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The Chromatin Remodeling Protein ATRX in Development and Maintenance of Mouse Skeletal Tissues

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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(Thesis format: Integrated Article)

by

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Abstract

Alpha-thalassemia X-linked mental retardation (ATR-X) syndrome is a rare genetic disorder associated with severe developmental delay, mental retardation and craniofacial dysmorphism. This syndrome is caused by mutations in the *ATRX* gene which encodes a member of the SWI/SNF family of chromatin remodeling proteins. ATR-X patients exhibit dwarfism and skeletal defects, including hand and foot deformities. I hypothesized that the skeletal deformities in ATR-X syndrome are due to a direct role of ATRX in the development of the skeleton. My objective was to characterise skeletal phenotypes observed in three animal models conditionally deficient for ATRX in different skeletal tissues. Mice lacking the *Atrx* gene in forelimb mesenchyme, cartilage, or bone were generated using the Cre-lox system with Cre-recombinase under control of *Prx1*, *Col2a1* or *Col1a2* promoters, respectively. Mice lacking ATRX in cartilage or bone displayed limited skeletal phenotypes and did not recapitulate the defects seen in ATR-X syndrome. Loss of ATRX protein earlier during limb development lead to shortening of the distal phalanges which correlated with increased DNA damage and apoptotic cell death in the mesenchyme of developing digits. This phenotype resembles brachydactyly in human ATR-X syndrome patients and suggests that ATRX is involved in maintaining genome integrity in limb tissue. Aging mice lacking ATRX in skeletal tissues did not show any increased susceptibility to osteoarthritis. In summary, this study suggests that skeleton-intrinsic roles of ATRX are responsible for some but not all of the skeletal deformities observed in ATR-X syndrome.

Keywords

Atrx, development, mouse model, chondrocyte, skeleton, dwarfism, brachydactyly, forelimb, DNA damage, osteoarthritis, aging

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Dedication

This dissertation is dedicated to my parents. Their love, encouragement, and measureless support have sustained me throughout my life.

Co-Authorship Statement

Sections of Chapter 1 are adapted from Solomon LA, Bérubé NG, Beier F (2008) Transcriptional regulators of chondrocyte hypertrophy. *Birth Defects Res C Embryo Today*. 2008 Jun 10; 84(2):123-130. Text and images reproduced here with permission from John Wiley and Sons (See Appendix A)

Chapter 2 is adapted from Solomon LA, Li JR, Bérubé NG, Beier F (2009) Loss of ATRX in Chondrocytes Has Minimal Effects on Skeletal Development. *PLoS ONE* 4(9): e7106. doi:10.1371/journal.pone.0007106. Figures and text are reproduced here in accordance with the Creative Commons Attribution License. Growth plate measurements and some figure preparation was conducted by JR. Li. All other experiments were performed by L. Solomon in the laboratories of Dr. F. Beier and Dr. N. Bérubé. The publication was written by L. Solomon with suggestions from Dr. F. Beier and Dr. N. Bérubé.

Chapter 3 is adapted from Solomon LA, Russell RA, Watson LA, Beier F, Bérubé NG (2013) Targeted loss of the ATR-X syndrome protein in the limb mesenchyme of mice causes brachydactyly. *Human Molecular Genetics*. doi:10.1093/hmg/ddt351. This chapter was written by L. Solomon with suggestions from Dr. F. Beier and Dr. N. Bérubé. Staining for activated caspase 3 was performed by A. Watson. Quantification of immunofluorescence and mouse genotyping was conducted by B. Russell. All other experiments were performed by L. Solomon in the laboratories of Dr. F. Beier and Dr. N. Bérubé.

Chapter 4, entitled: Loss of ATRX does not confer susceptibility to osteoarthritis, was written by L. Solomon with suggestions from Dr. F. Beier and Dr. N. Bérubé. Blinded OARSI scoring and Safranin-O stains were performed by B. Russell. D. Makar produced all

characterisations of the Atrx^{col2} mouse presented in 4-1 and Supplementary Figure 4-1. All other experiments were performed by L. Solomon in the laboratories of Dr. F. Beier and Dr. N. Bérubé.

