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FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Emma Goodall

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)

GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Characterizing the Enhanced Neuronal Apoptosis in ATRX Knockout mice

TITRE DE LA THÈSE / TITLE OF THESIS

D. Picketts

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

D. Bulman

R. Kothary

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Characterizing the enhanced neuronal apoptosis in ATRX knockout mice

Emma Goodall

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements for the degree of
Master of Science

Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

Your file Votre référence
ISBN: 978-0-494-61305-4
Our file Notre référence
ISBN: 978-0-494-61305-4

Abstract

Mutations in ATRX, a SWI/SNF-like chromatin remodeling protein, cause the very rare ATR-X mental retardation syndrome (OMIM# 30032). The ATR-X syndrome is characterized by global developmental delay and 95% of cases are diagnosed with severe to profound mental retardation. Previous experiments have established a developmental requirement for ATRX and have suggested a role for ATRX in corticogenesis. Conditional forebrain specific $ATRX^{-/-}$ mice were shown to have a loss of cortical mass, which resulted from a 12-fold increase in apoptosis during early stages of corticogenesis.

We show that the loss of ATRX results in a loss of neurons but does not affect the differentiation of astrocytes. Furthermore, we demonstrate enhanced p53-mediated apoptosis in the ATRX knockout model. Consistent with this, we demonstrated elevated activity levels of caspase-9 and -3, key apoptotic factors. Additionally, we show an upregulation of the pro-apoptotic genes Bid and Peg3 in ATRX knockout samples. Together these results suggest that ATRX may play a critical role in promoting neuronal survival by inhibiting p53 mediated apoptosis.

Acknowledgements

I would like to thank my supervisor, Dr. David Picketts, for the opportunity to study under his guidance and for the remarkable learning experiences that have accompanied my graduate studies. Similarly, I would also like to thank the members of my advisory committee, Dr. Lynn Megeney and Dr. Ruth Slack, whose suggestions and direction were greatly appreciated.

During my time in the lab, my experience was enriched by the friendship and encouragement offered by all lab mates: Chelsea Corcoran, Maureen Curtin, Marilyne Delorme, Mike Huh, Chantal Medina, Tina Price-O'Dea, Steve Rennick, Matt Todd, and Darren Yip. I would especially like to thank Maureen Curtin, Marilyne Delorme and Chantal Medina for the laughter and amusement that made the bad days bearable and the good days great.

Finally, I would like to thank my parents and Lucas for their infinite support, enthusiasm and confidence in my skills.

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List of Abbreviations

5-FU: 5-Fluorouracil

ADD: Atypical PHD domain common to ATRX, DNMT3a and DNMT3b

ADP: Adenosine diphosphate

ALS: Amyotrophic lateral sclerosis

ART: Assissted reproductive technologies

ATMDS: Alpha thalassemia myelodysplastic syndrome, a preleukemic condition

ATP: Adenosine triphosphate

ATRX: Alpha-thalassemia X-linked mental retardation gene

ATR-X: Alpha-thalassemia X-linked mental retardation syndrome

ATRX KO: *Foxg1*-cre;ATRX $^{f/y}$ mouse

BDNF: Brain-derived neurotrophic factor

BFLS: Börjeson–Forssman–Lehmann syndrome

BPTF: Bromodomain PHD finger transcription factor

BrdU: Bromodeoxyuridine

BRG1: Brahma-related gene 1

CHD family: Chromodomain/helicase/DNA binding domain family

DAPI: 4',6-diamidino-2-phenylindole

DD: Death domain

DED: Death effector domain

DISC: Death inducing signalling complex

DNA: Deoxyribonucleic acid

DS: Down Syndrome

FADD: FAS-associated death domain

FoxG1: Forkhead box G1

GABA: Gamma-aminobutyric acid

GFP: Green fluorescent protein

H1: Histone 1

H2A: Histone 2a

H2B: Histone 2B

H3: Histone 3

H4: Histone 4

HAT: Histone acetyltransferase

HbH: Hemoglobin H tetramers

HD: Homeodomain

HDAC: Histone deacetylase

HOX: Homeobox

IHC: Immunohistochemistry

ISWI: Imitation Switch

MeCP2: MethylCpG-binding protein 2

MOMP: Mitochondrial outer membrane permeability

NURF: Nucleosome remodeling factor

PBS: Phosphate buffered saline

PFA: Paraformaldehyde

PHD: Plant homeo domain

PML: Promyelocytic leukemia protein

PML-NB: Promyelocytic leukemia nuclear bodies

Q-RT PCR: quantitative reverse transcriptase-polymerase chain reaction

RNA: Ribonucleic acid

SNF2: sucrose non-fermenting 2

TNF: Tumour necrosis factor

TNFR: Tumour necrosis factor receptor

TRADD: TNFR1-associated death domain

TRD: Transcription repression domain

TUNEL: Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling

XLMR: X linked mental retardation

1.0 Introduction

1.1 Chromatin Overview and Basic Structure

Every eukaryotic cell must overcome the challenge of housing the massive quantity of DNA in the tiny space that is the cell's nucleus. In its most condensed state, the human genome achieves compaction on the order of 10 000 fold exhibited by mitotic chromosomes [Alberts *et al.,* 2002]. This feat is made possible through DNA-protein interactions and successive stages of folding and coiling that result in a nucleoprotein complex known as chromatin.

Nucleosomes are the basic building blocks that constitute chromatin and are repeating units of 147 base pairs of DNA wrapped tightly, approximately 1.7 times, around a histone protein core [Kornber, 1974; Luger *et al.,* 1997]. The globular histone core is in fact an octamer comprised of two copies each of the highly evolutionarily conserved H2A, H2B, H3 and H4 histone proteins. A common structural motif, the histone fold, allows these proteins to assume the specific conformation of one $(H3\text{-}H4)_2$ tetramer flanked by two H2A-H2B dimers. These individual nucleosome core particles are linked together by variably small stretches of DNA and the linker histone protein, H1, to form nucleosomes [Happel and Doenecke, 2009]. The descriptive term "beads on a string", referring to the nucleosomes as beads linked by DNA string, has long been used to represent this primary chromatin structure (Figure 1) [Alberts *et al.,* 2002].

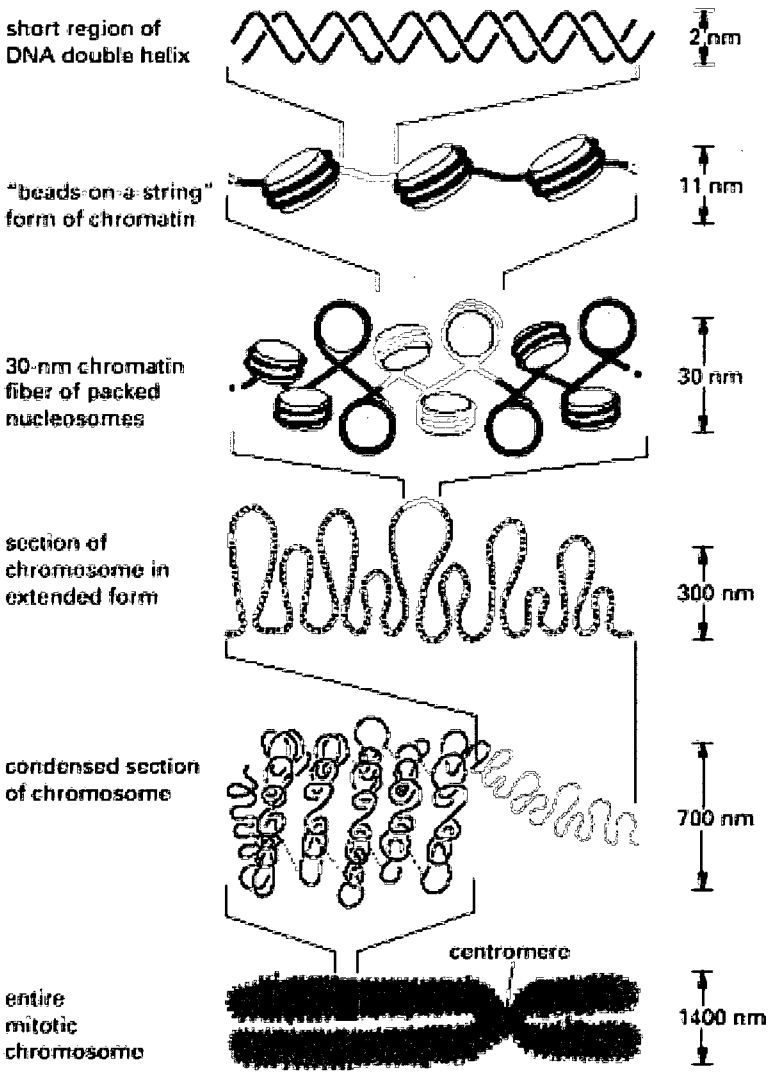


Figure 1. Illustration of hierarchical DNA packaging to form chromatin.
Small segments of 147 bp of double stranded DNA wrap around histone octamers in helical turns to form the 'beads on a string' structure. Long sequences of nucleosomes are connected by variable lengths of linker DNA and bound by histone H1 to facilitate higher order compaction. The linear string of nucleosomes is further condensed to form the 30-nm chromatin fiber. Finally, the chromatin fiber forms a series of loops in order to achieve the level of condensation commonly associated with chromatin. Figure from Alberts *et al* 2002.

Linear sequences of nucleosomes are further condensed into a higher-order chromatin structure known as the 30 nm fibre [Thoma *et al.,* 1979]. It is believed that the positively charged linker histone H1 is of particular importance in achieving this structure by pulling nucleosomes together through contacts with negatively charged DNA to form an interlocking organization [Alberts *et al.,* 2002;Happel and Doenecke, 2009;Thoma *et al*,1979]. Additionally, the histone tails extending from the core octamer are also thought to serve in connecting one nucleosome to another, although an exact mechanism is not yet understood [Alberts *et al.,* 2002; Happel and Doenecke, 2009].

1.2 Chromatin Modification

Despite the compact structure of chromatin, its architecture is remarkably dynamic and is able to condense and decondense to modulate the role of DNA in cell cycle progression, transcription, replication, and repair. Chromatin remodeling can occur by a variety of mechanisms broadly categorized as covalent histone modifications and ATP-dependent chromatin remodeling. These modifications are considered to be epigenetic as they succeed in altering gene expression without disrupting the DNA sequence. Specific epigenetic marks are involved in genome compartmentalization that facilitates the two transcriptional states, active euchromatin and inactive heterochromatin [Bartova *et al.,* 2008; Kouzarides, 2007]. As such, epigenetic chromatin remodeling is fundamental to genome function.

1.2.1 Covalent Histone Modification

Histone proteins are not only important in the formation of the nucleosome protein core but are also essential for achieving conformational changes in chromatin structure. Each core histone protein has a highly conserved N-terminal tail which extends out of the histone octamer and contains an outstanding number of sites susceptible to covalent modifications [Alberts *et al.,* 2002; Happel and Doenecke, 2009]. Studies have identified more than 60 residues on histone tails that have been modified and at least eight distinct forms of post-translational modification including: methylation, phosphorylation, acetylation, ubiquitination, sumoylation, ADP-ribosylation, deimination and proline isomerization [Kouzaride, 2007;Strahl and Allis, 2000]. While the stability of individual nucleosomes does not seem to be directly affected by covalent modifications, the opposite is true of the 30 nm fibre and higher-order structures [Alberts *et al.,* 2002; Kornberg and Lorch, 1999]. The specific role carried out by each modification in basic cellular functions is not only dictated by the modification type, but also by the position it occupies on the histone tail which leads to massive possiblities for functional responses [Kouzarides, 2007]. Such modifications form the basis of the 'histone code' hypothesis which predicts that the post-translational modifications of histones are interpreted, alone or in combination, by other proteins to control the structure and/or function of the chromatin fibre thereby, directing specific and distinct biological processes [Strahl and Allis, 2000].

Covalent histone modifications can be largely divided into two classes based on the mode by which they function (Table 1). Generally, modifications can serve to disrupt nucleosomal connections and loosen chromatin structure or modifications can facilitate or inhibit the recruitment of non-histone proteins [Alberts *et al.,* 2002; Kouzarides, 2007; Strahl and Allis, 2000]. Acetylation is perhaps one of the best known modifications and serves to loosen the chromatin structure. The addition of an acetyl group on a lysine residue neutralizes the residue's basic charge thereby altering the histone charge and the overall chromatin architecture [Alberts *et al.,* 2002; Kouzarides, 2007; Wolffe, 1992]. Consequently, acetylation contributes to chromatin decondensation, exposing chromatin loops in preparation for active transcription [Bartova *et al.,* 2008]. Not surprisingly, deacetylation is associated with transcriptional repression, and hypoacetylation is associated with constitutively inactive heterochromatin [Kouzarides, 2007; Kornberg and Lorch, 1999]. Typically, H3K9Ac and H3K4me2 are associated with active genes whereas H3K27me3 and H3K9me2 are associated with inactive genes [Bartova *et al.,* 2008]. Understanding the consequences of these modifications is the challenge in deciphering the histone code.

The second category of histone modifications, those which promote the recruitment of non-histone proteins, also contributes to the dynamic nature of DNA architecture. Specific modifications are able to bind to histone tails via distinctive domains [Kouzarides, 2007], such as the bromodomain which binds to acetylated lysine residues [Saha *et al.,* 2006]. Histones H3 and H4 have been especially well

Table 1. Summary of Histone Tail Modifications
Histone tail modifications are associated with specific functions as described in the table above. Table modified from Kouzarides,T. 2007.

Chromatin Modifications	Residues Modified	Functions Regulation
Acetylation	K-ac	Transcription, repair, replication, condensation
Methylation (lysines)	K-me1 K-me2 K-me3	Transcription, repair
Methylation (arginines)	R-me1 R-me2a Rme2s	Transcription
Phosphorylation	S-ph T-ph	Transcription, repair, condensation
Ubiquitylation	K-ub	Transcription, repair
Sumoylation	K-su	Transcription
ADP ribosylation	E-ar	Transcription
Deimination	R > Cit	Transcription
Proline Isomerization	P-cis > P-trans	Transcription

studied and a summary of proteins known to bind preferentially to modified residues is shown in Figure 2. Trimethylation of lysine 4 on H3 (H3K4me3) recruits the BPTF component of the NURF chromatin remodeling complex and binding occurs through a PHD domain. Recruitment of this ATPase causes activation of *HOXC8* gene expression, a murine transcription factor important for neuromuscular and skeletal development [Wysocka *et al.,* 2006; Lei *et al.,* 2005]. Histone modifications can also act to inhibit protein binding on chromatin. This is demonstrated by a single methyl group on the same lysine residue, H3K4, which prevents the binding of the NuRD (nucleosome remodeling and deacetylase) complex, known to be involved in gene repression [Denslow and Wade, 2007].

1.2.2 ATP-Dependent Chromatin Remodeling

Although covalent histone modifications are successful in altering the biochemical properties of chromatin and in modulating the recruitment of additional non-histone proteins, they are not sufficient to physically displace nucleosomes within chromatin [Johnson *et al.,* 2005]. It is necessary for nucleosomes to be repositioned along the DNA, via dissociation, sliding or relocation of individual nucleosomes, in order to alter the balance between permissive euchromatin and repressive heterochromatin states [Johnson *et al.,* 2005]. In this way, chromatin remodeling is essential to regulate the access of transcription factors to specific genes and also to regulate the access of DNA-repair factors to DNA breaks in chromatin [Saha *et al.,* 2006]. The enzymatic activity of all chromatin remodelers depends on ATP hydrolysis and

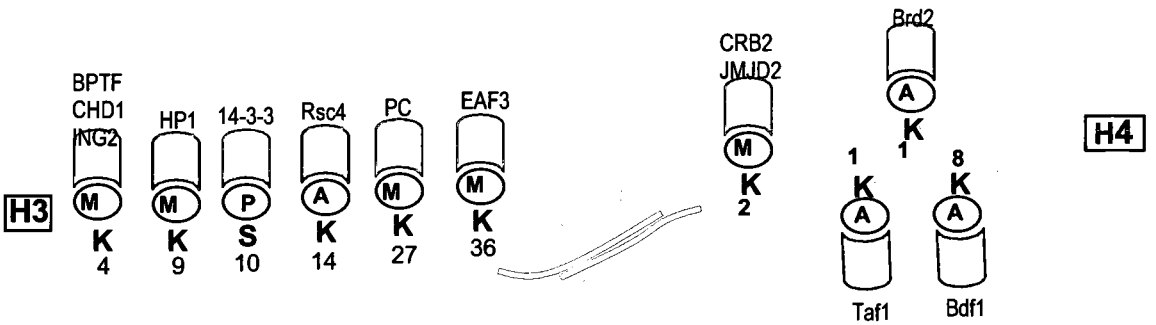


Figure 2. Preferential protein binding to modifications on histones H3 and H4. Specific modifications on histones H3 and H4 have been especially well studied and are associated with preferential protein binding. A summary of proteins known to bind preferentially to modified residues is seen in the figure above. Figure modified from Kouzarides, T. 2007.

consequently, all complexes contain a motif related to the ATPase/helicase superfamily which is referred to as the SNF2 domain [Saha *et al.,* 2006; Johnson *et al.,* 2005]. However, distinct families of chromatin remodelers have been classified based on ATPase domain and the presence of other chromatin interaction domains. To this effect, eight families of chromatin remodeling complexes have been identified in eukaryotes but two families, SWI/SNF and ISWI, have been particularly well studied [Schnitzler, 2008].

The largest known family of chromatin remodeling complexes is the imitation-switch (ISWI) family [Brown *et al.,* 2007] and these complexes generally contain two to four subunits [Racki and Narlikar, 2008]. In addition to the SNF2 ATPase domain, ISWI members have an evolutionarily conserved SANT domain, important for DNA and histone tail binding, and a SANT-like SLIDE domain known to interact with DNA (Figure 3) [Saha *et al.,* 2006; Schnitzler, 2008]. Both the SANT and SLIDE domains are found in the C-terminal end of the ISWI protein. Although the ISWI protein was first identified in *Drosophila*, it has since been identified in complexes in yeast, *Xenopus*, *Arabidopsis* and mammals [Brown *et al.,* 2007]. Studies have identified a role for ISWI in chromatin assembly, specifically organizing and ordering nucleosomes to promote repression [Saha *et al.,* 2006; Brown *et al.,* 2007; Burgio *et al.,* 2008]. Many studies have also identified important developmental roles of the ISWI ATPase and these are reviewed by Brown et al [Brown *et al.,* 2007]. Null mutations in *Drosophila* are lethal, and studies in *Xenopus* demonstrate an essential ISWI requirement for surviving early development

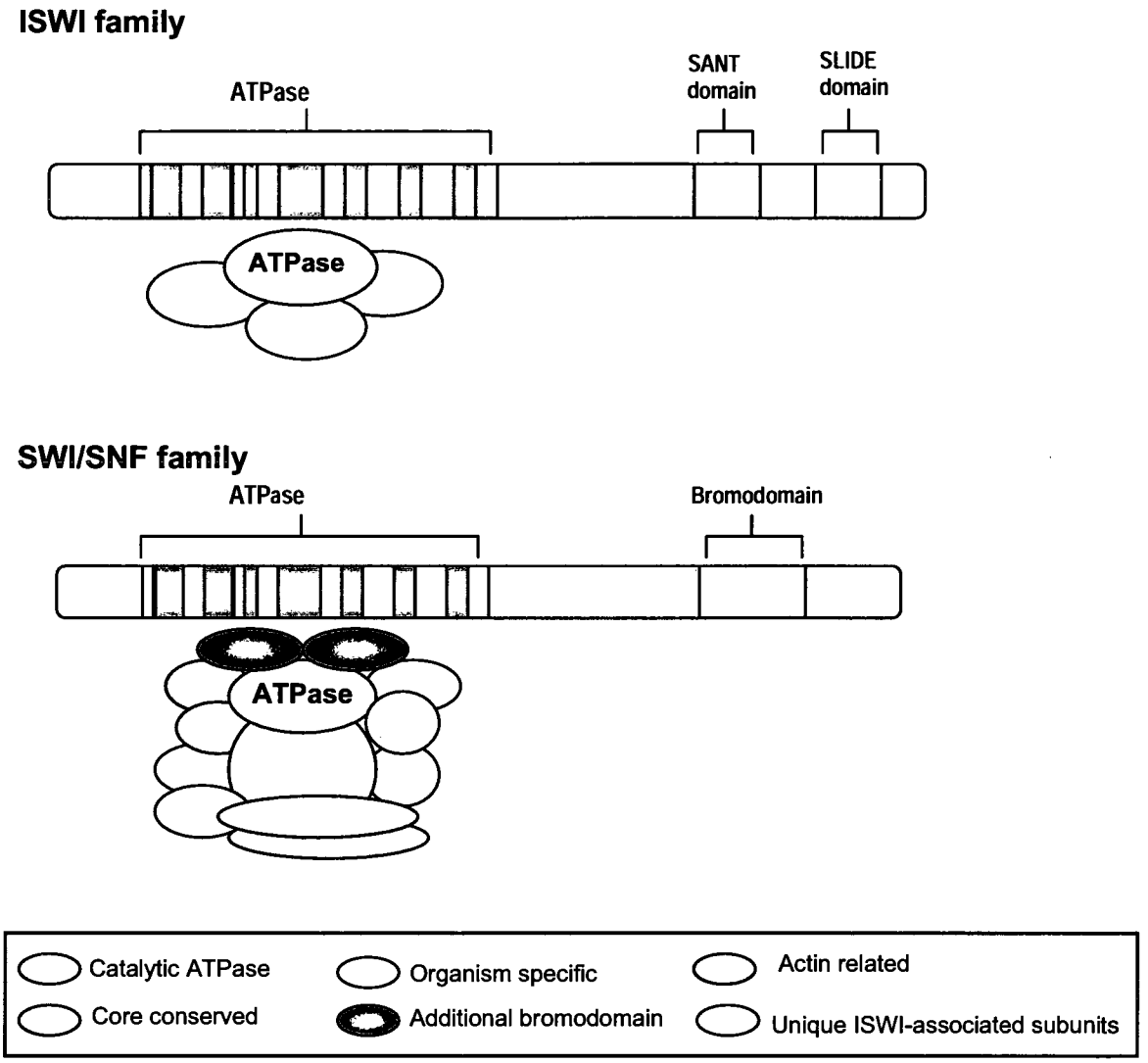


Figure 3. A comparative schematic of the defining features of ISWI and SWI/SNF proteins.
Although the SWI/SNF and ISWI families of chromatin remodelers share a very similar ATPase domain, variation exists in the additional subunits. SWI/SNF members contain bromodomains and 8-15 subunits. ISWI members contain fewere subunits, 2-4, in addition to C-terminal SANT and SLIDE domains. Figure modified from Saha, A *et al.*, 2006.

