none were found, suggesting it is not involved in DNA repair (Gibbons *et al.*, 1995a).

1.2.2 – Cellular functions and modifications of the ATRX protein.

The ATRX gene product is expressed in most tissues, and may have many functions within the cell. Patients with ATR-X syndrome exhibit multiple congenital

abnormalities in a wide spectrum of tissues and systems, suggesting mutations in the ATRX gene may exert pleiotropic effects during the course of development (Gibbons *et al.*, 1995a & c). ATRX is thought to be involved in the regulation of gene expression since mutations give rise to alpha-thalassemia via the down-regulation of α-globin gene expression, whereas β-globin gene expression is unaffected in ATRX mutations. The β-globin gene lies in an area of closed heterochromatin, which opens in a tissue specific manner in red blood cells under the control of a remote locus control region, whereas the α-globin gene is in an open region of transcriptionally active euchromatin regulated by a remote tissue-specific enhancer (Gibbons *et al.*, 1995a). This suggests a positive regulatory role for the ATRX protein by interaction with chromatin in the α-globin gene region. However, ATRX has also been shown to interact with heterochromatin protein 1 (HP1) through a coil-coiled motif just outside the PHD-like zinc-finger region (Le Douarin *et al.*, 1996; Berube *et al.*, 2000). HP1 is involved in establishing regions of heterochromatin, contributing to gene silencing and supra-nucleosomal chromatin structure (Lachner *et al.*, 2001). Therefore, the ATRX/HP1 interaction is suggestive of a negative regulatory role for the ATRX protein. The interaction of ATRX with HP1 occurs during mitosis, when they are bound to condensed chromatin (Berube *et al.*, 2000). ATRX has also been shown to associate with the nuclear matrix, and to interact with the Polycomb group protein EZH2 (homologue to Drosophila chromatin remodelling protein that regulates homeotic gene expression) by way of the same coil-coiled motif with which HP1 interacts (Cardoso *et al.*, 1998; Berube *et al.*, 2000; Cardoso *et al.*, 2000).

ATRX has been shown to localize at pericentromeric heterochromatin and bind to the short arms of acrocentric chromosomes, which contain arrays of GC-rich ribosomal DNA (rDNA) (McDowell *et al.*, 1999). Mutations in ATRX appear to cause changes in the methylation patterns of these rDNA arrays (decreased methylation), as well as in DYZ2 repeats on the Y-chromosome (increased methylation) (Gibbons *et al.*, 2000). The changes in methylation patterns in ATR-X patients suggests there is a link between chromatin remodelling, DNA methylation, and regulation of gene expression through influences on CpG-rich regions of the genome (Gibbons *et al.*, 2000). However, there is no methyltransferase domain region in the ATRX protein, but the PHD domain shows very close homology to that of the DNMT family of methyltransferases in humans, providing a link to both methyltransferase machinery and histone deacetylase (HDAC) complexes (Gibbons *et al.*, 2000). The DNMT family of methyltransferases are required for genomic methylation patterns through direct interaction with HDACs, and transcriptional repression and heterochromatin formation (Bachman *et al.*, 2001). Therefore, ATRX may provide a chromatin remodelling function for genes that establish and maintain gene silencing due to methylation, and when mutated, the altered function of ATRX may change methylation patterns in various ways throughout the genome (Gibbons *et al.*, 2000).

ATRX appears to be active throughout the cell cycle. During interphase, ATRX is found in the nucleus in bundles associated with the nuclear matrix, which appear as a speckled pattern when viewed by immunofluorescence. At the start of mitosis, the speckling pattern is greatly reduced, and by metaphase and anaphase, ATRX localizes to condensed chromatin; by telophase ATRX is entirely co-localized with chromatin

(Berube *et al.*, 2000). ATRX is phosphorylated at the start of mitosis (G_2-M), probably as a means to be solublized, and thus released from the nuclear matrix to allow mitosis to progress, it then appears to be dephosphorylated as the cell exits mitosis (Berube *et al.*, 2000). One study showed that ATRX, when mutated in the PHD/zinc-finger domain, did not form the characteristic speckling pattern in the nucleus, but rather showed a diffuse staining pattern, or a cytoplasmic perinuclear pattern (Cardoso *et al.*, 2000).

The evidence presented thus far suggests that ATRX has a role in controlling gene expression through the modification of chromatin structure, as well as roles in chromosomal inheritance, cytokinesis, as a linker molecule to establish nuclear domains during interphase that can regulate transcription, replication, and splicing, as well as acting with other proteins to regulate chromosome segregation and cell division (Berube *et al.*, 2000). ATRX likely regulates many genes including some involved in neural development. ATRX has been shown to exist in a large complex comprising 5-6 other polypeptides, reaching a molecular mass of 700-2000 kDa, thus changes in ATRX levels (either increased or decreased) may significantly alter the end-results of ATRX function (Berube *et al.*, 2002). Changing expression levels of ATRX in transgenic mice was shown to cause growth retardation, neural tube defects, abnormal brain growth and organization, high incidences of embryonic and perinatal death, seizures, facial abnormalities, and abnormal behaviour (Berube *et al.*, 2002). As mentioned above, ATRX is phosphorylated at the G_2-M phase of the cell cycle; thus, overexpression of ATRX may cause a block at this point, as the level of unphosphorylated ATRX present may overwhelm the system. One last point of interest is that ATRX has been shown to have ATPase activity, yet no chromatin remodeling activity has been shown to date.

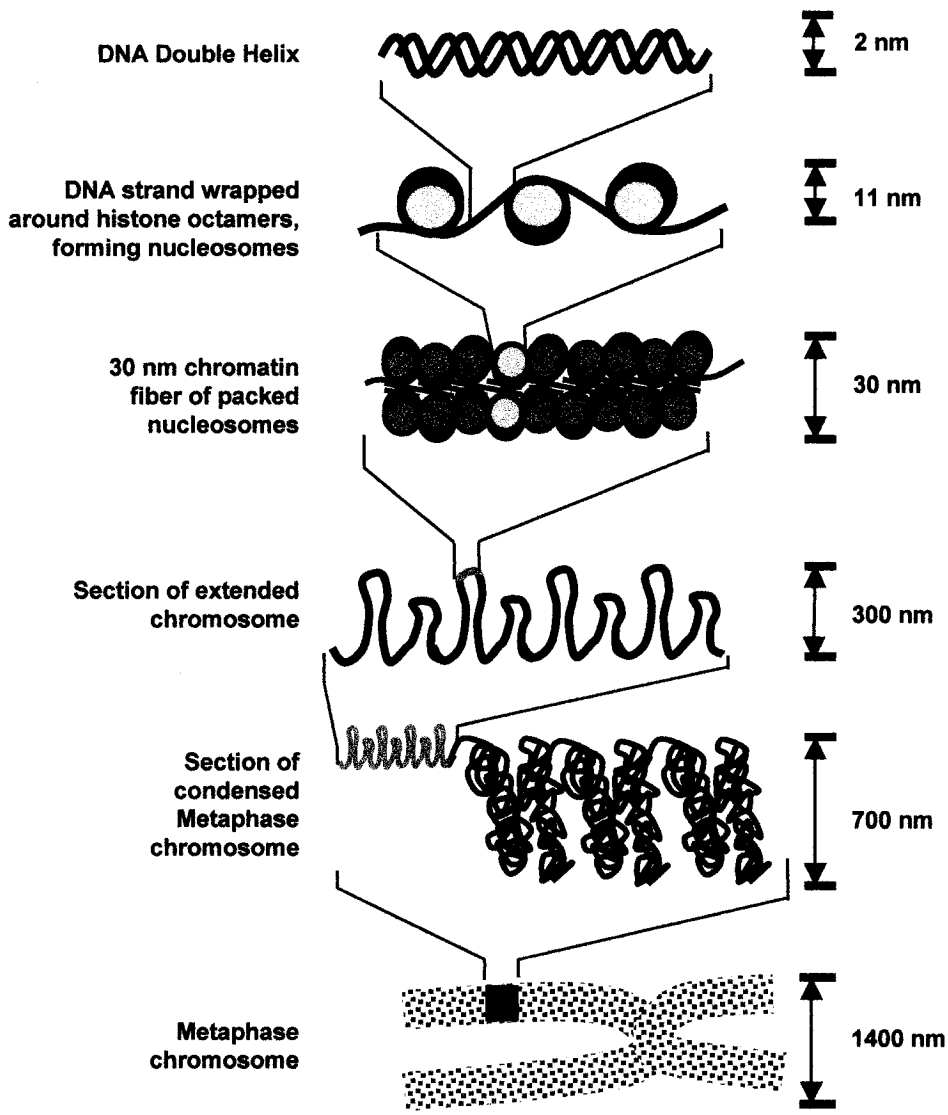
1.3 – Chromatin Remodelling

1.3.1 - Chromatin Structure, Organization, and Function

DNA is organized in a hierarchal manner ranging from the 2 nanometer (nm) double helix to the 1400 nm condensed metaphase chromosome (Figure 3) (Alberts *et al.*, 1994). Of interest here is the manner by which DNA is organized into chromatin, the nucleoprotein fibres that make up the eukaryotic chromosome (Zubay, 1998). The basic DNA strand is wrapped around histone octamers to form a beads-on-a-string structure, which is approximately 11 nm thick (Alberts *et al.*, 1994). This structure is referred to as the nucleosome, and makes up the primary repeating unit of chromatin (Cairns, 1998). The histone (H) octamer is made up of a tetramer core of two H3 proteins and two H4 proteins, flanked by two H2A-H2B dimers, around which 146 base pairs (bp) of DNA are wrapped in 1.65 left-handed superhelical turns (Cairns, 1998; Bjorklund *et al.*, 1999; Sterner and Berger, 2000; Deckert and Struhl, 2001). Separating each nucleosome are stretches of linker DNA that can be up to 100 bp in length, and contain bound nuclear proteins that may have a role in packing nucleosomal arrays into the higher order, 30 nm chromatin fibre (Redner *et al.*, 1999). Also, it is reported that bound to the linker DNA are lysine-rich linker histones H1 and H5, which are thought to be important in the compacting of chromatin structure (Bjorklund *et al.*, 1999; Zlatanova *et al.*, 2000).

Figure 3. Chromatin structure and packaging.

Shown are the hierarchal levels of chromatin packaging, from the 2 nm strand of the DNA double helix, up to the fully condensed metaphase chromosome with 1400 nm-thick arms. Adapted from *Molecular Biology of the Cell*, ©1998 by Alberts, Bray, Johnson, Lewis, Raff, Roberts, Walter. Published by Garland Publishing, a member of the Taylor & Francis Group. http://www.garlandscience.com/ECB/about.html. Source of picture from which figure was adapted: http://www.accessexcellence.org/AB/GG/chroma_packg.html.


DNA Double Helix
2 nm
DNA strand wrapped around histone octamers, forming nucleosomes
11 nm
30 nm chromatin fiber of packed nucleosomes
30 nm
Section of extended chromosome
300 nm
Section of condensed Metaphase chromosome
700 nm
Metaphase chromosome
1400 nm

The higher order structure of chromatin is an important factor in the regulation of gene expression. In highly compacted chromatin, genes are inaccessible to transcription machinery, which serves a transcriptionally repressive function. Chromatin that is densely compacted, and contains transcriptionally inactive genes, is called heterochromatin; chromatin that is more open in structure, and contains transcriptionally active genes is called euchromatin (Kimura *et al.*, 2002). Post-translational modifications are important in the control of chromatin condensation and remodelling (Zhang *et al.*, 1998). Two of the most important post-translational modifications are the acetylation and methylation of the amino-terminal tails of the core histone proteins (Bernstein *et al.*, 2002). Histone tails interact with chromatin components, and play an important role in transcriptional activation and repression (Bjorklund *et al.*, 1999).

Acetylation occurs on lysine (K) residues of the H3 (K9, K14, K18, K23) and H4 (K5, K8, K12, K16) histone tails (acetylation of different residues may have different effects), causing a neutralization of the lysine positive charge, resulting in an overall change in histone tail charge distribution (Wan *et al.*, 2001; Winkler *et al.*, 2002). This change in charge distribution causes a full or partial dissociation of the negatively charged DNA from the nucleosome, resulting in a remodelling of chromatin structure, and allowing transcription machinery to access the DNA template (Redner *et al.*, 1999; Sterner and Berger, 2000). Generally, areas of euchromatin that are transcriptionally active are highly acetylated, the converse being true for areas of heterochromatin that are transcriptionally inactive (Deckert and Struhl, 2001; Kruhlak *et al.*, 2001). The acetylation of histone proteins is carried out by a group of enzymes called histone acetyltransferases (HATs), who transfer the acetyl group from acetyl-coenzyme A

(aCoA) to the amino group of the above-mentioned lysine residues (Sterner and Berger, 2000). HATs are recruited to transcription sites by transcription factors (TFs), and may be gene-specific (that is, a specific HAT is targeted to a specific gene), or they may act globally as transcription co-activators (Ng and Bird, 1999; Kruhlak *et al.*, 2001). The HAT counterparts are a group of enzymes called histone deacetylases (HDACs), which are implicated in transcriptional repression and gene silencing (Wang *et al.*, 2000; Kruhlak *et al.*, 2001). HDACs remove the acetyl groups from histone tails, thus exposing the positive charges of the lysine residues and restoring the tight DNA/nucleosome winding interactions, resulting in genes being inaccessible to transcription machinery (Razin, 1998).

Another way genes are regulated by modifications in chromatin structure is by methylation, which is thought to have a transcriptional repressing or gene silencing function by limiting promoter accessibility or recognition (Razin, 1998; Meehan, 2003). Methylation commonly occurs on DNA or on histone tails. DNA Methylation has an important role in gene regulation, imprinting, X-chromosome inactivation, and tumour suppression; and occurs on the cytosine (C) and guanine (G) dinucleotides (CpGs) (Ng and Bird, 1999; Xie *et al.*, 1999; Rea *et al.*, 2000). Areas rich in CpGs are called CpG islands. CpG islands are generally associated with promoters, and have a low methylation status (Ng and Bird, 1999). Important to the methylation of DNA are the DNA methyltransferases (DNMTs) and the methyl-CpG binding proteins (MBDs), both of which associate with HDACs in their repressive functions (Razin, 1998; Ng and Bird, 1999; Bachman *et al*, 2001; Aapola *et al.*, 2002; Meehan, 2003).

There are three DNMT families in mammalian cells, DNMT1 (expressed in proliferating cells and associated with DNA replication foci), DNMT2 (expressed in most tissues at low levels, but functionality is unclear), and DNMT3 (consists of DNMT3A, DNMT3B, and DNMT3C), which are highly expressed in embryonic stem cells, but down-regulated after differentiation (Xie *et al.*, 1999). To repress transcription, DNMT3A and DNMT3B maintain heterochromatin by methylation, using a PHD-like motif (which is homologous to the one on ATRX) during the cell cycle through an interaction with HP1 and MBDs (Bachman *et al.*, 2001).

The ubiquitously expressed MeCP2 protein (gene product of the X-linked *MeCP2* gene), is the best known of the MBDs, and is thought to be a global transcription repressor (Wan *et al.*, 2001). It can bind to a single methylated CpG pair through its methyl-CpG binding domain, leading to gene repression using a transcription repression domain (TRD), and by recruiting HDAC-containing repressor complexes (Ng and Bird, 1999; Meehan, 2003). Defects in MeCP2 can be very serious, as CpGs are very frequent in the genome (one every 150bp), and may lead to hyperacetylation throughout the entire genome (Wan *et al.*, 2001). Mutations in MeCP2 are implicated in XLMR syndromes (the most well known being Rett Syndrome), suggesting gene regulation by methylation is important in central nervous system (CNS) development and/or stable brain function (Couvert *et al.*, 2001; Chen *et al.*, 2001; Guy *et al.*, 2001; Wan *et al.*, 2001).

Histone methylation, another form of transcriptional repression, follows the histone code, which theorizes that certain domains (such as chromodomains and bromodomains) recognize modified histone residues (Rea *et al.*, 2000; Bannister *et al.*, 2001; Lachner *et al.*, 2001). For example, it has been shown that the chromodomain of

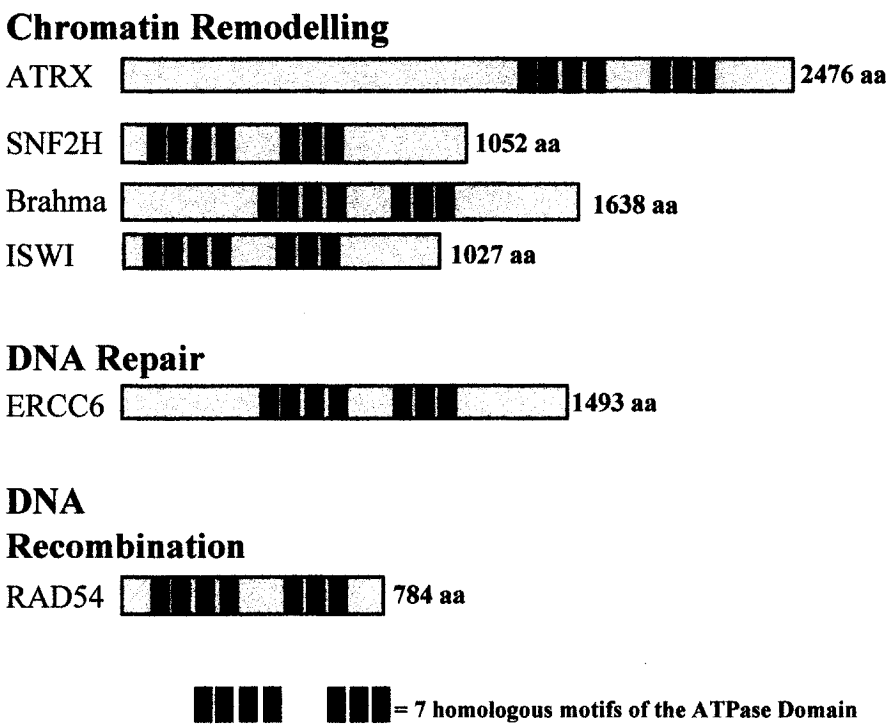
HP1 is able to recognize, and subsequently bind to H3 when it is methylated at K9 (Rea *et al.*, 2000; Bannister *et al.*, 2001; Lachner *et al.*, 2001). Post-translational modifications are important in the remodelling of chromatin structure, but another important mechanism in chromatin remodelling is the utilization of energy from ATP hydrolysis to change the location or conformation of nucleosomes (Zhang *et al.*, 1998; Kingston and Narlikar, 1999).

1.3.2 – The SWI/SNF Family of DNA-Dependant ATPases and Putative Helicases

The SWI/SNF family of DNA-dependant ATPases and putative helicases are characterized by 7 homologous motifs similar to a helicase domain (Figure 4). They usually exhibit ATPase activity that is DNA and/or nucleosome dependent. Members of this family appear to be involved in a wide range of cellular functions, but all appear to have the ability to remodel chromatin (Vignali *et al.*, 2000). The SWI/SNF family proteins, first discovered in yeast, were named corresponding to the phenotypes of the mutants in which they were found (SWI = mating-type switching; SNF = sucrose non-fermenting) (Carlson and Laurent, 1994). The SWI/SNF family is divided into three subfamilies; SWI2/SNF2 (DNA/nucleosome-stimulated ATPase activity, SWI/SNF homology extending outside the 7 homologous motifs, and contain a C-terminal bromodomain), ISWI (nucleosome-stimulated ATPase activity, SWI/SNF homology only within the 7 homologous motifs, and contain a C-terminal SANT domain), and CHD (have characteristic 7 homologous motifs, but also have a chromodomain) (Neely and Workman, 2002). ISWI proteins function in nucleosome remodeling and

Figure 4. The SWI/SNF Family

Shown are examples of members of the SWI/SNF family of DNA-dependant ATPases and putative helicases. Indicated are the locations of the ATPase domains, which contain the seven homologous helicase motifs that are conserved within this family.


Chromatin Remodelling
ATRX
2476 aa
SNF2H
1052 aa
Brahma
1638 aa
ISWI
1027 aa
DNA Repair
ERCC6
1493 aa
DNA Recombination
RAD54
784 aa
= 7 homologous motifs of the ATPase Domain

rearrangement, as well as chromatin assembly (Naar *et al.*, 2001; Neely and Workman, 2002). CHD proteins are generally associated with heterochromatin interactions (Neely and Workman, 2002).

Members of the SWI/SNF protein family exist within multiprotein complexes, having up to 15 or more subunits, and molecular weights reaching 2 MDa (Kadam *et al.*, 2000). Each of these complexes is thought to regulate specific subsets of genes through the modulation of chromatin structure, some in a positive manner, others negatively. However, the identities of SWI/SNF target genes, and the mechanisms that target a particular SWI/SNF complex to a gene, have not been elucidated (Angelov *et al.*, 2000). Although, a recent study has identified about 80 genes that are activated and two that are repressed by BRG-1, the catalytic component of the SWI/SNF complex A (BAF) (Liu *et al.*, 2001).

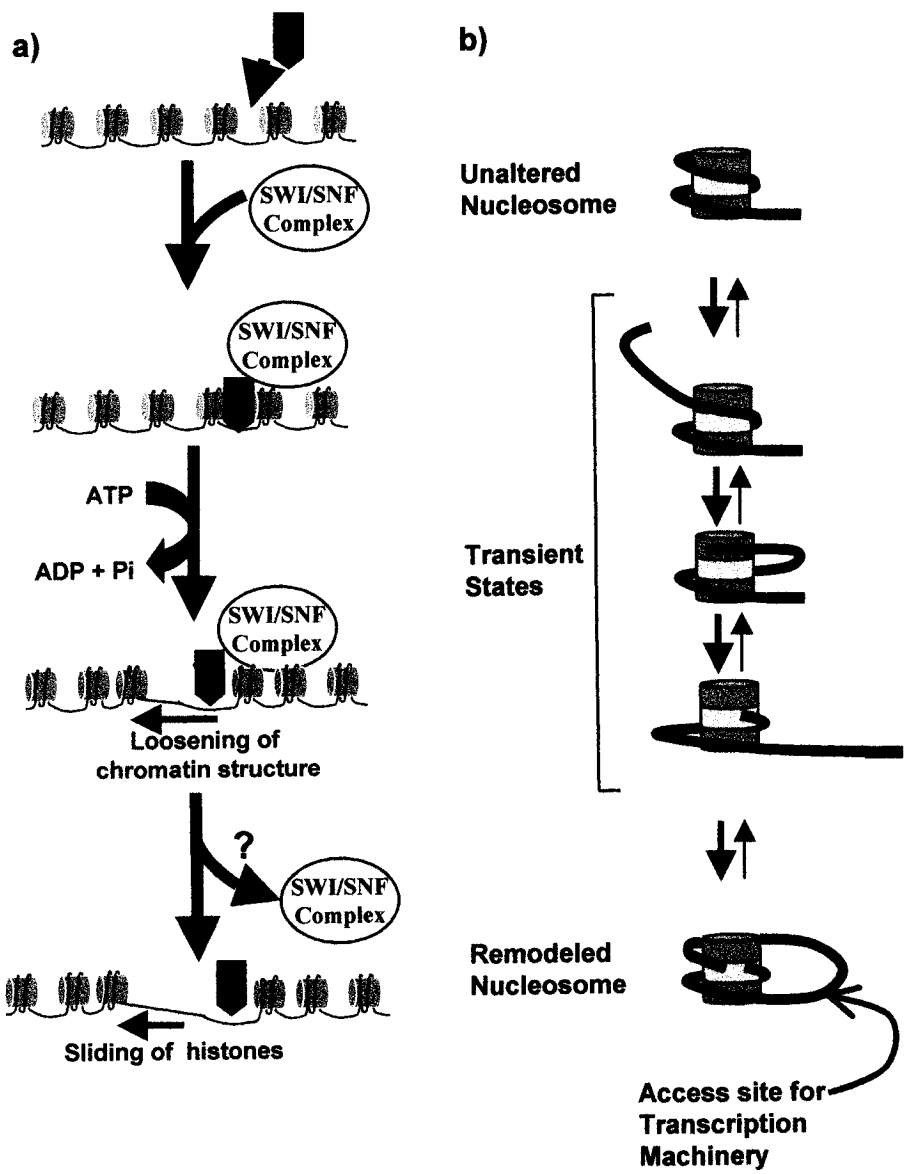
It is proposed that SWI/SNF complexes function in concert with DNA-binding transcription factors (TFs) in order to regulate chromatin structure and transcription (Wu and Winston, 1997). The SWI/SNF complexes generally act as typical enzymes, such that they provide a means by which to lower activation barriers between different nucleosomal states by use of energy from ATP-hydrolysis (Kingston and Narlikar, 1999). The SWI/SNF complex may weaken and disrupt DNA-histone interactions, allowing the access of TFs, such that a synergistic effect after the release of histone tails may be created as multiple TFs bind the DNA (Angelov *et al.*, 2000). Specialized domains, such as zinc-finger DNA-binding domains of transcription factors, may target SWI/SNF complexes to specific genes, serving a gene specific role in transcription factor-directed chromatin remodelling (Kadam *et al.*, 2000).