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CHAPTER ONE

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List of Abbreviations

γ-H2AX: gamma-histone H2A

APase: Alkaline Phosphatase

ATM: Ataxia telangiectasia mutated

ATP: Adenosine triphosphate

ATPase: Adenosine triphosphatase

ATR: Ataxia and telangiectasia and Rad3 related

ATRX, ATR-X: Alpha thalassemia/mental retardation X-Linked

BDA: Brachydactyly type A

BMD: Bone mineral density

BMP: Bone Morphogenic Protein

BSA: Bovine Serum Albumin

cKO: Conditional knockout

Col1: Type-I collagen

Col2: Type-II collagen

CTRL: Control

DAPI: 4’6-diamidino-2-phenylindole

DMEM: Dulbecco’s modified eagle medium

DNA: Deoxyribonucleic acid

HDAC: Histone deacetylase

E: Embryonic day

ECL: Enhanced chemiluminescence

ECM: Extracellular matrix

EDTA: Ethylenediamine tetracetic acid

FBS: Fetal bovine serum

h: Hours

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hox: Homeobox

HRP: Horseradish peroxidase

HU: Hydroxyurea

kb: Kilobase

kDa: Kilodalton

MicroCT: Micro-computed tomography

MMP: Matrix metalloproteinase

mRNA: Messenger RNA

n: number

OARSI: Osteoarthritis Research Society International

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PCR: Plymerase chain reaction

Pen/Strep: Penicillin/Streptomycin

PFA: Paraformaldehyde

PHD: Plant homeodomain

Prx: Paired related homeobox 1

rDNA: Ribosomal DNA

RIPA: Radioimmunoprecipitation assay

RNA: Ribonucleic acid

RT: Reverse transcriptase

RT-PCR: Quantitative real time PCR

SD: Standard Deviation

SDS: Sodium Dodecyl Sulphate

SHOX: Short stature homeobox

siRNA: small interfering RNA

SNF2: Sucrose non-fermenting 2

SWI/SNF: Switching defective/sucrose non-fermenting

TH/IGF1 axis: Thyroid hormone/ Insulin-like growth factor 1 axis

ZPA: Zone of polarising activity

Chapter 1

1 Introduction

1.1 ATRX

1.1.1 ATR-X Syndrome

ATR-X Syndrome (Alpha-Thalassemia Mental Retardation, X-linked) is an X-linked human disorder associated with severe psychomotor and mental retardation, characteristic facial features, urogenital abnormalities, skeletal deformities, and alpha-thalassemia (MIM# 301040) (Figure1-1) (Gibbons et al., 1995a). Patients present with global developmental delay, learning problems, limited speech, and mental retardation (Gibbons et al., 1995b). One original diagnostic tool used to define ATR-X syndrome was the presence of a type of anemia, called α-thalassemia, which is caused by a lack of alpha-globin expression and results in β-globin inclusions in peripheral red blood cells (Gibbons, 2006). These tetrameric inclusions of β-globin lead to a non-functional and unstable form of haemoglobin that precipitates as haemoglobin H (HbH) tetramers in older red cell populations (Higgs et al., 2009). Not all cases of ATR-X syndrome present with HbH inclusions, although there may still be a down-regulation of α-globin expression [Reviewed in (Gibbons, 2006)].

Characteristic facial features such as epicanthic folds, flat nasal bridge, small upturned nose, and midface hypoplasia are seen in 94% of patients, and 66% of patients display dwarfism (Gibbons et al., 2000a). ATR-X syndrome is rare, with just over 200 cases reported, and there is limited availability of patient samples for study. Almost all affected patients are male (XY karyotype) due to the X-linked nature of the disease (Gibbons et al., 2008). Female carriers are unaffected with normal intellect and physical appearance due to the skewed pattern of X-inactivation favouring silencing of the mutant allele (Gibbons et al., 1992).

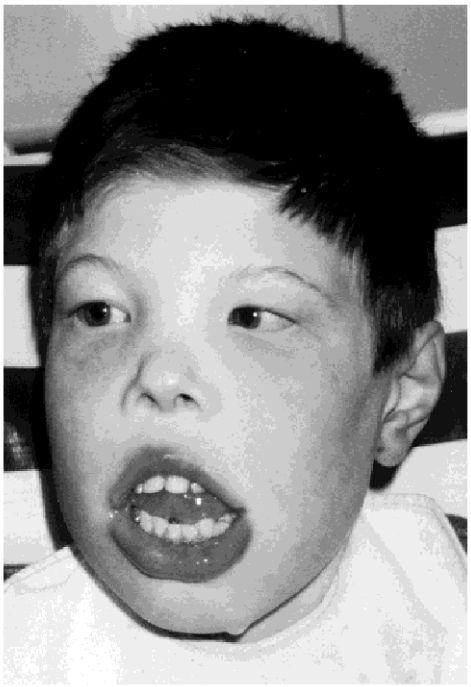
ATR-X syndrome bears similarity to other X-linked mental retardation syndromes, described together as 'X-linked mental retardation-hypotonic facies syndromes' (MIM: #309580). These syndromes include Carpenter-Waziri, Holmes-Gang, Juberg-Marsidi,

TABLE I. Clinical Findings in 145 Patients With ATR-X Syndrome

Clinical finding	Total[a]	%
Profound mental retardation	137/144[b]	95
Characteristic face	116/124	94
Skeletal abnormalities	110/121	91
HbH inclusions	106/122	87
Neonatal hypotonia	78/92	85
Genital abnormalities	103/129	80
Microcephaly	90/119	76
Gut dysmotility	78/104	75
Short stature	63/96	66
Seizures	46/131	35
Cardiac defects	24/135	18
Renal/urinary abnormalities	19/136	14

[a]Total represents the number of patients on whom appropriate information is available and includes patients who do not have α thalassemia but in whom *ATRX* mutations have been identified.