[Guschin *et al.*, 2000; Poot *et al.*, 2000]. The expression of the two mammalian homologs, *SNF2H* and *SNF2L*, varies slightly across mice and humans but is expressed in nervous tissues and gonads in both species [Lazzaro and Picketts, 2001; Barak *et al.*, 2004]. Homozygous *Snf2h* mutant mice are embryonic lethal at the peri-implantation stage, further demonstrating the importance of the evolutionarily conserved ISWI proteins in organism development [Stopka and Skoultchi, 2004].

The SWI/SNF family of chromatin remodelers was first identified in, and named for, yeast deficient in mating type switching (SWItching mutants) and sucrose nonfermenting (SNF) mutants [Brown *et al.*, 2007]. Since this time, highly conserved homologs have been identified in a wide variety of eukaryotes including *Arabidopsis, Drosophila, Xenopus*, zebrafish, chicken, and mammals [Brown *et al.*, 2007]. The defining feature of the SWI/SNF family is a bromodomain, a motif known to bind acetylated histone tails, near the C-terminus of the ATPase subunit (Figure 3) [Saha *et al.*, 2006]. Generally, SWI/SNF remodelers promote transcription factor binding and play a role in nucleosome disorder and subsequent reorganization [Martens and Winston, 2003] however, some studies suggest a role for SWI/SNF complexes in gene repression [Harikrishnan *et al.*, 2005]. Studies have implicated SWI/SNF complexes in a variety of cellular processes, particularly during development and these are well reviewed by Brown *et al.*, 2007. The general importance of these remodelers was demonstrated by the requirement for the mammalian SWI/SNF member Brg1 during mouse development as Brg1 null mice die during embryogenesis [Bultlman *et al.*, 2000]. In humans BRG1 is known to be

an important component in the regulation of cell cycle progression and has been shown to work with Rb to regulate E2F1, a critical transcription factor for the control of cell cycle progression and apoptosis [Martens and Winston, 2003; Napolitano *et al.,* 2007]. In rat mesenchymal cells, Brg1 plays a role in cell growth arrest, senescence and apoptosis [Napolitano *et al.,* 2007]. Additional studies have emphasized the different roles that Brg1 and Brm SWI/SNF members have in p53 mediated apoptosis and cell cycle exit [Xu *et al.,* 2007], highlighting the importance of this chromatin remodeling family in genome function.

1.3 Chromatin Remodeling and Disease Pathology

While many epigenetic errors have been linked to disease pathology, such as imprinting errors in the development of Angelman and Prader-Willi syndromes, a specific role for chromatin remodeling and disease has been, and continues to be, discerned. In fact, a wide variety of human diseases, including cancer, developmental delay and skeletal abnormalities, have been linked to aberrant chromatin remodeling, thus, highlighting the importance of this function in gene expression and DNA repair [Cho *et al.,* 2004].

Many studies have suggested that, under normal circumstances, chromatin remodeling complexes might inhibit cancer formation by regulating gene transcription involved in cell proliferation [Cho *et al.,* 2004]. Consequently, mutations in chromatin remodeling genes have been associated with many types of cancer. Mutations in the SWI/SNF member *BRG1,* are known to contribute to the

development of lung, breast and prostate cancer [Cho *et al.,* 2004]. Further studies have shown that BRG1 interacts with BRCA1 [Bochar *et al.,* 2000]. The development of chronic myeloid leukemia and rhabdoid tumours have been linked to mutations in the *SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily B, member 1* (*SMARCB1*) gene [Cho *et al.,* 2004].

Rett syndrome, an X-linked neurodevelopmental syndrome, results in altered histone modifications, specifically hyperacetylation of histone H4 [Waggoner, 2007; Wan *et al.,* 2001]. Affected individuals generally have normal development for 6 months followed by regression resulting in severe mental retardation, progressive microcephaly, poor locomotion and poor or absent speech [Colvin *et al.,* 2004]. Molecular studies have identified mutations in the *methyl-CpG-binding protein 2* (*MeCP2*) gene as the underlying cause of Rett syndrome [Waggoner, 2007; Cho *et al.,* 2004]. Two functional domains, the methyl-binding domain and a transcription-repression domain (TRD), allow MeCP2 to function as a transcriptional repressor [Waggoner, 2007]. These domains respectively allow interaction with methylated CpG sites and with a multi protein transcription repression complex which mediates repression via deactylation of core histones [Waggoner, 2007]. Additional MeCP2 activities include its ability to recruit histone methyltransferases which promotes chromatin compaction [Waggoner, 2007; Martinowich *et al.,* 2003]. Despite the ubiquitous expression of MeCP2, the Rett syndrome phenotype is principally neurological. This is most likely due to the fact that brain derived neurotrophic factor

(BDNF), essential for neuronal plasticity, memory and learning, is a MeCP2 target [Martinowich *et al.,* 2003].

Not surprisingly, studies have linked different disease phenotypes to mutations in a common gene. CREBBP (cyclic-AMP response element binding protein (CREB) binding protein) functions as a histone acetyltransferase (HAT). Specifically, CREBBP is involved in histone H3 acetylation, and as such promotes transcription by decondensation of chromatin and recruitment of multiple transcription regulators. [Waggoner, 2007; Cho *et al.,* 2004]. Mutations in the *CREBBP* gene cause Rubinstein-Tabi syndrome (RTS) which presents with growth and mental retardation, facial abnormalities and heart defects [Murata *et al.,* 2001]. Additionally, CREBBP has been implicated in Huntington disease and in aggressive acute myeloid leukemia [Cho *et al.,* 2004]. These examples highlight the burgeoning number of epigenetic regulators mutated in human disease, as summarized in Table 2.

1.4 X-Linked Mental Retardation

Mental retardation is estimated to affect 2-3% of the general population in western countries, with an excess of 30% affected males compared to females [Froyen *et al.,* 2007; Raymond, 2006]. Currently, 1546 entries for "mental retardation" have been recorded in Online Mendelian Inheritance in Man and 422, or 27%, of these are connected to the X chromosome. Combined, this suggests a disproportionately influential role for genes on the X chromosome in the development of cognitive

Table 2. A summary of epigenetic regulators known to associate with and modify chromatin. In some cases, mutation in genes encoding proteins involved in epigenetic regulation give rise to human disease. Table modified from Higgs *et al.*, 2007.

Non histone chromatin protein	Example	Disease
DNA methyltransferases	DNMT1, DNMT3A, DNMT3B	ICF Syndrome (DNMT3B)
Methyl-binding proteins	MBD1-4, MeCP2 Kaiso	Rett Syndrome (MeCP2)
Histone acetyltransferases	CBP, p300	Rubenstein-Taybi Syndrome (CBP, p300)
Histone deacetylases	HDAC1-9	
Histone methyltransferases	Suv39H1/2, EZH2	Sotos Syndrome (NSD1)
Histone kinase	RSK2	Coffin-Lowry Syndrome (RSK2)
Histone demethylases	LSD1, JHDM1	
Chromatin remodeling proteins	BRG, BRM, CHD1-9	Schimke Immunosseous Dysplasia (SMARCAL1) Cockayne Syndrome (ERCC6) ATR-X Syndrome (ATRX) CHARGE Syndrome (CHD7)
Histone binding	Hp1α,β,γ	
Polycomb group proteins	Pc, Suz12	

function in humans. Consequently, efforts to identify specific disease-associated genes have been strongly focused on the X chromosome [Froyen *et al.,* 2007; Skuse, 2005]. This research niche has coined the term X-linked mental retardation (XLMR) which encompasses a large and heterogeneous group of conditions, each presenting with some degree of cognitive impairment, resulting from one or more mutations in one or more genes on the X chromosome. Over 80 genes have been identified in XLMR and span the entire X chromosome and are illustrated in Figure 4 [Chiurazzi *et al.,* 2008].

XLMR can be broadly classified into two categories: the first is syndromic XLMR where patients consistently present with multiple congenital abnormalities and defects in tissues and organs in addition to the brain; the second category is nonsyndromic XLMR where mental retardation is the only consistent clinical presentation [Raymond, 2006; Chiurazzi *et al.,* 2008]. Although hundreds of XLMR conditions have been reported, only a subset of these have had causative genes identified and cloned [Chiurazzi *et al.,* 2008]. Interestingly, several XLMR syndromes have been linked to disordered epigenetic regulation, like chromatin remodeling. In fact, Rett syndrome and Coffin-Lowry syndrome have been linked to disregulated DNA methylation and histone phosphorylation respectively [Ausio *et al.,* 2003] while JARID1C-related XLMR is caused by mutations in the histone H3K4 demethylases *SMCX/JARID1C* and BFLS syndrome is caused by mutations in the plant homeodomain (PHD)-like finger *(PHF6)* [Lower *et al.,* 2002; Iwase *et al.,* 2007; Santos *et al.,* 2006]. Furthermore, multiple inherited mental retardation

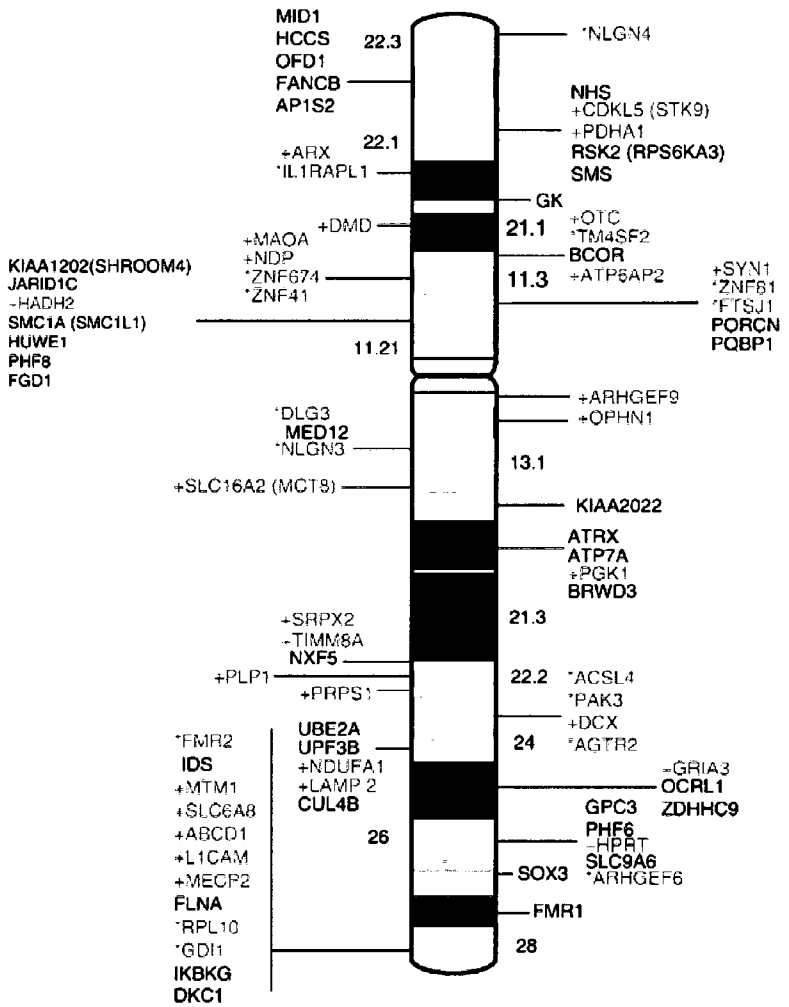


Figure 4. Distribution of known XLMR genes along the X chromosome.
The position of the 82 known XLMR genes on the X chromosome is depicted in the figure above. Genes written in black are known to cause syndromes, genes written in grey preceded by an asterisk are associated with nonspecific mental retardation and genes written in grey preceded by a + sign are involved in neuromuscular disorders. Reprinted by permission from Macmillan Publishers Ltd: European Journal of Human Genetics 16(4):422-34, copyright 2008.

syndromes have been linked to different mutations within the *ATRX* gene including Carpenter–Waziri syndrome, Juberg–Marsidi syndrome, Smith–Fineman–Myers syndrome, Sutherland–Haan syndrome and the well studied ATR-X syndrome [Raymond, 2006; Picketts *et al.,* 1996].

1.5 ATRX

1.5.1. Clinical features of X-linked alpha-thalassemia mental retardation syndrome (ATR-X)

ATR-X syndrome (OMIM# 30032) is a very rare form of mental retardation, caused by mutations in the *ATRX* gene, and is estimated to affect only 1-9/1,000,000 individuals in the general population [Gibbons, 2006]. To date over 200 affected individuals have been identified in 182 families worldwide [Gibbons *et al.,* 2008]. Due to the localization of the *ATRX* gene on the X chromosome, affected individuals are predominantly male [Gibbons *et al.,* 2008]. Presentation of the syndrome in females is exceptionally rare due to the naturally occurring act of X inactivation. Generally, the X-inactivation pattern is random and results in an approximately equal proportion of maternally and paternally inherited chromosomes being inactivated. However, in the presence of a mutated X-linked gene, skewed activation preferentially inactivates the defective chromosome, thereby preventing phenotypic expression of the mutation in the female [Plenge *et al.,* 2002]. This has been reported in studies of female carriers of *ATRX* mutations who present without any characteristics of the syndrome [Gibbons *et al.,* 2008; Gibbons *et al*, 1992; Gibbons and Higgs, 2000]. An exception to this rule has been identified in a woman

presenting with mild mental retardation and demonstrated non-skewed X inactivation [Wada *et al.,* 2005]. Additionally, one fully affected female individual has been identified and she presents with a totally skewed X-inactivation pattern whereby the mutated maternal allele is the active allele [Badens *et al.,* 2006]. This rare occurrence was attributed to the use of *in vitro* fertilization which, among other assisted reproductive technologies (ART) has been implicated in an increased incidence of epigenetic disorders in children born from ART [Niemitz and Feinberg, 2004].

The ATR-X syndrome is characterized by global developmental delay and 95% of cases are diagnosed with severe to profound mental retardation. The majority of patients do not develop speech, and very few develop expressive skills of any sort. General hypotonia is observed and may contribute to the facial anomalies which include flat nasal bridges, upswept frontal hair, small triangular upturned noses, tented upper lips and full, everted lower lips often associated with dribbling (Figure 5). In 80% of cases a genital abnormality is present and can range from relatively mild irregularities such as undescended testes to the very severe presentation of male pseudohermaphrodism [Gibbons, 2006]. An additional hallmark of the syndrome is the alpha thalassemia form of anemia which is present in 90% of patients [Gibbons *et al.,* 2008]. This anemia results from reduced expression of alpha globin which leads to an excess accumulation of beta globin chains which tend to form tetramers called Hemoglobin H (HbH) [Gibbons *et al.,* 2008]. Additional abnormalities which present sporadically in cases include some skeletal findings such as clinodactyly,

Figure 5. An individual affected by ATR-X syndrome.
Eight year old ATR-X patient presenting with the characteristic facial features of ATR-X syndrome. Note the upswept frontal hair line, hypertelorism, epicanthic folds, flat nasal bridge, small triangular upturned nose, tented upper lip, everted lower lip and hypotonic facies. Figure modified from Gibbons, RJ., 2006.

scoliosis, visual impairments, renal abnormalities and cardiac abnormalities [Gibbons, 2006; Gibbons *et al.,* 2008; Medina *et al.,* 2009].

1.5.2. Molecular Genetics of ATR-X Syndrome

Constitutional mutations in the *ATRX* gene, localized to Xq13, are the underlying cause of ATR-X syndrome [Gibbons *et al.,* 2008]. Interestingly, acquired *ATRX* mutations have been recognized as the cause of a preleukemic condition, primarily identified in elderly men, known as alpha thalassemia myelodysplastic syndrome (ATMDS) [Gibbons *et al.,* 2008; Gibbons *et al.,* 2003].

The *ATRX* gene spans 300 kb of genomic DNA separated into 36 exons [Picketts *et al.,* 1996]. Alternative splicing gives rise to multiple transcripts; a full length 280 kD protein, a truncated 180 kD isoform, ATRXt, consisting of the first 11 exons and a smaller truncated isoform in which exon 6 is spliced out (Figure 6) [Picketts *et al.,* 1996, Garrick *et al.,* 2004]. The full length protein contains an N-terminal ADD (atypical PHD domain common to ATRX, DNMT3a and DNMT3b) zinc finger domain and a C-terminal SNF2 ATPase/helicase motif, defining its membership in the SWI2/SNF2 family of chromatin remodeling proteins. The majority (80%) of mutations in the *ATRX* gene are found in either the ADD domain (~50%) or the ATPase/helicase (~30%) domain emphasizing the importance of these two highly conserved regions for protein function [Gibbons *et al.,* 2008]. Similar clinical symptoms arise from mutations within either domain and suggest that mutations cause a loss of function [Gibbons, 2006]. The majority of mutations are

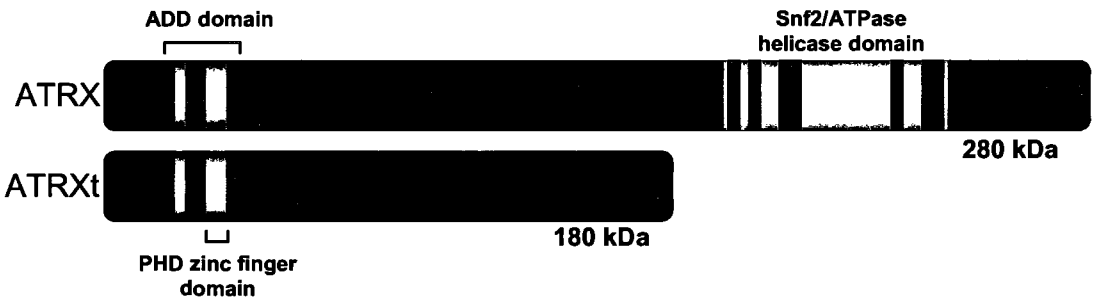


Figure 6. Schematic presentation of the ATRX proteins
Alternative splicing gives rise to multiple ATRX transcripts; a full length 280 kD protein, a truncated 180 kD isoform, ATRXt. The full length protein contains an N-terminal ADD (atypical PHD domain common to ATRX, DNMT3a and DNMT3b) zinc finger domain and a C-terminal SNF2 ATPase/helicase motif, defining its membership in the SWI2/SNF2 family of chromatin remodeling proteins.

missense mutations and two in particular, c563A>G and c736C>T, have been commonly identified, in affected families [Gibbons *et al.,* 2008]. Additional mutations have been identified including deletions and more recently an intragenic duplication and are summarized in Table 3 [Gibbons *et al.,* 2008; Thienpont *et al.,* 2007].