A general mechanism proposed for SWI/SNF chromatin remodelling is as follows; the complex is recruited to a transcription site by TFs, the complex binds to the nucleosomal array in a DNA dependent manner (independent of ATP), the energy from subsequent ATP hydrolysis disrupts histone-DNA interactions, nucleosomal conformations change as a result of the disturbed interactions, chromatin is remodelled (ie. nucleosomes may slide along the DNA strand in *cis*, or move to another DNA molecule in *trans*), gene expression may be up-regulated or repressed (Figure 5a) (Vignali *et al.*, 2000). It is not entirely certain whether the SWI/SNF complex remains bound to the promoter during this sequence of activity, but it has been reported that binding can be initiated and retained, either by TFs, or in an aCoA-dependent manner by HATs (Hassan *et al.*, 2001). Another study has shown that the energy of ATP-hydrolysis can be used by SWI/SNF complexes to generate superhelical torsion in the DNA strand, disrupting histone-DNA interactions as a result of the torsional strain, leading to accessibility of transcription machinery (Havas *et al.*, 2000; this theory is reviewed with great detail by Peterson, 2002).

Another theory of SWI/SNF chromatin remodelling involves peeling DNA from the edges of nucleosomes, thus allowing it to form a loop outward from the nucleosome, and enabling access of transcription machinery (Kassabov *et al.*, 2003). Earlier work supports this theory, showing that there is a decrease in DNA content from SWI/SNF-remodelled nucleosomes as a result of unwrapping of nucleosomal DNA (Bazett-Jones *et al.*, 1999). The Bazett-Jones *et al.* study also showed that one SWI/SNF complex can cause the disruption of numerous nucleosomes along a nucleosomal array. The model

Figure 5. Mechanisms of chromatin remodelling by SWI/SNF complexes.

(a) Transcription factors recruit the SWI/SNF complex to the promoter, after which the complex utilizes energy from ATP hydrolysis to loosen chromatin structure. Thus allowing access of transcription machinery. It is not known whether the SWI/SNF complex remains attached throughout the entire mechanism. Adapted from Vignali *et al.*, 2000. (b) The SWI/SNF complex peels DNA from the nucleosome edge, causing an unstable bulge of about 50 bp of DNA to form and subsequently rebind to the nucleosome at a different position. DNA is unable to return to its original position due to steric hindrance of the bound SWI/SNF complex, causing further bulging and spooling out of the DNA from the histone. The spooled DNA reattaches to the histone on a distant point on the spooled-out DNA strand, resulting in an altered, stable loop conformation that allows entry or exit of activators or repressors (The red box outlined in black, tracks the movement of a specific portion of the DNA strand). Adapted from Kassabov *et al.*, 2003.


a)
SWI/SNF Complex
SWI/SNF Complex
ATP
ADP + Pi
SWI/SNF Complex
Loosening of chromatin structure
?
SWI/SNF Complex
Sliding of histones
b)
Unaltered Nucleosome
Transient States
Remodeled Nucleosome
Access site for Transcription Machinery

proposed by the Kassabov *et al.* study correlates well with the Bazett-Jones *et al.* study, and proposes that the SWI/SNF complex peels DNA from the nucleosome edge, causing an unstable bulge of about 50 bp of DNA to form and subsequently rebind to the nucleosome at a different position. DNA is unable to return to its original position due to steric hindrance of the bound SWI/SNF complex, causing further bulging and spooling out of the DNA from the histone. The spooled DNA reattaches to the histone at a distant point on the spooled-out DNA strand, resulting in an altered, stable loop conformation that allows entry or exit of activators or repressors (Figure 5b).

1.4 – The Promyeolytic Leukemia Nuclear Body and Daxx.

1.4.1 - The promyeolytic leukemia nuclear body

The promyeolytic leukemia (PML) nuclear body [also called promyeolytic oncogenic domain (POD); nuclear domain 10 (ND10)] is associated with the nuclear matrix in nuclear bundles, and is present in most normal and cultured cells (Boisvert *et al.*, 2000). They are made up of PML protein, a 70 kDa RBCC motif protein (contains a RING-finger motif, two cysteine-rich regions called B-boxes, and a coiled-coil region), and are believed to be involved in numerous functions within the nucleus including transcriptional regulation, apoptosis, growth and tumour suppression, and response to viral infection and heat shock (Mattson *et al.*, 2001; Khan *et al.*, 2001; Eskiw and Bazett-Jones, 2002). The PML transcript contains about 20 potential splice variants, yielding

PML proteins that range in size from 48 to 97 kDa, all of which likely possess a unique function within the cell (Eskiw and Bazett-Jones, 2002). For example, the overexpression of a splice variant of PML (called PML3) has been shown to increase the phosphorylation and acetylation state of p53, leading to decreased cell survival (Eskiw and Bazett-Jones, 2002).

PODs were originally described to have been rich in RNA, and therefore a site of nascent RNA synthesis, but more recent research has found that they do not actually accumulate RNA, but rather, they may provide a favourable nuclear environment for gene expression (Boisvert *et al.*, 2000). PODs are shown to be tightly associated with transcription sites containing high degrees of hyperphosphorylated mRNA, and in the G1 phase of the cell cycle, greater than 70% of PODs contain active transcription foci, increasing to 80% upon interferon-γ exposure, suggesting PODs are recruited to active transcription sites where they may act as a scaffold for specific gene-expression factors (Kiesslich *et al.*, 2002). The same study also showed the cAMP response element-binding protein (CBP), a transcriptional coactivator and histone acetyltransferase, also colocalized with the hyperphosphorylated mRNA and PML at G1. CBP has been shown to move rapidly in and out of PODs, and it is thought that CBP may be recruited to the outer edges of PODs to create domains that are high in acetylated and transcriptionally active chromatin (Boisvert *et al.*, 2001).

The PML protein was identified as the fusion partner of the retinoic acid receptor α (RARα) as a result of the t(15:17) chromosome translocation in acute promyeolytic leukemia (APL), forming a product that modulates downstream target gene activity and disrupts PODs, leading to cell transformation (Doucas *et al.*, 1999; Boisvert *et al.*, 2001).

Introduction of all-trans retinoic acid disrupts the fusion protein, and PODs reform, providing evidence of their role in tumour suppression (Ferbeyre *et al.*, 2000). In fact, all-trans retinoic acid is used as a chemotherapeutic agent in APL patients (Eskiw and Bazett-Jones, 2002). Normally, PML is involved in apoptosis and transcriptional activation through its interaction with RAR, and transcriptional repression through its interaction with the corepressors c-Ski, N-CoR, mSin3, and HDAC1, all mediated by a tumour suppressor called Mad (Khan *et al.*, 2001). The PML-RARα complex blocks all these functions, possibly leading to the development of APL.

Further suggesting the many roles of PML is the fact that PODs have been shown to accumulate many other cellular proteins, such as Sp100 (an auto-antigen identified in primary biliary cirrhosis, and may have a structural or architectural role), SUMO-1 (a ubiquitin-like protein that modulates intracellular localization of proteins, and is thought to be important for POD assembly), HAUSP (removes ubiquitin from proteins before degradation), HSP70 (a heatshock protein), RB (a tumour suppressor involved in *ras*-mediated senescence), Topors (a topoisomerase 1-binding protein), NBS1 and TRF1 (proteins involved in telomere lengthening), and Daxx (a transcriptional corepressor and apoptosis protein) (Doucas *et al.*, 1999; Ishov *et al.*, 1999; Boisvert *et al.*, 2000; Ferbeyre *et al.*, 2000; Mattson *et al.*, 2000; Wu *et al.*, 2000; Eskiw and Bazett-Jones, 2002; Rasheed *et al.*, 2002).

1.4.2 – Daxx

Daxx is a ubiquitously expressed protein, with a molecular weight of 120 kDa, which is thought to act as a transcriptional co-repressor and a promoter of apoptosis (Hollenbach *et al.*, 2002). Daxx was first identified as a signaling protein that binds to the death domain of Fas, a cell surface receptor responsible for inducing apoptosis (Yang *et al.*, 1997). This group showed that overexpression of Daxx enhances Fas-mediated apoptosis and activates the Jun N-terminal kinase (JNK) pathway. Daxx has also been shown to repress the transcriptional activity of Pax3 through interactions with the Pax3 homeodomain recognition sequence, octapeptide domain, and N-terminal flanking amino acids (Hollenbach *et al.*, 1999). Daxx also interacts with Pax5, a transcription factor important in early B-cell development, and a transcription activator or repressor in late B-cell development (Emelyanov *et al.*, 2002). These studies suggest that Daxx, acting as a transcriptional coactivator, may affect the role of other Pax genes during development.

Daxx is associated with many proteins important in transcriptional repression, such as HDACs, core histones (H2A, H2B, H3, and H4), DNA methyltransferases, and Dek, a protein that changes DNA topology within chromatin after histone tails are deacetylated, preventing the access of transcription factors (Ishov *et al.*, 1999; Hollenbach *et al.*, 2002). Daxx is dephosphorylated prior to associating with transcription factors at active transcription sites, after which it associates with acetylated core histones and Dek (Hollenbach *et al.*, 2002). At this point, Daxx becomes associated with HDAC2, leading to the deacetylation of nucleosomal histone tails, and subsequent repression of transcription (Figure 6) (Hollenbach *et al.*, 2002). The transcriptional

repressive effects of Daxx have been shown to decrease upon overexpression of MSP58, a nucleolar protein reported to interact with p120, a proliferation-related nucleolar protein detectable in the early G1 phase of the cell cycle, with expression peaking early in the S phase (Ren *et al.*, 1998; Lin *et al.*, 2002). Most importantly, Daxx is often found in the nucleus, colocalized with PODs (Ishov *et al.*, 1999).

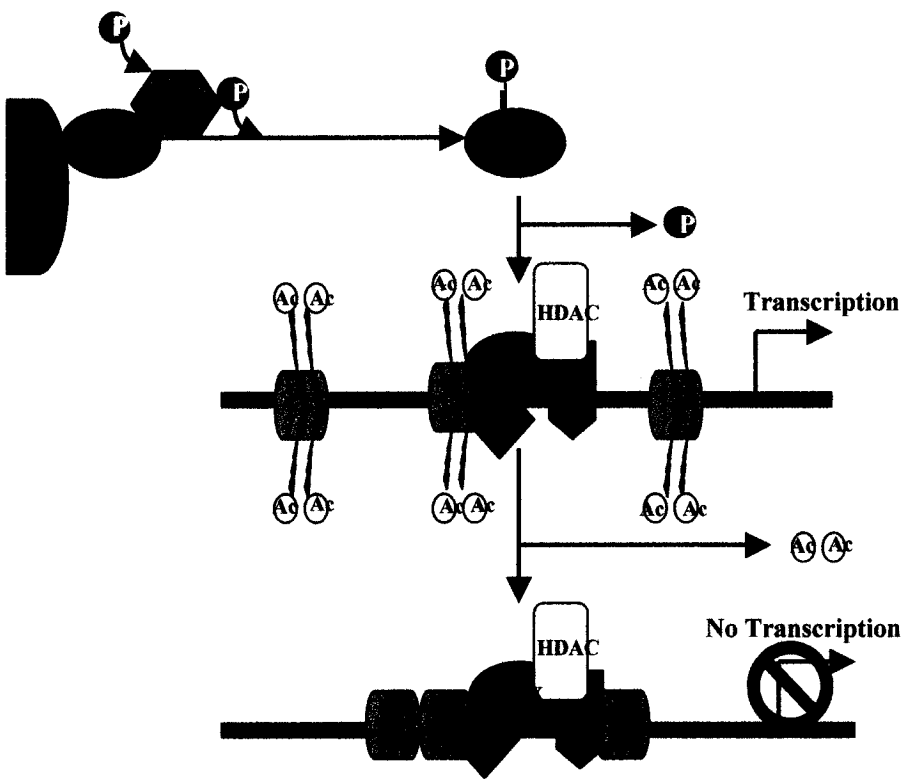
1.4.3 – Interactions of PML and Daxx

Daxx and PML are associated in the nucleus, and like PML, Daxx exists in multiple isoforms of 120, 90, and 70 kDa (Hollenbach *et al.*, 2002). PML appears to specifically interact with the phosphorylated 120-kDa isoform of Daxx (Hollenbach *et al.*, 2002). Daxx has been shown to be colocalized to condensed chromatin when PML is absent from the cell (Ishov *et al.*, 1999). As mentioned earlier, PML is modified by SUMO-1. It has been shown that this modification of PML is required to recruit Daxx from heterochromatin to PODs, and it is thought that Daxx may act as an adaptor to recruit other components of PODs that do not directly interact with PML (Ishov *et al.*, 1999). Another suggestion is that PML may activate and relocalize Daxx to nuclear bodies as part of the Fas-induced apoptosis mechanism (Hollenbach *et al.*, 2002; Lin *et al.*, 2002; Lopez *et al.*, 2002). It was mentioned earlier that Daxx associates with HDACs to act as a transcriptional repressor. This mechanism requires that Daxx is relocalized from the PODs to chromatin by homeodomain-interacting protein kinase 1 (HIPK1), where;

1) HIPK1 relocalizes Daxx from PODs to HDACs (HIPK1 is autophosphorylated during this transaction);

Figure 6. Schematic diagram of mechanism by which Daxx and promyeolytic leukemia bodies interact.

HIPK1 relocalizes Daxx from PODs to HDACs (HIPK1 is autophosphorylated during this transaction). HIPK1 phosphorylates Daxx at serine 669, an important event in modulating the transcriptional repressor function of Daxx. Daxx is dephosphorylated prior to associating with transcription factors at active transcription sites, after which it associates with acetylated core histones and Dek. At this point, Daxx becomes associated with HDAC2, leading to the deacetylation of nucleosomal histone tails, and subsequent repression of transcription. Adapted from Hollenbach *et al.*, 2002.


P
P
P
P
HDAC
Transcription
Ac
HDAC
No Transcription

2) HIPK1 phosphorylates Daxx at serine 669, an important event in modulating the transcriptional repressor function of Daxx (Ecsedy *et al.*, 2003) (refer to Figure 6).

The Daxx/PML interaction has recently been shown to be important in glucocorticoid receptor (GR) function (Lin *et al.*, 2003). This group showed that recruitment by PODs sequesters Daxx from the GR/coactivators complex, thus alleviating the transcriptional repression of Daxx (Lin *et al.*, 2003).

The PML/Daxx interaction is apparently important in the function of gene regulation, particularly the repression aspect. Many other proteins are likely involved in this interaction (including ATRX), so it should be interesting to see what evolves from future research in this area.

1.5 – The Tet-Off and Tet-On Gene Expression System

Tetracycline-controlled inducible gene expression systems provide a means by which genes can be investigated in mammalian cell lines in a tightly regulated fashion, and also allows genes to be expressed at up to five levels of magnitude above normal expression levels (Gossen and Bujard, 1992). The system used in this study was primarily the Tet-Off Gene Expression System from Clontech (Palo Alto, CA). However, both the Tet-Off and the Tet-On systems will be described here. Each system is comprised of two vector plasmids; a regulatory plasmid (pTet-Off or pTet-On, respectively) and a response plasmid (pTRE and it's modified descendants, all of which work in either system; pTRE2 will be the modification discussed from here on in). The Tet-Off system was first

described in 1992 by Manfred Gossen and Hermann Bujard (Gossen and Bujard, 1992). Gossen's group described the Tet-On system in 1995 (Gossen *et al.*, 1995). The regulatory plasmid of the Tet-Off system expresses a fusion protein comprised of the first 207 amino acids of the *Escherichia coli* Tn*10* tetracycline-resistance operon control element, and the last 127 amino acids of the activation domain of virion protein 16 (VP16) from the Herpes simplex virus, all under the control of a cytomegalovirus (CMV) immediate early promoter (P_{CMV}) (Gossen and Bujard, 1992). The resulting fusion protein product is called the tetracycline-controlled transactivator (tTA) (Gossen and Bujard, 1992). The pTet-Off regulatory plasmid also confers neomycin resistance to transfected cells.

In *E. coli*, the Tn*10* tetracycline-resistance operon encodes the genes responsible for conferring tetracycline resistance, and is negatively regulated by the tetracycline repressor (*tet*R), which is the control element used in the Tet-Off system (Gossen and Bujard, 1992). Normally, *tet*R is bound to the operators (*tet*O) in the promoter region of the Tn*10* tetracycline-resistance operon, but when tetracycline is present in the cell, the drug binds to *tet*R, releasing it from the operator and allowing the resistance genes to be transcribed (Gossen and Bujard, 1992). The addition of the VP16 activation domain in the Tet-Off system converts *tet*R from a transcriptional repressor into a transcriptional activator (Gossen and Bujard, 1992).

The response plasmid in the Tet-Off/On system (pTRE2) contains a multiple cloning site in which the desired gene-of-interest can be inserted. Expression of the inserted gene-of-interest is under the control of the tetracycline response element (TRE), driven by a minimal CMV promoter (P_{minCMV}) (Gossen and Bujard, 1992; Furth *et al.*,

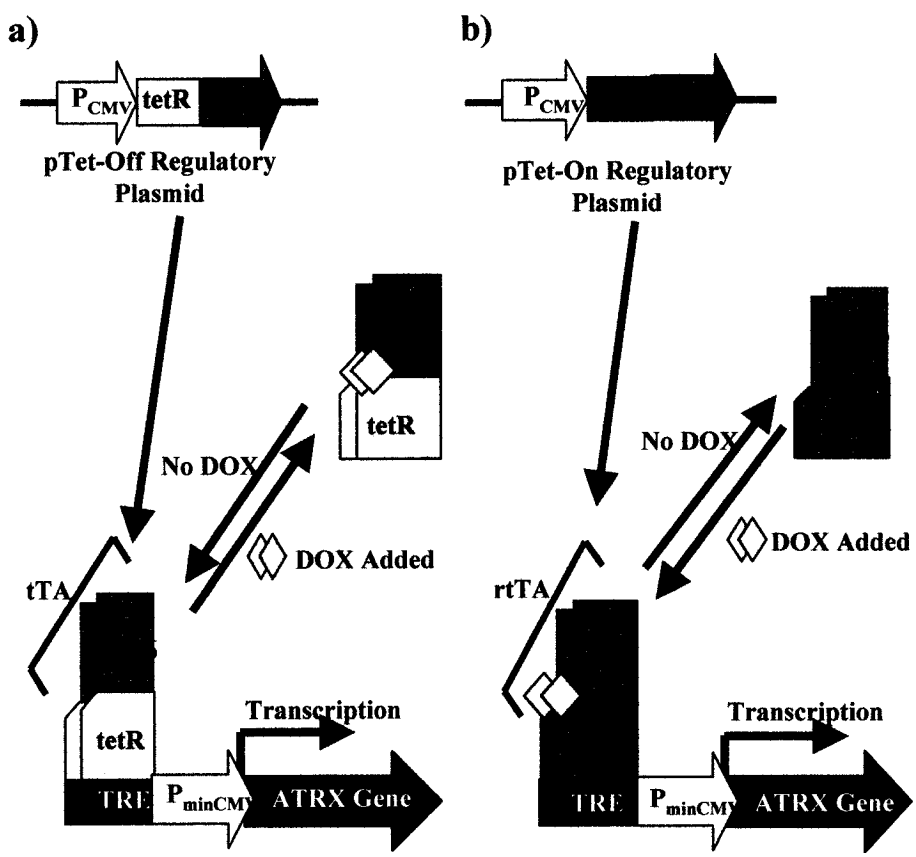
1994). The TRE is located upstream of the P_{minCMV}, and is made up of seven direct repeats of a 42 base pair sequence containing the *tet*O (Gossen and Bujard, 1992; Furth *et al.*, 1994). The minimal promoter used on pTRE2, lacks the strong enhancer elements normally associated with P_{CMV} in order to prevent background expression of the transgene in the absence of tTA binding (Furth *et al.*, 1994). The pTRE2 plasmid is available with the gene for hygromycin B (hygB) resistance built in (pTRE2hyg), otherwise it is necessary to cotransfect the pTRE2 plasmid with pTK-hyg, a plasmid that confers hygB resistance to transfected cells.

When both the regulatory plasmid, and the response plasmid are present in a cell, the expression of the gene-of-interest is initiated when the tTA binds to the TRE, and activates the P_{minCMV}. The tTA can only bind to the TRE in the absence of tetracycline. When tetracycline is present in the cell, it binds to the tTA, causing it to dissociate from the TRE, shutting down expression of the gene-of-interest (Figure 7a) (Gossen and Bujard, 1992; Clontech Laboratories, Inc., 2001). The Tet-On system has basically the same elements as the Tet-Off system. The difference being that the tTA has four amino acid mutations in the *tet*R element (Glu71Lys, Asp95Asn, Leu101Ser, and Gly102Asp), which converts it into a reverse *tet*R or r*tet*R (thus the fusion protein expressed by the Tet-On regulatory plasmid is designated as rtTA). This change reverses the affinity of the regulatory fusion protein for the TRE, such that, in the presence of tetracycline, the rtTA binds to the TRE, and the expression of the gene-of-interest is initiated (Figure 7b) (Gossen *et al.*, 1995; Clontech Laboratories, Inc., 2001).

Logistically speaking, the Tet-On system is probably a better choice, as there is no need to maintain tetracycline in the media as in the Tet-Off system. The drug can be

Figure 7. Schematic diagram of the Tet-Off and Tet-On gene regulation system.

(a) The expression of the gene-of-interest (in this case the ATRX gene) in the Tet-Off system is initiated when the tTA binds to the TRE, and activates the P_{minCMV}. The tTA can only bind to the TRE in the absence of tetracycline or its derivative doxycycline (Dox). When tetracycline is present in the cell, it binds to the tTA, causing it to dissociate from the TRE, shutting down expression of the gene-of-interest. (b) The Tet-On system has basically the same elements as the Tet-Off system. The difference being that the tTA has four amino acid mutations in the *tet*R element (Glu71Lys, Asp95Asn, Leu101Ser, and Gly102Asp), which converts it into a reverse *tet*R or r*tet*R (thus the fusion protein expressed by the Tet-On regulatory plasmid is designated as rtTA). This change reverses the affinity of the regulatory fusion protein for the TRE, such that, in the presence of tetracycline, the rtTA binds to the TRE, and the expression of the gene-of-interest is initiated. Adapted from the *Tet-Off and Tet-On Gene Expression Systems User Manual*, published 01 February 2001 by Clontech Laboratories, Inc. Palo Alto, CA.


a)
P_{CMV}
tetR
pTet-Off Regulatory Plasmid
tetR
No DOX
DOX Added
tTA
tetR
Transcription
TRE
P_{minCMV}
ATRX Gene
b)
P_{CMV}
pTet-On Regulatory Plasmid
No DOX
DOX Added
rtTA
Transcription
TRE
P_{minCMV}
ATRX Gene

added when expression is desired. This would also be necessary in an animal model, as it would be easier to add the drug to the animal's body, rather than remove it, in order to attain expression. It would also be advantageous in situations requiring rapid induction because induction may be slower in the Tet-Off system, as traces of drug may still be present.

The Tet-Off/On system has advantages over other inducible gene systems, such as the *lac*R/O-based system, which uses isopropyl β-D-thiogalactopyranoside (IPTG) as an inducer. In that system, IPTG is quickly taken up by the cell, and remains stable for a long time, but its inducing ability is moderate and it acts slowly and inefficiently (Gossen and Bujard, 1992). In the Tet-Off/On system, the regulator (*tet*R) is very specific to the TRE, tetracycline has a very high affinity for *tet*R, and induction has been shown to be much quicker than the *lac*R/O-based system, among others (Gossen and Bujard, 1992). Other advantages of the Tet-Off/On system are; low background expression and no pleiotropic effects (due to their prokaryotic origin), a well-characterized effector (tetracycline has been well characterized as an inducer, and is non-toxic in mammalian cells at the levels used), high induction levels, and high absolute expression (Blau and Rossi, 1999; Clontech Laboratories, Inc., 2001 and references therein).

A number of tetracycline derivatives were tested to see which was the most potent effector (Gossen *et al.*, 1995). Those tested were doxycycline (Dox), chlortetracycline, oxytetracycline, anhydrotetracycline, and tetracycline. It was found that Dox and anhydrotetracycline were of the highest potency, with Dox being slightly higher (required 0.01 to 1 μg/ml to attain complete activation or inactivation, whereas tetracycline requires

1-2 μg/ml), thus Dox is the effector of choice in the Tet-Off/On systems (Gossen *et al.*, 1995; Clontech Laboratories, Inc., 2001).