[b]One patient too young (<1 yr) to assess degree of mental retardation.



Thirteen year-old boy with ATR-X syndrome showing the typical facial appearance.

Figure 1-1 Molecular clinical spectrum of ATR-X syndrome

Reproduced with permission from: Gibbons et al. (2000) Am. J. Med. Genet. (Semin. Med. Genet.) 97:204–212

and Smith-Fineman-Myers syndromes and X-linked mental retardation with spastic paraplegia (Abidi et al., 1999; Abidi et al., 2005; Lossi et al., 1999; Stevenson et al., 2000; Villard et al., 2000; Villard et al., 1996). Studies have revealed that some of these syndromes can be considered ATR-X syndrome, but absence of HbH inclusions excluded them from the initial diagnosis. The presence of these inclusions is variable even between ATR-X cases with the same mutation. A common 736C>T mutation seen in 32 cases of ATR-X syndrome presents with varying frequency of HbH inclusions, ranging from 0 to 14% (Gibbons, 2006). This variability between identical mutations leads to difficulties in making phenotype/genotype correlations and suggests the role for ATRX in α-globin expression differs between patients.

1.1.2 ATRX Gene and Protein

The remodeling of chromatin influences the packaging and accessibility of DNA within nucleosomes. While nucleosomes themselves are structurally stable, nucleosome modifying and remodeling complexes confer specific properties to the chromatin by modifying epigenetic marks on particular histones [Reviewed in (Saha et al., 2006)]. In eukaryotes, there are at least five families of chromatin remodeling proteins; SWI/SNF, ISWI, NURD/Mi-2/CHD, INO80 and SWR1 (Saha et al., 2006). SWI/SNF proteins exist in large nucleosome remodeling complexes with evolutionarily distinct subfamilies and functions (Carlson et al., 1994; Eisen et al., 1995). Purified SWI/SNF complexes directly interact with nucleosomal DNA and enhance transcription by the hydrolysis of ATP to open the chromatin or increase accessibility (Cote et al., 1994). SNF2 proteins utilize ATP hydrolysis to power molecular motors that alter interactions between nucleic acids and proteins to affect DNA wrapping around the nucleosome (Clapier et al., 2009). This unwrapping function allows SWI/SNF complexes to disrupt the arrangement of nucleosomes and thereby regulate gene transcription by altering DNA accessibility (Peterson et al., 2000). SWI/SNF complexes can also alter chromatin structure through nucleic acid twisting to generate superhelical torsion, which distorts helices and alters the local chromatin topology (Havas et al., 2000). Complexes containing these proteins play roles in transcriptional regulation, DNA repair, and regulation of mitotic recombination [Reviewed in (Allard et al., 2004)].

ATR-X syndrome is caused by mutations in the *ATRX* gene (Gibbons et al., 1997; Gibbons et al., 1995b). *ATRX* is located on the X-chromosome, is composed of 36 exons spanning over 300 kilobases (kb), and encodes a potential protein of 2492 amino acids (Villard et al., 1997). An alternative splicing event involving exon 11 generates a shorter protein, ATRXt, which lacks the C-terminal domains (Garrick et al., 2004; Villard et al., 1997). Comparative genetic analysis between mouse *Atrx* and human *ATRX* shows a high degree (85% overall identity) of homology and the mouse and human proteins have a similar weight of 280 kDa (Picketts et al., 1998). Conservation between human and mouse ATRX is highest within two conserved functional motifs which code for a Plant Homeo Domain (PHD)-like zinc finger at the N-terminus and a catalytic ATPase domain at the C-terminus (Figure 1-2) (Gibbons et al., 1997; Picketts et al., 1996; Picketts et al., 1998). Mutations to the regions coding these domains can cause a reduction in protein levels or diminish enzymatic function (Gibbons et al., 1995b; Picketts et al., 1996). Furthermore, most disease-causing mutations identified in ATR-X patients are within these conserved domains, confirming their requirement in normal development (Gibbons et al., 2008). Complete loss of function is not observed in ATR-X syndrome, which suggests complete loss of ATRX protein in humans is lethal (Gibbons et al., 1995b; Picketts et al., 1996).