Attempts to make genotype/phenotype correlations have been made but thus far, only weak associations have been identified. Results from a cohort study demonstrate a link between mutations in the ADD domain and severe psychomotor impairment and severe urogenital abnormalities [Badens *et al.,* 2006b]. However, a different study found mutations in the C-terminus were linked to severe genital abnormalities including micropenis and ambiguous genitalia suggesting a new genotype/phenotype correlation [Gibbons *et al.,* 2008]. Further studies are required to understand the full role of *ATRX* gene mutations in the aetiology of the ATR-X syndrome.

1.5.3. Localization and Function of the ATRX protein

Although mutations in the *ATRX* gene are known to cause the ATR-X syndrome, the exact function of the ATRX protein remains unclear. The homology of ATRX with other chromatin remodeling proteins suggests a potential role for gene regulation via epigenetic mechanisms. A nuclear localization signal (NLS) has been identified in the N-terminal region of the ATRX protein, and antibody studies have demonstrated extensive punctate expression in the nucleus [Picketts *et al.,* 1996; Bérubé *et al.,*

Table 3. A summary of the distribution and classification of identified ATRX mutations. Table modified from Gibbons *et al.*, 2008.

Distribution of Mutations			
Region of Gene	***Number of different mutations***	***Number of independent occurrences***	***% of Total***
ADD domain (exons 8-10)	42	95	49
Helicase domain (exons 17-31)	52	57	30

Classifications of Mutations in ATRX	
Mutation	***Number***
Missense	81
Nonsense	8
Splicing	18
Small deletion	8
Small insertion	3
Small deletion/insertion	1
Large deletion (>1 kb)	4
Large insertion (>1 kb)	1
Large duplication	2

2000; Cardoso *et al.,* 2000]. ATRX has been shown to associate with pericentromeric heterochromtin through physical interaction between the ATRX N-terminal PHD domain and heterochromatin protein 1α (HP1α), a protein known to be involved in regulating epigenetic gene silencing by promoting and maintaining chromatin condensation [Cardoso *et al.,* 2000; McDowell *et al,* 1999]. Interestingly, an additional study in mouse cells has revealed that the localization of ATRX at heterochromatic foci requires interaction with MeCP2, a protein abundant in neurons and known to recruit transcriptional repressors [Nan *et al.,* 2007]. Mutations in the human *MECP2* gene cause the X-linked mental retardation syndrome Rett syndrome, and these disease causing mutations were found to inhibit the interaction between ATRX and MeCP2 [Nan *et al.,* 2007]. Thus, it would appear that the ATRX-MeCP2 interaction is critical to ATRX localization, at least in the brain.

In addition to associating with heterochromatin, ATRX also associates with promyelocytic leukemia nuclear bodies (PML-NBs)[McDowell *et al.,* 1999; Nan *et al.,* 2007; Xue *et al.,* 2003]. Bérubé *et al.,* (2008) identified two domains and two functional nuclear localization sequences required for ATRX subnuclear localization. One of the two identified domains, which encompassed several motifs of the SNF2 domain, was found to be required for PML-NB localization [Bérubé *et al.,* 2008]. Interestingly, several patient mutations have been identified within that domain and *in vitro* experiments demonstrated that cells carrying these mutations failed to localize to or be retained at PML nuclear bodies [Bérubé *et al.,* 2008]. These results indicated that clinical ATR-X mutations negatively impact ATRX localization to

PML nuclear bodies and suggest that this localization is crucial for normal ATRX nuclear activity [Bérubé *et al.,* 2008]. ATRX has also been shown to interact with the death domain-associated (Daxx) protein, an apoptotic regulator and a component of promyelocytic leukemia protein (PML) nuclear bodies [Xue *et al.,* 2003; Tang *et al.,* 2004]. Furthermore, *in vitro* studies demonstrate ATP-dependent chromatin remodeling activity in this complex which, like PML-NB localization, is attenuated by mutations found in individuals with ATR-X syndrome [Xue *et al.,* 2003; Tang *et al.,* 2004; Bérubé *et al.,* 2008]. While these results are evidence for the role of ATRX as an ATP-dependent chromatin remodeler, further studies suggest it might modify chromatin structure by altering DNA methylation patterns. This hypothesis stems from the interaction of ATRX at ribosomal DNA (rDNA) repeats as demonstrated by the binding of ATRX to the short arms of human acrocentric chromosomes, a region rich in ribosomal DNA repeats [McDowell *et al.,* 1999]. In patient lymphoblasts, hypomethylation at these rDNA repeats has been identified, suggesting a role for ATRX in regulating methylation patterns [McDowell *et al.,* 1999].

Several studies have indicated a role for ATRX in cell cycle progression. In *C. elegans*, the gene *xnp-1* has been identified as a homolog to the human ATRX gene [Villard *et al.,* 1999]. *Xnp-1* was crucial for embryogenesis, and further experiments demonstrated a potential link to the *C. elegans* Rb homolog, lin-35 [Cardoso *et al.,* 2005]. Early larval arrest was observed in *xnp-1, lin-35* double mutants however, while the size of the arrested larvae did not increase, no defect in

cell divisions was detected [Cardoso *et al.,* 2005]. This suggests that ATRX might have a role in cell cycle regulation through a link to Rb.

Mammalian models have also provided evidence that ATRX might have a role in cell cycle progression. Using mouse oocytes, changes in ATRX phosphorylation were demonstrated at the onset of meiosis I and II. Although the elimination of ATRX by RNAi did not alter the progression of meiosis, it did cause misalignment of chromosomes at meiosis II [de la Fuente *et al.,* 2004]. Additional studies have implicated cell cycle-dependent ATRX phosphorylation in cellular localization. *In vitro* cell studies showed that serine residues were phosphorylated at mitosis and that ATRX associated with chromosomes [Bérubé *et al.,* 2000]. At interphase the protein was not phosphorylated and associated with the nuclear matrix [Bérubé *et al.,* 2000]. Finally, evidence from studies in HeLa cells and mouse neuroprogenitors suggested a requirement for ATRX in normal mitotic progression [Ritchie *et al.,* 2008]. Without ATRX, the transition from prometaphase to metaphase was prolonged and sister chromatids were deficient in cohesion and congression at the metaphase plate [Ritchie *et al.,* 2008].

The use of animal models has provided extensive information about the biological function of the ATRX protein. Expression of ATRX is detectable at embryonic day 7.0 (E7.0) in mouse development, and a global knockout model for full length protein causes embryonic lethality, indicating a developmental requirement for ATRX [Stayton *et al., 1994;* Garrick *et al.,* 2006]. This embryonic

lethality occurs before E9.5 and results from the abnormal differentiation of the extraembryonic trophoblast, one of the first terminally differentiated lineages [Garrick *et al.,* 2006]. While initially this result does not seem to accurately mimic the human ATR-X syndrome, since affected individuals are known to live into the third and fourth decade, it is likely attributable to a complete loss of the ATRX protein. In contrast, patient mutations do not abolish activity but are likely functional hypomorphs [Gibbons *et al.,* 2008]. Although it is currently unproven, it is possible that true null mutations in the human *ATRX* gene may result in embryonic lethality [Gibbons *et al.,* 2008].

In addition to the global knockout mouse model, overexpression studies have also contributed to our understanding of ATRX in development. Transgenic mice that overexpress full length human ATRX have an increased incidence of pre- and perinatal lethality [Bérubé *et al.,* 2002]. Upon closer examination, embryos were found to suffer abnormal growth and organization of the neuroepithelial layer and ventricular zone, suggesting a role for ATRX in corticogenesis [Bérubé *et al.,* 2002]. Those animals that did survive beyond birth demonstrated mild cranofacial dysmorphy and epileptic seizures, both traits of the human syndrome [Bérubé *et al.,* 2002]. Recent studies in *Drosophila* have also indicated a role for ATRX in neuronal development and survival [Hong *et al.,* 2009]. Neuronal expression of dXNP, the *Drosophila* ATRX homolog, induced apoptosis and also increased the activity levels of pro-apoptotic factors known to activate caspases [Hong *et al.,* 2009].

These studies suggest a role for ATRX in neurodevelopment and further investigation has taken advantage of a conditional knockout model generated using the Cre-loxP system. A forebrain specific conditional ATRX knockout was generated by controlling Cre recombinase expression with the *Forkhead box G1* (*FoxG1*) gene. While these animals did survive to birth they were smaller and suffered early post-natal lethality and were unable to survive beyond 48 hours (Figure 7) [Bérubé *et al.,* 2005]. This was likely caused by deficient suckling, although it remains to be proven. Closer examination of the forebrain revealed a substantially smaller frontal cortex and missing dentate gyrus [Bérubé *et al.,* 2005]. Interestingly, BrdU experiments revealed normal embryonic neurogenesis in the cortical progenitor pool of knockout animals. However, a 12-fold increase in apoptosis was identified by TUNEL assay in E11.5 knockout embryos as shown in Figure 7. This data suggests a requirement for ATRX for survival during corticogenesis, perhaps by inhibiting apoptosis [Bérubé *et al.,* 2005].

1.6 Apoptosis

Although the idea of controlled, intentional cell death was introduced in 1964, the term 'apoptosis' was not used until 1972 to describe the morphological processes that lead to the very precise act of cellular self destruction [Lockshin and Williams, 1964; Kerr *et al.,* 1972]. Apoptosis is a widespread phenomenon and has seemingly been evolutionarily conserved as various forms of regulated cell death have been identified in prokaryotes, unicellular and complex eukaryotes [Amiesen, 2002]. Decades of studies have revealed the importance of apoptosis in the development

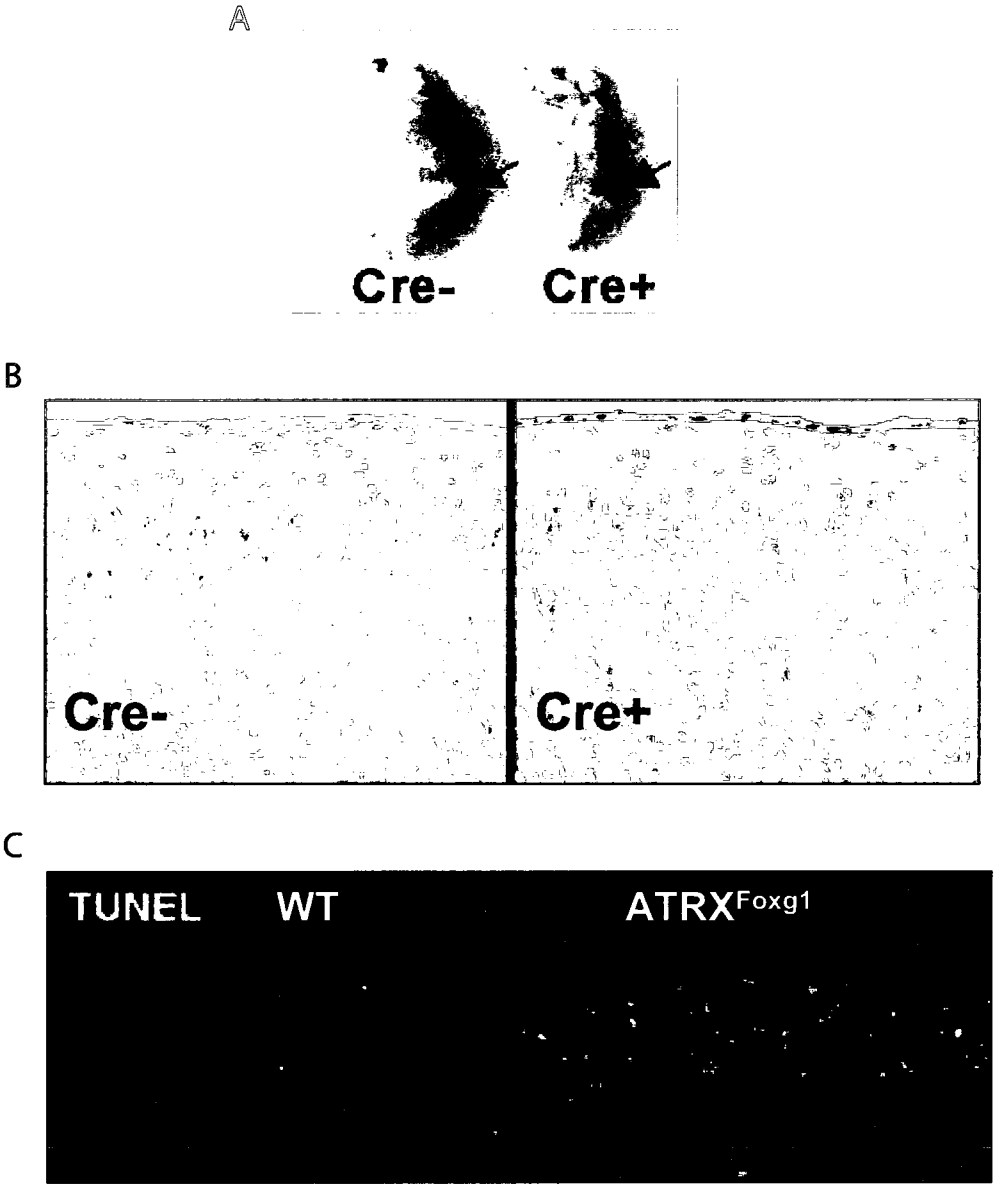


Figure 7. Phenotypes associated with conditional forebrain specific Atrx knockout mice

(A) Postnatal Atrx knockout mice are smaller than their wildtype littermates. Arrows indicate milk in the stomachs of the mouse pups and demonstrate a lack of milk in Cre+ pups. **(B)** Cortical hypocellularity was demonstrated in Atrx knockout forebrains and was attributed to **(C)** a 12-fold increase in apoptotic cells identified by TUNEL assay. Figure modified from Bérubé *et al.*, 2005.

and maintenance of organisms and apoptosis has been implicated in embryogenesis, morphogenesis, homeostasis, and in the destruction of defective and harmful cells [Lockshin and Zakeri, 2001]. The process of apoptosis can be triggered by a wide variety of stimuli, from outside or inside the cell, including cell surface receptor ligands, DNA damage, exposure to drugs or irradiation, and contradictory cell cycle signaling. With such diverse triggers and biological functions, it is not surprising that the initiation and execution of apoptosis is not restricted to one biological pathway. Two major apoptotic pathways, intrinsic and extrinsic, have been identified and are well characterized.

1.6.1 An overview of the intrinsic and extrinsic pathways

Apoptosis can be broadly broken into two biochemical pathways, intrinsic and extrinsic, based on the apoptotic stimuli and the transduction of the ensuing death signal. The extrinsic pathway is induced when death ligands bind to, trimerize and activate, death-receptors such as when tumor necrosis factor (TNF) binds to its receptor, TNFR1 [Muppidi *et al.*, 2004]. While many distinct death receptors are known, they collectively form the TNFR superfamily [Ashkenazi, 2002]. Each receptor consists of unique extracellular subdomains, rich in cysteine, which facilitates highly specific ligand binding [Naismith and Sprang, 1998]. Activation of death receptors causes the subsequent recruitment of adaptor molecules such as TNFR1-associated death domain (TRADD) and FAS-associated death domain (FADD) [Muppidi *et al.*, 2004]. Conserved death domains (DD) in the cytoplasmic component of the receptor recruit the DD contained in adaptor molecules [Naismith

and Sprang, 1998]. Some adaptor molecules also contain a death effector domain (DED) which is integral to sequestering the initiator caspase, procaspase 8 [Denault and Salvesen, 2002]. The complex of death receptor, ligand and adaptor molecules forms the death inducing signaling complex (DISC) to which procaspase 8 molecules bind. In this formation, the procaspase 8 molecules are able to activate one another via autoproteolysis [Denault and Salvesen, 2002]. Active caspase 8 is then able to activate executioner caspases, caspase 3 and caspase 7 which effect cell death.

The intrinsic pathway is initiated from within the cell in response to stress signals generated by events such as DNA damage, hypoxia or a defective cell cycle [Chipuk and Green, 2006]. These stress signals engage the activity of pro-apoptotic members of the Bcl-2 family of proteins [Green and Kroemer, 2004]. Members of the Bcl-2 family can be defined by the presence of conserved Bcl-2 homology (BH) domains and pro-apoptotic members are further divided into two subfamilies [Green and Kroemer, 2004]. Members of the Bax subfamily, including Bax and Bak, are multidomain proteins and contain BH1, BH2 and BH3 domains [Green and Kroemer, 2004]. The second subfamily is defined by, and named for, the presence of only the BH3 domain and includes the members Bid, Bad, Noxa, and Puma to name a few [Green and Kroemer, 2004]. Although multiple theories regarding the exact role of Bcl-2 pro-apoptotic proteins are debated, it is widely accepted that these proteins function by regulating mitochondrial membrane integrity and the release of mitochondrial proteins into the cytoplasm [Green and Kroemer, 2004; Kroemer and Reed, 2000]. Upon stress signals, pro-apoptotic Bcl-2 members translocate from the

cytosol to the mitochondrial membrane where they oligomerize through direct or indirect activation by the BH3-only members and form pores in the mitochondrial membrane that induce mitochondiral outer-membrane permeabilization (MOMP) [Green and Kroemer, 2004; Kroemer and Reed, 2000]. As a consquence of MOMP, cytochrome c is released from the mitochondrial inner membrane space into the cytosol where it causes oligomerization of apoptotic protease activating factor 1 (APAF1) and associates with procaspase 9 to form the apopotosome [Denault and Salvesen, 2002]. The apoptosome functions by cleaving and activating initiator procaspase 9 [Denault and Salvesen, 2002]. Consequently, caspase 9 is able to activate executioner capases 3 and 7, via proteolytic processing, which ultimately effect apoptosis [Denault and Salvesen, 2002].

Interestingly, the BH3-only protein Bid (BH3-interacting domain death agonist) can act as a point of convergence for the intrinsic and extrinsic pathways as illustrated in Figure 8. In some cases, the death signal resulting from an activated receptor in the extrinsic pathway does not generate a caspase signaling cascade strong enough to execute cell death. In these cases, signal amplification occurs when active caspase 8 cleaves Bid allowing the truncated, active form, tBid, to translocate to the mitochondria [Luo *et al.,* 1998]. Once localized to the mitochondria, tBid activates Bax and Bak which initiates MOMP, cytochrome c release and the rest of the intrinsic cascade ensues [Luo *et al.,* 1998].

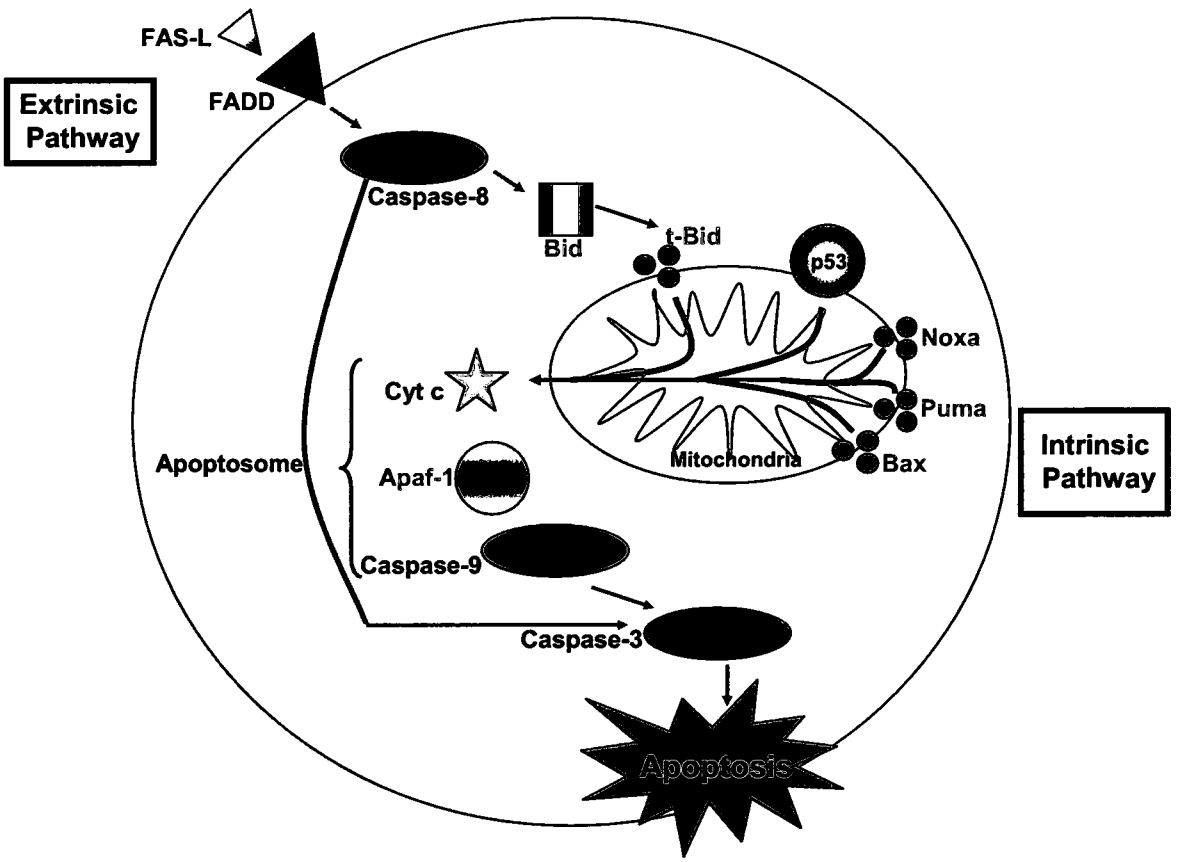


Figure 8. A schematic overview of intrinsic and extrinsic apoptosis
Death via the extrinsic pathway relies on activation of the initiator caspase 8. Cell death via the intrinsic pathway can be activated by multiple stimuli and leads to the release of pro-apoptotic factors from the mitochondria. Both the intrinsic and extrinsic pathways lead to the activation of the executioner caspse 3. Figure modified from Haupt *et al.*, 2003.