The Tet-Off/On system has proved to be a milestone in the study of mammalian gene expression. It has numerous applications beyond what was attempted in this thesis. The Tet-Off/On system could be used in human gene therapy, as the system meets two major criteria required in this type of therapy:

1) **Regulators must be non-toxic to mammals** - tetracycline and its derivatives are non-toxic at doses used in these systems, and have been proved so by years of use in human and animal patients;

2) **Regulators must be nonimmunogenic** - no immune response was reported in mice who were delivered the tetR-VP16 fusion protein by an *ex vivo* approach.

(Bohl *et al.*, 1997; Blau and Rossi, 1999)

The Tet-Off/On system has been used successfully in transgenic mice, and has been used to study numerous diseases, including Huntington's disease and diabetes, as well as being tested for its suitability as an agent in gene therapy (Kistner *et al.*, 1996; Yamamoto *et al.*, 2000; Unterrainer *et al.*, 2000; Lottman *et al.*, 2001).

1.6 – Rationale and research objectives

ATR-X syndrome is a tragic, debilitating genetic disease that causes great physical and emotional suffering for patients and their families. It is caused by mutations in the ATRX gene, which alters or eliminates the proper function of the ATRX gene

product. Although not much is known about the function of the ATRX gene product, it appears to be a member of the SWI/SNF family of DNA-dependant ATPases and putative helicases. Due to the phenotypic manifestations of the disease, it is likely that ATRX is an important regulator of developmental genes, particularly those involved in neural development. The aim of this work is to determine important interactions of ATRX within the cell, with the hope of elucidating possible ATRX functions, and speculating on how specific patient mutations may alter these functions. To address this question, the following objectives are proposed:

[1] To develop stable cell lines with ATRX under the control of a tetracycline-regulated gene expression system (specifically, the Tet-Off system).

[2] To determine if the ATRX nuclear-staining pattern comprising nuclear speckles colocalizes with Daxx protein and PML bodies through transient transfection, overexpressed ATRX protein, and examination of endogenous proteins.

[3] To identify the PML-interacting domain within the ATRX protein, using vector constructs containing fragments of the ATRX protein.

[4] To determine if patient mutations in these areas of the ATRX protein affect its interaction with PML bodies.

With this information it is hoped that some of the functions of ATRX can be elucidated, leading to a better understanding of the neuropathology of this complex disease.

2 - Materials and Methods

2.1 – Materials

2.1.1 – General Materials

The general chemicals used in buffers and solutions were obtained from Sigma-Aldrich Canada Ltd., Oakville, ON; Fisher Scientific, Toronto, ON; EM Science, Gibbstown NJ; BDH Incorporated, Toronto, ON; or Invitrogen Life Technologies, Carlsbad, CA. Agarose for DNA gels was obtained from Invitrogen Life Technologies, Carlsbad, CA. BSA was obtained from Fisher Scientific, Toronto, ON. Ethidium bromide, DAPI, PMSF, Tween 20, and β-mercaptoethanol were obtained from Sigma-Aldrich Canada Ltd., Oakville, ON. Bacto-agar, bacto-tryptone and yeast extract, used in LB broth, were obtained from VWR International, Ville Mont-Royal, QC. All cell culture plates, dishes, flasks, and tubes were obtained from Becton Dickinson Labware, Franklin Lakes, NJ; Fisher Scientific, Toronto, ON; Simport, Beloeil, QC; DiaMed Lab Supplies, Mississauga, ON; and Nalg Nunc International, Denmark. Sources of all other materials are indicated in the text as they are used.

2.1.2 – Antibodies

Primary antibodies used were RabbitαHA.11 (Berkeley Antibody Company, Richmond, CA), Rabbit αATRX H-300 (Santa Cruz Biotechnologies, Paso Robles, CA), Sheep αfxnp5 (Berube *et al.*, 2000), Mouse αPML PG-M3 (Santa Cruz Biotechnologies, Paso Robles, CA), Rabbit αDaxx M-112 (Santa Cruz Biotechnologies, Paso Robles, CA), and Goat αDaxx CT (Upstate Biotechnology, Lake Placid, NY). Secondary antibodies used were αRabbit.FITC (Jackson ImmunoResearch Laboratories, West Grove, PA),

αSheep.FITC (Jackson ImmunoResearch Laboratories, West Grove, PA), αRabbit.Alexa594 (Molecular Probes, Eugene, OR), αRabbit.Alexa488 (Molecular Probes, Eugene, OR), αMouse.Alexa594 (Molecular Probes, Eugene, OR), αSheep.Alexa488 (Molecular Probes, Eugene, OR), αRabbit.HRP (Sigma-Aldrich Canada Ltd., Oakville, ON), αMouse.HRP (Sigma-Aldrich Canada Ltd., Oakville, ON), αSheep.HRP (Sigma-Aldrich Canada Ltd., Oakville, ON), and αGoat.HRP (Sigma-Aldrich Canada Ltd., Oakville, ON).

2.2 – Methods

2.2.1 – Maintenance of cell lines

Hela (human cervical carcinoma) cells were maintained at 37°C in 5% CO_2 in Dulbecco's Minimal Essential Medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (CanSera, Rexdale, ON). Untransfected NIH 3T3 (mouse fibroblast) Tet-Off cells (Clontech Laboratories, Inc. Palo Alto, CA) were maintained at 37°C in 5% CO_2 in DMEM supplemented with 10% Tet-Approved FBS (Clontech Laboratories, Inc. Palo Alto, CA) and 100 μg/ml geneticin (G418) (Invitrogen Life Technologies, Carlsbad, CA). Stable NIH 3T3 Tet-Off clones were maintained in the same media as the untransfected cells, except media was also supplemented with 200 μg/ml hygromycin B (hygB) (Roche Diagnostics Corporation, Indianapolis, IN). All cells were maintained as a monolayer in various dishes and flasks (depending on intended purpose), and were split 10:1 once a week, or when they reached 100% confluence, whichever came first. Splitting of cells consisted of

washing cells once with phosphate-buffered saline (PBS) (1 mM KH_2PO_4, 154 mM NaCl, 5.8 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.5), incubating with trypsin-EDTA (Invitrogen Life Technologies, Carlsbad, CA) for approximately 5 minutes, resuspending in complete maintenance media, keeping 1/10 of the volume of cell/media resuspension, and bringing culture volume up to its original value with complete maintenance media.

2.2.2 – Construction of pTRE2hyg-ATRXHA.

To construct the pTRE2hyg-ATRXHA vector, the full-length ATRX cDNA, tagged with an influenza hemagglutinin (HA) epitope (amino acid sequence YPYDVPDYA), from the pCAGGS-SL-ATRXHA vector (*BssHII* - *NotI* fragment), was inserted into the *MluI* - *NotI* sites of the pTRE2hyg vector multicloning site (MCS) (*BssHII* and *MluI* are compatible, cohesive ends).

To excise each fragment from their respective vector, they were digested with the appropriate restriction enzyme as follows; to a 1.5 ml eppendorf tube were added 10 µg DNA, 2 µl of each appropriate restriction enzyme (indicated above; *MluI* and *BssHII* from New England Biolabs, Mississauga, ON; *NotI* from MBI Fermentas, Burlington, ON), 5 µl 10X bovine serum albumin (BSA) solution (New England Biolabs, Mississauga, ON), 5 µl of appropriate 10X restriction enzyme digest buffer (restriction enzyme digest buffer #3 for pCAGGS-SL-ATRXHA digest, restriction enzyme digest buffer #3 for pTRE2hyg digest, both buffers obtained from New England Biolabs, Mississauga, ON), 1 µl of shrimp alkaline phosphatase (SAP) (to pCAGGS-SL-ATRXHA reaction only, obtained from Roche Diagnostics Corporation, Indianapolis, IN), and ddH_2O up to 50 µl. Digest mixture was incubated at 37°C for approximately 2

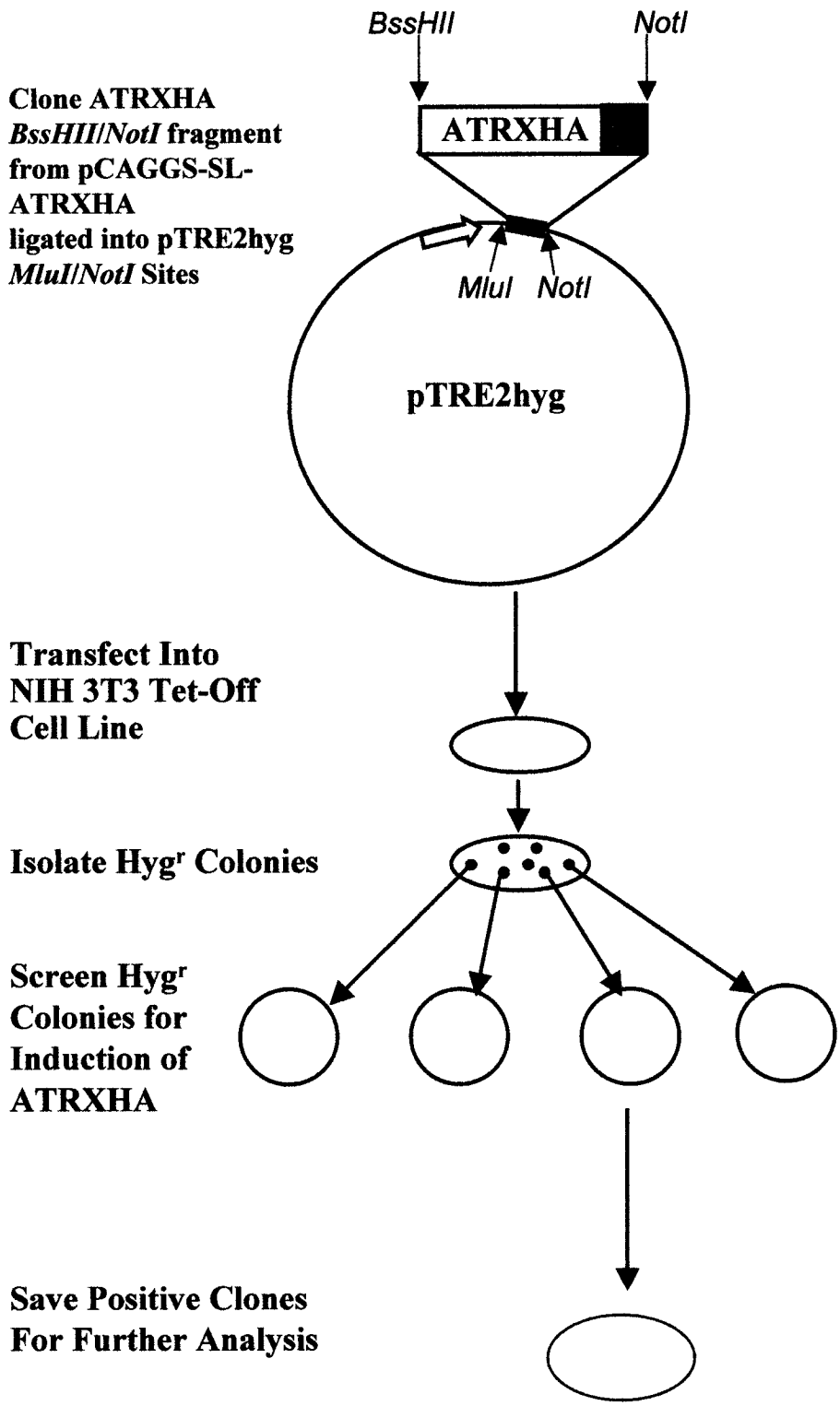
hours, after which 10 µl agarose gel loading buffer (trace of bromophenol blue, trace of xylene cyanol, 200 mg/ml Ficoll 400) were added to each digest, and samples were loaded onto a 1% agarose gel (supplemented with 1 µg/ml ethidium bromide), and run using a Bio-Rad Mini Sub-Cell GT gel apparatus (Bio Rad Laboratories, Hercules, CA) at 120V for approximately 1.5 hours, in TAE buffer [40 mM tris-acetate, 1 mM ethylenediaminetetraacetic acid (EDTA)]. To identify band sizes, 1 kb plus DNA ladder (Invitrogen Life Technologies, Carlsbad, CA) was run in one lane. Gels were viewed under an ultraviolet lamp, and the bands corresponding to the correct base pair (bp) sizes were cut out of the gel using a razor blade (ATRXHA ≈ 7.5 kbp; pTRE2hyg ≈ 5.3 kbp). DNA was purified from the agarose gel pieces using the GFX Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ) as per instructions included with the kit. Fragments were ligated together using the Rapid DNA Ligation Kit (Roche Diagnostics Corporation, Indianapolis, IN) following manufacturer's intructions. Ligation mixture was transformed into STBL-2 competent cells using the heat shock method as follows; in a 15 ml culture tube, 1 µl of ligation mixture was mixed with 100 µl STBL-2 competent cells and placed on ice for 20 minutes, mixture was then incubated at 42°C for 45 seconds and allowed to cool on ice for 2 minutes, 1 ml of Luria-Bertani (LB) broth (1.0% bacto-tryptone, 0.5% yeast extract, 171 mM NaCl) was added to the mixture and the culture tube was incubated at 37°C for 1 hour, with shaking, for recovery. After the recovery period, 150 µl of transformation broth were plated on 100 mm bacterial culture dishes containing 1.5% bacto-agar in LB broth supplemented with 100 µg/ml carbenicillin (Sigma-Aldrich Canada Ltd., Oakville, ON), and incubated overnight at 37°C. After overnight incubation, colonies from plates were selected for screening by touching a

sterile toothpick to a given colony, and placing toothpick in 3 ml of LB broth, supplemented with 100 μg/ml carbenicillin, in a 15 ml culture tube, and incubating overnight at 30°C with shaking. After overnight incubation, plasmid DNA was purified from cultures using GFX mini plasmid prep kit (Amersham Biosciences, Piscataway, NJ) as directed by the supplier. Mini preps were digested with *EcoRI* as follows; to a 1.5 ml eppendorf tube were added, 10 μl of DNA preparation, 2 μl *EcoRI* (MBI Fermentas, Burlington, ON), 5 μl 10X O^+ restriction enzyme digest buffer (MBI Fermentas, Burlington, ON), ddH_2O up to 50 μl. Digest mixture was incubated at 37°C for approximately 2 hours, after which samples were analyzed by agarose gel electrophoresis, as described above, to determine whether or not clones were positive for the correct pTRE2hyg-ATRXHA *EcoRI* banding pattern.

NOTE: cloning and selection strategy is outlined in Figure 8.

Figure 8. NIH 3T3 Tet-Off Stable Clones: Diagram of cloning and selection strategy.

The 7.5 kbp *NotI – BssHII* fragment from pCAGGS-SL-ATRXHA, which represents the cDNA for HA-tagged ATRX, is cloned into the *NotI – MluI* site of the pTRE2hyg multicloning site. The *BssHII* and *MluI* restriction sites are compatible, cohesive ends. The pTRE2hyg-ATRXHA construct is stably transfected into NIH 3T3 Tet-Off cells. Hygromycin B-resistant colonies are selected and cultured, then analyzed for incorporation of the pTRE2hyg-ATRXHA construct, and expression of ATRXHA. Adapted from the *Tet-Off and Tet-On Gene Expression Systems User Manual*, published 01 February 2001 by Clontech Laboratories, Inc. Palo Alto, CA.


Clone ATRXHA
BssHII/NotI fragment
from pCAGGS-SL-
ATRXHA
ligated into pTRE2hyg
MluI/NotI Sites
BssHII
NotI
ATRXHA
MluI
NotI
pTRE2hyg
Transfect Into
NIH 3T3 Tet-Off
Cell Line
Isolate Hygr Colonies
Screen Hygr
Colonies for
Induction of
ATRXHA
Save Positive Clones
For Further Analysis

2.2.3 – Transient and stable transfections of pTRE2hyg-ATRXHA into NIH 3T3 Tet-Off cells.

Prior to transfection, cells were plated on coverslips in six-well tissue culture dishes (for transient transfections), or in five 100 mm tissue culture dishes (for stable transfections), and were grown to approximately 80% confluency. To transfect cells in six-well dishes, 200 µl DMEM, 24 µl Lipofectamine reagent (Invitrogen Life Technologies, Carlsbad, CA), and 1 µg DNA midi-prep (pTRE2hyg-ATRXHA) were mixed for each well to be transfected, and incubated at room temperature for exactly 30 minutes. After incubation, 800 µl of 37°C DMEM were added to the incubation mixture for each well to be transfected. To each well, 1 ml of the final transfection mixture was added (Note: cells were washed two times with 37°C DMEM prior to addition of final incubation mixture). Cells were incubated at 37°C in 5% CO_2 for 4.5 hours, after which 1 ml of DMEM, supplemented with 20% tet-approved FBS, was added, and cells were returned to the 37°C, 5% CO_2 incubator for recovery. Cells were maintained in this media until desired induction time had past in order for cells to be ready for analysis (usually 48 hours). The same procedure was followed for 100 mm dishes except, 800 µl DMEM, 30 µl Lipofectamine, and 3 µg DNA midi-prep, were used for each dish, and 6.2 ml of 37°C DMEM were added to the incubation mixture after the initial 30 minute incubation. Following the 4.5-hour incubation, 7 ml of DMEM supplemented with 20% tet-approved FBS were added to each transfected dish. For stable transfections, media was changed approximately 24 hours post-transfection, and 5 µg/ml doxycycline (Dox) (Clontech, Palo Alto, CA), 100 µg/ml G418 and 200 µg/ml hygB were added to the 100 mm culture dishes. Stably transfected cells were maintained in culture, with media change every 4 days (including antibiotics), until mass cell death (approximately 2

weeks). After mass cell death, individual surviving colonies were selected and transferred to 12-well culture dishes (one colony per well), and expanded as needed to 6-well culture dishes, then to T-25 tissue culture flasks, and finally to T-75 tissue culture flasks. The nomenclature used to identify the stable clones is as follows; the clone is given a number *x-y,* where *x* = the plate number from which it was obtained, and *y* = an arbitrary, unique number assigned to each colony in the dish. Aliquots of cells were frozen for liquid nitrogen (LN_2) stocks in 1 ml of an 80% tet-approved FBS, 10% DMEM, and 10% dimethyl sulfoxide (DMSO) solution (with approximately 10^6 cells/ml) using Nunc cryotube vials (Nalg Nunc International, Denmark). Cells were analyzed for expression (see section 2.2.4 and 2.2.5), and stable clones that showed good expression were serially diluted 1:1000 into 100 mm plates to repeat the selection process. The nomenclature followed here was, each colony was designated as *x-yz*, where *x-y* = the original colony designation from which it was derived, and *z* = an arbitrary, unique letter assigned to each colony (for example, if the colony came from the dish in which the original colony #1-2 was diluted, it could be designated #1-2a). These cells were then analyzed for expression, and aliquots were frozen in LN_2 stocks. Transfection procedures were adapted from Berube *et al.*, 2000 and Clontech Laboratories, Inc., 2001.

2.2.4 – Inducing Tet-Off stable clones.

To induce stable Tet-Off clones, cells were washed three times with PBS, and complete media (minus Dox) was added to the culture. Cells were maintained in this media until desired induction time had past (usually 24, 48, 72, or 96 hours), and were subsequently prepared for analysis. This procedure was done with cells in 6-well dishes

or 100 mm plates, adjusting volumes as appropriate (3 ml of media/6-well dish, 14 ml of media for 100 mm plates),

2.2.5 – Analysis of Tet-Off stable clones and transiently transfected cells.

To analyze expression in Tet-Off stable clones, the following techniques were utilized:

2.2.5a – Immunofluorescence

Following induction, cells in six well dishes were fixed to the coverslips at 0, 24, 48, 72 or 96 hours post-induction as follows: each well of cells was washed three times with 3 ml of PBS, the cells were then fixed to coverslips for ten minutes at –20°C with a 3:1 ethanol: methanol solution, followed by three more washes with 3 ml of PBS. If not used immediately, fixed cells were kept in 3 ml PBS at 4°C for a maximum of 12 hours prior to use. Prior to antibody staining, fixed cells were blocked in a solution of 2% BSA in PBS for ten minutes at room temperature, then incubated for 1 hour at room temperature with the appropriate primary antibodies diluted 1:100 in 2% BSA in PBS. Cells were washed three times in PBS as indicated above, then incubated for 1 hour at room temperature with the appropriate secondary antibodies diluted 1:100 in 2% BSA in PBS. Cells were washed three times in PBS as indicated above, then stained with 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS for 3 minutes at room temperature to detect nuclear DNA, then washed three times in PBS as indicated above. Coverslips were mounted on slides with Vectashield Mounting Medium for Fluorescence (Vector Laboratories Inc. Burlingame, CA), and viewed with a Zeiss Axiophot photomicroscope

using the 40X objective lens. Immunofluorescence procedure adapted from Berube *et al.*, 2000.

2.2.5b – Western Blot Analysis.

Induced cells were harvested from 100 mm tissue culture dishes as follows: media was removed from dish, and cells were washed once with PBS, cells were trypsinized (trypsin-EDTA from Invitrogen Life Technologies, Carlsbad, CA) for five minutes at 37°C, resuspended in 10 ml DMEM supplemented with 10% FBS, transferred to a 15 ml Falcon tube, and pelleted in a Heraeus Instruments Megafuge 1.0 at 1500 rpm for 5 minutes at room temperature, media was aspirated and cells were resuspended in 10 ml cold (4°C) PBS supplemented with protease inhibitors (CompleteMini, EDTA-free; Boehringer Mannheim, Laval, QC) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF).

Protein extraction was performed as follows: cells in cold PBS were pelleted as indicated above. Pelleted cells were then resuspended in 1 ml of the cold PBS solution, transferred to a 1.5 ml Eppendorf tube, and pelleted at 5000 rpm for 5 minutes in a Desaga MC2 bench top centrifuge. Pelleted cells were lysed by resuspension in RIPA buffer (50 mM Tris pH 8.0; 150 mM NaCl; 1% NP40; 0.2% SDS; 1% sodium deoxycholate) supplemented with protease inhibitors (CompleteMini, EDTA-free; Boehringer Mannheim, Laval, QC) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Volume of RIPA buffer was adjusted to the size of the pellet (usually about 200 µl). Lysis mixture was placed on ice for 10 minutes, then 15 U/100 µl of DNaseI (Amersham Biotech Inc., Piscataway, NJ) were added, and mixture was incubated for 5 minutes at 37°C. Mixture was then passed through a 25 gauge needle several times, followed by the

addition of another 15 U/100 µl of DNaseI, and incubation for 5 minutes at 37°C. Mixture was centrifuged at 14000 rpm for 5 minutes at 4°C in an Eppendorf 5417R benchtop centrifuge. Clarified extract was retained and quantitated by Lowry assay using the DC Protein Assay Kit (Bio Rad Laboratories, Hercules, CA) as per instructions supplied with the kit.

For western analysis, 30 µg of whole cell protein (in RIPA buffer) from clarified extracts and 20 µl sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (100 mM Tris pH 6.8, 4% SDS, 20% glycerol, bromphenol blue, 2 µl β-mercaptoethanol) were incubated 2 minutes at 100°C, then loaded on a 6% SDS-PAGE gel (Separation Gel = 6% acrylamide:bis-acrylamide, 375 mM Tris pH 8.8, 0.1% SDS, 2 mM EDTA, 0.1% N,N,N',N'- tetramethylethylenediamine (TEMED), 0.03% ammonium persulfate ; Loading Gel = 6% acrylamide:bis-acrylamide, 125 mM Tris pH 6.8, 0.1% SDS, 2 mM EDTA, 0.25% TEMED, 0.025% ammonium persulfate). Samples were run at 80V until Full Range Rainbow Recombinant Protein Molecular Weight Marker RPN800 (Amersham Biosciences, Uppsla, Sweden) entered the separation gel (approximately 30 minutes), after which the samples were run at 130V for approximately 2 hours. SDS-PAGE gels were run in SDS-PAGE tank buffer (50 mM Tris-Base, 384 mM glycine, 0.1% SDS, 2 mM EDTA). Protein samples were transferred from the gel to an Immobilon-P transfer membrane (Millipore, Bedford, MA) as follows: a transfer stack was made in the following order; 3 sheets 3MM chromatography paper (Whatman, Maidstone, England) cut to the exact dimensions of the gel, and soaked in 10 mM CAPS buffer pH 11, Immobilon-P cut to the exact dimensions of the gel and soaked first in methanol, then in 10 mM CAPS buffer pH 11, the 6% SDS-PAGE gel with the protein

samples, and finally 3 more sheets 3MM chromatography paper cut to the exact dimensions of the gel, and soaked in 10 mM CAPS buffer pH 11. The samples were transferred for 2 hours at 50 mA per gel being transferred (ie. 50 mA for one gel, 100 mA for two gels), in a Hoefer Semiphor transfer apparatus, model TE77 (Pharmacia Biotech, San Fransisco, CA). After transfer, membranes were air dried, and placed in TBST buffer (100 mM Tris pH 8.0, 1.5 M NaCl, 0.5% Tween 20) with 5% skim milk powder to block overnight. After blocking, membranes were incubated for 1-2 hours at room temperature, with shaking, in 10 ml TBST buffer with 5% skim milk powder and the appropriate primary antibodies (diluted in blocking solution as follows: Rabbit αHA.11, 1:1000; Rabbit αATRX, 1:500; Sheepαfxnp5, 1:1000). After incubation, membranes were washed three times, with shaking for ten minutes each time, in TBST buffer. Membranes were incubated for 1-2 hours at room temperature, with shaking, in 10 ml TBST buffer with 5% skim milk powder and the appropriate secondary antibodies (diluted in blocking solution as follows: αRabbit.HRP, 1:1000; αSheep.HRP, 1:5000). After incubation, membranes were washed three times, with shaking for ten minutes each time, in TBST buffer. After washes, membranes were treated with enhanced chemoluminescence (ECL) reagent (4 ml 100 mM Tris pH 8.5, 8.8 µl 90 mM coumaric acid, 20 µl 250 mM Luminol, 1.2 µl H_2O_2) for two minutes, then exposed to Kodak Biomax MR Scientific Imaging Film (Amersham Biosciences, Baie d'Urfé, QC) for 1 minute and 30 minutes. If a membrane needed to be reprobed with the same, or a different set of antibodies, it was placed in membrane stripping buffer (100 mM β-mercaptoetanol, 2% SDS, 62.5 mM Tris pH 6.7) for 30 minutes at 56°C, then washed two times, ten minutes each time, in a large quantity of TBST buffer (approximately 50-100 ml). The western analysis procedure was

then repeated, starting from the overnight blocking step. Protein extraction and western analysis adapted from Berube *et al.*, 2000.