1.1.3 Functions of ATRX

1.1.3.1 Biochemical Functions of ATRX

A number of ATR-X mutations are concentrated in the PHD-like domain, suggesting this region has an important functional role in vivo (Gibbons et al., 1997). The PHD-like zinc finger has homology with the DNMT3 family of DNA methytransferases, leading to the specific name ATRX-DNMT3-DNMT3L (ADD) domain (Aapola et al., 2000; Argentaro et al., 2007; Xie et al., 1999). Characteristic of a protein containing an ADD domain, ATRX appears to influence methylation of DNA but

Figure 1-2 Major domains of the ATRX protein

Major domains of the full length ATRX and truncated ATRXt protein: the PHD zinc finger-like domain (ADD), SNF2 chromatin remodeling domain (Helicase domain), the P box a (P), and glutamine-rich region (Q).

whether this is a direct effect or not is unknown. Specifically, EBV-transformed lymphocytes from ATR-X patients demonstrate alterations to the methylation pattern of ribosomal DNA (rDNA), Y chromosome-specific and subtelomeric repeats of DNA (Gibbons et al., 2000b). The C-terminal catalytic domain of ATRX has seven conserved collinear helicase domains, and is homologous to the RAD54-like subfamily of SNF2 family of chromatin remodeling proteins (Flaus et al., 2006; Picketts et al., 1996). RAD54 is a DNA translocase that plays an important role in DNA strand exchange for the recombinational DNA repair (RAD52) pathway (Shah et al., 2010; Solinger et al., 2002).

The conservation of important functional domains of ATRX suggests it has chromatin-remodeling functions (Aasland et al., 1995). SNF2 proteins are involved in DNA replication, ATP-dependent chromatin remodeling, and processing of DNA damage (Chai et al., 2005; Cote et al., 1994; Flanagan et al., 1999). In vitro studies of recombinant ATRX have demonstrated that the SWI/SNF domain possesses ATPase activity that is stimulated by mononucleosomes (Tang et al., 2004). ATP hydrolysis is also required for the translocase activity of ATRX, allowing it to unwind a third strand of DNA wrapped around a double helix, in what is known as a triple-helix displacement assay (Mitson et al., 2011a; Xue et al., 2003). Some mutations to the SNF2 domain of ATRX weaken its ability to mechanically slide along double-stranded DNA in vitro, an activity termed DNA translocation (Mitson et al., 2011b).

Evidence suggests that a complex containing ATRX has a role in chromatin assembly at tandem repeat regions, including G-rich regions and G-quadruplex structures (Law et al., 2010). A G-quadruplex is a structure that occurs in single-stranded G-rich DNA, where the DNA self-associates in a four-stranded complex of guanines binding to each other (Sen et al., 1988). These structures are predicted to occur frequently in areas of the genome targeted by ATRX, including telomeres, rDNA, G-rich minisatellites, and CpG-rich promoters (Law et al., 2010). The binding of ATRX to G-quadruplex structures may be a mechanism by which ATRX influences the expression of α-globin in erythroid cells (Law et al., 2010). It is predicted that ATRX recognizes unusual and difficult-to-replicate DNA configurations, like the G-quadruplex, and converts them into normal forms of

DNA through incorporation of histone variants (Law et al., 2010; Watson et al., 2013). Stabilizing G-quadruplexes with the ligand telomestatin exacerbated the DNA damage phenotype and decreased the survival of ATRX-null mouse neuroprogenitors, confirming a requirement for ATRX in replication through these structures (Watson et al., 2013).

ATRX may interact with heterochromatin through direct contact with DNA. Synthetic peptides of the C-rich zinc finger domains of ATRX were shown to effectively associate with double-stranded DNA in a gel-shift assay (Cardoso et al., 2000). ATRX can also bind directly to G-quadruplexes in vitro (Law et al., 2010). Together, these studies suggest another mechanism by which ATRX can influence DNA, and also target repetitive regions.