1.6.2 p53 mediated apoptosis

Many of the initial intrinsic apoptotic signals originate in the nucleus, as a consequence of DNA damage [Denault and Salvesen, 2002]. DNA damage, in addition to aberrant hyperproliferative signals, cell-cycle re-entry, cytotoxic stimuli and cellular stress, is known to activate the p53 network [Jacobs *et al.,* 2006]. Under normal cellular circumstances, p53 protein levels are very low due to rapid degradation [Jacobs *et al.,* 2006]. This degredation is accomplished by the E3 ubiquitin ligase Mdm2 which binds to and ubiquitinates p53 consequently marking the protein for degradation [Jacobs *et al.,*2006]. During periods of cellular stress, Mdm2 is unable to bind to, and destabilize p53, due to conformational changes in p53 and phosphorylation of specific residues [Jacobs *et al.,* 2006]. Consequently, p53 is released from Mdm2, stabilized, activated and able to promote apoptosis, generally via the intrinsic pathway, however a role for p53 in the extrinsic pathway has been identified [Bennett *et al.,* 1998]. Active p53, which accumulates in the nucleus, has been shown to upregulate pro-apoptotic members of the Bcl-2 family including Bax, Bid, Noxa and Puma [Chipuk and Green, 2006; Sax *et al.,* 2002]. Interestingly, Daxx is known to modulate Mdm2 activity and the disruption of the normal Daxx-Mdm2 interaction is believed to contribute to p53 activation in response to DNA damage [Tang *et al.,* 2006]. ATRX also forms a complex with Daxx, it is possible that this complex could connect ATRX to p53 regulation.

In addition to its nuclear function, p53 transcriptional independent mechanisms have been identified in apoptosis. Active p53 can accumulate in the cytosol where it can activate pro-apoptotic proteins Bax and Bak, and directly bind anti-apoptotic members of the Bcl-2 family which leads to MOMP [Chipuk *et al.,* 2003; Chipuk *et al.,* 2004; Schuler and Green, 2005]. Additionally, p53 can localize at the mitochondrion where it activates pro-apoptotic proteins and subsequent MOMP [Mihara *et al.,* 2003; Marchenko *et al.,* 2000; Chipuk *et al.,* 2004]. Chipuk *et al.,* (2005) propose a model where cytosolic p53 exists in a complex with anti-apoptotic Bcl-2 family member Bcl-xl in the absence of cellular stress. However, in response to cellular stress, p53 is activated leading to stabilization and increased nuclear p53 levels. These increased p53 levels are believed to lead to the transcriptional activation of PUMA which then binds to Bcl-xl, releasing p53. The free p53 then directly activates Bax leading to MOMP, cytochrome c release and activation of the remaining intrinsic apoptotic cascade [Chipuk *et al.,* 2005]. Clearly the role of p53 in apoptosis is complex, multifaceted and continues to evolve in light of new research.

1.6.3 Apoptosis in the brain: function and dysfunction

Programmed cell death, particularly p53-dependent apoptosis, has been implicated in neuronal development and in response to disease and damage in the adult nervous system [Jacobs *et al.,* 2006; Kuan *et al.,* 2000; Culmsee and Mattson, 2005]. It is well established that apoptosis is a naturally occurring process that eliminates approximately half of the neurons produced in the nervous system to ensure the appropriate establishment of neural connections [Jacobs *et al.,* 2006]. This process of

neuronal pruning effectively matches the size of neuronal subpopulations to the corresponding target fields and eliminates neurons with inadequate or erroneous extensions [Kuan *et al.,* 2000; Jacobs *et al.,* 2006]. Interactions between the pro- and anti-apoptotic Bcl-2 family members seem to be responsible for the apoptotic sensitivity during neuronal development [Kuan *et al.,* 2000]. In particular, the anti-apoptotic Bcl-xl and pro-apoptotic Bax seem to be key apoptotic regulators and disruption to both proteins causes a severe phenotype and disruption in neuronal development. Bcl-xl deficiency results in massive neuronal cell death while decreased *Bax* expression caused a dramatic decrease in cell death and resulted in increased neurons in the telencephalon [Roth *et al.,* 1996; Deckwerth *et al.,* 1996]. Additionally, there is a role for p53-dependent apoptosis during development. Some $p53^{-/-}$ mice are viable although they are highly susceptible to spontaneous tumor formation [Donehower *et al.,* 1992; Armstrong *et al.,* 1995; Sah *et al.,* 1995]. However, a closer examination suggests that a high percentage of $p53^{-/-}$ embryos presented with mid-brain exencephaly, overproduction of neural tube tissue causing malformation and failure to achieve neural tube closure and consequently died *in utero* [Donehower *et al.,* 1992; Armstrong *et al.,* 1995; Sah *et al.,* 1995]. Furthermore, the model system of peripheral neurons of the superior cervical ganglion has provided strong evidence for p53-mediated apoptosis during the period of naturally occurring cell death. Overexpression of p53 was sufficient to induce death in this model, and depletion of p53 inferred enhanced survival in sympathetic neurons upon trophic factor withdrawal [Sah *et al.,* 1995; Slack *et al.,* 1996].

Apoptosis is not, however, limited to neuronal development but also has a key role in cell death as a response to acute injury in the mature nervous system and in neurodegenerative disease pathology [Jacobs *et al.,* 2006]. Elevated levels of p53 in apoptotic neurons has been associated with excitotoxic damage following seizure, the area surrounding the ischemic core after middle cerebral artery occlusion, and post traumatic brain injury [Vogel and Prada, 1998; Sakhi *et al.,* 1994; Chopp *et al.,* 1992]. Neurodegenerative disease pathology has also been associated with apoptosis and p53 mediated death has been linked to the progressive cell death phenotype that is characteristic of Parkinson's disease, Huntington's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS) in mouse models as well as in postmortem tissue [Napieralski *et al.,* 1999; de la Monte *et al.,* 1997; Bae *et al.,* 2005; Biswas *et al.,* 2005; LaFerla *et al.,* 1996]. With such widespread function in neuronal development and maintenance it is no surprise that understanding the complexities of apoptotic pathways, like the p53 pathway is the focus of intense studies and the cornerstone of hope for new therapies.

1.7 Rationale and Research Objectives

Epigenetic modification of chromatin has emerged as an important regulator of cellular processes such as proliferation and differentiation. Not surprisingly, deregulation of the epigenome has been associated with many human diseases. One example of this is ATR-X syndrome, a severe form of mental retardation caused by mutations in the *ATRX* gene, which codes for an ATP dependent chromatin remodeling protein.

The early requirement for ATRX during embryogenesis, as well as its importance for neuronal differentiation and proper brain development, has been demonstrated by *in vivo* studies and *in vitro* studies have proposed a role in cell cycle progression and survival during differentiation. Conditional forebrain specific $ATRX^{-/-}$ mice were shown to have a loss of cortical mass, which resulted from a 12-fold increase in apoptosis during early stages of corticogenesis. Additional studies using ATRX wildtype and $ATRX^{-/-}$ cortical progenitor cells found no difference during growth, however the $ATRX^{-/-}$ cultures incurred more death than the wildtype counterparts when induced to differentiate. More recent *in vitro* studies subjected $ATRX^{-/-}$ and ATRX wildtype macrophage cultures to a variety of apoptotic stimuli, including 5-fluorouracil (5-FU) which is known to stabilize p53. Results from these studies showed increased susceptibility to death induced by 5-FU in the ATRX-/- population, a phenotype that was successfully rescued by either the reintroduction of ATRX or the ablation of p53. Combined, these results suggest that ATRX is required for events associated with differentiation and that there is a link between ATRX and regulation of p53 dependent apoptosis. It is **hypothesized that ATRX, a chromatin remodeling protein, regulates neurogenesis by promoting neuronal survival by inhibition of cell death pathways, specifically the p53 pathway**. To address this hypothesis, the specific objectives of this project include:

1. Characterization of the differentiation of $ATRX^{-/-}$ neurospheres.
2. Investigation of the active apoptotic pathway invoked in $ATRX^{-/-}$ mouse forebrains.

2.0 Materials and Methods

2.1 General Materials

All plasticware used during experiments, including cell culture flasks, dishes and microcentrifuge tubes, was purchased from Fisher Scientific (Ottawa, ON). Common chemicals such as TRIS, NaCl, KCl, Na_2PO_4, KH_2PO_4, Glycine, SDS (sodium dodecyl sulfate), EDTA (ethylenediaminetetraacetic acid) used for buffer preparation were obtained from Fisher Scientific (Ottawa, ON), Sigma-Aldrich (Oakville, ON), and Invitrogen (Burlington, ON). Additional experimental reagents including agarose, ethidium bromide, and Triton-X-100 were acquired from Invitrogen (Burlington, ON) and Fisher Scientific (Ottawa, ON). Ethanol, methanol and sterile HPLC water were obtained from Fisher Scientific (Ottawa, ON). Centrifugation during tissue culture protocols was performed using a table top Heraeus Instruments Megafue 1.0, rotor #2740. The supplier information for additional materials and reagents used is stated throughout the methods section as they appropriate.

2.2 Animal Husbandry and Tissue Preparation

To generate the appropriate ATRX knockout embryos, two transgenic parental lines were bred. ATRX floxed females ($ATRX^{f/f}$) were maintained on a C57BL6 strain and mated to $ATRX^{wt/y}$;$Foxg1cre^{+}$ males, also maintained on a C57BL6 strain. Animals were allowed an unlimited diet of 18% Protein Rodent Diet pellets (Harlan Tekland Global Diets, Madison WI) and water. Females were housed in pairs and

males were housed individually. For the purpose of timed matings, females were placed in the male's cage and replaced in their home cage the next morning.

All animal experiments were approved by the University of Ottawa's Animal Care ethics committee and were in accordance with the guidelines set out by the Canadian Council on Animal Care.

2.2.1 Tissue Preparation for Sectioning

Timed matings were used to generate embryos at embryonic day 12.5 (E12.5), 15.5 and 17.5. Identification of vaginal plugs, or the day of separation, was considered embryonic day 0.5 (E0.5). At appropriate time points, pregnant mice were anaesthetized by CO_2 and sacrificed by cervical dislocation. The uterus was removed from the pregnant female and the embryos were removed from their embryonic sacs, separated from their placentas and placed in 1X Hanks' Balanced Salt Solution (HBSS). When preparing tissue to be used for sectioning, the heads were removed from the embryos and placed in 4% paraformaldehyde (PFA) 0.1M phosphate buffered saline (PBS) (0.14 M NaCl, 2.5 mM KCl, 0.2 M Na_2HPO_4 and 0.2 M KH_2HPO_4 at pH 7.4) for fixation overnight at 4°C. The heads were subsequently subjected to three 10 minute washes in PBS and then cryoprotected in a 30% sucrose/PBS solution overnight at 4°C. Finally, the heads were embedded in a 1:1 solution of 30% sucrose and O.C.T. Compound (Tissue-Tek®, Japan), flash frozen on liquid nitrogen and stored at -80°C. A small sample of tissue from each embryo was reserved for genotyping.

2.2.2 Tissue Preparation for protein or RNA extraction

When preparing tissue for protein or RNA extraction, embryos were generated and obtained as described above (section 2.1.1). After removal from the uterus and embryonic sac, the cortices from individual embryos were dissected from the brain in 1X HBSS using a Leica MZ95 dissection microscope. The cortices were collected as whole tissue samples from individual embryos in cell culture tubes, flash frozen in liquid nitrogen and stored at -80°C. A small sample of tissue from each embryo was reserved for genotyping.

2.3 Embryo Genotyping

A small tissue sample was reserved from each embryo at the time of dissection. DNA was extracted using the "hotshot" alkaline lysis method [Truett *et al.,* 2000]. Embryo tissue was incubated at 95°C for 30 minutes in 75 µl of alkaline lysis reagent (25 mM NaOH, 0.2 mM disodium EDTA, pH 12) then cooled over 8 minutes to 4°C. The lysate was then neutralized with 75 µl of neutralizing reagent (40 mM Tris-HCl).

PCR genotyping for sex determination was performed on the cell lysate using primers that amplified regions of the *SRY* (*sex-determining region Y*) gene and *FABP1* (*fatty acid binding protein 1, liver*) gene. Consequently, PCR product from male embryos would show two amplification bands whereas female embryos only showed FABP1 amplification. The following primer pairs were used:

SRY-F 5'-TTG TCT AGA GAG CAT GGA GGG CCA TGT CAA -3'

SRY-R 5'CCA CTC CTC TGT GAC ACT TTA GCC CTC CGA -3'

FABP1-F 5'TGG ACA GGA CTG GAC CTC TGC TTT CCT AGA -3'

FABP1-R 5'CTAG AGC TTT GCC ACA TCA CAG GTC ATT CAG -3'

The PCR mix contained 5 µl of 10X PCR buffer, 1 µl of 10 mM dNTPs, 1.5 µl of 50 mM $MgCl_2$, 0.25 µl of each 10 mM primer (4), 0.3 µl of Taq polymerase (Invitrogen, Burlington ON) and 40.2 µl of water and 1 µl of the cell lysate. Reactions were incubated at 95°C for 20 sec, 57°C for 20 sec, and 72°C for 40 sec for a total of 35 cycles. A separate PCR was prepared to determine Cre status using the following primers:

Cre-F 5'-ATG CTT CTG TCC GTT TGC CG -3'

Cre-R 5'-GGG CGT AGA CAT CTG GGT AG -3'

The reaction conditions were the same as those used for the sex determining reaction, however 0.5 µl of each 10 mM primer was used. Following the PCR reaction, 10 µl of the PCR product was visualized after electrophoresis on a 1.5% agarose gel in 1x TAE with 0.5 µl /mL of ethidium bromide was photographed under UV light.

2.4 Primary Neurosphere Cultures

2.4.1 Neurosphere Isolation

Embryos were generated and harvested as described above in sections 2.1.1. Cortices from individual E12.5 embryos were placed into 1 mL of complete neurosphere proliferation medium and kept on ice until all dissections were complete and the

neurosphere isolation protocol was initiated. A small sample of tissue from each embryo was reserved for genotyping. Complete neurosphere proliferation medium was prepared as per the manufacturer's protocol (StemCell Technologies, Vancouver BC). One 50 mL bottle of NeuroCult® NSC Proliferation Supplements (StemCell Technologies, 05701) was added to 450 mL of NeuroCult® NSC Basal Medium (StemCell Technologies, 05700). Recombinant human epidermal growth factor, rhEGF, (Invitrogen,) was added to give a final concentration of 20 ng/mL. Antibiotic-antimycotic was added to give a final concentration of 0.5%.

Harvested embryonic cortices were triturated using a wide bore pipette tip (BioRad, 223-9028) in 1 mL of complete proliferation medium until a homogenous solution was achieved. This cell suspension was added to 24 mL of complete proliferation media, pre-warmed to 37°C, in a T-75 flask and cultured in a 37°C, 5% CO_2 incubator for seven to ten days before being passaged or used for experiment.

2.4.2 Neurosphere Proliferation and Maintenance

Neurosphere cultures were maintained in complete proliferation media until spheres formed and confluence was achieved. For newly isolated cultures this took 7 to 10 days from the date of isolation. Established cultures were passaged every 3 days. Cell suspensions containing neurospheres in proliferation media were collected in 50 mL tubes and centrifuged at 800 rpm (revolutions per minute) in a table top Heraeus Instruments Megafuge 1.0 for 5 mintues. The media was aspirated and chemical dissociation was performed using the NeuroCult Chemical Dissociation Kit (StemCell Technologies, 05707) as per the manufacturer's protocol. Cells were

either seeded for differentiation or replaced in T-75 flasks with 20 mL of fresh proliferation medium and cultured in a 37°C, 5% CO_2 incubator. Cultures were discarded after 5 passages.

To freeze neurospheres, cells were pelleted by centrifugation for 5 minutes at 800 rpm. Cells from one confluent T-75 flask were used to make 5 aliquots of 1 mL. The old proliferation media was aspirated and the cells were resuspended and subsequently frozen in complete proliferation media including 10% DMSO. Cells were flash frozen in liquid nitrogen and stored at -80°C.

To establish neurosphere cultures from frozen stock, vials were thawed quickly and gently in a 37°C water bath. The contents of each frozen aliquot was added to 14 mL of sterile PBS and centrifuged for 5 minutes at 800 rpm. The PBS was aspirated and the cells were then resuspended in 20 mL of fresh, warm complete proliferation media in a T-75 flask and cultured in a 37°C, 5% CO_2 incubator.

2.4.3 Neurosphere Differentiation

Ten cm tissue culture plates (Sarstedt, 83.1802) or individual glass coverslips placed in the wells of a 6-well tissue culture dish (Costar 3516) were pre-treated with 1 x Poly-D-Lysine and 15 mg/mL laminin in 1x PBS at room temperature for 3 hours in a laminar flow hood. The remaining solution was aspirated and the surface was allowed to dry before neurospheres were plated. Neurospheres were centrifuged at 800 rpm for 5 minutes, media was aspirated and the cell pellet was resuspended and subsequently plated in warm, complete differentiation media. Complete neurosphere differentiation media was prepared as per the manufacturer's protocol. One 50 mL

bottle of NeuroCult® NSC Differentiation Supplements (StemCell Technologies, 05703) was added to 450 mL of NeuroCult® NSC Basal Medium (StemCell Technologies, 05700). Antibiotic-antimycotic was added to give a final concentration of 1%.

Neurospheres were cultured for 1, 3 or 7 days in a 37°C, 5% CO_2 incubator. The differentiation media was refreshed every 2 to 3 days or if a colour change, indicating a change in pH, was observed.

2.5 Western Blot Analysis

2.5.1 Protein Extraction from tissue

Harvested cortices were retrieved from -80°C storage and put on ice. 200 µL of fresh RIPA buffer (radio immuno precipitation assay) (1X PBS, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, protease inhibitor CompleteMini EDTA-free in ddH_2O) was added to each tube of cortical tissue. Cortical tissue was mechanically homogenized at high speed for 30 seconds in RIPA buffer using the Tissue Tearor™ (Biospec Products, Inc.) homogenizer and left on ice for 45 minutes incubation. The cell suspension was centrifuged at 10 000g for 20 minutes at 4°C. The supernatant was subsequently removed and aliquoted for storage at -80°C. The protein samples were quantified using the Bio-Rad Protein Assay reagent (Bio-Rad, Mississauga, ON) and Eppendorf BioPhotometer [Bradford, 1976].

2.5.2 Protein Extraction from Cells

Media from dishes of adherent neurospheres was aspirated and the cells were rinsed with 1X PBS. A rubber policeman was used to scrape the cells from each dish and the cells were collected in 1X PBS in a 10 mL tube. The neurospheres were centrifuged at 800 RPM for 5 minutes. The 1X PBS was aspirated and the cell pellet was resuspended in an appropriate volume, based on pellet size, (200-500 μL) of fresh RIPA buffer (1X PBS, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, protease inhibitor CompleteMini EDTA-free in ddH_2O) and left to incubate for 45 minutes on ice. The cell suspension was centrifuged at 10 000g for 20 minutes at 4°C. The supernatant was subsequently removed and aliquoted for storage at -80°C. The protein samples were quantified using the Bio-Rad Protein Assay reagent (Bio-Rad, Mississauga, ON) and Eppendorf BioPhotometer [Bradford, 1976].