2.2.5c – Polymerase chain reaction and reverse transcriptase polymerase chain reaction analysis.

Stable Tet-Off clones were analyzed for incorporation of the pTRE2hyg-ATRXHA construct, and expression of ATRXHA messenger RNA (mRNA), by polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR) respectively. Genomic DNA for PCR was purifed by phenol:chloroform extraction as follows for each clone; cells induced in 100 mm plates were trypsinized and harvested as described in section 2.2.5b, cells were resuspended in approximately 100 µl PBS and 5 ml tail lysis buffer (50 mM Tris pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS), 25 µl of 20 µg/ml of proteinase K (Roche Diagnostics Corporation, Indianapolis, IN) were added, and mixture was incubated at 56°C for 24 to 48 hours. DNA was extracted twice in a 1:1 phenol:chloroform solution, using a 15 ml Falcon tube, and aqueous phase was retained. DNA was precipitated by mixing aqueous phase with 500 µl 5 M NaCl and 10 ml 100% EtOH. DNA was pelleted Heraeus Instruments Megafuge 1.0 centrifuge at 4600 rpm for 20 minutes. Pellet was washed twice and stored overnight in 70% EtOH, then resuspended in 500 µl ddH_2O. DNA was quantitated by measuring absorbance at 260 nm and 280 nm in a Beckman DU-600 spectrophotometer.

PCR was carried out as follows: each reaction contained approximately 100 ng genomic DNA template, 4 µl of 10 mM dNTPs (MBI Fermentas, Burlington, ON) mixture, 1 µl of each 20 µM primer solution (forward primer: Xnp74 = 5'-

TCTCCTGGCACGAATATAAAGCAC-3'; reverse primer: ATRX/HA-tag = 5'-TAATCTGGAACATCGTATGGGTA-3'; from Cortec DNA Service Laboratories, Kingston, ON), 2.5 units *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, CA), 5 µl 10X PCR buffer (Invitrogen, Carlsbad, CA), $MgCl_2$ to a concentration of 15 mM, and brought up to 50 µl with ddH_2O. Reactions were placed in a PTC-200 Peltier Thermo Cycler PCR machine (MJ Research, Watertown, MA), and run through the following cycle; an initial 2 min at 95°C, then 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute. A final extension at 72°C for 10 minutes was run before the overall cycle ended with an infinite18°C setting period. Reactions were analyzed by agarose gel electrophoresis as described earlier (except each lane contained 20 µl of reaction mixture and 10 µl agarose gel loading buffer).

ATRXHA mRNA was extracted for RT-PCR from induced cells using the RNeasy Mini Kit (Qiagen, Mississauga, ON) as per directions supplied by manufacturer. RNA was quantitated by adding 2 µl of extract to 500 µl diethyl pyrocarbonate (DEPC) water (0.1% DEPC in ddH_2O), and measuring absorbance at 260 nm and 280 nm in a Beckman DU-600 spectrophotometer. RT-PCR was performed as follows; 5 µg total RNA and 300 ng random primers were mixed in 25 µl ddH_2O, and denatured for 10 minutes at 65°C in a PTC-200 Peltier Thermo Cycler PCR machine (MJ Research, Watertown, MA), followed by immediate cooling on ice. To each reaction was added 20 µl 5X expand RT buffer (Boehringer Mannheim, Laval, QC), 10 µl of 100 mM DTT, 40 µl 10 mM dNTPs mix, 50 units Superscript reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA), and brought up to 75 µl with ddH_2O. Reaction was incubated for first strand cDNA synthesis at 30°C for 10 minutes, followed by 42°C for

45 minutes in PTC-200 Peltier Thermo Cycler PCR machine (MJ Research, Watertown, MA). After first strand cDNA synthesis, PCR was performed as described above, except 5 µl of first strand cDNA reaction mixture was used instead of the 2 µg genomic DNA template. The RT-PCR reactions were done in duplicate, where one set had reverse transcriptase omitted. PCR and RT-PCR procedures were adapted from manufacturer's instructions supplied with equipment and kits.

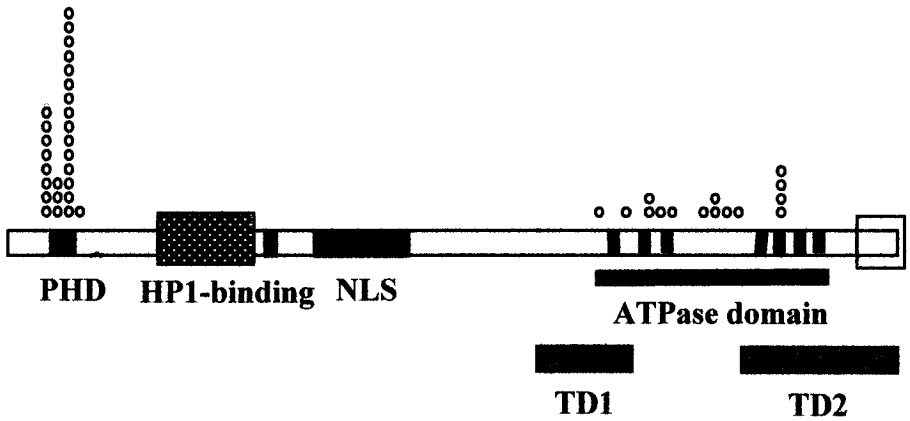
2.2.6 – Analysis of ATRX interactions with PML and Daxx.

2.2.6a - Transient transfection of cells with L2, L19, L19ped3, L19ped16, L19ped23, L19ped26, dsRED-PML, or GFP-PML.

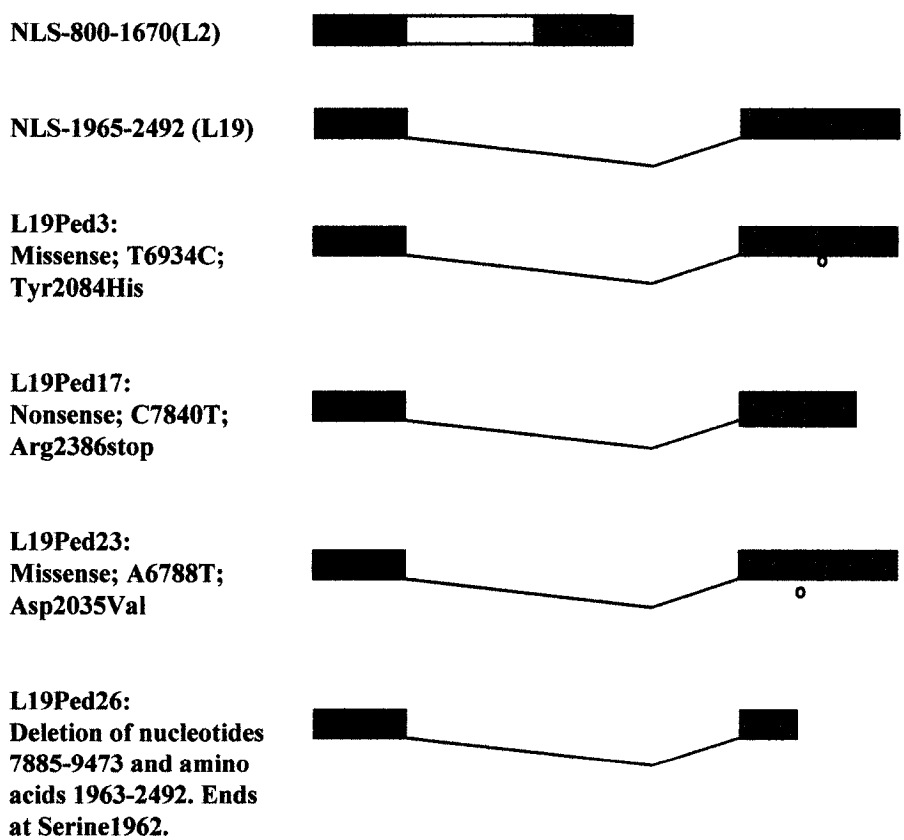
Transient tranfections were performed as described in 2.2.3, except the following constructs were used; L2GFP, L19GFP, L19ped3GFP, L19ped17GFP, L19ped23GFP, L19ped26GFP; constructed and provided by Dr. Nathalie Berube, Ottawa, ON (See Figure 9); dsRED-PML and GFP-PML; a gift of Dr. David P. Bazett-Jones, Toronto, ON. All transfections were in HeLa or NIH 3T3 cells. Also, NIH 3T3 Tet-Off stable clones 1-2c and 1-3d were transfected with EGFP-PML, and ATRXHA expression was induced for 48 hours post-transfection (as previously described). Following transfection, cells in six well dishes were fixed to the coverslips at 18 hours post-transfection as described in 2.2.5a (except NIH 3T3 Tet-Off stable clones 1-2c and 1-3d). Fixed cells were kept in 3 ml PBS at 4°C for no more than 12 hours prior to use. Cells were prepared for immunofluorescence as described in 2.2.5a. To detect GFP and dsRED fusion proteins, no antibodies were required. To detect endogenous proteins, slides were treated with the appropriate primary antibodies at the following dilutions: Rabbit αATRX H-300, 1:100;

Figure 9. Schematic diagram of EGFP-ATRX constructs and patient mutations.

Shown are the locations of the nuclear localization signal (NLS), and the important targeting domains 1 and 2. Below ATRX diagram are schematic diagrams of constructs used in ATRX, PML, and Daxx co-localization studies. Construct L2 contains targeting domain 1 (TD1), and construct L19 contains targeting domain 2 (TD2). Variations of L19 containing patient mutations are also shown. Mutation Ped3 represents a missense mutation where nucleotide 6934 is changed from thymidine to cytidine, resulting in a change of amino acid 2084 from tyrosine to histidine, Ped17 represents a nonsense mutation where nucleotide 7840 is changed from cytidine to thymidine, resulting in a change of amino acid 2386 from arginine to a stop codon, Ped23 represents a missense mutation where nucleotide 6788 is changed from adenosine to thymidine, resulting in a change of amino acid 2035 from asparagine to valine, and Ped26 represents a deletion of nucleotides 7885 to 9473 and amino acids 1963-2492. Ends at Serine1962.
(Picketts *et al.*, 1996).



EGFP Deletion Constructs (used in PML/Daxx studies):



Mouse αPML PG-M3, 1:10; Rabbit αDaxx M-112, 1:50; followed by the appropriate secondary antibodies diluted 1:100.

2.2.6b - Cell harvesting and protein extraction.

Cells transfected with GFP and dsRED constructs were harvested, and protein was extracted and quantified, as described in 2.2.5b. Any wild-type cells used were also subject to the same procedures of harvesting, extraction, and quantitation.

2.2.6c - Immunoprecipitation of ATRX and PML

To immunoprecipitate (IP) ATRX and PML, whole cell protein from clarified extracts (amount used depended on yield of extraction process) was added to 1 ml RIPA buffer supplemented with protease inhibitors (CompleteMini, EDTA-free; Boehringer Mannheim, Laval, QC) and 0.2 mM PMSF in a 1.5 ml Eppendorf tube (three tubes used for each sample, one for ATRX, one for PML, and one for sheep IgG). To each tube were added, 20 µl Protein G Sepharose beads (Amersham Biosciences, Uppsla, Sweden), and 5 µl of the appropriate antibody [rabbit αATRX H-300 (Santa Cruz Biotechnologies, Paso Robles, CA) or sheep αfxnp5 (Affinity Biologicals Incorporated, Hamilton, ON) for ATRX, and mouse αPML PG-M3 (Santa Cruz Biotechnologies, Paso Robles, CA) for PML]. Samples were incubated overnight with rotation at 4°C. The next day, beads were washed five times with 500 µl cold RIPA buffer supplemented with protease inhibitors and 0.2 mM PMSF (each wash consists of resuspending beads in buffer, spinning at 14000 rpm for 5 minutes at 4°C in Eppendorf 5417R benchtop centrifuge, and removing supernatant). Beads were then resuspended in 10 µl RIPA buffer supplemented with

protease inhibitors and 0.2 mM PMSF, and 6 µl SDS-PAGE loading buffer (100 mM Tris pH 6.8, 4% SDS, 20% glycerol, bromophenol blue, 2 µl β-mercaptoethanol), then incubated 3 minutes at 100°C, and centrifuged at 13000 rpm for 3 minutes in a Desaga MC2 bench top centrifuge. Supernatant was kept for immediate use in western blotting analysis.

2.2.6d - SDS-PAGE and Western Blotting

For IP and co-IP western analysis, the entire volumes of supernatant from IP reactions were subject to SDS-PAGE and Western blotting analysis described in 2.2.5b. The appropriate primary antibodies were used at the following dilutions: Rabbit αATRX H-300, 1:100; Mouse αPML PG-M3, 1:100; Rabbit αDaxx M-112, 1:100; Goat αDaxx CT, 1:500; Sheepαfxnp5, 1:1000. The appropriate secondary antibodies were used at the following dilutions: αRabbit.HRP, 1:1000; αMouse.HRP, 1:1000; αSheep.HRP, 1:5000; αGoat.HRP, 1:1000.

3 – Results

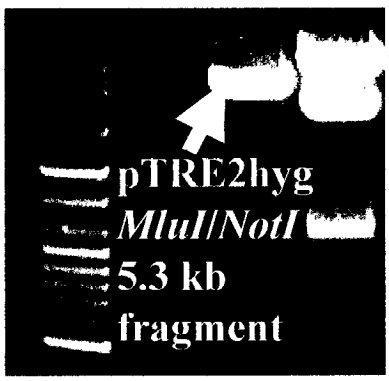
3.1 – ATRXHA was cloned into pTRE2hyg.

ATRXHA overexpression experiments were initiated by constructing a tetracycline response plasmid. The ATRXHA cDNA was excised from pCAGGS-SL-ATRXHA at the *BssHII – NotI* sites and ligated into the *MluI – NotI* sites of the pTRE2hyg vector plasmid (Clontech Laboratories, Inc., Palo Alto, CA). The *BssHII* and *MluI* restriction sites represent compatible cohesive ends. The *BssHII – NotI* digest of pCAGGS-SL-ATRXHA gave three bands, of which the 7.5 kilobase pair (kb) band corresponded to the size of ATRXHA and was excised (Figure 10a). The *MluI – NotI* digest of pTRE2hyg gave one band of 5.2 kb, corresponding to the size of the plasmid, and was excised (Figure 10a). After ligating the fragments, transforming STBL-2 cells, and purifying the plasmid DNA, *EcoRI* digests revealed that 19 of the 30 samples screened had the banding pattern corresponding to pTRE2hyg-ATRXHA (3.6 kb, 2.5 kb, 2.3 kb, 1.6 kb, 1.1 kb, 1.0 kb, and 0.6 kb) (Figure 10b, lanes 5, 8, 10, 11, 13, and 14; Figure 10c, lanes 2, 4, 5, 6, 7, 8, 9, 10, 12, 14, 15, 16, and 17). Of the samples that were negative, 3 samples revealed the *EcoRI* digest banding pattern of the empty pTRE2hyg vector (Figure 10b, lanes 9 and 12; Figure 10c, lane 13), one sample appeared to have the banding pattern of an undigested or single cut pTRE2hyg empty vector (Figure 10b, lane 7), and 7 samples had an *EcoRI* digest banding pattern that was similar to pTRE2hyg-ATRXHA, except the 2.3 kb band had dropped to approximately 1.8 kb, and the 1.0 kb band was absent (Figure 10b, lanes 2, 3, 4, 6, and 15; Figure 10c, lanes 3 and 11).

Figure 10. ATRXHA was cloned into pTRE2hyg.

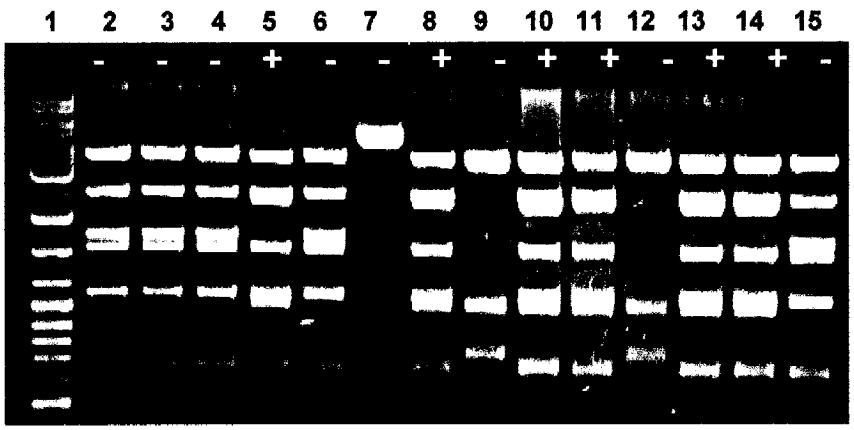
(a) The 5.3 kb *MluI – NotI* band of pTRE2hyg, and the *BssHII – NotI* band of pCAGGS-SL-ATRXHA (corresponding to ATRXHA cDNA), were excised from the gel and ligated together. *EcoRI* digests of DNA from plasmid mini preps of isolated transformed STBL-2 colonies, revealed that 19 of the 30 samples screened had the banding pattern corresponding to pTRE2hyg-ATRXHA (3.6 kb, 2.5 kb, 2.3 kb, 1.6 kb, 1.1 kb, 1.0 kb, and 0.6 kb) (b) lanes 5, 8, 10, 11, 13, and 14; (c) lanes 2, 4, 5, 6, 7, 8, 9, 10, 12, 14, 15, 16, and 17.

a)

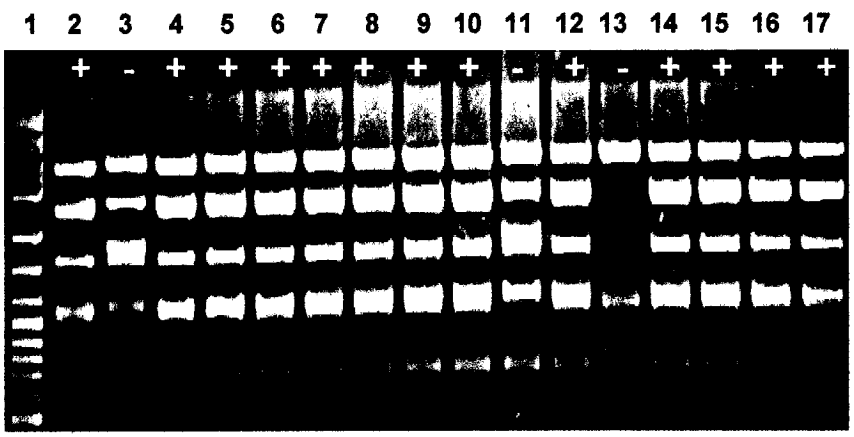


ATRXHA
BssHII/NotI
7.5 kb fragment

b)



c)

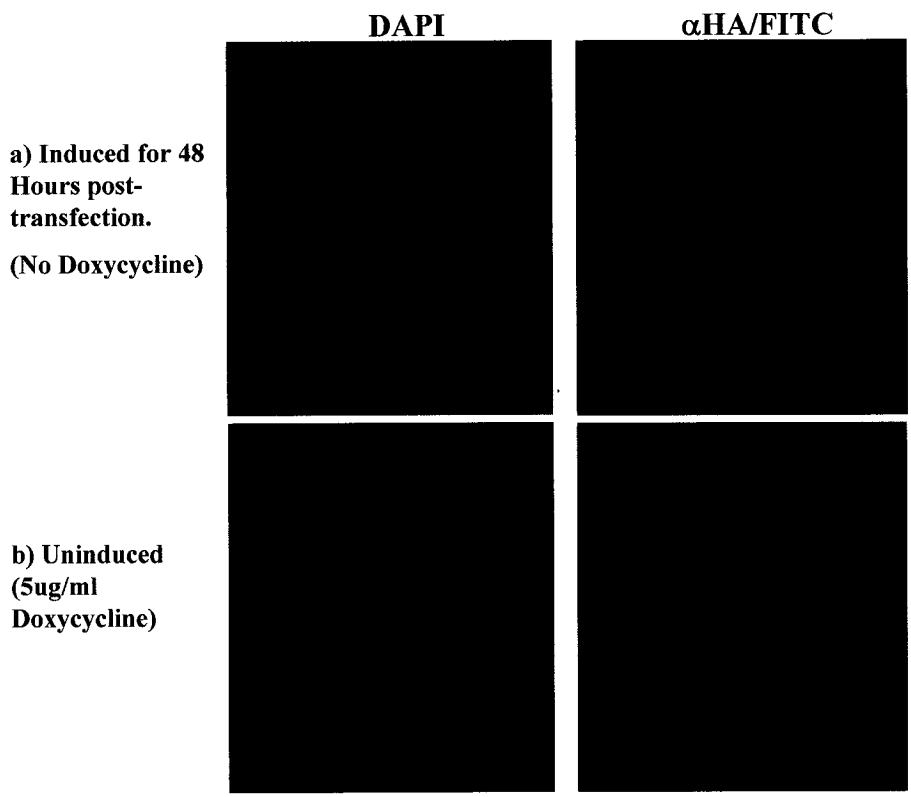


3.2 – pTRE2hyg-ATRXHA expresses ATRXHA when transiently transfected into NIH 3T3 Tet-Off cells.

To determine whether or not the pTRE2hyg-ATRXHA construct could be induced in the Tet-Off system to express ATRXHA, plasmid DNA was transiently transfected into NIH 3T3 cells stably transfected with the pTet-Off regulatory plasmid (Clontech Laboratories, Inc., Palo Alto, CA). When NIH 3T3 Tet-Off cells, transiently transfected with pTRE2hyg-ATRXHA, were grown in the absence of doxycycline (Dox), it was possible to detect by immunofluorescence (IF), using the Rabbit αHA.11 primary antibody and the αRabbit.FITC secondary antibody, a punctate nuclear pattern of speckling which is characteristic of endogenous ATRX, suggesting the HA-tagged form of ATRX behaves like the endogenous form (Figure 11a). In contrast, when NIH 3T3 Tet-Off cells, transiently transfected with pTRE2hyg-ATRXHA, were grown in the presence of Dox, it was not possible to detect an HA/FITC signal (Figure 11b). These results show that both the regulatory and response plasmids of the Tet-Off system are operational in these cells.

Figure 11. pTRE2hyg-ATRXHA expresses ATRXHA when transiently transfected into NIH 3T3 Tet-Off cells and grown in the absence of doxycycline.

(a) NIH 3T3 Tet-Off cells induced 48 hours, post-transient transfection with pTRE2hyg-ATRXHA, in the absence of doxycycline, were stained with αHA/FITC. Seen by immunofluorescence analysis was a nuclear staining pattern similar to that of ATRX. (b) NIH 3T3 Tet-Off cells, grown post-transient transfection with pTRE2hyg-ATRXHA, in the presence of doxycycline, were stained with αHA/FITC with no visible signal. These results suggest that the Tet-Off system is operational in these cells.