1.1.3.2 Transcriptional Regulation

Considering the biochemical functions of ATRX, it is expected to play significant roles in DNA recombination, DNA repair, and regulation of transcription. When mutations reduce the function of ATRX in humans, expression levels of α-globin may be reduced (Gibbons et al., 1995b). This observation provides a genetic and functional component linking *ATRX* mutations with the down-regulation of α-globin gene expression leading to alpha thalassemia (Gibbons et al., 1995b). Since this initial observation, ATRX has been shown to affect the expression of other genes. Microarray analysis of gene expression within the embryonic forebrains of mice which lacked ATRX beginning at E8.5 showed changes in the expression of ancestral pseudoautosomal genes (Levy et al., 2008). Genes with significantly altered expression in the forebrains of E13.5 Atrx-deficient mice also include genes required for brain development such as *Gbx2* and *Neurod4* (Levy et al., 2008). ATRX-deficient postnatal mouse forebrain also demonstrates defective repression of a number of imprinted genes, including *H19* and *Igf2* (Kernohan et al., 2010). Expression analysis on ATR-X patient peripheral blood mononuclear cells has shown a significant decrease in the expression of GTPase regulator associated with the focal adhesion kinase (*GRAF1*) and also confirmed a decrease in α-globin expression in some cases (Barresi et al., 2010). Therefore, ATRX is predicted to play a role in controlling the regulation of numerous genes.

A role of ATRX in transcriptional control is also suggested by the putative association with the human homolog of the polycomb protein Drosophila enhancer of zeste 2 (EZH2) (Cardoso et al., 1998). Using a yeast-two hybrid screen between various heterochromatin-associated proteins and ATRX, the C-terminal SET domain of EZH2 was found to interact with ATRX (Cardoso et al., 1998). This interaction may play a role in forming repressive complexes, and regulating homeotic gene expression in a developmental or tissue-specific context [Reviewed in (Mahmoudi et al., 2001)]. Yeast-two hybrid screens have also demonstrated a possible interaction between SWI/SNF domains and murine heterochromatin protein 1α (HP1α), which has been verified by coimmunofluorescence between ATRX and HP1α (Le Douarin et al., 1996; McDowell et al., 1999). This interaction was confirmed by co-immunoprecipitation in human cells, and has been assigned to a putative PxVxL motif (LYVKL) which interacts with the HP1α chromoshadow domain (Berube et al., 2000; Lechner et al., 2005). ATRX has also been predicted to associate with histone deacetylase 4 (HDAC4) through a yeast two-hybrid screen using an N-terminal fragment of HDAC4 (Wang et al., 2005).

Wide-scale genome studies have been conducted to identify sequences for ATRX binding in ES cells (Law et al., 2010). ATRX predominantly localizes to GC-rich sequences, including CpG islands, repetitive sequences, pericentromeric heterochromatin and telomeres. Among these targets are several sites where ATRX binds to directly influence gene expression, including the *Rhox5* promoter in Sertoli cells, the *H19* imprinting control region in brain, and VNTR repeats near α-globin (Bagheri-Fam et al., 2011; Kernohan et al., 2010; Law et al., 2010). The localisation of ATRX to ancestral pseudoautosomal regions of the genome is also speculated to be due to the repetitive nature of sequences surrounding these regions (Levy et al., 2008). Like tandem repeats, pseudoautosomal genes are rich in repetitive sequences and suggest a common epigenetic environment for ATRX to control expression of genes (Levy et al., 2008). Finally, ATRX has been reported to associate with the inactive X-chromosome during embryonic stem cell differentiation and X-inactivation (Baumann et al., 2009).

1.1.3.3 Protein Interactions of ATRX

SWI/SNF chromatin remodeling complexes are large and contain multiple subunits, so ATRX function may be elucidated by identifying other members of the complex. Early studies identified an interaction between ATRX and DAXX (Death-associated protein 6), a death domain associated protein involved in transcription and cell cycle regulation (Ishov et al., 2004; Tang et al., 2004; Xue et al., 2003). ATRX and DAXX reside in promyelocytic leukaemia (PML) bodies, at heterochromatin, and at telomeres (Goldberg et al., 2010; Tang et al., 2004; Xue et al., 2003). Immunopurification of DAXX from HeLa cell nuclear extract causes most of the ATRX protein to purify out of the extract at near equimolar amounts (Xue et al., 2003). This interaction between ATRX and DAXX was confirmed by immunoprecipitation in human 293T cells extracts, and mapped to the NH2-terminal PAH domains of DAXX and the middle region of ATRX (Tang et al., 2004).

Recombinant DAXX has been shown to preferentially bind Histone 3.3 (H3.3) and deposit histone tetramers onto DNA (Drané et al., 2010). This assembly complex includes ATRX, suggesting that ATRX may assist in depositing histones, specifically H3.3 (Drané et al., 2010). ATRX is required for repression of telomeric RNA and maintenance of H3.3 localisation at the telomeres in mouse ES cells (Goldberg et al., 2010). ATRX co-localizes with telomeric H3.3, and this localization is lost when the K4 residue of H3.3 is mutated (Wong et al., 2010). When this co-localization is perturbed by RNAi depletion of ATRX, mouse ES cells display reduced HP1α incorporation and have telomere dysfunctions (Wong et al., 2010). Telomere dysfunction phenotypes can be caused by loss of specific post-translational histone modifications such as trimethylation at lysine 9 of H3 (H3K9me3) and trimethylation at lysine 20 of H4 (H4K20me3) and can lead to chromosomal instability and aberrantly increased telomere length (Garcia-Cao et al., 2004; Wong et al., 2009). Functionally, ATRX has been demonstrated to read methylation states of histone tail peptides. The ADD domain of ATRX can bind to H3 tails, with a specificity for H3K9me3 and unmethylated lysine 4 (H3K4me0) (Dhayalan et al., 2011).