2.5.3 Protein Gel and Transfer Conditions

Protein samples, 20 μg, were mixed with ¼ total volume of 4X NuPage® SDS Loading buffer (Invitrogen, Burlington, ON) and heated at 100°C for 5 minutes. Samples were loaded into pre-made Nupage® Novex® 3-8% Tris-Acetate gel (Invitrogen, Burlington, ON). The gel was electrophoresed at 150 volts for 1.5 hours, or until the dye front reached the gel foot, using the XCell *SureLock*™ Mini-Cell with 1X NuPAGE® Tris-Acetate SDS Running Buffer (Invitrogen, Burlington, ON). The proteins in the gel were transferred onto a PVDF Immobilon–P Transfer Membrane (Millipore, Billerica, MA) using a TRANS-BLOT® SD SEMI-DRY

TRANSFER CELL (Bio-Rad, Mississauga, ON) in 1X transfer buffer (50 mM Tris base, 40 mM Glycine, 10% SDS, 20% MeOH) for 3 hours at 50 mAmp.

Alternatively, for better separation of smaller proteins, samples were loaded into a pre-made Nupage® Novex® 4-12% Bis-Tris gel (Invitrogen, Burlington, ON). The gel was electrophoresed at 200 volts for 1 hour using the XCell *SureLock*™ Mini-Cell and freshly prepared MOPS running buffer. The proteins in the gel were transferred onto a PVDF Immobilon–P Transfer Membrane (Millipore, Billerica, MA) via wet transfer in a BioRad Mini-Blot Transfer Cell for 50 minutes at 50 volts. Protocols were obtained from the 3rd edition of "Short Protocols in Molecular Biology", edited by Ausubel *et al.,* 1995.

2.5.4 Immunoblot Conditions

Following the transfer, the membrane was blocked in 5% milk/TBST overnight or for 3 hours. Subsequently, the membrane was incubated with the primary antibody diluted in 5% milk/TBST in a plastic bag on a shaking platform overnight at 4° (or as indicated) then washed 3 times in TBST for 10 minutes. The membrane was incubated with a secondary antibody diluted in 5% milk/TBST conjugated to HRP (horseradish peroxidase) for 1 hour, washed 3 times in TBST for 10 minutes and rinsed twice in PBS. The ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire UK) was used for detection following the manufacturer's protocol. The membrane was exposed to CL-XPosure film (Thermo scientific, Rockford, IL) and developed in the Konica-Minolta SRX-101A Tabletop film processor. Initially, films were exposed for 2 minutes after which the exposure

time was adjusted based on the clarity of the picture. The following antibodies were used:

- Anti-ATRX 39F mouse monoclonal raised against a GST fusion protein of ATRX amino acids 85-319, dilution 1:4 (gift from Dr Douglas Higgs, Weatherall Institute of Molecular Medecine, Oxford, UK); sheep-anti-mouse-HRP (1:2500)
- Anti-β-Actin mouse monoclonal, dilution 1:15000 (Sigma, Oakville, ON); sheep-anti-mouse-HRP (1:2500)

2.6 Fluorometric Caspase Activity Assays

Protein was extracted, and quantified, from embryonic cortical tissue as described in section 2.5.1. Experiments were prepared in 96 well tissue culture dishes (Costar 3596) on ice. For each sample prepared in duplicate, 20 μg of protein was added to freshly prepared caspase activity buffer (25mM HEPES, 10% sucrose, 1mM EDTA, 0.1% CHAPS, 10 mM DTT in ddH_2O) for a total volume of 199 μL per well [modified from Oberdoester and Rabin, 1999]. The reaction was initated by the addition of 1 μL of 10 mM fluorescent substrate to each well. A ThermoLabsystems Fluoroskan Ascent FL fluorometer using an excitation filter set to 380 nm and an emission filter set to 460 nm was used to read the absorbance of each well every 5 minutes over a 2 hour period. The results were exported and saved as a Microsoft Excel file for further analysis. The fluorescent substrates used were:

- Caspase 3 substrate, Ac-DEVD-AMC (BioMol P411)
- Caspase 8 substrate, Ac-IETD-AMC (BioMol P432)

- Caspase 9 substrate, Ac-LEHD.AMC (BioMol P444)

As a positive control, N1E115 neuroblastoma cells were treated with 1 μM staurosporine (Sigma, Oakville, ON) in DMEM and 10% fetal bovine serum for 6 hours in a 37°C, 5% CO_2 incubator. Separate plates of N1E115 cells were treated with 20 μg/mL of anti-Fas (BD Pharmingen™) in DMEM and 10% fetal bovine serum for 4 hours in a 37°C, 5% CO_2 incubator. Protein extraction and quantification occurred as described above (2.5.1). Protein from the staurosporine treated cells was used as a positive control for the intrinsic pathway, protein from the anti-Fas treatment was used a positive control for the extrinsic pathway. As a negative control, 199 μL of caspase activity buffer and 1 μL of fluorescent substrate were combined. For analysis, the arbitrarty fluorescence value of the negative control was subtracted from the experimental fluorescence values. For clarity in the figures, values were shown at 30 minute intervals.

2.7 Immunocytochemistry

Adherent neurospheres on coverslips were fixed using 4% PFA for one hour at room temperature. Coverslips were then washed three times for 10 minutes in 1X PBS and stored in ddH_2O at 4°C. Cells were permeabilized by incubating coverslips in 0.3% Triton X-100 in 1X PBS for 5 minutes at room temperature and then washed twice for 5 minutes in 1X PBS. Samples were incubated with primary antibodies, diluted in 10% goat serum in 1X PBS, overnight in a humid chamber at 4°C. Following three 5 minute washes in 1X PBS, samples were incubated in secondary antibodies diluted in 2% goat serum in 1X PBS for one hour, at room temperature in a dark humid

chamber. The combination and dilutions of primary and secondary antibodies used were:

- rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) (1/100, StemCell Technologies), Alexa 594 Red-conjugated donkey anti-rabbit-IgG (1/100, Molecular Probes)
- anti-nestin (1/50, StemCell Technologies), Alexa 594 Red-conjugated donkey anti-mouse-IgG (1/1000, Molecular Probes)
- anti-neuronal call III β-tubulin (Tuj1) (1/1000, StemCell Technologies), Alexa 488 Green-conjugated donkey anti-mouse-IgG (1/100, Molecular Probes)
- rabbit polyclonal anti-ATRX (1/200, Santa Cruz Biotechnology), Alexa 594 Red-conjugated donkey anti-rabbit-IgG (1/2500, Molecular Probes)

Cell nuclei were counterstained with DAPI for 3 minutes (10 μg/mL) and the coverlips were mounted on Superfrost Plus coated slides (Fisher Scientific, USA) with DAKO fluorescence protector. Slides were were analyzed on a Zeiss Axioplan microscope and digital images were taken using an AxioVision 6.05 (Zeiss) camera and processed using Adobe® Photoshop.

2.8 Immunohistochemistry

Embedded tissues, as prepared in section 2.2.1, were sectioned on a Leica 1850 cryostat at 10 μM. Sections were transferred onto Superfrost Plus coated slides (Fisher Scientific, USA), air dried for 3 hours at room temperature and stored with dessicant at -80°C. For immunofluorescence studies, tissue sections were dehydrated

in 70% ethanol for 5 minutes and re-hydrated in PBS using three 10 minute washes. Cryosections were blocked in 20% bovine calf serum (Sigma)/0.03% Triton-X 100 in PBS for 1 hour and incubated overnight at 4°C with the primary antibody in a humidified box. Following three 5 minute washes in 1X PBS, samples were incubated in secondary antibodies diluted in 1X PBS for one hour, at room temperature in a dark humid chamber. Nuclei were counterstained with DAPI for 3 minutes (10 μg/mL). The dilutions used for primary and secondary antibodies were with the following:

- rabbit polyclonal anti-ATRX (1/100, Santa Cruz Biotechnology),
- anti-cleaved caspase 3 (1:200, Cell Signaling 9661)

2.9 5-Fluorouracil assay

The apoptotic stimuli 5-fluoruracil (5-FU) was used to induce p53 mediated apoptosis in differentiated neurospheres. Neurospheres were isolated, cultured and differentiated as described in sections 2.4.1, 2.4.2 and 2.4.3 respectively. After seven days in differentiation conditions, the media was removed by aspiration and replaced with fresh differentiation media containing 0.5 μM 5-FU and the cells were returned to a 37°C, 5% CO_2 incubator for 18 hours. After 18 hours of exposure the media was aspirated, the cells were fixed, as described in section 2.4.3, and subsequently processed by TUNEL assay.

2.10 TUNEL assay

Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed on adherent neurospheres using the *In Situ* Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, after fixation (section 2.6) cells were permeabilised for 5 minutes in 0.01% Triton-X, 0.1% Sodium citrate (Sigma) at room temperature. Cells were washed for 10 minutes with 1X PBS and incubated in a mixture of 5 µl of enzyme solution and 45 µl labeling solution for 1 hour at 37°C in a humid chamber according to the manufacturer's protocol. After three 10 minute PBS washes, cell nuclei were counterstained with DAPI for 5 minutes (10 µg/mL). Cells were mounted with DAKO fluorescence protector. As a positive control, cells were treated with 10 units/mL DNase1 in 50 mM Tris-HCl, pH 7.5, 1 mg/mL BSA on ice for 20 minutes. After the DNase1 treatment, cells were rinsed three times in 1X PBS for 5 minutes, and were then subject to the TUNEL treatment as described above.

2.11 Q-RT-PCR

Total RNA was isolated from embryonic cortical tissue using the RNeasy kit (Qiagen) according to the manufacturer's protocol. First strand cDNA was synthesized from 1 ug of total RNA using 300 ng of random primers and a reverse transcription cocktail containing 5x first strand buffer, 100 mM DTT, 25 mM dNTPs and Superscript II RT (Invitrogen). Q-PCR was performed using an Stratageme Mx 3000 with Absolute SYBR Green Q-PCR Master Mix (Abgene) under the following

conditions: 10 minutes at 95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 59°C and 30 seconds at 72°C. A final elongation step of 1 minute at 95°C, 30 seconds at 55°C and 30 seconds at 95°C ensued. Using the standard curve corresponding threshold method of quantification, PCR product formation was monitored in real time (Mx4000 multiplex quantitative PCR system; Stratagene) and the threshold cycles were determined using the Mx4000 software. All data was normalized to GAPDH and 18S RNA expression levels. Threshold cycle variance in four biological replicates was tested for significance using a two-sample Student's *t*-test with equal variance. The following primer sequences were used:

Bid F- 5'-AGC TAG CCG CAC AGT TCA TG- 3'

Bid R 5'-AGC TGT TCT CTG GGA CCT GTC-3'

GAPDH F 5'-TGA AGG GCT CGT TGA TGG-3'

GAPDH R 5'-AAA ATG GTG AAG GTC GGT GT-3'

18S F 5'-CGG CTA CCA CAT CCA AGG-3'

18S R 5'-CTG GAA TTA CCG CGG CT-3'

ATRX F 5'-TGC ATT CTT GCC CAC TGT ATG G-3'

ATRX R 5'-CGT GCT GAA ATC CAG TTT GTC ACA C-3'

Peg3 F 5'-TTGGAGACAACTGGCAAGAG-3'

Peg3 R 5'-CCAACGGAGTGGTGAGTTTT-3'

These primers amplified the *Bid* and *Peg3* genes, both known to be involved in apoptosis, the *ATRX* gene and two housekeeping genes, *GapdH* and *18S*.

3.0 Results

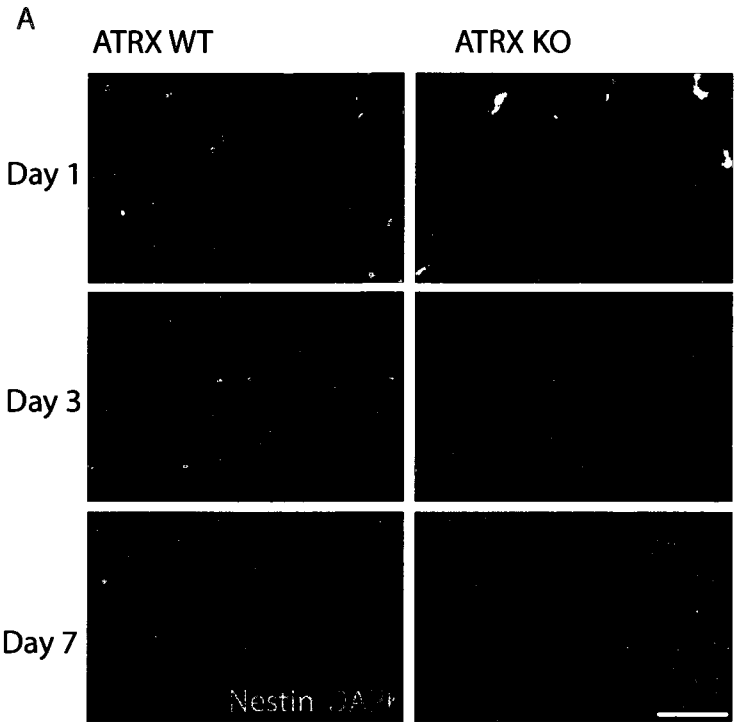
3.1 Characterizing Neurosphere Differentiation

ATRX is a chromatin remodeling gene with a putative role in differentiation. To assess the biological role of *ATRX* during neuronal differentiation a conditional forebrain knockout model was used to circumvent the embryonic lethality associated with global *ATRX* ablation [Garrick *et al.,* 2006]. Using the Cre-recombination system and the *Foxg1* promoter to drive Cre recombinase expression, embryos were generated by mating Foxg1-Cre+ males with ATRX floxed females. The resulting $Atrx^{f/y}Cre^{+}$ embryos were deficient for Atrx in the forebrain and, for clarity, are referred to as Atrx knockout (KO) animals. These mice survive to birth, have a reduced cortical mass but display normal cortical lamination [Bérubé *et al.,* 2005]. Atrx KO and wildtype embryos, at embryonic day 12.5, were used to generate primary neurosphere cultures to characterize neuronal differentiation. Previous studies demonstrated that neurospheres from Atrx KO and wildtype embryos had similar growth properties. However, the Atrx KO cultures showed an increased number of TUNEL positive cells upon differentiation [Bérubé *et al.,* 2005].

3.1.1 Lack of ATRX does not affect neurosphere progenitor status

To determine which cell types required Atrx for survival, we used a differentiation timecourse and stained cells for neuronal, glial and progenitor cell markers. Nestin is an intermediate filament protein that is most highly expressed in neural tissues,

although it is also present in other embryonic and fetal tissues, including cardiomyocytes and pancreatic epithelial progenitor cells [Wiese *et al.,* 2004]. Nestin is unequivocally recognized as a marker of neural progenitor cells and is used here for this purpose [Wiese *et al.,* 2004]. Following one day of differentiation, neurospheres were fixed, stained and the number of nestin positive cells was counted as a percentage of the total cell population. No differences were identified and both populations had a very high level of Nestin expression, nearly 90 %, reflecting the early stage of differentiation (Figure 9). It is likely that after only 24 hours in differentiation conditions the majority of cells would be completing the final round of mitosis. Investigation after three days of differentiation again revealed no differences in Nestin expression between the ATRX KO and wildtype populations (Figure 9). Not surprisingly, both populations had decreased expression profiles compared to that of day 1 differentiation, which is in keeping with progressive differentiation. Finally, analysis at day seven again revealed no differences in Nestin staining when comparing ATRX KO and wildtype neurospheres (Figure 9). At this advanced stage of differentiation, the neurosphere cultures were almost devoid of all Nestin staining suggesting that few progenitors remained and most cells were induced to differentiate (Figure 9A). This result, which demonstrates equivalent nestin expression in ATRX KO and wildtype neurospheres, is consistent with previous accounts of normal neurogenesis in ATRX KO cortical progenitors [Bérubé *et al.,* 2005]. This was examined using BrdU labeling as well as staining with the mitotic marker phosphohistone H3 [Bérubé *et al.,* 2005]. Combined, these results suggest that ATRX KO neurospheres proliferate normally and exhibit comparable



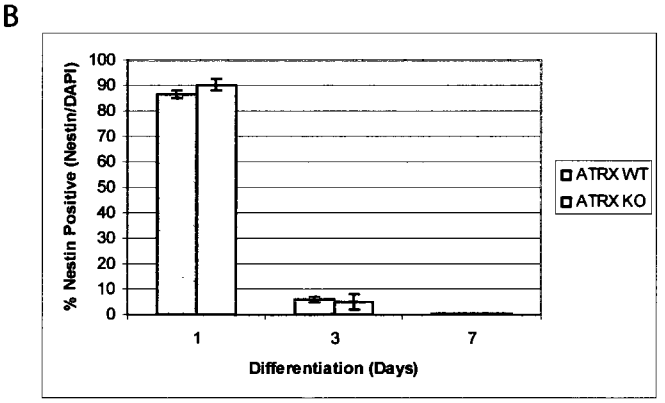


Figure 9. Nestin expression in ATRX KO and ATRX WT neurospheres. Wildtype and ATRX KO neurospheres immunostained for DAPI (blue) and Nestin (red), a marker of neuroprogenitor cells, after 1, 3 and 7 days of differentiaion **(A)**. No significant difference in Nestin expression was identified at any differentiation time point when comparing ATRX wildtype and ATRX KO neurospheres **(B)**. Error bars represent standard error of the mean (SEM). Scale bar represents 50 μM. (N=4, Student T-Test analysis).

Nestin progression, across seven days of differentiation, when compared to ATRX wildtype neurospheres.

3.1.2 Lack of ATRX does not affect the formation of astrocytes

Glial fibrillary acidic protein (GFAP) progressively replaces Nestin over the course of differentiation and is the primary intermediate filament in astrocytes [Cho and Messing, 2009]. As such, GFAP is used as a marker of gliogenesis [Cho and Messing, 2009]. As in the case of the Nestin expression studies, GFAP expression was evaluated in ATRX KO and wildtype neurospheres across a differentiation timecourse. After one day of differentiation, GFAP was expressed at similarly low levels in the ATRX KO and wildtype cultures (Figure 10). As differentiation progressed, GFAP expression increased equally in both cultures and reached peak expression after three days of differentiation (Figure 10). A slight decrease in the percentage of GFAP positive cells occurred after seven days of differentiation, however the majority of the cell population, greater than 70%, was GFAP positive (Figure 10). No morphological differences were identified in the ATRX KO and wildtype cultures based on qualitative analysis. These results suggest that the absence of ATRX does not obstruct the progression from proliferating cells to differentiated astrocytes.

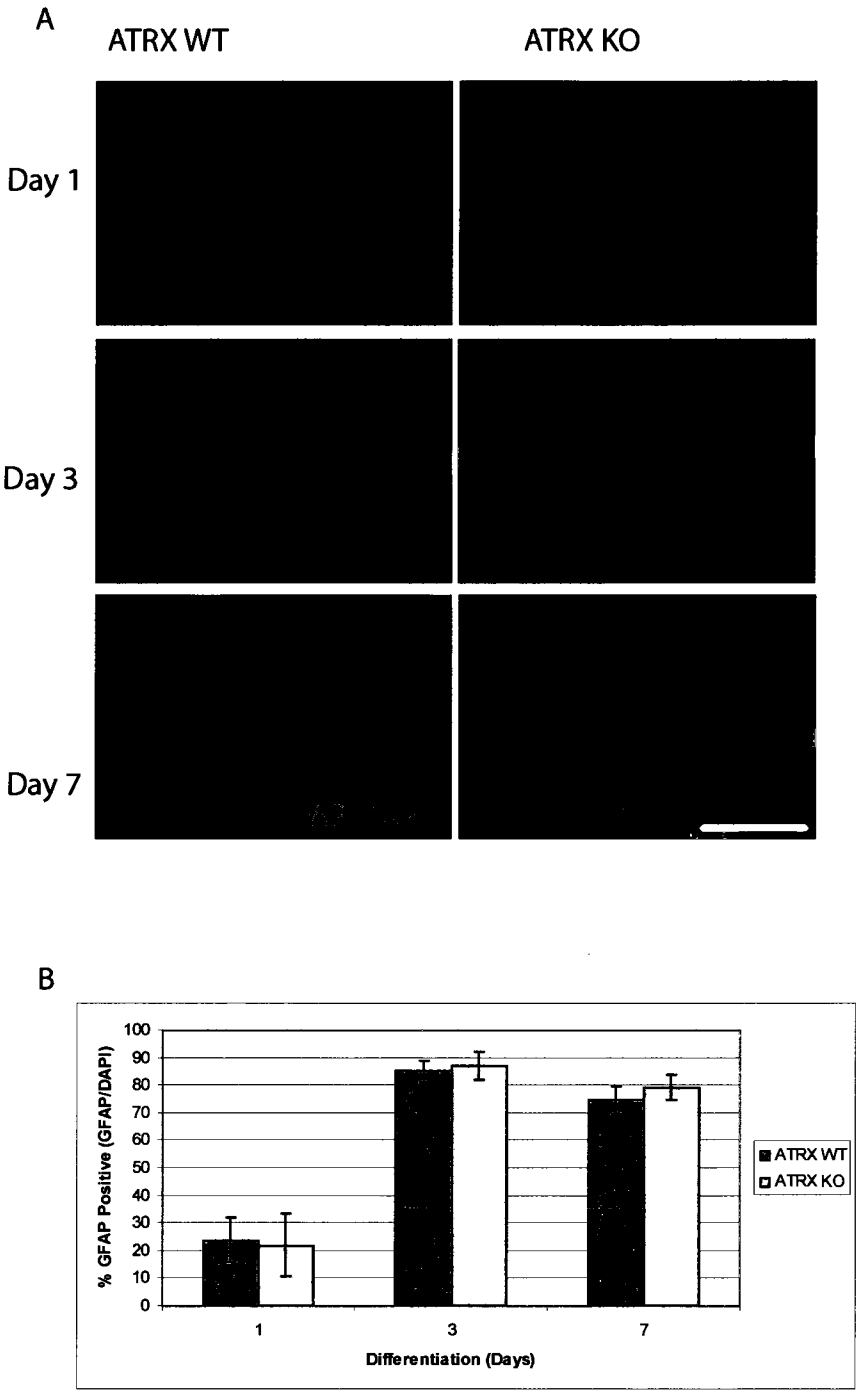


Figure 10. GFAP expression in ATRX KO and ATRX WT neurospheres. Wildtype and ATRX KO neurospheres immunostained for DAPI (blue) and the astrocyte marker GFAP (red) after 1, 3 and 7 days of differentiaion (A). No significant difference in GFAP expression was identified at any differentiation time point when comparing ATRX wildtype and ATRX KO neurospheres (B). Error bars represent SEM. Scale bar represents 50 μM. (N=4, Student T-Test analysis).