DAPI
αHA/FITC
a) Induced for 48 Hours post-transfection.
(No Doxycycline)
b) Uninduced (5ug/ml Doxycycline)

3.3 – NIH 3T3 Tet-Off stable clones integrate pTRE2hyg-ATRXHA, and express ATRXHA.

Initial attempts at creating stable clones revealed that ATRXHA was expressed, and was detectable by IF in clones 1-2, 1-3, 1-4, 1-9, 1-10 (not shown), 1-11, 1-12, 1-14, and 4-1 (Shown at 96 hours post-induction) (Figure 12). However, cell populations were heterogeneous, where approximately 50% of cells, at best, were expressing ATRXHA (Table 1). After serial dilutions of cell populations, and reselecting isolated colonies in an attempt to improve the homogeneity of the cell lines, it was found that although ATRXHA was expressed, and was detectable by IF in clones 1-2a, 1-2b, 1-2c, 1-2d, 1-2e, 1-3b, 1-3c, 1-3d, 1-3e, and 1-3f (shown at 96 hours post induction) (Figure 13), the numbers of cells expressing ATRXHA had improved only marginally, and in some cases decreased (Table 2). To determine if the pTRE2hyg-ATRXHA construct had actually been integrated into the cellular genome, genomic DNA was isolated and subject to PCR analysis (see Materials and Methods). PCR analysis of selected stable clones produced the expected product of approximately 900 bp in clones 1-2, 1-2b, 1-2c, 1-2d, 1-2e, 1-3, 1-3d, 1-3e, 1-4, 1-9, 1-10, 1-11, and 1-14 (Figure 14). To look at ATRXHA mRNA expression, a representative clone with good ATRXHA expression (1-2b) was subject to RT-PCR analysis (the same primer pair was used as in the PCR analysis). RT-PCR analysis showed that ATRXHA mRNA transcript was being expressed, starting at 48 hours and continuing until 96 hours, as is evident by the presence of the expected 900 bp band (Figure 15). To determine if and when ATRXHA protein was being expressed, whole cell protein extract from a selection of clones was subject to Western blot analysis. This analysis showed that parent cell line 1-2 expressed ATRXHA at 24, 48, and 72

Figure 12. NIH 3T3 Tet-Off cell lines stably transfected with pTRE2hyg-ATRXHA express ATRXHA when grown in the absence of doxycycline.

Shown are eight of the nine cell lines, from clones selected during initial screening process, that consistently express ATRXHA when grown in the absence of doxycycline. Cells are shown at 96 hours after the removal of doxycycline. Slides were scanned for a field in which a maximal number of cells were expressing. Table 1 lists counts for overall percentages of cells expressing.

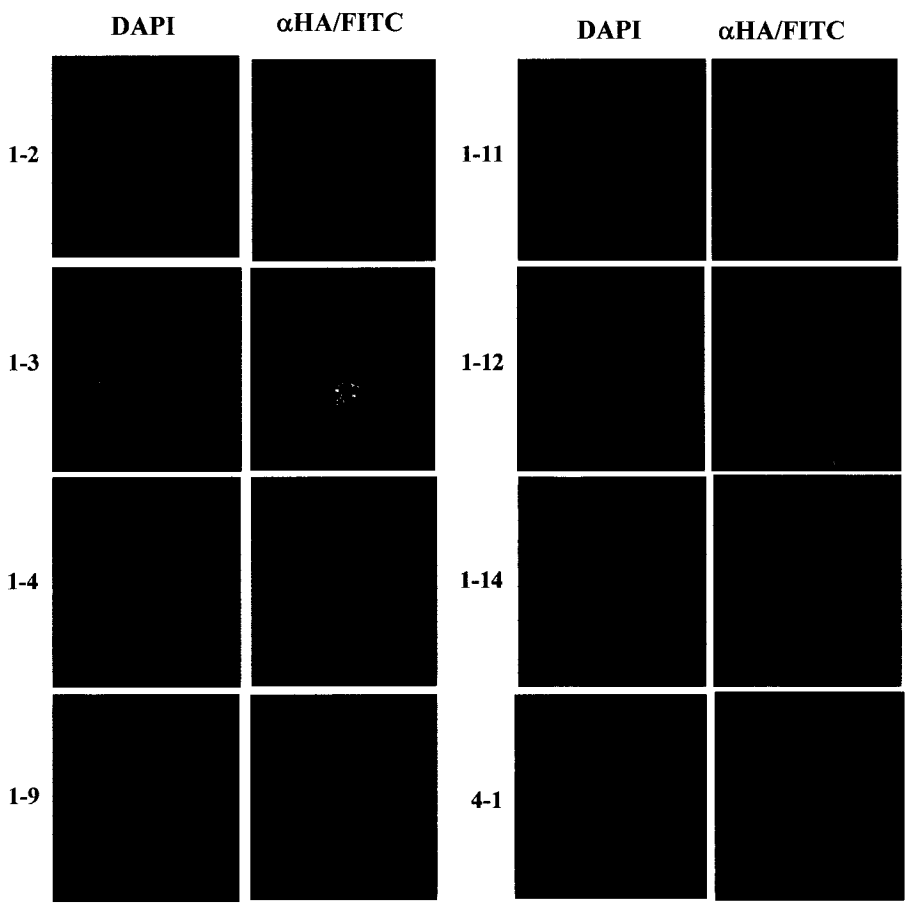

DAPI
αHA/FITC
DAPI
αHA/FITC
1-2
1-3
1-4
1-9
1-11
1-12
1-14
4-1

Table 1. Summary of clones selected during initial pTRE2hyg-ATRXHA stable transfection screening process.

Clone #	ATRXHA Expression	Cells Expressing ATRXHA
1-1	-	N/A
1-2	+	~50%
1-3	+	~60%
1-4	+	~60%
1-5	-	N/A
1-6	-	N/A
1-7	-	N/A
1-8	-	N/A
1-9	+	<10%
1-10	+	<10%
1-11	+	~60%
1-12	+	~10%
1-13	-	N/A
1-14	+	~40%
1-15	-	N/A
1-16	-	N/A
3-1	-	N/A
3-2	-	N/A
3-3	-	N/A
3-4	-	N/A
4-1	+	<10%
4-2	-	N/A
4-3	-	N/A
4-4	-	N/A
4-5	-	N/A
5-1	-	N/A
5-2	-	N/A
5-3	-	N/A
5-4	-	N/A

Figure 13. Cell lines derived from initial NIH 3T3 Tet-Off cell lines stably transfected with pTRE2hyg-ATRXHA.

Shown are the ten cell lines, derived by serial dilution from clones selected during initial screening process, which consistently express ATRXHA when grown in the absence of doxycycline. Cells are shown at 96 hours after the removal of doxycycline. Slides were scanned for a field in which a maximal number of cells were expressing. Table 2 lists counts for overall percentages of cells expressing.

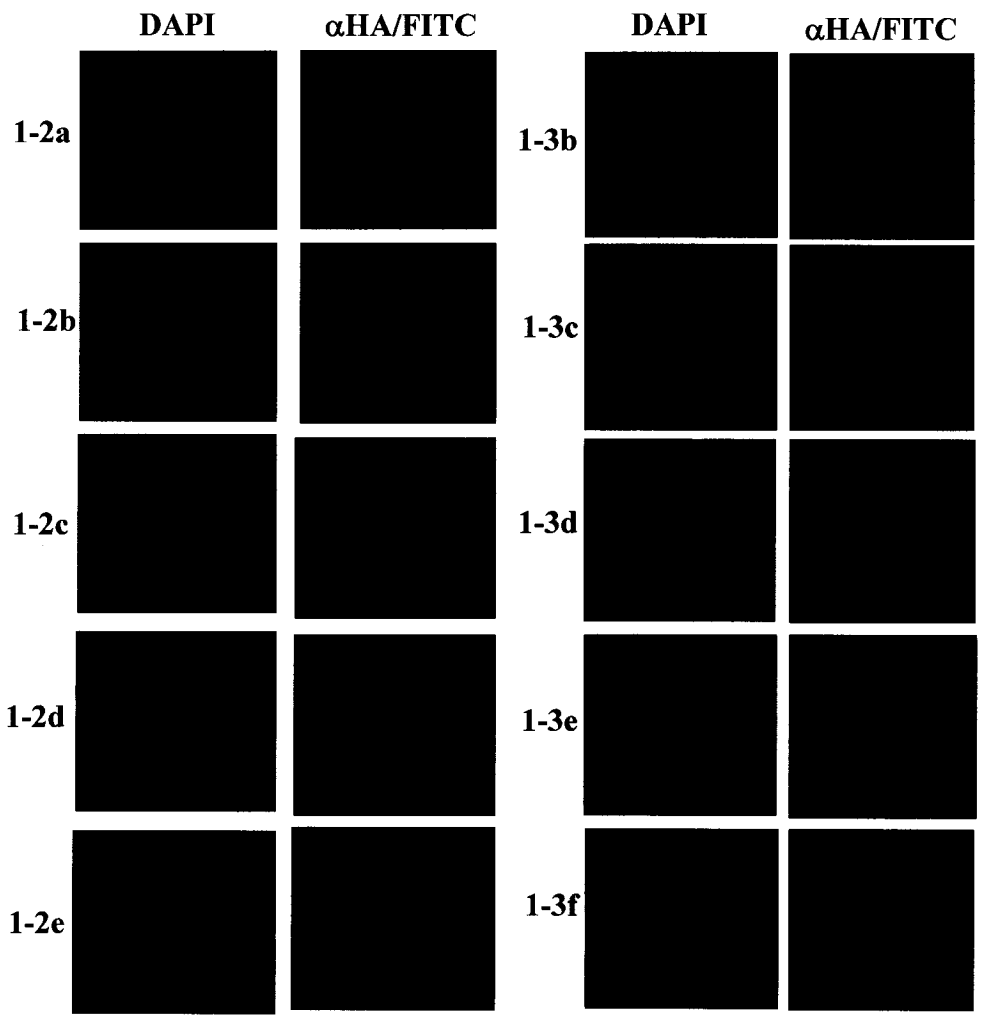

DAPI
αHA/FITC
DAPI
αHA/FITC
1-2a
1-2b
1-2c
1-2d
1-2e
1-3b
1-3c
1-3d
1-3e
1-3f

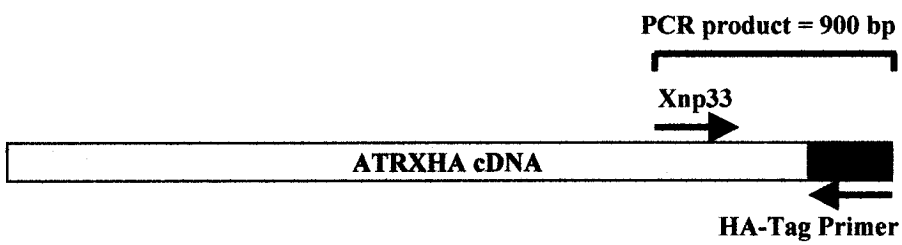
Table 2. Summary of cell lines derived by serial dilution from clones selected during initial pTRE2hyg-ATRXHA stable transfection screening process.

Of the twelve cell lines obtained, only two did not express ATRXHA. However, the serial dilutions do not appear to have improved the homogenosity of the cultures.

Clone #	ATRXHA Expression	Cells Expressing ATRXHA
1-2a	+	>50%
1-2b	+	~50%
1-2c	+	~50%
1-2d	+	~50%
1-2e	+	~50%
1-2f	-	N/A
1-3a	-	N/A
1-3b	+	~40%
1-3c	+	<10%
1-3d	+	~50%
1-3e	+	>60%
1-3f	+	>60%

Figure 14. Integration of pTRE2hyg-ATRXHA into genomic DNA.

PCR analysis of stable cell lines demonstrates pTRE2hyg-ATRXHA integrated into genomic DNA. Lanes: 1 = 1 kb plus DNA ladder; 2 = clone #1-2; 3 = clone #1-2b; 4 = clone #1-2c; 5 = clone #1-2d; 6 = clone #1-2e; 7 = clone #1-3; 8 = clone #1-3d; 9 = clone #1-3e; 10 = clone #1-4; 11 = clone #1-9; 12 = clone #1-10; 13 = clone #1-11; 14 = clone #1-14; 15 = positive control (pTRE2hyg-ATRXHA plasmid DNA); 16 = positive control (pCAGGS-SL-ATRXHA plasmid DNA); 17 = negative control (no DNA template). Lanes 2 to 14 are positive for the expected PCR product of approximately 900 bp. Lanes 15 and 16 show plasmid DNA of pTRE2hyg-ATRXHA and pCAGGS-SL-ATRXHA produced the same 900 bp band. These results show that pTRE2hyg-ATRXHA is integrated into the genomic DNA of all tested clones.


PCR product = 900 bp
Xnp33
ATRXHA cDNA
HA-Tag Primer

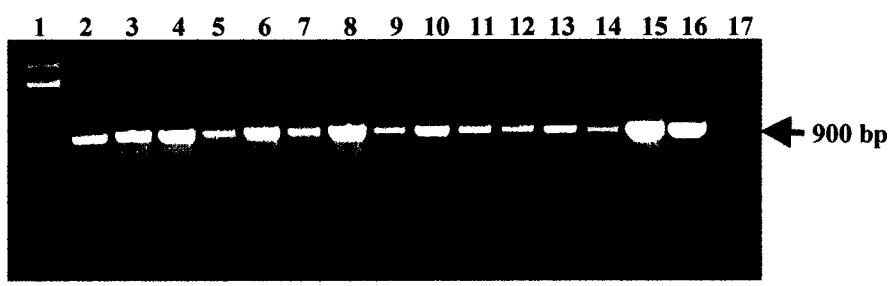
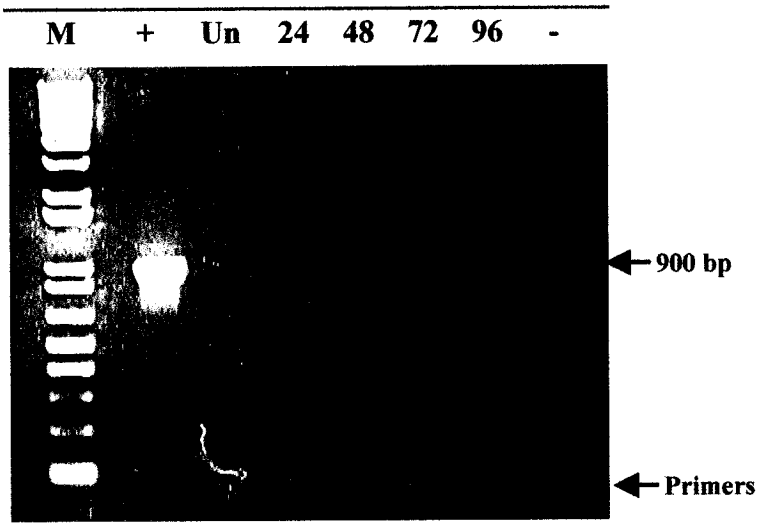

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
900 bp

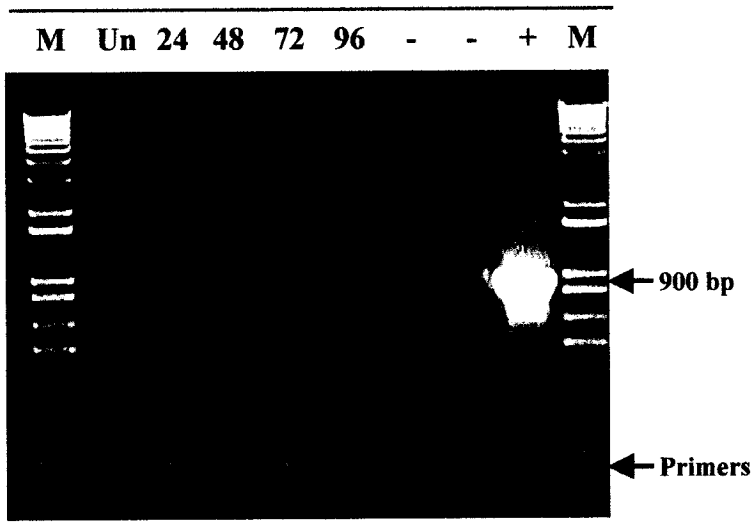
Figure 15. ATRXHA mRNA is detected after 48 hours of induction.

To look at ATRXHA mRNA expression, a representative clone with good ATRXHA expression (1-2b) was subject to RT-PCR analysis (the same primer pair was used as in the PCR analysis). RT-PCR analysis of clone 1-2b shows that expression of ATRXHA mRNA starts somewhere between 24 and 48 hours, and continues until, but is starting to decrease at 96 hours. Identical primers were used as those in the PCR analysis shown in Figure 14. Thus, a band of 900 bp is the expected product.

1-2b RT-PCR



1-2b No RT



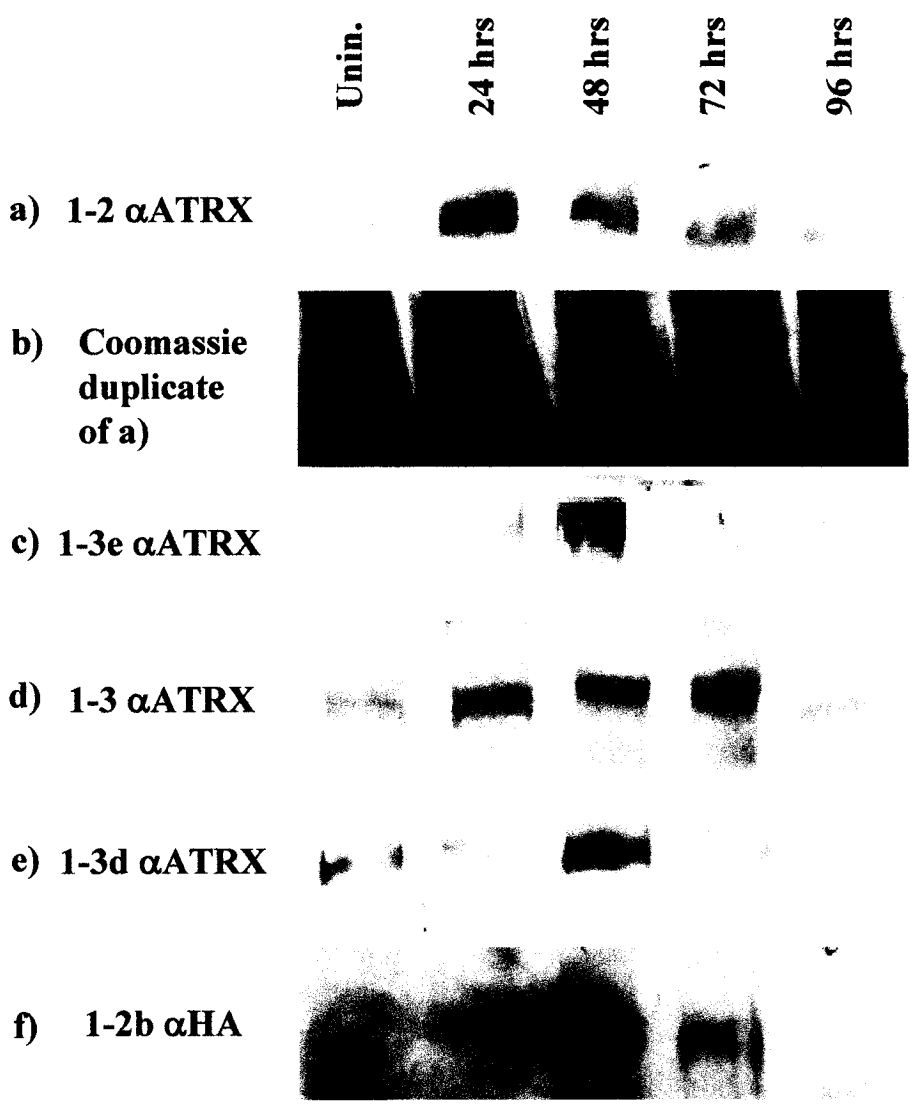
hours after removal of doxycycline from media (Figure 16a and 16b). Immunoprecipitation of ATRX in clone 1-3e shows ATRX expression to be maximal at 48 hours (Figure 16c). Western analysis of cell line 1-3 shows ATRX expression at 24, 48, and 72 hours after removal of doxycycline from media (Figure 16d). Clones 1-2b and 1-3d appeared to have a maximal expression at 48 hours (Figure 16e and 16f). These results show that ATRX protein was being expressed at different levels at different time points. The pictures in Figures 16a-c are valid with respect to time point expression analysis. However, although gel-loading technique was consistent with respect to amount of protein loaded, Figures 16d-f do not have loading controls. Therefore, these gels can only verify the presence of ATRX and ATRXHA.

3.4 – Induced ATRXHA colocalizes with transiently transfected EGFP-PML.

It has been shown that ATRX localizes to PML bodies within the nucleus (Gibbons *et al.*, 2003). To verify that ATRXHA will behave like endogenous ATRX, and colocalize to PML bodies, NIH 3T3 Tet-Off stable clones were transiently transfected with EGFP-PML, then induced to express ATRXHA. When NIH 3T3 Tet-Off stable cell lines 1-2b and 1-3d were transiently transfected with EGFP-PML, and induced to express ATRXHA, it was observed by IF that the two proteins colocalized with each other in the nucleus, forming the distinct punctate nuclear speckling pattern characteristic of both proteins (Figure 17). These results demonstrate that ATRXHA colocalizes with PML in the nucleus in a similar fashion to that of endogenous ATRX.

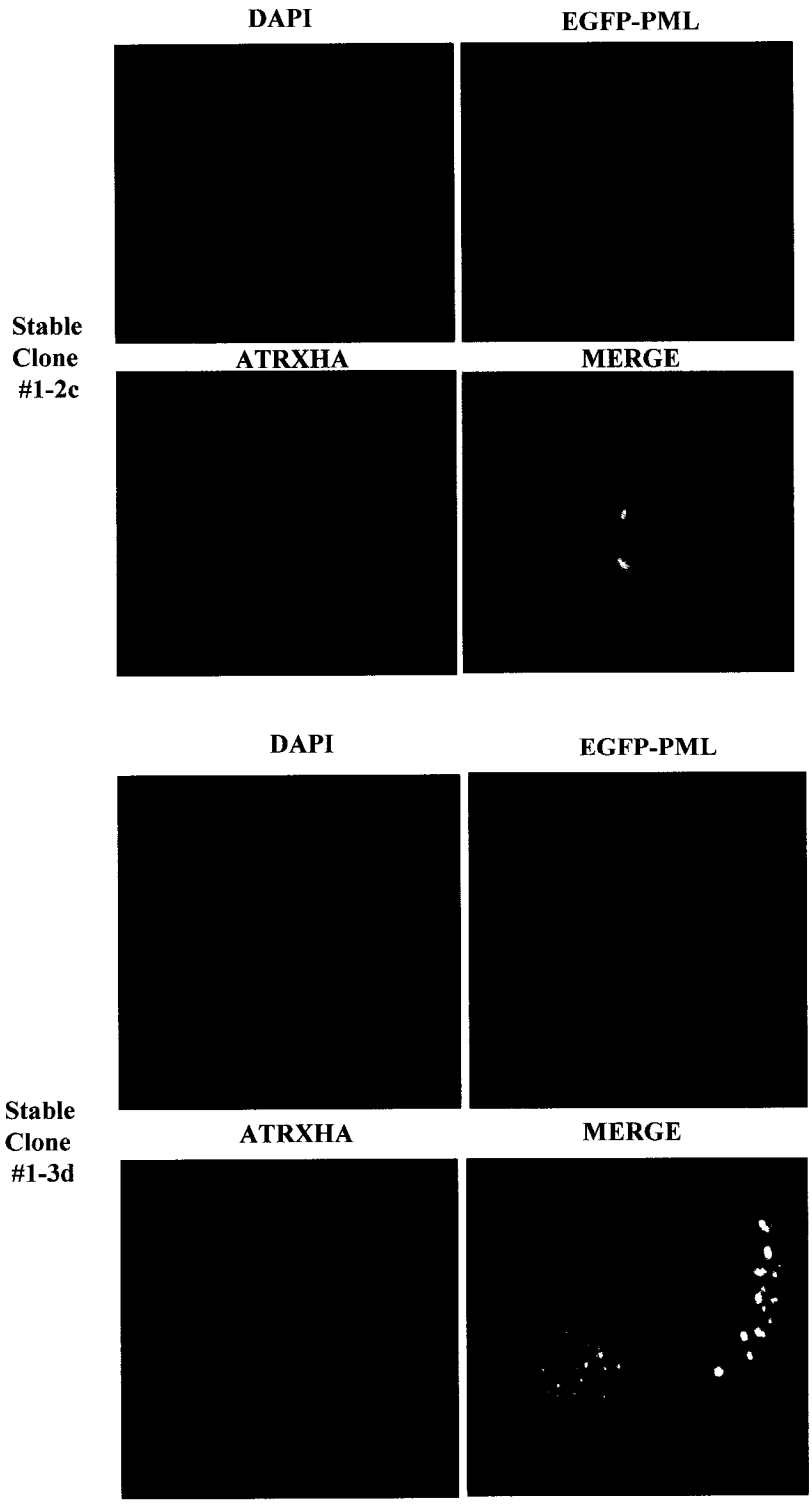
Figure 16. Western blot analysis of stable clones.