An interaction between ATRX and the methyl-CPG binding protein MeCP2 has been identified in which MeCP2 associates directly with the helicase domain of ATRX and recruits it to heterochromatin (Nan et al., 2007). Mutations in the *MECP2* gene cause the progressive neurodevelopmental disorder Rett syndrome, which bears some similarities to ATR-X syndrome (Amir et al., 1999; Dotti et al., 2002). Rett syndrome is characterized by normal development until 6-18 months of age, then a gradual loss of speech and purposeful hand use (Amir et al., 1999). Patients develop microcephaly, seizures, autism, ataxia, intermittent hyperventilation, and stereotypic repetitive hand movement (Amir et al., 1999). *Mecp2* mutation or deficiency in mouse perturbs its interaction with ATRX, and leads to disrupted localization of ATRX to pericentromeric heterochromatin (Baker et al., 2013; Nan et al., 2007). In a mouse model of Rett syndrome, loss of an AT-hook domain of MeCP2 abrogates recruitment of ATRX and was the difference between death or survival of the animals, suggesting this interaction might be important (Baker et al., 2013). Furthermore, it was also found that the ATRX-MeCP2 complex localizes to imprinted genes, where it also associates with the cohesin complex (Kernohan et al., 2010).

1.1.3.4 ATRX and the Cell Cycle

During interphase, ATRX is observed by immunofluorescence to have a speckled pattern within the nucleus and concentrate at sites of pericentromeric heterochromatin (McDowell et al., 1999). The localisation of ATRX relative to the chromatin is altered throughout the cell cycle in human cancer cell lines (Berube et al., 2000). Using indirect immunofluorescence and cell fractionation, ATRX was shown to localise with nuclear matrix during interphase, but associate with condensed chromatin at the beginning of M-phase (Berube et al., 2000). During mitosis, ATRX becomes phosphorylated and associates with HP1α, binding centromeric DNA at metaphase and anaphase, and having a less distinct association pattern during telophase (Berube et al., 2000). These data suggest that ATRX moves throughout the cell in a cell-cycle dependant fashion. Loss of ATRX in myoblasts leads to changes in expression of cell cycle genes and aberrant cell cycle gene regulation (Huh et al., 2012). Similarly, cell cycle progression is aberrant in ATRX-deficient Sertoli cells, which demonstrate a 38% increase in phospho-histone H3

(PH3), a marker of late G2 phase and mitosis compared to controls (Bagheri-Fam et al., 2011). A requirement for ATRX throughout the cell cycle has also been demonstrated in HeLa cells, where depletion of ATRX by siRNA leads to chromosome cohesion and congression defects during mitosis (Ritchie et al., 2008). Chromosome segregation and meiotic spindle formation defects are also seen in mouse oocytes depleted of ATRX (De La Fuente et al., 2004).

1.1.3.5 ATRX and Genomic Stability

Depletion studies of ATRX have demonstrated that ATRX loss leads to increased replicative stress at telomeres (Huh et al., 2012; Watson et al., 2013; Wong et al., 2010). Recently, important roles for ATRX in genomic integrity have been demonstrated in oocytes, muscle, tumour cell lines, and brain (Baumann et al., 2010; Huh et al., 2012; Lovejoy et al., 2012; Watson et al., 2013). When ATRX is depleted in cells, chromatid defects are observed including lobulated nuclei, formation of micronuclei, and chromatin bridges (Baumann et al., 2010; De La Fuente et al., 2004; Ritchie et al., 2008). These studies present an important role for ATRX in mitotic cell division and maintenance of genomic integrity.