3.1.3 Neuronal differentiation requires ATRX

The mature neuronal marker class III beta-tubulin (Tuj1) was used to assess the differentiation of mature neurons in ATRX KO and wildtype neurosphere cultures. After only one day in differentiation conditions, both cultures had a comparably low percentage of Tuj1 positive cells, approximately 1% of the total cell population (Figure 11). However, after three days of differentiation the ATRX KO neurospheres demonstrated a 3.1-fold reduction in Tuj1 positive cell population compared to the wildtype cultures (Figure 11). Statistical analysis using a two-tailed student's T-test revealed that this difference is indeed statistically different ($p \leq 0.006$). This statistically significant increase in Tuj1 positive cells in ATRX wildtype neurospheres was maintained after 7 days of differentiation *in vitro*; wildtype cultures demonstrated 7.1% Tuj1 positive cells compared to 2.2% Tuj1 positive cells in the ATRX KO cultures ($p \leq 0.03$) (Figure 11). Interestingly, qualitative observations revealed no morphological differences in the two cultures which implies that ATRK KO neurospheres can achieve the same neuronal maturation as wildtype neurospheres. The significant reduction in Tuj1 positive cells in the ATRX KO cultures suggests a role for ATRX in neuronal survival during differentiation.

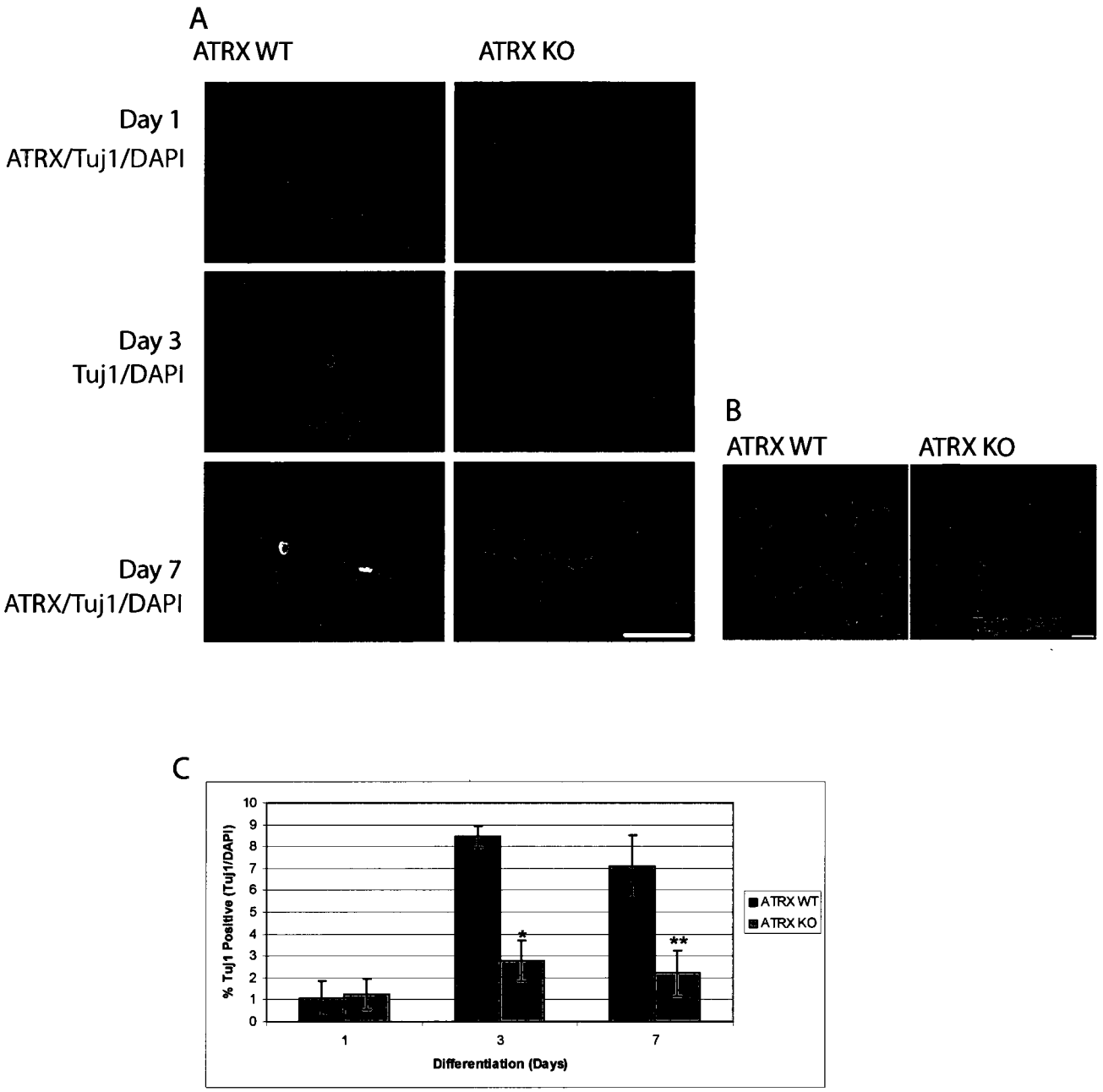


Figure 11. Tuj1 expression in ATRX KO and ATRX WT neurospheres
Wildtype and ATRX KO neurospheres stained for DAPI (blue) and the neuronal marker Tuj1 (green) after 1, 3 and 7 days of differentiaion. Simultaneous immunostaining for Atrx (red) was performed after 1 and 7 days of differentiation to confirm Atrx status **(A)**. No significant difference in Tuj1 expression was identified after 1 day of differentiation when comparing ATRX wildtype and ATRX KO neurospheres **(A,C)**. However, after 3 and 7 days of differentiation, ATRX KO neurospheres showed a statistically significant decrease in Tuj1 positive cells compared to wildtype cells **(A,C)**. A large field of view clearly demonstrates the Tuj1 deficit in the ATRX KO population after 7 days of differentiation **(B)**. Error bars represent SEM. Scale bar in panel A represents 50 μM; scale bar in panel B represents 100 μM. (*$p \leq 0.05$, **$p \leq 0.01$, N=4, Student T-Test analysis).

3.2 Investigation into the active apoptotic pathway in ATRX KO mice

Although immunocytochemistry studies suggested that ATRX was not required for neurosphere proliferation or gliogenesis, the reduced Tuj1 positive cell population in the ATRX KO culture did suggest it was important for neuronal differentiation. Previous work identified a 12-fold increase in apoptosis in ATRX KO forebrain tissue and macrophage studies suggested a potential role for p53 mediated apoptosis [Bérubé *et al.,* 2005, unpublished data]. Accordingly we sought to identify the active apoptotic pathway in ATRX KO neurospheres.

3.2.1 ATRX KO neurospheres show increased sensitivity to 5-fluorouracil

Since macrophage studies revealed that ATRX KO cells suffered more death upon exposure to the nucleotide analogue 5-fluorouracil (5-FU), we rationalized that this pathway should be investigated in the neurosphere cultures. After seven days in differentiation media neurospheres were exposed to 5-FU, fixed and stained with the TUNEL assay to identify apoptotic cells (Figure 12). Remarkably, quantification of TUNEL positive cells as a percentage of the total cell population revealed that ATRX KO cultures suffered nearly four times the amount of apoptosis as the ATRX wildtype neurospheres (Figure 12). After performing the assay on neurospheres harvested from three separate littermate embryos, we observed that 2.1% ± 0.1% of

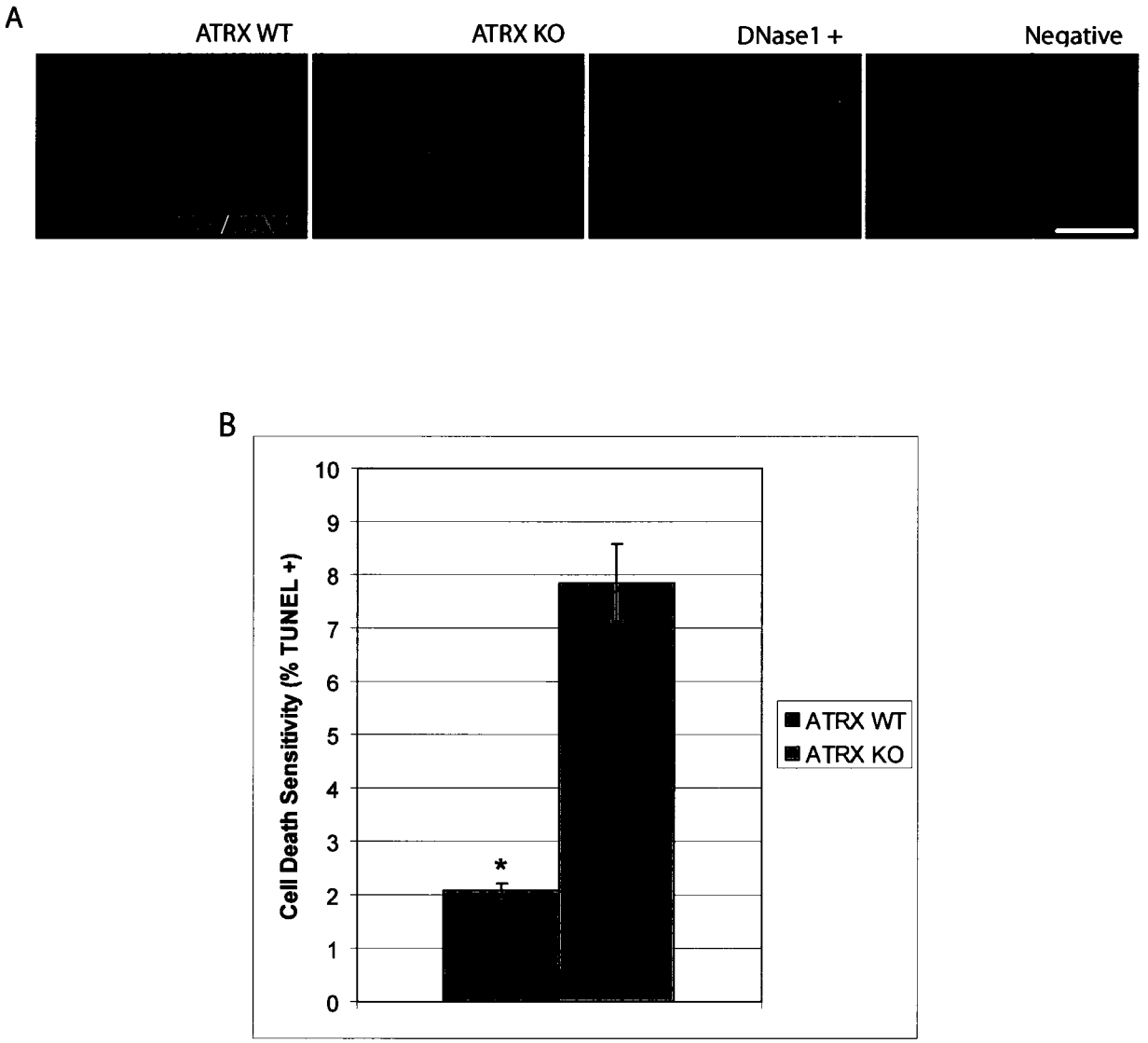


Figure 12. Analysis of cell death in ATRX KO and wildtype neuronal cultures after exposure to 5-fluorouracil.

(A) Wildtype and ATRX KO neurospheres were differentiated for 7 days, treated with 5-FU (0.5 μM for 18 hours), then fixed and stained for DAPI (blue) and TUNEL (red). **(B)** The proportion of TUNEL positive cells to the total number of cells were quantified and the ATRX KO population demonstrated a statistically significant increase in the number of TUNEL positive cells compared to their wildtype counterparts. Cells treated with DNAseI were used as a positive control and cells left untreated were labelled negative. Scale bar represents 50 μM. (*$p \leq 0.001$, N=3, Student T-Test analysis).

the wildtype neurosphere population was TUNEL positive, whereas 7.8% ± 0.7% of the ATRX KO neurosphere population was TUNEL positive. Moreover, this was a statistically significant change as determined by a two-tailed student's T-Test ($p<0.01$). This result suggests that in the absence of ATRX, neurospheres are more sensitive to the apoptotic stimuli 5-FU which consequently implicates p53 in the apoptotic phenotype.

3.2.2 Increased caspase 3 activity was identified in ATRX KO brains

Apoptosis mediated by p53 is almost exclusively implemented via the intrinsic pathway which culminates with the activity of executioner caspases, caspase 3 and 7 [Chipuk and Green, 2006]. Since increased apoptosis was identified *in vivo* in the forebrain tissue of ATRX KO mice and *in vitro* 5-FU studies implicated p53 in the apoptosis phenotype, we proposed to examine whether the intrinsic pathway was active. As an initial step, we evaluated caspase 3 activity in protein extracted from cortical tissue of E12.5 ATRX KO and wildtype mice using a fluorometric enzyme assay. As a positive control protein extracted from N1E115 neuroblastoma cells treated with staurosporine was analyzed and a negative control consisted of the assay buffer and enzyme in the absence of protein. Predictably, the positive control samples demonstrated very high caspase 3 activity (43.2 arbitrary fluorescence units (a.f.u.)) while the negative control provided a measure of auto fluorescence (2.9

a.f.u) that was subtracted from the experimental values during statistical analysis. The ATRX status of each embryo was confirmed by Western Blot protein analysis.

Nearly double the amount of caspase 3 activity was detected after 2 hours in the ATRX KO protein samples compared to the ATRX wildtype samples and this difference was statistically significant (8.4 ± 1.3 a.f.u. versus 2.3 ± 0.04 a.f.u, $p \leq 0.01$) (Figure 13A,B). Protein from five separate E12.5 ATRX KO samples was analyzed. Three separate E12.5 ATRX wildtype samples were analyzed in duplicate. In addition, we examined caspase 3 activity by staining E12.5 tissue sections using an antibody against cleaved caspase 3. This complementary analysis supported the findings of the fluorometic assay and confirmed greater caspase 3 activity in the ATRX KO tissue sections. Additionally, this result was corroborated by results from Seah *et al.*, 2008.

Previous studies identified a greater amount of apoptosis in ATRX KO forebrain tissue during earlier embryogenesis, when E11.5 and E15.5 samples were compared [Chipuk and Green, 2006]. Similarly, caspase 3 activity assays performed on cortical protein extracted from three separate E17.5 embryos, analyzed in duplicate, revealed statistically significantly less caspase 3 activity than that detected in the E12.5 ATRX KO samples (3.9 ± 0.08 a.f.u. versus 8.4 ± 1.3 a.f.u, $p \leq 0.04$) (Figure 13). Additionally, a statistically significant increase in caspase 3 activity was identified in the E17.5 ATRX KO samples compared to the E17.5 wildtype samples, (3.9 ± 0.08 a.f.u. versus 2.5 ± 0.14 a.f.u., $p<0.01$) (Figure 13).

These results provide evidence for greater caspase 3 activity in the cortical tissue of ATRX KO embryos compared to ATRX wildtype tissue.

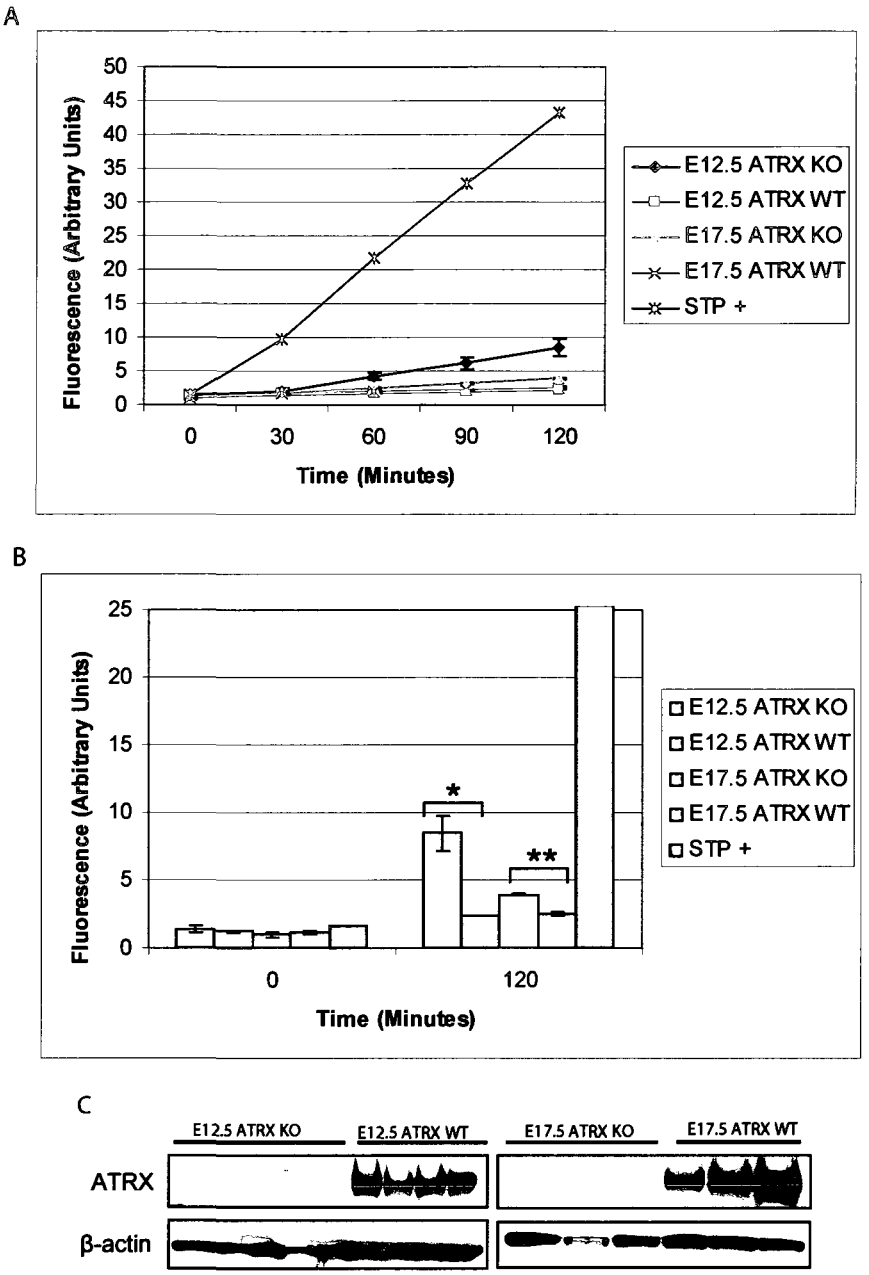


Figure 13. Caspase 3 activity in ATRX KO and ATRX WT cortical tissue
(A) A fluorometric enzymatic caspase 3 activity assay was performed on ATRX KO and wildtype cortical protein extracted from E12.5 and E17.5 embryos. Measurements taken at 5 minute intervals for 2 hours were plotted. **(B)** Analysis of the results from this assay showed there was a statistically significant increase in caspase 3 activity in E12.5 ATRX KO samples versus wildtype (*$p \leq 0.01$) and in E17.5 ATRX KO samples compared to wildtypes (**$p \leq 0.001$). Protein extracted from N1E115 cells treated with staurosporine was used as a positive control. **(C)** Confirmation of ATRX inactivation was monitored by Western blot and demonstrates complete loss of Atrx protein in the KO samples. Error bars represent SEM. (N E12.5 ATRX KO =5, NE12.5 ATRX WT =3, NE17.5 ATRX KO =3, NE17.5 ATRX WT =3, Student T-Test analysis).