(a) Stable clone 1-2 is shown to be expressing ATRXHA protein at 24, 48, and 72 hours, but seems to return to approximately endogenous levels at 96 hours. (b) Coomassie of duplicate gel of (a) demonstrates equal loading of protein. (c) Immunoprecipitation of ATRX from clone 1-3e suggests ATRXHA expression to have a significant peak at 48 hours, and a much lesser signal at 24 and 72 hours, but does not seem to have significant levels of expression at any other times. (d) Western analysis of cell line 1-3 shows ATRX expression at 24, 48, and 72 hours after removal of doxycycline from media. (e) and (f) Clones 1-2b and 1-3d appeared to have a maximal expression at 48 hours. These results show that ATRX protein was being expressed at different levels at different time points. The pictures in Figures 16a-c are valid with respect to time point expression analysis. However, although gel-loading technique was consistent with respect to amount of protein loaded, Figures 16d-f do not have loading controls. Therefore, these gels can only verify the presence of ATRX and ATRXHA.


Unin.
24 hrs
48 hrs
72 hrs
96 hrs
a) 1-2 αATRX
b) Coomassie duplicate of a)
c) 1-3e αATRX
d) 1-3 αATRX
e) 1-3d αATRX
f) 1-2b αHA

Figures 17. Transient transfection of EGFP-PML into stable clones 1-2c and 1-3d.

Stable cell lines were induced to express ATRXHA for 24 hours, then transiently transfected with EGFP-PML. In both cases, ATRXHA colocalized almost completely with EGFP-PML. Nuclear bundles were larger, and lesser in number than is normal for either protein endogenously.

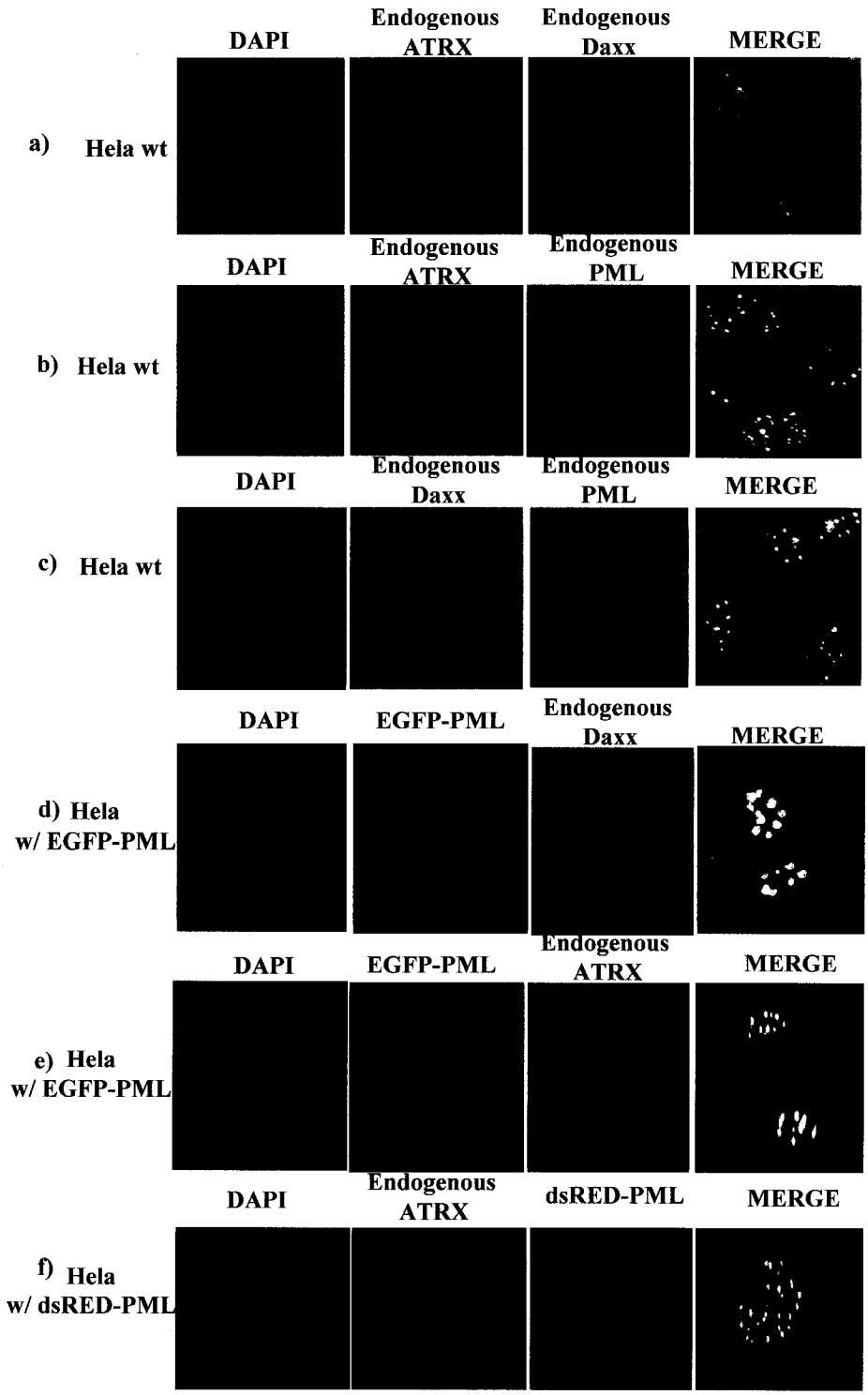

DAPI
EGFP-PML
Stable
Clone
#1-2c
ATRXHA
MERGE
DAPI
EGFP-PML
Stable
Clone
#1-3d
ATRXHA
MERGE

3.5 – Endogenous ATRX colocalizes with endogenous PML and Daxx, and with transiently transfected EGFP-PML and dsRED-PML.

The PML/Daxx interaction was explained earlier (see Introduction), and it has been mentioned that ATRX localizes to PML bodies in the nucleus (see 3.4 above). To verify all of these interactions, IF analysis of these endogenous proteins were performed in HeLa cells. Immunofluorescence analysis of HeLa cells showed that endogenous ATRX colocalizes with endogenous PML and endogenous Daxx (Figure 18a and 18b). However, the colocalization does not appear to be complete, as there are areas where there is ATRX alone, without PML or Daxx colocalization. PML and Daxx have been shown to colocalize with each other almost completely (Figure 18c). In contrast, when HeLa cells are transfected with either EGFP-PML or dsRED-PML, IF analysis shows that ATRX colocalizes with these proteins almost 100% (Figure 18d, 18e and 18f). It should also be mentioned that all these proteins form the distinct punctate nuclear speckling pattern, but in cells transfected with EGFP-PML or dsRED-PML, the speckles are much larger than is normal for PML or ATRX. As well, there also appears to be fewer speckles than is normal for PML or ATRX.

Figure 18. ATRX, Daxx, and PML colocalization in HeLa cells.

(a) and (b) ATRX appears to colocalize with both Daxx and PML, but neither appear to be an all inclusive colocalization. (c) Colocalization of Daxx and PML seems to be much more inclusive than either proteins' colocalization with ATRX. (d) (e) and (f) The colocalization of EGFP-PML with either ATRX or Daxx is very inclusive, as is that of dsRED-PML and ATRX. Although the characteristic punctate nuclear speckling pattern of all three proteins is present, the speckles are much larger than is normal, and there also appears to be fewer speckles.


DAPI
Endogenous ATRX
Endogenous Daxx
MERGE
a) Hela wt
DAPI
Endogenous ATRX
Endogenous PML
MERGE
b) Hela wt
DAPI
Endogenous Daxx
Endogenous PML
MERGE
c) Hela wt
DAPI
EGFP-PML
Endogenous Daxx
MERGE
d) Hela w/ EGFP-PML
DAPI
EGFP-PML
Endogenous ATRX
MERGE
e) Hela w/ EGFP-PML
DAPI
Endogenous ATRX
dsRED-PML
MERGE
f) Hela w/ dsRED-PML

3.6 – Targeting domain 1 (TD1) does not colocalize with PML and Daxx, whereas targeting domain 2 (TD2) does colocalize with PML, and to a lesser degree Daxx.

Vector constructs containing fragments of ATRX in a fusion protein with GFP were subject to IF analysis, and specific fragments of the protein resulted in the characteristic nuclear speckling pattern, two of these were designated as Targeting Domain 1 (TD1) and Targeting Domain 2 (TD2) (DJ Picketts, unpublished data). The GFP constructs (both containing the ATRX nuclear localization signal) were designated as L2 and L19 respectively. Immunofluorescence studies where the L2 (TD1) or L19 (TD2) GFP constructs were transiently transfected into HeLa cells by themselves, or co-transfected with dsRED-PML, revealed that L2 does not readily colocalize with transfected dsRED-PML or endogenous PML and Daxx in the nucleus (Figures 19a, 20a, and 21a). In contrast, L19 colocalizes readily with dsRED-PML and endogenous PML, and is able to form the distinct punctate nuclear speckling pattern (Figures 19b and 20b). However, L19 does not appear to have significant colocalization with endogenous Daxx (Figure 21b). These results confirm that TD1 and TD2 are probably responsible for the formation of the characteristic nuclear speckling pattern. Also, they confirm that TD1 is not the portion of ATRX that interacts with PML or Daxx, and that TD2 likely contains the PML-interacting domain, but may not contain the Daxx-interacting domain.

3.7 – Patient mutations appear to alter the targeting of ATRX to nuclear bundles, as well as the colocalization of ATRX with Daxx and PML.

Mutations in TD2 have been shown to reduce or alter the characteristic nuclear speckling pattern. The ATR-X patient mutations Ped3, Ped17, Ped23, and Ped26 are four mutations that have been shown to alter ATRX nuclear speckling (DJ Picketts, unpublished data). In patients, all four mutations result in typical ATR-X features with varying degrees of severity, such as genital abnormality (present in all, but most severe in Ped17 and Ped26), and HbH inclusions (interestingly Ped17 and Ped26 have the least severe forms of alpha-thalassemia, the most severe being Ped23 followed by Ped3) (Picketts *et al.*, 1996). To determine if the alteration in speckling is due to altered interactions with PML or Daxx, constructs were transiently transfected into HeLa cells (in some cases co-transfected with dsRED-PML), and their colocalization and nuclear localization patterns were observed. The same IF studies described in section 3.7 were performed with the L19 GFP constructs containing ATR-X patient mutations Ped3, Ped17, Ped23, and Ped26 (described in Material and Methods). All mutations appeared to affect colocalization of ATRX and endogenous Daxx, endogenous PML or transfected dsRED-PML to some degree (Figures 19c-f, 20c-f, and 21c-f). They did not affect nuclear localization (except for a slight affect in Ped17 when cotransfected with dsRED-PML, possibly because the presence of dsRED-PML affects this particular construct differently), but there did appear to be less nuclear bundle formation, as well as less PML bodies than normal in transfected cells. The constructs with Ped17 and Ped26 appeared to have the least amount of speckling. In fact, they were usually distributed evenly throughout the nucleus. In the cells stained for endogenous Daxx, L19Ped3, L19Ped17,

Figure 19. Targeting domain (TD) and patient mutation immunofluorescence studies with transiently transfected dsRED-PML in HeLa cells.

(a) L2 (TD1) does not readily colocalize with dsRED-PML, but maintains nuclear speckling pattern. (b) L19 fully colocalizes with dsRED-PML, and maintains nuclear speckling pattern. (c) These pictures show L19Ped3 having significant colocalization with dsRED-PML, but there appears to be less speckling, and some diffuse ATRX staining within the nucleus. (d) Here L19Ped17 appears to have reduced colocalization with dsRED-PML, and there appears to be less speckling, and some difficulty entering the nucleus. This difficulty may have something to do with the presence of dsRED-PML affecting this particular construct. (e) and (f) These pictures show L19Ped23 and L19Ped26 entering the nucleus, and having significant colocalization with dsRED-PML, although speckling is reduced, and there is evidence of some diffuse nuclear staining.

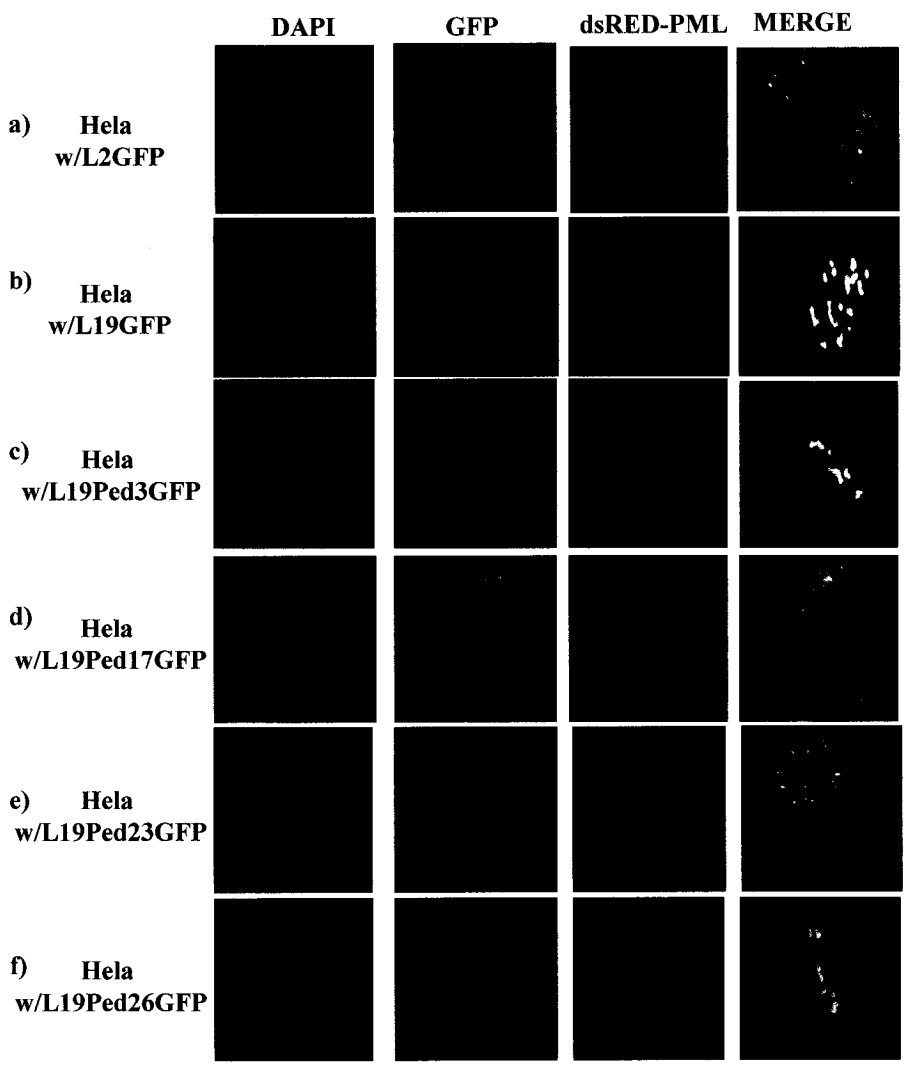

DAPI
GFP
dsRED-PML
MERGE
a) Hela w/L2GFP
b) Hela w/L19GFP
c) Hela w/L19Ped3GFP
d) Hela w/L19Ped17GFP
e) Hela w/L19Ped23GFP
f) Hela w/L19Ped26GFP

Figure 20. L19 and L19 patient mutation immunofluorescence studies with endogenous PML in HeLa cells.

(a) L2 does not readily colocalize with endogenous PML, but maintains the nuclear speckling pattern to some degree. (b) L19 is shown to enter the nucleus, form nuclear bundles, and to colocalize with endogenous PML. (c) and (d) These pictures show L19Ped3 and L19Ped17 are able to enter the nucleus, where L19Ped3 seems to be able to form nuclear bundles, but L19Ped17does not. L19Ped3 appears to colocalize with endogenous PML, whereas L19Ped17 does not having any significant colocalization. (e) and (f) These pictures show L19Ped23 and L19Ped26 entering the nucleus, but only L19Ped23 is shown to form nuclear bundles and colocalize with ATRX. Like Ped17, Ped26 shows a diffuse nuclear staining pattern.
Note that, in most cases, cells transfected with the GFP fusion protein constructs have reduced levels of endogenous PML.

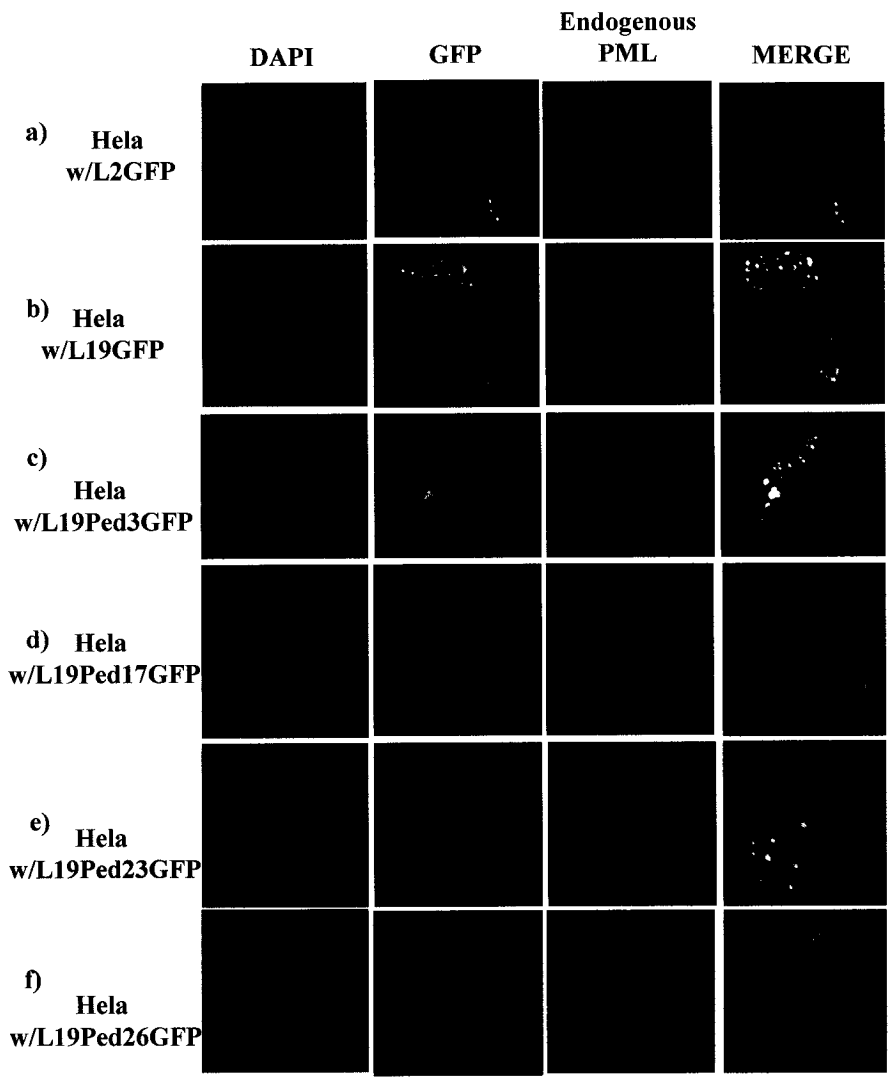
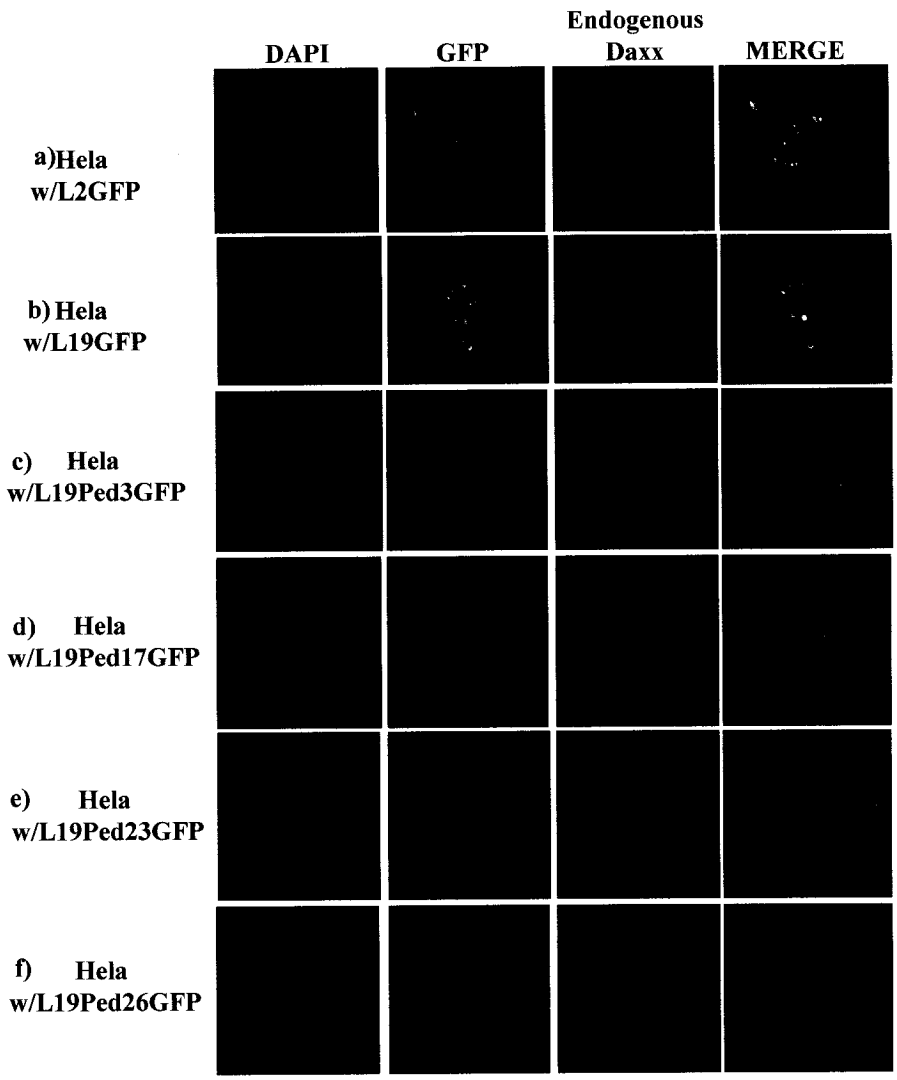

DAPI
GFP
Endogenous PML
MERGE
a) Hela w/L2GFP
b) Hela w/L19GFP
c) Hela w/L19Ped3GFP
d) Hela w/L19Ped17GFP
e) Hela w/L19Ped23GFP
f) Hela w/L19Ped26GFP

Figure 21. L19 and L19 patient mutation immunofluorescence studies with endogenous Daxx in HeLa cells.

(a) L2 does not readily colocalize with endogenous Daxx, but maintains the nuclear speckling pattern. (b) L19 is shown to enter the nucleus and form nuclear bundles, but it appears to have very little colocalization with endogenous Daxx. (c-f) These pictures show L19Ped3, L19Ped17, L19Ped23, and L19Ped26 to be spread throughout the nucleus, with very little, if any, colocalization with Daxx. In fact, Daxx appears to be sequestered to nucleoli in these pictures.


DAPI
GFP
Endogenous Daxx
MERGE
a)Hela w/L2GFP
b) Hela w/L19GFP
c) Hela w/L19Ped3GFP
d) Hela w/L19Ped17GFP
e) Hela w/L19Ped23GFP
f) Hela w/L19Ped26GFP

L19Ped23, and L19Ped26 were observed to be spread throughout the nucleus, with very little, if any, colocalization with Daxx. In fact, Daxx appears to be sequestered to nucleoli in these pictures. Cells co-transfected with dsRED-PML had more evidence of speckle formation, but speckles were abnormally large (compared to wild-type cells), and lower in number, suggesting dsRED-PML may draw in nuclear material in a non-specific manner.

When cotransfected with dsRED-PML into NIH 3T3 cells, L19 and L19 mutants did not appear to have much difficulty interacting with dsRED-PML (Figure 22). Although the speckling pattern of the mutants was not typical of endogenous, wild-type ATRX.

To determine if L19 and L19 mutants actually do affect endogenous PML levels, western blot analysis was performed on whole cell extract from transiently transfected cells. Western blot analysis suggests that L19 and the L19 mutants are expressed when transiently transfected into HeLa cells (Figure 23).