The association of ATRX to the telomeres of mouse ES cells during interphase has been linked to incorporation of histone H3.3, which is required for telomere function (Wong et al., 2010). Telomere dysfunction has been observed in cases of paediatric glioblastoma, where mutations in *ATRX* or *DAXX* were associated with alternative lengthening of telomeres (ALT) (Heaphy et al., 2011; Lovejoy et al., 2012; Schwartzentruber et al., 2012). ALT is a telomerase-independent process in which telomere recombination is used to counteract telomere attrition [Reviewed in (Cesare et al., 2010)]. ALT is associated with poorer outcomes in cancer, as normal telomere attrition is associated with telomere shortening and eventual senescence (Reddel, 2000). In panels of ALT cell lines, loss of *ATRX* was frequently found to be a predictor of ALT, as well as genome rearrangements, micronucleation, defects in cell cycle, and defects in double-strand break repair (Lovejoy et al., 2012). Since there is a requirement for ATRX to deposit HP1α and H3.3 at telomeres, loss of ATRX would alter protein loading at the telomeres and lead to telomere dysfunction (Lovejoy et al., 2012; Wong et al., 2010). This contribution of

ATRX to proper telomere function prevents ALT in cancer cells, demonstrating an important role for ATRX in maintaining normal telomere integrity (Lovejoy et al., 2012).

1.1.3.6 Mouse Models of ATR-X Syndrome

Gene knockout strategies have been used to understand functional roles of genes in the mouse and other organisms; however, conventional gene knockouts can lead to embryonic lethality and are not ideal for examining development of a specific tissue [Reviewed in (Zhang et al., 2012)]. The prokaryotic cre-lox site-specific recombination system has been used in eukaryotes to drive both site and time-specific gene recombination (Sauer, 1987). Cre is a viral protein which can promote recombination of loxP sites, which is now used to generate various animal models (Sternberg et al., 1981; Zhang et al., 2012). This system allows the specific manipulation of DNA based on the location and arrangement of two loxP sites (Hoess et al., 1982). When two loxP sites are arranged in the same direction on a strand of DNA, the activity of cre in a tissue of interest will excise the targeted sequences between these sites [Reviewed in (Zhang et al., 2012)].

Studies removing *Atrx* in mouse ES cells indicate that *Atrx* may be required for normal ES cell growth and expansion (Garrick et al., 2006). To bypass these effects and identify roles for *Atrx* in later-stage tissues, conditional alteration of Atrx using the Cre-loxP system has revealed specific roles for *Atrx* in the development of various tissues. Conditional deletion of *Atrx* at the morula stage using a ubiquitous GATA1-cre transgene has demonstrated that Atrx is required during early embryogenesis, as $Atrx^{Null}$ embryos demonstrate a dramatically reduced trophectoderm layer and growth restriction (Garrick et al., 2006). Conditional inactivation in the forebrain using Foxg1-cre leads to reduced brain size and excessive apoptosis (Bérubé et al., 2005). Loss of cells was also observed in the retinas of mice depleted of Atrx using a Pax6-cre system (Medina et al., 2009). Atrx has been conditionally inactivated in the testes of mice with an AMH-cre, leading to smaller testes with defects in cell cycle and spermatogenesis (Bagheri-Fam et al., 2011). These methods compliment micro-injection and siRNA methods, and provide a growing body of tissue-specific knockout animals that illustrate specific and direct properties of *Atrx* in mouse development.

Numerous knockout studies in mice have demonstrated a DNA-damage or cell-death phenotype in tissues of these animals (Bagheri-Fam et al., 2011; Bérubé et al., 2005; Huh et al., 2012; Watson et al., 2013). In vitro studies have established that some ATRX-deficient cells are more sensitive to DNA damaging agents, and more susceptible to cell death during proliferation (Conte et al., 2012; Watson et al., 2013). Cell death can be prevented by deleting the tumor suppressor protein p53 concomitantly with ATRX, although this does not completely rescue the phenotype of ATRX loss in the brain (Seah et al., 2008). Loss of ATRX in macrophages sensitizes them to DNA-damaging agents though the p53 pathway (Conte et al., 2012). Furthermore, when cell death of neuroprogenitors is prevented by p53 deletion, DNA damage continues to accumulate in these cells (Watson et al., 2013). Thus, loss of ATRX leads to DNA-damage, p53-mediated cell death, and eventual hypocellularity in some tissues including forebrain, muscle, and testes (Bérubé, Mangelsdorf et al. 2005; Huh, Price O'Dea et al. 2012; Bagheri-Fam, Argentaro et al. 2011).

Skeletal abnormalities in ATR-X patients range from relatively mild to severe. One-third of ATR-X patients have spinal deformities such as kyphosis or scoliosis (Gibbons et al., 1995a). The majority also have retarded physical and osseous development (delayed bone age), demonstrating fundamental problems in growth and bone maturity (Gibbons et al., 1995a). For some patients these skeletal abnormalities are apparent at birth, for others they manifest later in life around the time of the pubertal growth spurt (Gibbons et al., 2000a). Finally, hand and foot deformities are common, including fingers with fixed flexion deformities, clinodactyly, brachydactyly, tapering fingers, overlapping digits, syndactlyly of toes, and bifid thumb (Gibbons et al., 1995a).