Figure 13. Caspase 3 activity in ATRX KO and ATRX WT cortical tissue
(A) A fluorometric enzymatic caspase 3 activity assay was performed on ATRX KO and wildtype cortical protein extracted from E12.5 and E17.5 embryos. Measurements taken at 5 minute intervals for 2 hours were plotted. **(B)** Analysis of the results from this assay showed there was a statistically significant increase in caspase 3 activity in E12.5 ATRX KO samples versus wildtype (*$p \leq 0.01$) and in E17.5 ATRX KO samples compared to wildtypes (**$p \leq 0.001$). Protein extracted from N1E115 cells treated with staurosporine was used as a positive control. **(C)** Confirmation of ATRX inactivation was monitored by Western blot and demonstrates complete loss of Atrx protein in the KO samples. Error bars represent SEM. ($N_{E12.5\ ATRX\ KO}=5$, $N_{E12.5\ ATRX\ WT}=3$, $N_{E17.5\ ATRX\ KO}=3$, $N_{E17.5\ ATRX\ WT}=3$, Student T-Test analysis).

3.2.3 Increased caspase 9 activity was identified in ATRX KO brains

Although increased activity levels of caspase 3 were identified in the brains of ATRX KO animals, this does not directly implicate the intrinsic apoptotic pathway as caspase 3 is situated at the base of both the intrinsic and extrinsic pathways. Indeed, executioner caspase 3 can be cleaved and activated by upstream initiator caspase 8 in the extrinsic pathway or caspase 9 in the intrinsic pathway [Denault and Salvesen, 2002]. To further elucidate the active apoptotic pathway in ATRX KO brains, a fluorometric enzyme assay was performed to measure caspase 9 activity. Again protein extracted from staurosporine treated cells served as a positive control and a negative control consisted of the assay buffer and enzyme in the absence of protein. Protein samples from four separate ATRX KO and ATRX wildtype embryos were analyzed. Similar to the results from the caspase 3 assay, greater caspase 9 activity was measured in the E12.5 ATRX KO samples compared to the E12.5 ATRX wildtype samples (0.95 ± 0.09 a.f.u. versus 0.06 ± 0.05 a.f.u.) (Figure 14). Furthermore, this result was statistically significant, $p \leq 0.001$, based on analysis by a two-tailed student's T-Test. This result in combination with the caspase 3 activity assay suggests that the intrinsic apoptotic pathway is active in ATRX KO brains.

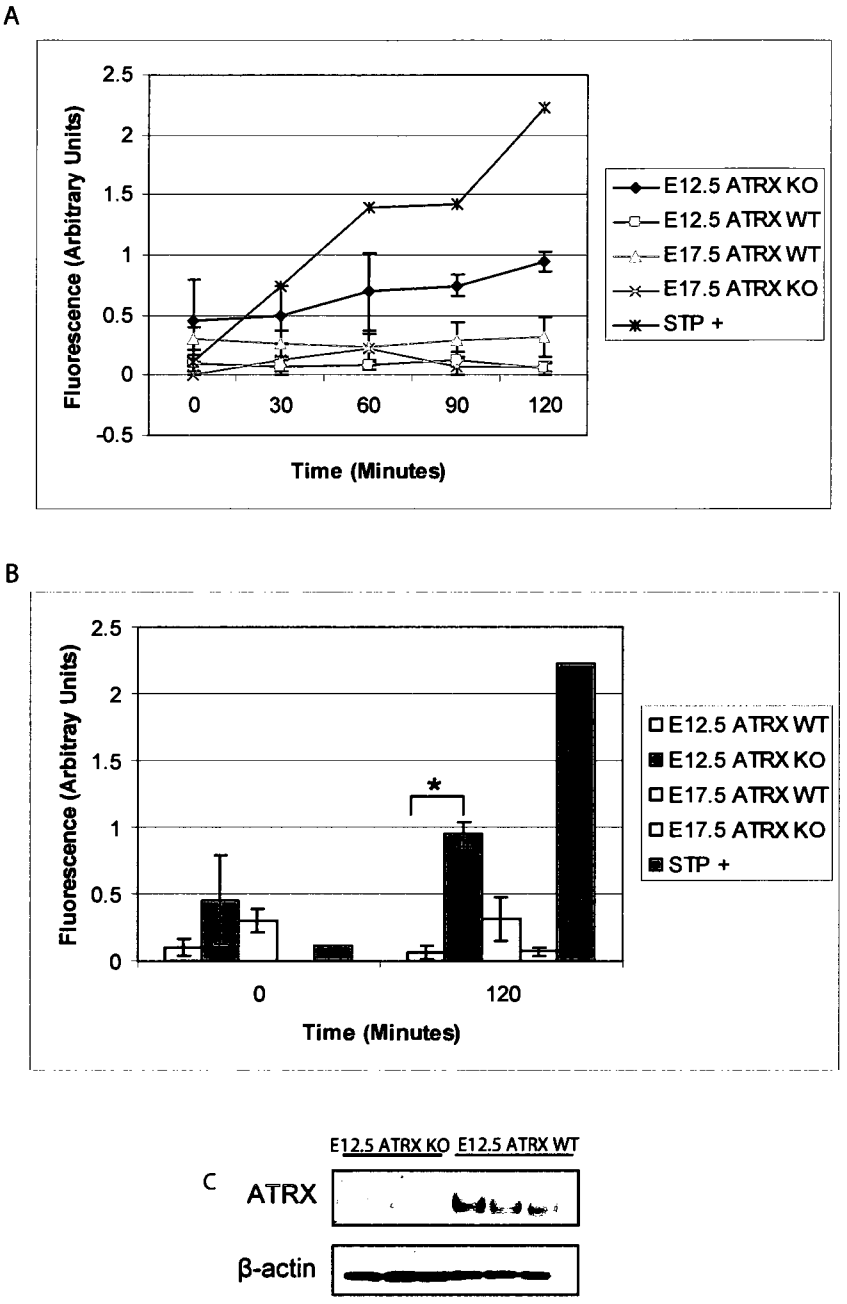


Figure 14. Caspase 9 activity in ATRX KO and ATRX WT cortical tissue
(A) A fluorometric enzymatic caspase 9 activity assay was performed on ATRX KO and wildtype cortical protein extracted from E12.5 and E17.5 embryos. Measurements taken at 5 minute intervals for 2 hours were plotted. **(B)** Analysis of the results from this assay showed there was a statistically significant increase in caspase 3 activity in E12.5 ATRX KO samples versus wildtype (*$p \leq 0.001$). Protein extracted from N1E115 cells treated with staurosporine was used as a positive control. **(C)** Confirmation of ATRX inactivation was monitored by Western blot and demonstrates complete loss of Atrx protein in the KO samples. The E17.5 protein samples were the same as those used in the caspase 3 activity assay. Error bars represent SEM. (N E12.5 ATRX KO =4, NE12.5 ATRX WT =4, NE17.5 ATRX KO =3, NE17.5 ATRX WT =3, Student T-Test analysis)

3.2.4 Caspase 8 activity was equivalent in ATRX KO brains compared to ATRX wildtype brains

To consolidate the results from the caspase 3 and caspase 9 activity assays, which indicated intrinsic apoptotic activity in ATRX KO brains, a caspase 8 activity assay was performed. For this experiment, protein extracted from N1E115 neuroblastoma cells treated with anti-Fas served as a positive control and a negative control consisted of the assay buffer and enzyme in the absence of protein. Protein from four separate ATRX KO and ATRX wildtype embryos was analyzed in duplicate. No difference in caspase 8 activity was identified when comparing E12.5 ATRX KO and ATRX wildtype cortical protein samples ($p \leq 0.9$) (Figure 15). Similarly cortical protein samples from three separate E17.5 embryos, analyzed in duplicate, also showed equivalent caspase 8 activity in ATRX KO and wildtype samples ($p \leq 0.2$). Since no activity difference was detected when comparing ATRX KO and wildtype samples of the same gestational age, these results suggest that caspase 8 is not contributing to the enhanced apoptosis observed in the ATRX KO model.

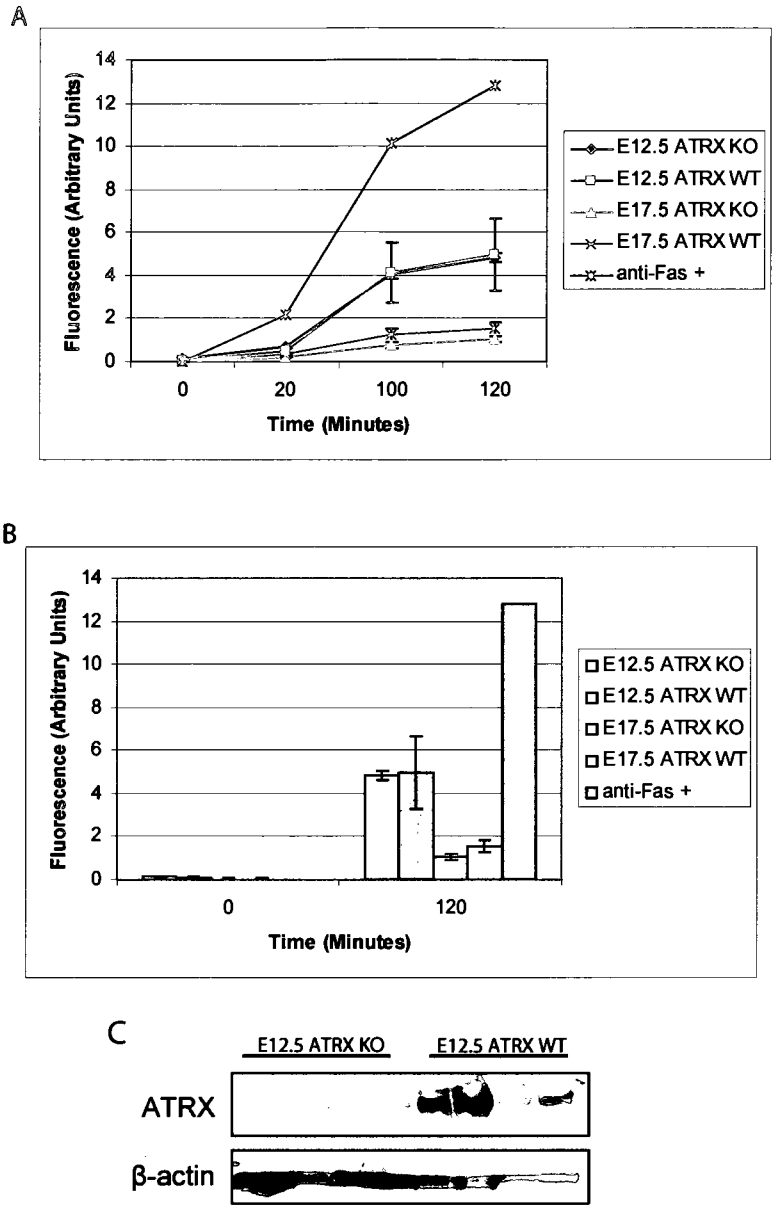


Figure 15. Caspase 8 activity in ATRX KO and ATRX WT cortical tissue

(A) A fluorometric enzymatic caspase 8 activity assay was performed on ATRX KO and wildtype cortical protein extracted from E12.5 and E17.5 embryos. Measurements taken at 5 minute intervals for 2 hours were plotted. **(B)** Analysis of the results from this assay showed there was no statistically significant increase in caspase 8 activity in E12.5 ATRX KO samples versus wildtype ($p \geq 0.94$) nor in E17.5 ATRX KO samples compared to wildtypes ($p \geq 0.24$). Protein extracted from N1E115 cells treated with anti-Fas was used as a positive control. **(C)** Confirmation of ATRX inactivation was monitored by Western blot and demonstrates complete loss of Atrx protein in the KO samples. The E17.5 protein samples were the same as those used in the caspase 3 activity assay. Error bars represent SEM. (N E12.5 ATRX KO =4, NE12.5 ATRX WT =4, NE17.5 ATRX KO =3, NE17.5 ATRX WT =3, Student T-Test analysis).

3.3 Downstream targets of p53

Evidence from the caspase activity assays in combination with the 5-FU assay suggests that the increased apoptosis observed in *in vivo* and *in vitro* ATRX KO models is carried out via p53 mediated cell death and the intrinsic apoptotic network. To strengthen this hypothesis, we rationalized that pro-apoptotic p53 target genes could be altered in ATRX KO embryos. The selection of such p53 target genes for analysis was assisted by previous unpublished microarray data. Microarray analysis performed on cortical cDNA from postnatal day 0.5 ATRX KO and wildtype pups indicated an upregulation in several p53 target genes including *Peg3 (paternally expressed gene 3)*, and *Bid (BH3interacting domain death agonist)* in the ATRX KO samples. These results were substantiated by quantitative RT-PCR analysis on RNA extracted from four separate E15.5 ATRX KO and wildtype samples. Although the results were not statistically significant, perhaps due to low sample size, expression of *Bid* was upregulated 1.7- fold relative to wildtype expression ($p \leq 0.29$). Expression of *Peg3* was statistically significantly upregulated 3.5-fold in ATRX KO samples relative to wildtype expression ($p \leq 0.01$) (Figure 16). As expected, expression of ATRX exon 18 was downregulated, in ATRX KO samples. These results contribute to the body of evidence which suggests that p53 mediated apoptosis is active in ATRX KO brains.

A

Fold Change (P<0.05; n=4)	Gene Symbol	Gene Name
+2.20	AK083432	PEG3 (Paternally expressed gene 3)
+1.46	Bid	BH3 interacting domain death agonist

B

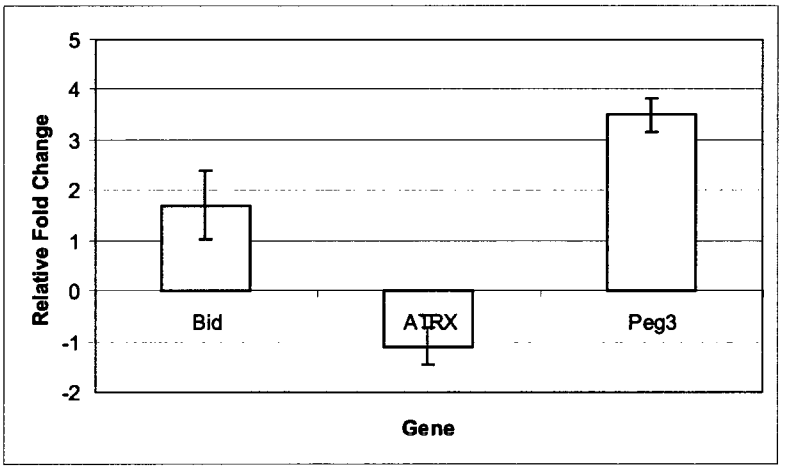


Figure 16. p53 Target genes are upregulated in ATRX KO cortical tissue
(A) Microarray results for P0.5 cortical extracts of wildtype and Atrx KO mice showed that two known pro-apoptotic p53 target genes were upgregulated. **(B)** Quantitative RT-PCR analysis was performed on RNA isolated from E15.5 cortical tissue for Bid, Peg3 and Atrx genes. The results are presented as the expression of each gene relative to wildtype levels ± standard error of the mean. All levels were standardized to GAPDH and 18S expression. Upregulation of Bid and Peg3 was identified in ATRX KO samples however this was not statistically significant. Expression of Atrx exon 18 was decreased by 1.1-fold in Atrx KO samples. (N=4, Student T-Test analysis).

3.4 Deleting p53 rescues the apoptosis phenotype in ATRX KO brains

To confirm that p53 mediated apoptosis was occurring in the ATRX KO brains, a second mouse model was used to generate $p53^{-/-}$ATRX KO embryos. Two matings were used and consisted of either $ATRX^{f/f}p53^{-/-}$ or $ATRX^{f/f}p53^{-/+}$ females mated to $ATRX^{wt/y}p53^{-/+}$;$Foxg1^{+/Cre}$ males. Embryos were harvested at E12.5, cryosectioned and examined by TUNEL for cell death. Interestingly, qualitative observations of TUNEL stained sections revealed fewer TUNEL positive cells in the $p53^{-/-}$ATRX KO tissue sections compared to the ATRX KO sections (Figure 17). This suggests that deleting p53 in the brains of ATRX KO mice rescues the apoptosis phenotype previously identified. Like the ATRX KO mice, the double knockout mice did not survive beyond birth. These results are consistent with a separate study showing that death of interneurons in the dentate gyrus of ATRK KO mice is dependent on p53 [Medina *et al.*, 2009].

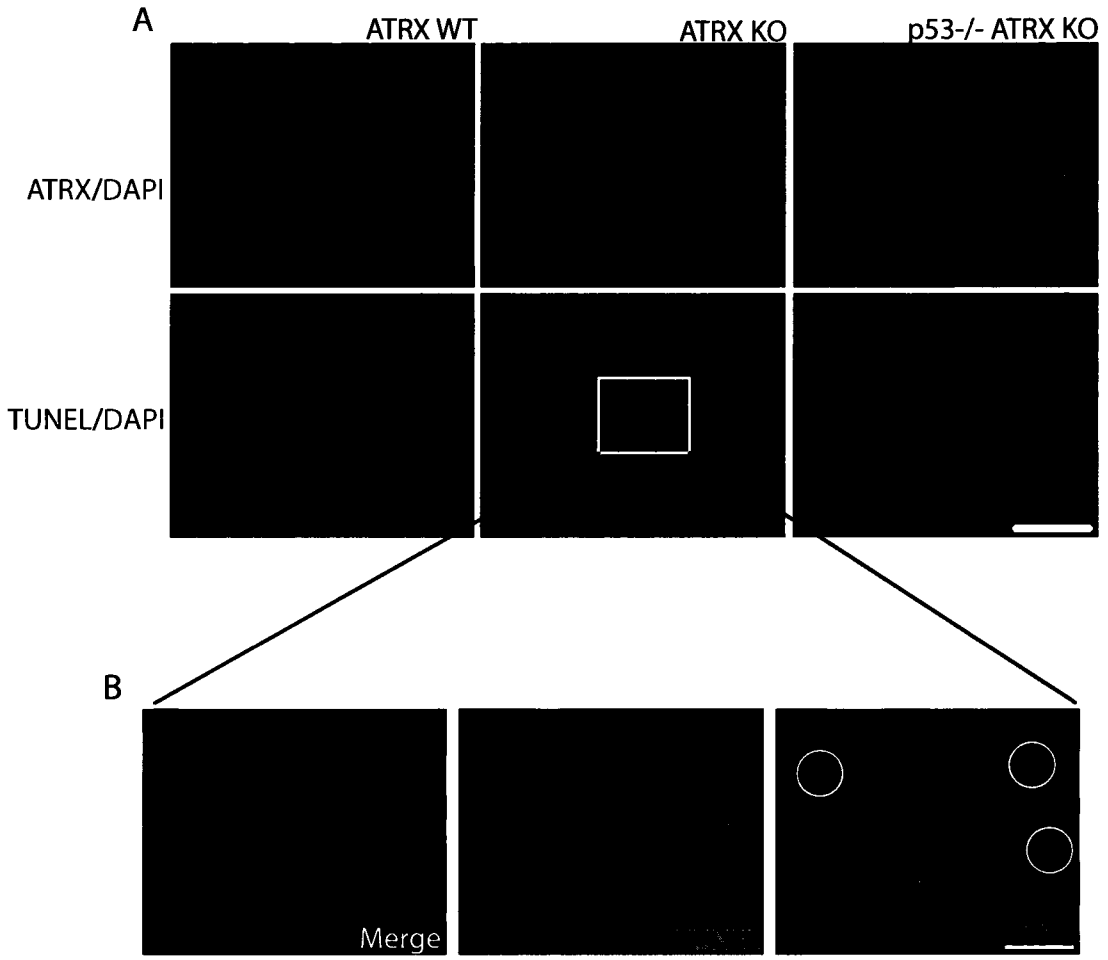


Figure 17. Deleting p53 from ATRX KO brains rescues apoptosis
(A) Forebrain sections from E12.5 littermates, stained for TUNEL (red) and DAPI (blue), and additional sections stained for ATRX (red). Increased apoptosis is seen by TUNEL staining in ATRX KO tissue but is rescued in p53-/-ATRX KO tissue. Scale bar represents 50 μM. **(B)** At higher magnification, DAPI staining reveals the shriveled morphology of TUNEL positive cells (white circles). Scale bar represents 20 μM. N=2

4.0 Discussion

Despite having identified mutations in the *ATRX* gene as the cause of the ATR-X syndrome, the multiple roles of ATRX protein remain to be fully understood. A global knockout mouse model demonstrated an early requirement for ATRX during embryogenesis and further studies using a forebrain specific conditional ATRX knockout demonstrated smaller frontal cortex and missing dentate gyrus, and increased apoptosis during corticogenesis [Garrick *et al.*, 2006; Bérubé *et al.*, 2005]. We questioned whether the enhanced apoptosis was cell-type specific and sought to identify the active apoptotic pathway. Using *in vitro* and *in vivo* Atrx KO models, we demonstrate that neurons were preferentially lost through p53 mediated apoptosis.