To determine if endogenous ATRX can colocalize with dsRED-PML in actual ATR-X patient cells, fibroblasts isolated from two different ATR-X patients were transiently transfected with dsRED-PML and analyzed by immunofluorescence. When transfected into these patient fibroblasts, dsRED-PML formed large nuclear bodies that colocalized with endogenous ATRX (Figure 24). In these patient cell lines, ATRX levels appear to be very low in untransfected cells. The mutation in cell line BH is a point mutation at nucleotide 6720 that changes a Glycine residue to an Arginine. The mutation in cell line 851302 is a point mutation at nucleotide 5376 that changes an Isoleucine to a Threonine residue. These both occur in the ATPase domain.

Figure 22. Targeting domain (TD) and patient mutation immunofluorescence studies with transiently transfected dsRED-PML in NIH 3T3 cells.

In all cases, L19 and L19 mutants did not appear to have difficulty entering the nucleus, forming nuclear bundles, and colocalizing with dsRED-PML. Although the speckling pattern of the mutants was still not typical of endogenous, wild-type ATRX.

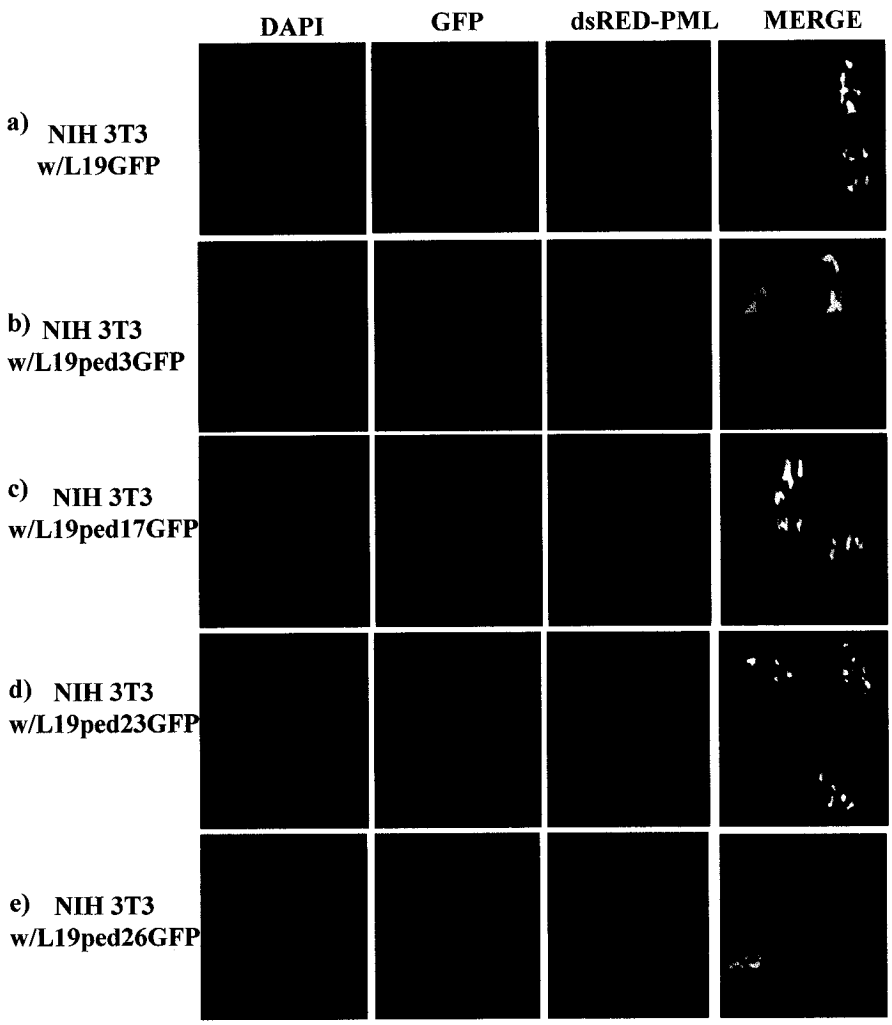

DAPI
GFP
dsRED-PML
MERGE
a) NIH 3T3 w/L19GFP
b) NIH 3T3 w/L19ped3GFP
c) NIH 3T3 w/L19ped17GFP
d) NIH 3T3 w/L19ped23GFP
e) NIH 3T3 w/L19ped26GFP

Figure 23. Western blot analysis of HeLa cells transfected with L19 and the L19 mutants.

This western blot analysis shows that L19 and the L19 mutants are expressed when transiently transfected into HeLa cells.

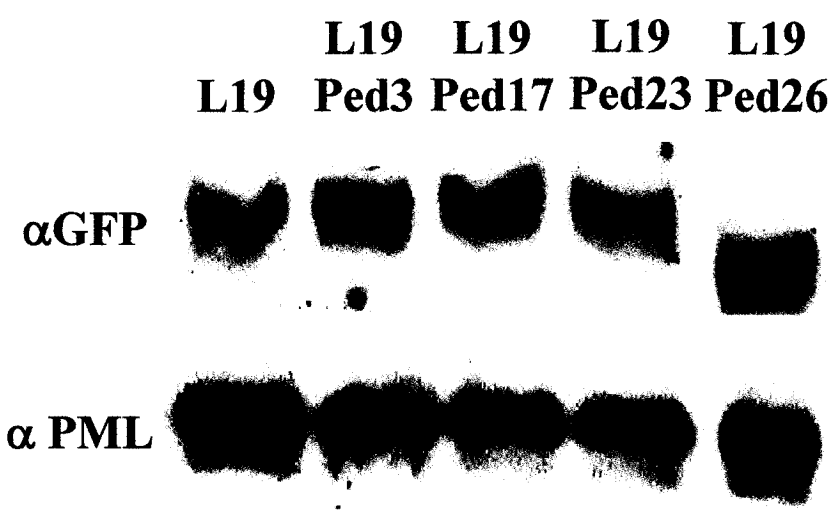
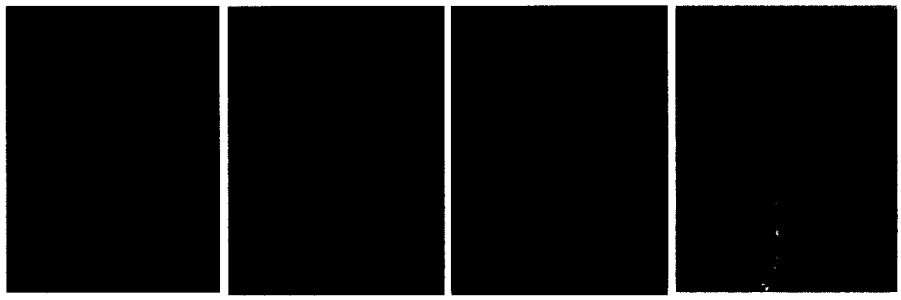

L19
L19 Ped3
L19 Ped17
L19 Ped23
L19 Ped26
αGFP
α PML

Figure 24. Colocalization of ATRX and dsRED-PML in patient cell lines.

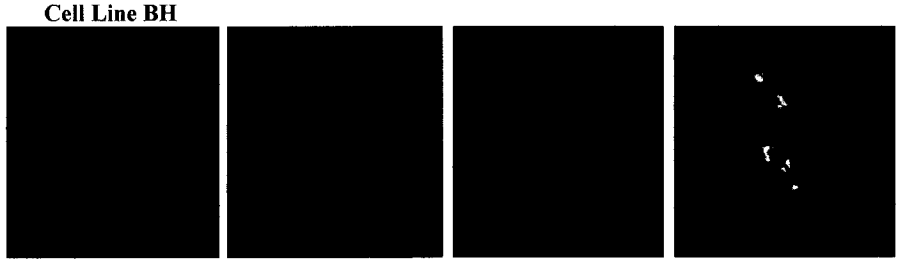
When transfected into patient fibroblasts, dsRED-PML formed large nuclear bodies that colocalized with endogenous ATRX. In these patient cell lines, ATRX appears to be very low in quantity in untransfected cells. The mutation in cell line BH is a point mutation at nucleotide 6720 that changes a Glycine residue to an Arginine. The mutation in cell line 851302 is a point mutation at nucleotide 5376 that changes an Isoleucine to a Threonine residue. These both occur in the ATPase domain.

a) Patient Fibroblast
Cell Line BH

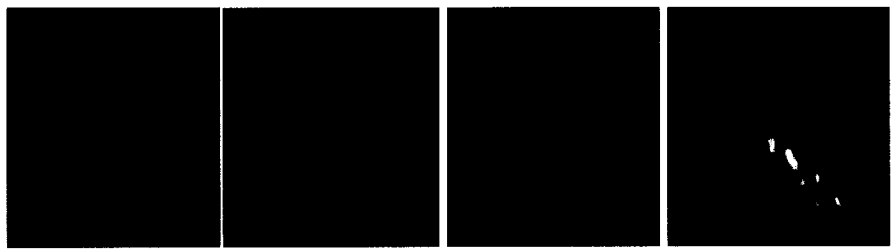
DAPI ATRX dsRED-PML MERGE



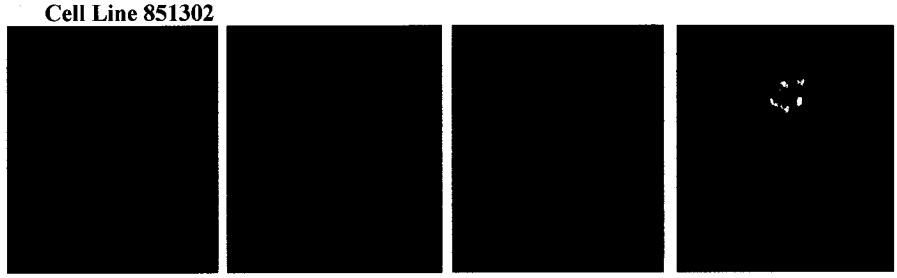
b) Patient Fibroblast
Cell Line BH



c) Patient Fibroblast
Cell Line 851302



d) Patient Fibroblast
Cell Line 851302



4.0 – Discussion

The overall purpose of this project was to contribute to what little is known about the ATRX protein and to begin to elucidate its functions within the cell. It is likely that the ATRX protein functions in gene regulation, as ATR-X patients often have alpha-thalassemia, which is attributed to a drastic down-regulation of the α-globin gene (Gibbons *et al.*, 1995a). More specifically, the ATRX protein probably has a major role in regulating developmental genes, as is evident by the very nature of ATR-X syndrome. ATRX is a member of the SWI/SNF family of global transcription regulators, and like most members of this family, ATRX exists in a multi-protein complex (Vignali *et al.*, 2000; Berube *et al.*, 2002). Numerous avenues were explored here in an attempt to study the ATRX gene. NIH 3T3 stable cell lines expressing ATRX in a tetracycline-regulated system were developed as a tool with which to study effects of ATRX overexpression in mouse cells. Evolving from the work in the Tet-Off stable cell lines was the study of possible ATRX interactions with other known nuclear proteins (Daxx and PML), which both exhibit a similar punctate nuclear speckling pattern when observed by immunofluorescence, and are thought to have a role in transcription regulation.

4.1 – Developed NIH 3T3 stable cell lines with HA-tagged ATRX (ATRXHA) under the control of the Tet-Off gene expression system.

An initial attempt was made to develop stable cell lines using the Tet-On gene expression system in HeLa cells, but this proved unsuccessful. It was possible to detect ATRXHA expression in transiently transfected cells, but it was not possible to develop stable clones that would express ATRXHA (see Appendix for a brief summary of this work). It is likely that the transgene incorporated into an area of the genome that was transcriptionally silent in these cells, thus, stable cell lines with adequate levels of expression were never observed. Development of stable Tet-Off cell lines proved to be more successful, as is evident in the results of this work.

The initial cloning of ATRXHA into pTRE2hyg proved difficult and timely, as ATRX inherently tends to rearrange itself when grown at 37°C in bacterial plasmids, creating a deletion in the gene (DJ Picketts, unpublished data). To resolve this issue, constructs must be propagated in bacterial cells grown at 30°C, and interestingly, when colonies are selected, usually the smaller colonies are positive for the correct construct (DJ Picketts, unpublished data). A deletion is apparent in Figure 10b and 10c; the negative lanes (i.e. the negative lanes that are not just empty plasmid) have the exact banding pattern as the positive lanes, except for one missing band, and one band that is substantially smaller in size.

ATRXHA was expressed very well in transiently transfected NIH 3T3 Tet-Off cells (see Figure 11), a result that was also observed in the Tet-On cell lines (see Appendix). When stable transfections were performed, it was observed that ATRXHA was indeed being expressed in a number of cell lines (see Figure 12). However, there

seemed to be a very heterogeneous population of cells where, in most cases, half the cells or less were expressing the transgene (see Table 1). An attempt to increase homogeneosity by serial dilution did not increase, and in some cases decreased, the number of cells expressing ATRXHA when induced (see Figure 13 and Table 2). It could be that all cells do in fact have the transgene incorporated, but promoter silencing or cell cycle effects may prevent us from ever being able to see greater than 50% to 60% of cells expressing. For example, ATRXHA may be overexpressed and the cells become blocked at the G_2-M phase of the cell cycle. This is the point where ATRX is phosphorylated as a means to be released from the nuclear matrix (Berube *et al.*, 2000). Therefore, it may be that the system becomes quenched with unphosphorylated ATRX, causing a block. Cell cycle studies were attempted with no conclusive or reproducible results (see Appendix for an example of an attempted cell cycle study). The most likely reason for the inability to generate homogeneous stable cell lines is, the viral promoters of the Tet-Off/On systems are prone to silencing by methylation, and one or both of the two elements of the system may cease to be expressed (Hoffman *et al.*, 1996). Cell lines transfected with a tet-regulated system have been reported to drift over time in a manner dependant on cell type, with HeLa cells being reported to be the most stable responders to the tet system (Blau and Rossi, 1999). Another problem inherent in development of transgenic lines is the random nature of gene integration, whereby a transgene may be incorporated into an area of repressed transcriptional activity, or may be subject to effects mediated by cis regulatory elements around the area of integration (Shockett and Schatz, 1996). Whatever the case, it does not appear that a stable Tet-Off/On cell line can be developed that uniformly expresses ATRXHA throughout the cell population.

4.2 - The ATRX nuclear-staining pattern comprising nuclear speckles colocalizes with Daxx protein and PML bodies through transient transfection, overexpressed ATRX protein, and examination of endogenous proteins.

Daxx and PML are both nuclear proteins that are associated with transcription regulation, and exhibit a similar speckled nuclear-staining pattern as that of ATRX. Initially, to determine if ATRX and PML had any possible interaction, both proteins were overexpressed in NIH 3T3 Tet-Off cell lines stably transfected with pTRE2hyg-ATRXHA, and transiently transfected with EGFP-PML. It was observed that their nuclear speckles colocalized within the nucleus (see Figure 17). Further immunofluorescence experiments in HeLa cells demonstrated that endogenous ATRX colocalizes with both PML and Daxx (see Figure 18). Interestingly, the endogenous ATRX speckles never seem to colocalize 100% of the time with either endogenous Daxx or PML, whereas endogenous Daxx and endogenous PML speckles always seem to colocalize with each other. When EGFP-PML or dsRED-PML are transfected into HeLa cells, ATRX always colocalizes completely with these proteins, with the nuclear speckles appearing larger and lower in number than normal. It is likely that overexpressed dsRED-PML or EGFP-PML draws ATRX into the nuclear bodies. At this point, everything in the nucleus is probably congealed into these unnaturally large masses of PML protein. The nuclei of cells with overexpressed EGFP-PML or dsRED-PML generally appear malformed and unhealthy. Therefore, it is probably the case that everything is drawn into these massive PML bodies, causing the cell to die. So, as interesting as it is to see these interactions, one cannot conclude that ATRX and PML have a definite natural interaction based on the dsRED-PML or EGFP-PML results. From the results of the endogenous

protein studies, it can be suggested that ATRX, Daxx and PML may associate for some functions in the nucleus, but it cannot be concluded that they are important members of a common multi-protein complex. Rather, ATRX probably interacts more strongly with one than the other, and the colocalization of ATRX with either, may be through the interaction of PML and Daxx. Co-immunoprecipitation studies of ATRX, Daxx, and PML were attempted, but were of minimal success. However, what is seen suggests Daxx co-IPed with both PML and ATRX, but no co-IP of PML with ATRX or vice-versa was observed (see Appendix E.1 and E.2; also discussed further in section 4.4).

4.3 – Identification of the Daxx and/or PML-interacting domain within the ATRX protein, using vector constructs containing fragments of the ATRX protein.

There are two domains within the ATRX protein that may be possible candidates for PML-interacting domains (DJ Picketts, unpublished data). These are aptly named Targeting Domain 1 (TD1) and Targeting Domain 2 (TD2) (see Figure 9). Vector constructs were developed with fragments of the ATRX protein containing these domains: L2GFP containing TD1, and L19GFP containing TD2. When transiently transfected along with dsRED-PML into HeLa cells or NIH 3T3 cells, only the L19GFP protein colocalized completely, in a similar fashion to that of endogenous ATRX, with dsRED-PML (see Figures 19, 20, and 22).

Similar results were obtained when GFP constructs were transfected into HeLa cells, and cells were stained for endogenous PML. The L19GFP protein colocalized with PML in a similar fashion to that of endogenous ATRX, whereas the L2GFP protein never

colocalized with PML (see Figure 20). The same experiment was performed, except the cells were stained for Daxx, which essentially echoed the results of the endogenous PML study in terms of L2GFP, but L19GFP did not show overly convincing colocalization with Daxx (see Figure 21). Therefore, based on these observations, the portion of ATRX that interacts with PML is contained within TD2. The portion that interacts with Daxx is still uncertain.

4.4 - Patient mutations in the TD2 area of the ATRX protein affect its interaction with PML bodies and Daxx

Mutations in the ATRX gene are responsible for ATR-X syndrome, and a number of these mutations fall within the TD2 domain (see Figure 9). Thus, is the severe phenotype of ATR-X syndrome that is caused by these mutations, due in part to hindered interactions with Daxx or PML? Four patient mutations that are found in the TD2 domain were tested in this study (Ped3, Ped17, Ped23, and Ped26) to determine if a mutation in TD2 (L19GFP) affects colocalization with ATRX. In patients, all four mutations result in typical ATR-X features with varying degrees of severity, such as genital abnormality (present in all, but most severe in Ped17 and Ped26), and HbH inclusions (interestingly Ped17 and Ped26 have the least severe forms of alpha-thalassemia, the most severe being Ped23 followed by Ped3) (Picketts *et al.*, 1996).

When L19GFP with Ped3 or Ped23 is transfected into Hela cells, both appear to colocalize with PML and dsRED-PML (see Figures 19c, 19e, 20c and 20e). The pictures in 19c, 19e, 20b, and 20d show that Ped3 and Ped23 do not hinder the ability of L19GFP

to form a speckled nuclear pattern. When transfected into NIH 3T3 cells, L19GFP with Ped3 or Ped23 colocalize with dsRED-PML, but the speckling pattern is decreased (see Figures 22b and 22d). When Ped3 and Ped23 are transfected into cells and stained for Daxx, it seems as though Daxx is sequestered to the nucleoli (Figure 21c and 21e). Daxx is reported to be sequestered to the nucleoli by MSP58 (a 58-kDa microspherule protein) as a means to remove its transcription-repressing effect (Lin and Shih, 2002). As well, both Daxx and PML are reported to be sequestered to the nucleoli as an alternate degradation pathway from the proteasome (Mattson *et al.*, 2001). Therefore, it is possible that overexpression of Ped 3 or Ped23 either, removes the transcriptionally repressive effect of Daxx in the nucleus, or inhibits the proteasome, resulting in Daxx being sent to the nucleoli for degradation. More than likely it is the first explanation, because PML was not sequestered to the nucleoli in the presence of Ped3 or Ped23, which it probably would be if proteasome function were inhibited. The Ped3 and Ped23 mutations retain a lot of their natural ability to colocalize with PML. This observation, coupled with the fact that the Ped3 and Ped23 mutations result in more severe cases of alpha-thalassemia, suggests that although the function of these proteins to interact with PML may not be drastically altered, their actual function in gene regulation is probably hindered. ATRX may be recruited to sites of transcription by PML bodies, where it probably has a role in transcriptional activation, and Daxx is probably sequestered to the nucleoli to remove its inhibitory effect. However, the mutations in Ped3 and Ped23 (tyrosine to histidine, and asparagine to valine respectively) hinder the ability of ATRX to fulfill its role in the mechanism. Therefore, the gene that was supposed to be upregulated is not properly expressed. This may explain the more severe alpha-

thalassemia in patients with these mutations. Also, Daxx sequestration to the nucleoli appears to be excessive in these cells, possibly removing Daxx's inhibitory effects throughout the nucleus, resulting in a cascade of gene expression problems in the cell. It has also been reported that Daxx may have an anti-apoptotic function, as Daxx-null mutants show an increase in apoptosis (Ecsedy *et al.*, 2003). Therefore, it is possible that ATRX has a role in this pathway, and if it is absent or not functioning properly, Daxx is sequestered to the nucleoli, and is unable to fulfill its anti-apoptotic role. This possibly explains why cells transfected with these constructs begin to die shortly after transfection.

When L19GFP with Ped17 or Ped26 is transfected into Hela cells, it does not appear to colocalize with endogenous PML in either case (see Figures 20d and 20f). Both Ped17 and Ped26 appear to colocalize with dsRED-PML (see Figures 19d and 19f). When cotransfected with dsRED-PML, both seem to form some degree of speckling, but when transfected alone, no nuclear speckling is seen (see figure 19d, 19e, 20d, and 20f). Thus, when L19GFP contains the Ped17 or Ped26 mutations, the ability of L19GFP to form a speckled nuclear pattern is essentially obliterated. When L19GFP contains the Ped17 or Ped26 mutations, it does not appear to colocalize with Daxx, in fact, Daxx is sequestered to the nucleoli in these cells as well (see Figures 21c and 21e). When transfected into NIH 3T3 cells, L19GFP with Ped17 or Ped26 colocalize with dsRED-PML, but the speckling pattern is decreased (see Figures 22c and 22e). Therefore, the Ped17 and Ped26 mutations have lost most of their natural ability to colocalize with PML. It is interesting to note that patients with both of these mutations appear to have milder forms of alpha-thalassemia. It may be that these mutations simply do not severely hinder the function of ATRX in alpha-globin gene regulation. The Ped17 mutation is a

nonsense mutation, resulting in a stop codon at amino acid 2386 (arginine to stop), and the Ped26 mutation is a deletion of the last 1588 nucleotides of the ATRX gene, and results in almost no ATRX protein present in the cell, normal or abnormal (Picketts *et al.*, 1996). Both mutations disrupt one of the helicase motifs in the ATPase domain (whereas Ped3 and Ped23 do not) (Picketts *et al.*, 1996). In both cases, patients have severe genital abnormalities. Thus, it appears that an intact ATPase domain and an intact C-terminal region of ATRX are important in genital development (and apparently PML interactions).

One other thing to note is, cells transfected with L19GFP and its mutants appear to have a decreased amount of PML bodies. This is well demonstrated in Figures 20b, 20e, and 20f, but was a common observation in all cases. However, western blot analysis showed that PML is quite abundant in transfected cells (see Figure 23). It may be that there are not enough transfected cells to make a quantitative analysis. Fibroblast cell lines derived from human ATR-X patients were also transfected with dsRED-PML to see what may happen with ATRX/PML colocalization (see Figure 24). The observations in these experiments showed that ATRX levels were very low in untransfected cells, but ATRX was clearly present in cells transfected with dsRED-PML, and was fully colocalized. More than anything, these observations contribute to a theory that overexpressed dsRED-PML draws everything in the nucleus into its giant globular masses. Similar phenomena were reported by several groups (Ishov *et al.*, 1999; Lin and Shih, 2002; Lopez *et al.* 2002). Overall, it seems that any colocalization with dsRED-PML is not an accurate representation of the natural workings of the nucleus, and should be used only as a step to validate further testing.