4.1 ATRX in neuronal differentiation

Initial characterization of *Atrx* expression in the developing murine cortex demonstrated that the pattern of *Atrx* expression was parallel to the process of neuroprogenitor differentiation [Bérubé *et al.*, 2005]. To this effect, postmitotic cells exhibited the strongest *Atrx* signal when assessed by immunohistochemistry [Bérubé *et al.*, 2005]. This finding suggested a potential role for *Atrx* in the induction or maintenance of neuronal differentiation [Bérubé *et al.*, 2005]. To gain more insight into the role of Atrx in neuronal differentiation, we used primary neurosphere cultures harvested from Atrx KO and wildtype forebrains. The multipotent nature of neurospheres facilitated the evaluation of whether cells lacking Atrx retain the same

potential as wildtype cultures to proliferate and to differentiate into astrocytes and neurons.

Immunocytochemistry revealed no differences in the expression of Nestin, a marker of neural proliferation, when comparing Atrx KO and wildtype neurosphere cultures after 1, 3 and 7 days of differentiation. Interestingly, these results are in line with previous *in vivo* studies using the same conditional forebrain Atrx KO mouse model. Earlier studies demonstrated cortical hypocellularity in Atrx KO animals [Bérubé *et al.,* 2005]. Further BrdU experiments revealed normal proliferation with no obvious deficit in the Atrx KO animals [Bérubé *et al.,* 2005]. Combined, the results suggest that Atrx is not a critical component in the capacity of neuroprogenitors and neurospheres to proliferate.

Similarly, analysis of GFAP by immunocytochemistry revealed no differences in expression in the Atrx KO populations versus the wildtype population after 1, 3 and 7 days of differentiation conditions. These results indicate that, *in vitro*, the development of astrocytes is undisturbed in the absence of Atrx.

Remarkably, immunocytochemical studies using the mature neuronal marker Tuj1 revealed a striking deficit in Tuj1 positive cells in the Atrx KO cultures after 3 and 7 days of differentiation. In fact, a 3 fold reduction in the number of neurons was observed in the Atrx KO culture. This is not the first instance of neuronal loss associated with Atrx ablation. Previous studies showed that the conditional

inactivation of Atrx in the retina during development resulted in a post-natal loss of two types of interneurons, amacrine and horizontal cells [Medina *et al.,* 2009]. The dramatic reduction in neurons in the Atrx KO cultures, as identified by Tuj1 staining, could be a contributing factor in the pathology of the mental retardation associated with the human ATR-X syndrome. Although mental retardation syndromes vary phenotypically and clinically, a reduction in the number and density of neurons has been reported in Down Syndrome (DS) and is hypothesized to be a contributing factor to the associated mental retardation [Kai *et al.,* 2009].

While the Tuj1 immunocytochemical studies revealed fewer neurons in the Atrx KO cultures, more studies were required to understand why this discrepancy was occurring. Previously, significantly increased levels of cortical apoptosis were detected, *in vivo*, in cortical tissue from Atrx KO embryos and *in vitro* in neurosphere cultures, although this was not quantified [Bérubé *et al.,* 2005]. In addition, unpublished data from our laboratory demonstrated that terminally differentiated Atrx KO macrophages experienced more cell death than their wildtype counterparts when exposed to 5-FU, a nucleotide analog which can induce a DNA repair response and death via a p53-dependent mechanism. As such, this prompted us to examine whether differentiated neurons were also more susceptible to p53-induced cell death. Indeed, we observed nearly a 4-fold increase in TUNEL positive cells from the Atrx KO neurosphere cultures following 7 days of differentiation compared to the wildtype cultures (7.8% ± 0.7% and 2.1% ± 0.1% respectively), suggesting that similar pathways are compromised in neurons. Of note, studies using

a mouse model for DS demonstrated a higher incidence of apoptosis during neuronal differentiation [Kai *et al.,* 2009]. While ATR-X syndrome and DS are unique syndromes with different aetiologies, one caused by a lack of gene product and the other by trisomy, it is possible that the common presentation of mental retardation in both is related to a reduction in neurons resulting from enhanced apoptosis

4.2 ATRX in p53 mediated apoptosis

The 5-FU experiments demonstrated that Atrx KO cells exhibited an enhanced apoptotic phenotype. We rationalized that since 5-FU functions via p53, the observed apoptosis was, at least in part, likely mediated by the p53 pathway. Furthermore, p53 has been identified as a key molecular switch in neuronal apoptosis and has been implicated in a variety of neurodegenerative diseases including Parkinson's and Alzheimers [Jacobs *et al.,* 2006]. While the majority of p53 mediated apoptosis is carried out through the internal apoptotic pathway, p53 has also been implicated in the external pathway [Chipuk and Green, 2006; Bennett *et al.,* 1998]. This prompted a broad investigation into the role of each pathway in our Atrx KO neurosphere model by assessing the activity of caspases 3, 9, and 8. Elevated levels of executioner caspase 3 were detected in cortical protein from Atrx KO embryos compared to cortical protein from wildtype littermates. Furthermore, Atrx KO cortical protein demonstrated elevated levels of caspase 9, the initiator caspase upstream of caspase 3 in the internal apoptotic pathway, and showed no difference in activity levels of the external pathway initiator caspase 8. Taken together, these studies provide strong evidence that the enhanced apoptosis in the Atrx KO model occurs via the internal pathway.

A strong link between p53 and caspase 3 activity in models of neuronal apoptosis had previously been well established and prompted our immunohistochemical analysis of caspase 3 [Cregan *et al.,* 1999; Keramaris *et al.,* 2000; Haupt *et al.,* 2003]. Preliminary results showed more cells with positive cleaved caspase 3 staining in the Atrx KO tissue samples compared to wildtype littermates (data not shown). Similar findings were recently reported by Seah *et al.,* (2008) demonstrating greater casapse 3 staining in the telencephalon of Atrx KO embryos.

Furthermore, we initiated a complementary genetic rescue experiment, in which double knockout embryos for p53 and Atrx were generated. Cell death assessment by TUNEL, performed on forebrain tissue from two pairs of double knockout embryos and wildtype littermates, revealed that ablating p53 in conjunction with Atrx rescued the apoptosis phenotype of Atrx KO embryos (Figure18). Although we were only able to observe this in two double knockout embryos due to mating difficulties, our results were substantiated when Seah *et al.,* published analogous findings [Seah *et al.,* 2008]. Additionally, we investigated p53 stabilization by immunoblot but our results were non informative. Despite not succeeding in demonstrating p53 stabilization, our results are strengthened by results recently published from Seah *et al.,* 2008. This group was also conducting studies to assess the role of p53-dependent cell death in the same Atrx KO model after observing that Atrx loss in the developing forebrain results in the loss of a subset of GABAergic neurons [Seah *et al.,* 2008]. Using primary cortical neuroprogenitors and an inducible p53 Cignal Reporter Assay system, Seah *et al.,* (2008) demonstrated

increased p53 activity in ATRX-null cortical progenitors. Using an *in vivo* model, this group demonstrated that introducing a mutant p53 allele in Atrx KO mice resulted in a partial rescue of the reduced cortical size observed in Atrx KO mice. Combined, our preliminary results with these recently published data provide strong evidence that the enhanced apoptosis in the absence of Atrx is dependent on p53 protein.

Many downstream targets of p53 have been identified and are known to have a role in apoptosis [Chipuk and Green, 2006; Schuler and Green, 2005; Culmsee and Mattson, 2005; Haupt *et al.,* 2003]. Following stabilization, which occurs through phosphorylation or acetylation, p53 accumulates in the nucleus where it acts as a transcription factor [Denault and Salvesen, 2002; Tang *et al.,* 2006]. Specifically, p53 has been shown to regulate several pro-apoptotic genes including *Bid, Puma, Peg3, Noxa* and *Bax* [Sax *et al.,* 2002; Nakano and Vousden, 2001; Oda *et al.,* 2000; Miyashita and Reed, 1995; Johnson *et al.,* 2002]. Interestingly, unpublished microarray data from our laboratory demonstrated an upregulation in *Peg3*, and *Bid* gene expression in Atrx KO samples from neonates (P0.5). Our results from Q-RT-PCR experiments corroborated the microarray results, supporting the hypothesis that p53 mediated apoptosis is enhanced in Atrx KO animals and primary cell cultures and suggesting that the role of Atrx may be to repress p53 dependent pro-apoptotic gene expression during differentiation.

While our studies have established evidence of p53 mediated apoptosis in a conditional Atrx KO model, more studies are required to further elucidate the exact molecular apoptotic pathway that is engaged in the absence of Atrx. Not only is p53 known to interact with many pro-apoptotic genes, but two such genes identified as being upregulated in the absence of Atrx, *Bid* and *Peg3*, are multifaceted in their apopototic roles. Generally, the pro-apototic role of Bid is initiated when cytoplasmic Bid is truncated by caspase 8. Post-translational modification results in myristoylated tBid which translocates to the mitochondria [Haupt *et al.*, ,2003]. At the mitochondria, tBid inserts into the membrane and activates the oligomerization of Bax which in turn disrupts the mitochondrial membrane permeability and facilitates the release of cytochrome c and the ensuing downstream internal apoptotic pathway [Haupt *et al.*, 2003]. Bid provides a unique convergence point for the internal and external pathway. It is a transcriptional target of p53 and activates Bax which facilitates cytochrome c release and downstream caspase activation. However, traditional Bid activation relies on truncation by caspase 8, a member of the external apoptotic pathway [Sax *et al.*, 2002; Haupt *et al.*, 2003]. Although our results did demonstrate upregulated Bid levels in Atrx KO samples, no differences in caspase 8 activity levels were observed. Interestingly, recent studies have demonstrated that efficient translocation of full length Bid to the mitochondria can occur within a neuronal model [Konig *et al.*, 2007]. Furthermore, the translocation of full length Bid was associated with the release of pro-apoptotic cytochrome c, suggesting a functional role for full length Bid at the mitochondria [Konig *et al.*, 2007]. Additionally, recent studies investigating apoptosis resulting from the specific

chemotherapy agent vinorelbine have suggested a role for caspases 3 and 9 in Bid truncation [Hayakawa *et al.,* 2008]. An exact role for Bid in our model could be clarified by creating a knock out or knock down model on the Atrx KO background.

In addition to upregulated levels of Bid, our results demonstrated upregulated Peg3 levels, an identified mediator between p53 and Bax in neuronal death resulting from hypoxia and DNA damage [Deng and Wu, 2000; Johnson *et al.,* 2002]. Interestingly, the involvement of Bax is common to activated Bid and Peg3 and represents a gene of interest in our conditional Atrx KO model. Bax is a transcriptional target of p53 and it is required for p53 induced caspase 3 activtaion [Chipuk *et al.,* 2004; Cregan *et al.,* 1999]. Given that our results demonstrated increased caspase 3 activity in the Atrx KO samples, it would be interesting to assess if Bax is upregulated in our Atrx KO model. More studies are required to examine the specific role of Bax in our model.

Our results demonstrate upregulated expression of the pro-apoptotic genes *Bid* and *Peg3,* key molecules in the p53 apoptotis pathway, in the absence of Atrx. However, an exact biological role for Atrx remains unclear. There is a growing body of literature that proposes that the conserved biochemical apoptotic pathways are also essential for terminal cell differentiation [Fernando and Megeney, 2007]. Recently, it has been demonstrated that the executioner caspase 3 is not exclusively active during apoptosis but also has a pro-differentiation role [Fernando and Megeney, 2007]. Studies in which caspase 3 activity was blocked resulted in the inhibition of differentiation in neurons and neural glial progenitors, bone marrow

stromal cells and skeletal myogenesis [Rohn *et al.,* 2004; Miura *et al.,* 2004; Fernando *et al.,* 2002; Fernando *et al.,* 2005]. Additionally, parallels have been drawn between the morphological hallmarks of apoptosis, such as nuclear disruption, and some of the physical changes endured by cells during differentiation, such as nuclear extrusion in erythropoesis and lens epithelial cell differentiation [Fernando and Megeney, 2007]. In neuronal differentiation, the cytoskeletal rearrangements that facilitate neurite outgrowth are similar to those changes that accompany apoptosis [Fernando and Megeney, 2007]. Studies have even demonstrated a role for programmed DNA damage in differentiation [Sjakste and Sjakste, 2007]. Both single strand and double-strand DNA breaks have been identified in a number of differentiation models including early myoblast differentiation, monocyte, granulocyte and lymphocyte differentiation and murine spermatide elongation [Dawson and Lough, 1988; Coulton *et al.,* 1992; Khan and Francis, 1987; Matei *et al.,* 2006; Leduc *et al.,* 2008]. Given that DNA damage can also trigger an apoptotic response, it is clear that some mechanism exists to sway the cell towards apoptosis or differentiation. It is at this crucial biological point of divergence that Atrx could have a potential role. It is possible that Atrx is required to repress pro-apoptotic genes in the p53 pathway in an effort to avoid an apoptotic response to DNA damage. In the absence of Atrx, some cells activate these apoptotic genes inappropriately and undergo apoptosis as hypotheized in Figure 18. Interestingly, other genes involved in the control of chromatin reorganization and DNA repair have been implicated in neuronal differentiation. DNA topoisomerase IIβ is known to interact with proteins involved in chromatin remodeling and also possesses the ability to alter DNA

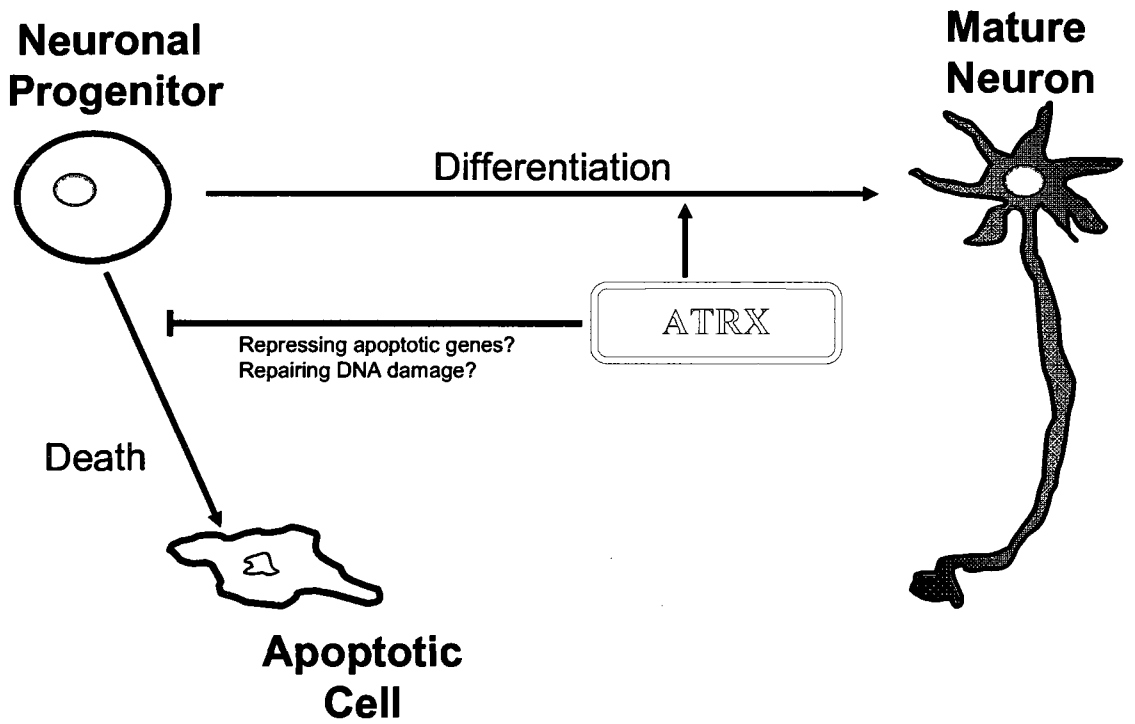


Figure 18. A schematic representation of the role ATRX plays in supporting neurogenesis
We propose a model in which ATRX facilitates the differentiation and survival of mature neurons by inhibiting apoptosis. This could be achieved by repressing pro-apoptotic genes or by facilitating DNA damage repair.

topology [Sugo *et al.,* 2004; Zhao *et al.,* 2008]. Inhibition of topoisomerase enzymes induces double strand DNA breaks and results in the phosphorylation and subsequent activation of p53 [Zhao *et al.,* 2008]. In mice deficient in topoisomerase IIβ, laminar organization of the neocortex and neuronal migration are defective [Lyu and Wang, 2003]. Another interesting mouse is that of mice lacking DNA polymerase β (Pol β), a key molecule in executing base excision repair [Sugo *et al.,* 2004]. Polβ knockout mice displayed a reduced body size and weight, died shortly after birth and postmitotic neurons in the central and peripheral nervous systems suffered substantial apoptosis [Sugo *et al.,* 2004]. Of particular note, a double knockout for Polβ and p53 mouse model rescued the neuronal apoptosis demonstrated in the Polβ mice, however some cytoarchitectural defects remained in the telencephalon and the early postnatal death was not rescued [Sugo *et al.,* 2004]. Proliferation and early differentiation of $Pol\beta^{-/-}$ $p53^{-/-}$ neural progenitors was normal which contrasted the high level of apoptosis seen in $Pol\beta^{-/-}$ $p53^{+/+}$ neocortices, particularly in cells positive for the neuron marker TujI. The authors of this study suggested that in the absence of Polβ, DNA damage can accumulate and trigger the p53 apoptotic pathway. Although an exact role for Polβ in neuronal differentiation was not identified, the authors hypothesized that Polβ might have a role in chromatin remodeling, similar to that demonstrated by topoisomerase IIβ [Sugo *et al.,* 2004]. Of course, Polβ might also be required to repair specific DNA damage generated during neuronal differentiation [Sugo *et al.,* 2004]. Similarly, the role of Atrx might be part of a comparable DNA repair pathway and in its absence, cells could become hypersensitive to p53 mediated apoptosis. Our results, in conjunction with these above, suggest a role for Atrx which

supports neuronal differentiation by facilitating DNA repair or repressing pro-apoptotic genes.

4.3 Conclusion

Neuronal apoptosis, particularly p53 dependent apoptosis, has been implicated in many neurodegenerative diseases as well as some developmental syndromes, and in response to damage in the adult nervous system [Jacobs *et al.,* 2006; Kuan *et al.,* 2000; Kai *et al.,* 2009]. Parkinson's disease, Huntington's disease, Alzheimer's disease, ALS, Down Syndrome and now the mental retardation syndrome ATR-X have all been connected to p53 mediated apoptosis [Jacobs *et al.,* 2006; Kuan *et al.,* 2000; Kai *et al.,* 2009]. Using primary cell lines and genetic rescue experiments, our results demonstrated enhanced p53-mediated apoptosis during times of genomic reorganization, *in vitro* and *in vivo*, in a conditional Atrx KO model. Furthermore, this was corroborated by similar experiments conducted by Seah *et al.,* (2008). The majority of p53-mediated apoptosis is achieved through the internal apoptotic cascade [Chipuk and Green, 2006]. Consistent with this, we demonstrated elevated activity levels of the internal pathway initiator caspase 9 and executioner caspase 3 in Atrx KO samples. No differences in caspase 8, an initiator caspase in the external apoptotic pathway, were identified when comparing Atrx KO and wildtype samples. Microarray data substantiated by Q-RT-PCR data indicated an upregulation of the pro-apoptotic genes *Bid* and *Peg3* in Atrx KO samples.

We propose a model in which ATRX, a chromatin remodeling protein, regulates neurogenesis by promoting neuronal survival by inhibition of p53 mediated apoptosis. Furthermore, we postulate that ATRX is involved in the promotion of

neuronal survival by one of two mechanisms, (i) by the repression of pro-apoptotic genes or (ii) by facilitating the repair of DNA damage before an apoptotic response could be initiated.

5.0 References

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