To determine whether ATRX exists in a complex, or has a strong interaction with Daxx and/or PML, an attempt was made to coimmunoprecipitate (co-IP) ATRX, PML and Daxx (see Appendix E.1 and E.2). Although it appears Daxx co-IPs with both ATRX and PML, neither ATRX nor PML co-IPed with each other. This would support the earlier suggestion that ATRX and Daxx are recruited to PML bodies, whereby they are brought together to perform some sort of mutual regulatory role. One such role may be part of the mechanism whereby Daxx is phosphorylated by HIPK-1, then dephosphorylated at some point so it can interact with Dek and HDACs to condense chromatin structure and repress transcription (Hollenbach *et al.*, 2002). As ATRX is known to be phosphorylated during the cell cycle (Berube *et al.*, 2000), it could be phosphorylated by HIPK-1 at the PML body, and interact with Daxx throughout the above mechanism. This would demonstrate the role of ATRX in gene silencing or transcriptional down-regulation. It could also be the case that the phosphate group removed from Daxx is transferred to ATRX. Conversely, ATRX may provide the phosphate group transferred to Daxx, mediated by HIPK-1 at the PML body site. It is apparent that ATRX interacts with PML and Daxx, but the exact role ATRX has within these interactions is yet to be determined. [Note: Since the time this thesis was completed, ATRX has been confirmed to exist in a complex with Daxx. It turns out Daxx is the subunit of the complex that physically interacts with PODs (Xue *et al.*, 2003; Tang *et al.*, 2004). These studies support the data found here. It was also shown in the Tang *et al.* study that the Daxx-interacting domains on ATRX are constituted by amino acids 1189 to 1326. Also, another Daxx-interacting domain exists from amino acids 321 to 865. This explains why Daxx did not colocalize with the EGFP constructs].

4.5 – Future Directions

Future work in the areas covered by this thesis should concentrate on optimizing the investigations of interactions between ATRX, Daxx, and PML. It should also be considered that there are many other proteins that could be involved in the mechanisms in which ATRX, Daxx, and PML interact. Immunofluorescence studies could be performed whereby all three proteins could be viewed, using different coloured stains, in an attempt to see if they colocalize at the same or different time and place. Studies could be performed utilizing individual tet-regulated cell lines that overexpress ATRX, Daxx, or PML, and cell lines that have a knockout of ATRX, Daxx, or PML. This way, it could be determined how the other two proteins behave when one is not present in the cell. Furthermore, if two of the proteins were not present in the cell, how would the remaining protein behave? Also, it would be interesting to obtain acute promyeolytic leukemia (APL) cell lines, who do not have PML bodies present in the nucleus [due to the PML-RARα fusion protein, as a result of the t(15:17) chromosome translocation in APL (Doucas *et al.*, 1999; Boisvert *et al.*, 2001)], and see how ATRX and Daxx behave before and after all-trans retinoic acid treatment (which restores PML bodies to APL cells). Colocalization studies could be performed in animal tissues, such as mouse brain, to determine if the behaviour of ATRX, Daxx, and PML reflects those results observed in cell culture.

Further work needs to be performed optimizing and characterizing the NIH 3T3 Tet-Off stable cell lines described in this paper. Whether or not it is possible to create a homogeneous population of cells expressing ATRX is unknown. As ATRX has proven to

be a difficult gene to work with, it will probably take a considerable amount of time and effort to develop reliable, tet-regulated, ATRX-expressing cell lines. If it does happen, the possibilities are exciting. Microarray analysis could be performed, whereby cells induced at various time points could be compared to those that are uninduced, and from this could be elucidated some of the ATRX target genes. Also, cell cycle analysis could be performed to determine the effects of overexpressed ATRX on cell cycle regulation. There are other ways in which the creation of stable tet-regulated cell lines could be initiated, that may or may not solve the problems presented in this study. To combat the randomness of transgene integration, one group suggested surrounding individual transcription units with matrix attachment regions, which apparently insulates stably integrated transgenes and vectors from the previously mentioned effects mediated by adjacent cis regulatory elements (Shockett and Schatz, 1996). Retroviral delivery has been reported to be a very effective method for creating tet-regulated cell lines (Hofmann *et al.*, 1996). To address the problem of having to create a double-stable cell line, one group reported success with the Tet-On system whereby they combined the two expression units of the system on a single DNA fragment (Lottmann *et al.*, 2001).

4.6 – Conclusions

Based on the work in this study, it seems unlikely that a reliable, tet-regulated, ATRX-expressing cell line can be developed using these methods. A vast amount of time was spent in an attempt to develop these cell lines, with minimal success. If it is possible, a considerable amount of time, effort, and patience will be required.

ATRX appears to colocalize with both PML and Daxx when viewed by immunofluorescence. However, based on the fact that colocalization is not 100%, and the results of the coimmunoprecipitation experiments, it is probable that all three proteins have relatively transient, short-lived interaction with each other. It seems that PML and ATRX have a much stronger interaction with Daxx than with each other, and that their interaction is probably related to a common mechanism they share with Daxx, such as the HIPK-1/Dek/HDAC transcription repression mechanism described earlier. The patient mutations in L19 that seem to have the most effect on PML colocalization are Ped17 and Ped26. Thus, it can be concluded that the PML interacting domain is located somewhere around the C-terminal of the ATRX protein. Whether or not TD2 contains the Daxx-interacting domain is still somewhat ambiguous. [See note on page 87 for a brief update on ATRX/Daxx/PML work that has been done since the completion of this thesis].

5.0 – References

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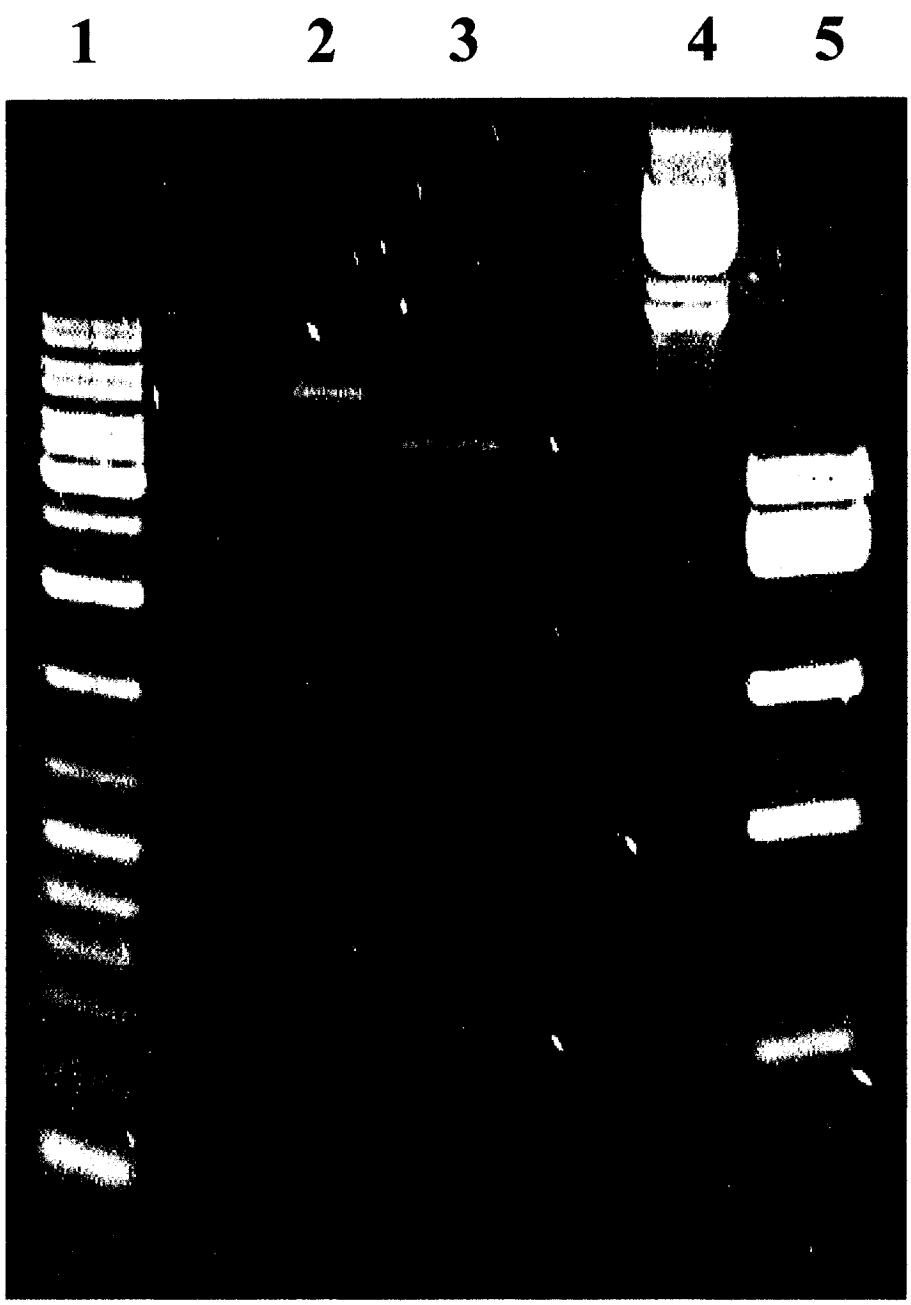
6.0 - Appendix

Appendix A – ATRXHA was cloned into pTRE2.

HA-tagged ATRX (7.5 kb *SmaI/NotI* fragment from pCAGG-SL-ATRX) was cloned into the *PvuII/NotI* sites of the pTRE2 expression vector (construct referred to as pTRE2-ATRXHA). Gel Lanes: 1 = DNA Ladder, 2 = pTRE2 undigested, 3 = pTRE2 *EcoRI* digest, 4 = pTRE2-ATRXHA undigested, 5 = pTRE2-ATRXHA *EcoRI* digest.

Expected bands:
pTRE2 *EcoRI* digest (kb) = 3.0 and 0.7
pTRE2-ATRXHA *EcoRI* digest (kb) = 3.0, 2.5, 2.3, 1.6, 1.1, and 0.7


1
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Appendix B – PCR of HeLa Tet-On cells stably transfected with pTRE2-ATRXHA.

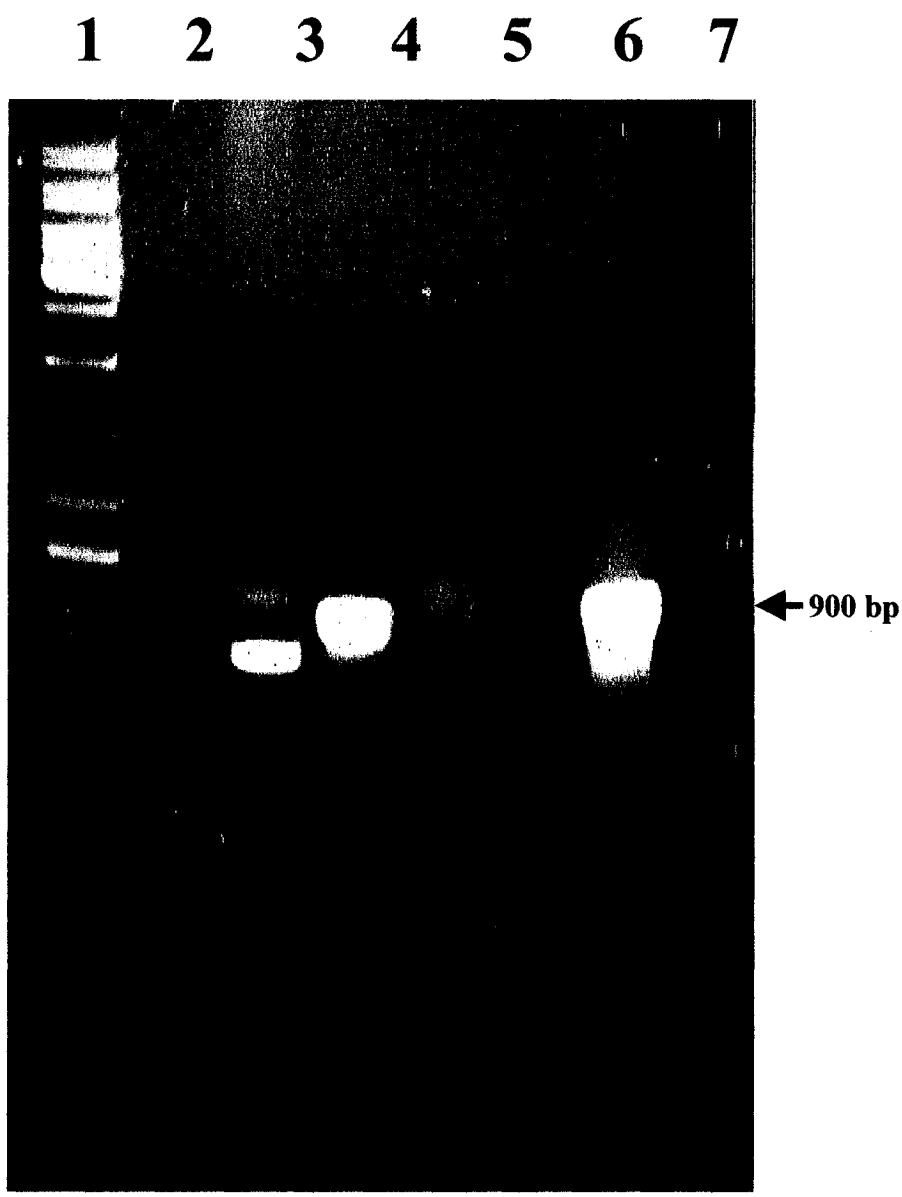
Results show pTRE2-ATRXHA integrated into the genome of some cell lines intact, while others appear to have suffered a deletion. Gel Lanes: 1 = DNA Ladder; Lane 2 = Negative control; Lane 3 = Negative result demonstrating deletion or gene rearrangement; Lane 4 = Hela Tet-On stable clone 10-9 genomic DNA positive for desired construct; Lane 5 = Negative result demonstrating deletion or gene rearrangement; Lane 6 = Positive control (pTRE2-ATRXHA plasmid DNA).

Primers used:

pTRE2 5'-PCR primer: 5'-CGCCTGGAGACGCCATC-3'

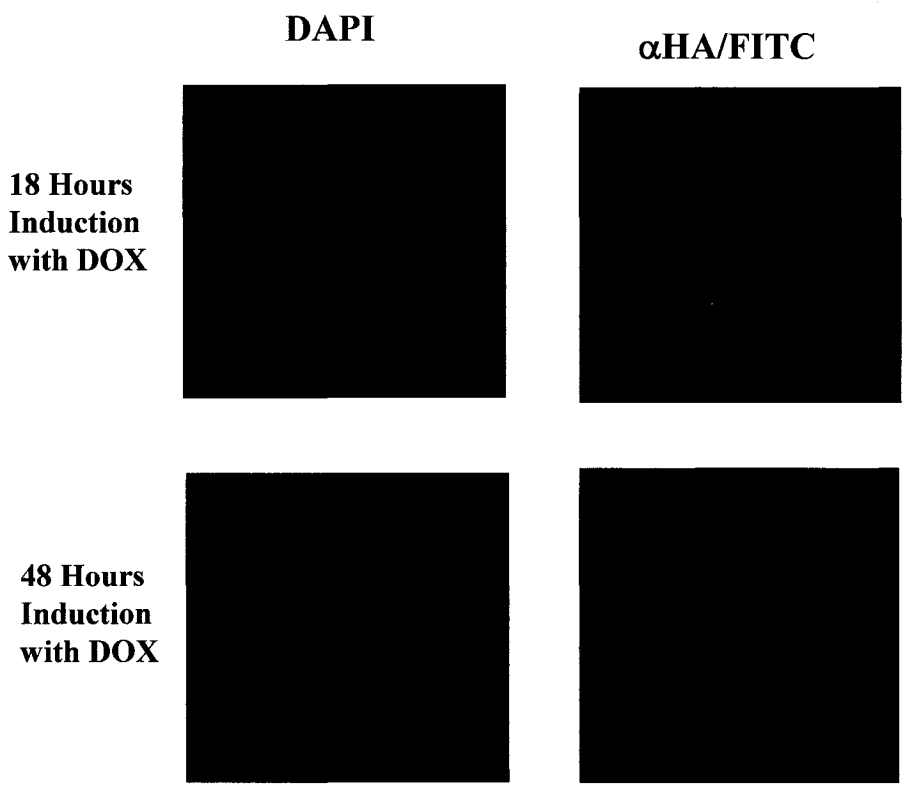
Xnp164 primer: 5'-GCTGTTACATGCAGTGA-3'

Expected PCR product = 900 bp


1 2 3 4 5 6 7
900 bp

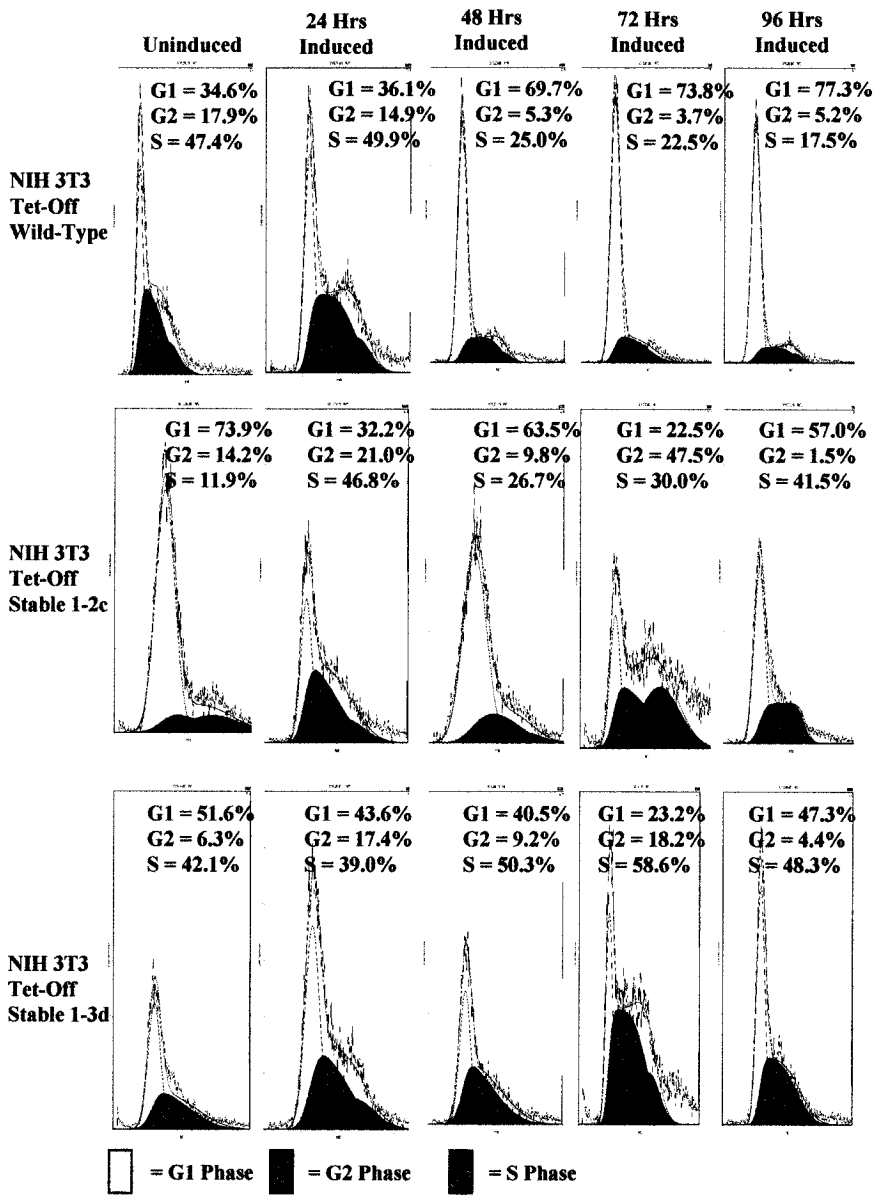
Appendix C – Transiently transfected HeLa Tet-On Cells express ATRXHA.

Hela Tet-On Cells express ATRXHA when transiently transfected with pTRE2-ATRXHA. However, it was not possible to obtain stable clones that expressed the ATRXHA transgene. It was concluded that lack of expression was caused by either lack of integration of transgene (i.e. only cotransfected antibiotic resistance vector pTK-hyg was integrated, allowing growth and subsequent selection of colonies), or in those that were known to have the transgene integrated; gene rearrangement or positional effects (for example, transgene was integrated in an area of silenced chromatin).


DAPI
αHA/FITC
18 Hours Induction with DOX
48 Hours Induction with DOX

Appendix D – Cell cycle analysis of NIH 3T3 Tet-Off stable clones.

In an attempt to determine if ATRXHA overexpression had any effects on the cell cycle, wild type (i.e. untransfected) NIH 3T3 Tet-Off cells, and stable clones 1-2c and 1-3d were subject to cell cycle analysis by flow cytometry. These results show that, in both cases, there are differences from wild-type cells undergoing mock induction. Unfortunately these results could never be reproduced, so the experiment was abandoned, but future work should investigate the effects of ATRX overexpression or knockout on the cell cycle. Cells were stained with propidium iodide for flow cytometry analysis.

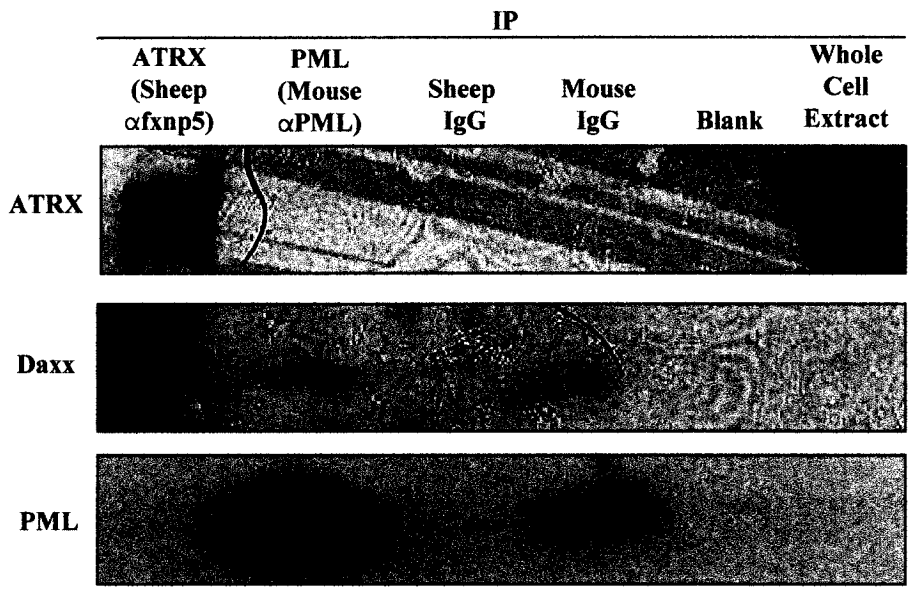

Uninduced
24 Hrs Induced
48 Hrs Induced
72 Hrs Induced
96 Hrs Induced
NIH 3T3 Tet-Off Wild-Type
G1 = 34.6%
G2 = 17.9%
S = 47.4%
G1 = 36.1%
G2 = 14.9%
S = 49.9%
G1 = 69.7%
G2 = 5.3%
S = 25.0%
G1 = 73.8%
G2 = 3.7%
S = 22.5%
G1 = 77.3%
G2 = 5.2%
S = 17.5%
NIH 3T3 Tet-Off Stable 1-2c
G1 = 73.9%
G2 = 14.2%
S = 11.9%
G1 = 32.2%
G2 = 21.0%
S = 46.8%
G1 = 63.5%
G2 = 9.8%
S = 26.7%
G1 = 22.5%
G2 = 47.5%
S = 30.0%
G1 = 57.0%
G2 = 1.5%
S = 41.5%
NIH 3T3 Tet-Off Stable 1-3d
G1 = 51.6%
G2 = 6.3%
S = 42.1%
G1 = 43.6%
G2 = 17.4%
S = 39.0%
G1 = 40.5%
G2 = 9.2%
S = 50.3%
G1 = 23.2%
G2 = 18.2%
S = 58.6%
G1 = 47.3%
G2 = 4.4%
S = 48.3%
= G1 Phase
= G2 Phase
= S Phase

Appendix E.1 – Daxx coimmunoprecipitates with ATRX and PML, but PML and ATRX do not appear to coimmunoprecipitate with each other.

To prove that ATRX has a significant interaction with PML and Daxx, it was attempted to coimmunoprecipitate (co-IP) ATRX with Daxx and PML. Coimmunoprecipitation studies were weak, but suggested that it is possible to co-IP Daxx with either ATRX or PML, but attempts to co-IP ATRX and PML were continuously unsuccessful (Appendix E.2). This suggests that ATRX may be in a complex with Daxx, but the ATRX/PML interaction is likely a temporary arrangement for a specific function, possibly mediated through Daxx. (See Note on page 87).

Appendix E.2. Co-immunoprecipitation of ATRX, Daxx, and PML.

These blots suggest that Daxx immunoprecipitates with either ATRX or PML, but ATRX and PML are never able to immunoprecipitate together, suggesting that ATRX may be in a complex with Daxx, but the ATRX/PML interaction is likely a temporary arrangement for a specific function, possibly mediated through Daxx. (See Materials and Methods for a description of the protocol).


IP
ATRX (Sheep αfxnp5)
PML (Mouse αPML)
Sheep IgG
Mouse IgG
Blank
Whole Cell Extract
ATRX
Daxx
PML