1.2 Development of the Appendicular Skeleton

The skeletal defects seen in ATR-X syndrome suggest an important role for ATRX in proper development of the skeleton. Studies of ATRX protein have revealed functions in DNA and chromatin binding, gene expression control, and maintenance of genomic integrity. ATRX participates in complexes with other important gene-regulating proteins. Furthermore, studies continue to reveal pathologies in ATRX-depleted tissues and organs, which may extend to the developing skeleton.

Mouse limb development begins with the formation of the limb bud, an outgrowth of undifferentiated mesenchyme cells from the body wall of the developing embryo. This outgrowth is controlled by fibroblast growth factor (FGF) signaling from the apical ectodermal ridge (AER) which directs growth in a proximodistal axis (shoulder to digits) (Niswander et al., 1993). Patterning is also directed by the zone of polarizing activity (ZPA) which directs positional information across the limb bud mesenchyme (Wolpert, 1969). This ZPA secretes molecules required to specify the anterio-posterior axis and perturbations to this zone lead to duplication or loss of structures in the limb (Tickle, 1981). One of the most important molecules produced specifically by the ZPA is sonic hedgehog (SHH), which is required for polarizing limb tissue and activation of *Hox* genes (Riddle et al., 1993).

Once these axes of limb outgrowth and patterning are established, the limb undergoes a SHH-directed proliferative expansion (Zhu et al., 2008). This expansion provides the necessary population of cells to contribute to all subsequent limb structures. Inhibition of early proliferation leads to an overall reduction in the size of skeletal elements in the limb (Ota et al., 2007). When SHH is perturbed, there can be defects in both the type and number of digits formed out of the limb bud (Tickle, 2006). Positional digit identity is determined by SHH and its transcriptional effectors GLI1, GLI2, and GLI3, and studies altering these effects have demonstrated loss of digits (Zeller, 2004). Digits are first formed as precartilaginous condensations within the limb bud, where mesenchymal cells are recruited to the site of digit formation and begin producing cartilage-specific collagens and proteoglycans (Hall et al., 1995). Following establishment of the unsegmented cartilage anlagen, future joints are established at sites where chondrocytes flatten and become non-chondrogenic (Craig et al., 1987). This joint interzone contributes to the formation of structures of the synovial joint, including the joint capsule, synovial fluid, and articular cartilage (Francis-West et al., 1999). Distal FGF signaling from the AER drives elongation of the digit, determining both the final length of the phalanges and their number (Sanz-Ezquerro et al., 2003).

The long bones of the mammalian skeleton develop through a process known as endochondral ossification. In this process, templates of bones are laid down as

condensations of mesenchymal cells, which differentiate into chondrocytes and form precursors to the bones (Figure 1-3) [Reviewed in (Kronenberg, 2003)]. In the case of digit bones, as well as the long bones of the appendicular skeleton, the cartilage template grows longitudinally and become ossified into bone. The differentiated chondrocytes are found in three populations that are fundamental to promoting bone growth: resting, proliferating, and hypertrophic (Hunziker, 1994). The proliferating cells continually replenish a population of cells that undergo terminal differentiation and hypertrophy, growing in size and producing a collagen matrix, with proliferation and hypertrophy both contributing to longitudinal growth (Noonan et al., 1998). This highly-regulated process determines the growth rate and final size of the bone (Breur et al., 1991). The center of the bone becomes invaded by blood vessels, and the hypertrophic cartilage cells die through apoptosis to be replaced by osteoblasts carried in from the bone collar [Reviewed in (Ducy et al., 2000)].

Osteoblasts are mesenchymal cells, which are only distinguishable from other types of fibroblasts by virtue of their ability to synthesise and deposit extracellular matrix as part of bone and other mineralised tissues (Ducy et al., 2000). Expression and DNA binding activity of Runx2 increase as osteoblasts mature, and this maturation is associated with production of proteins required to produce a mineralised matrix (Banerjee et al., 2001; Ducy et al., 2000).This matrix is composed primarily of type I collagen with other non-collagenous proteins responsible for induction or regulation of mineralisation [Reviewed in (Robey et al., 1993)]. Bone formation and mineralization by the osteoblast and bone remodeling by the osteoclast continue throughout life, maintaining mineral balance and bone physiology (Ducy et al., 2000). Perturbations in the differentiation to any of the three cell types (chondrocyte, osteoblast, or osteoclast) lead to numerous skeletal pathologies and defects in skeletal development, including dwarfisms and brachydactyly (Stricker et al., 2011).

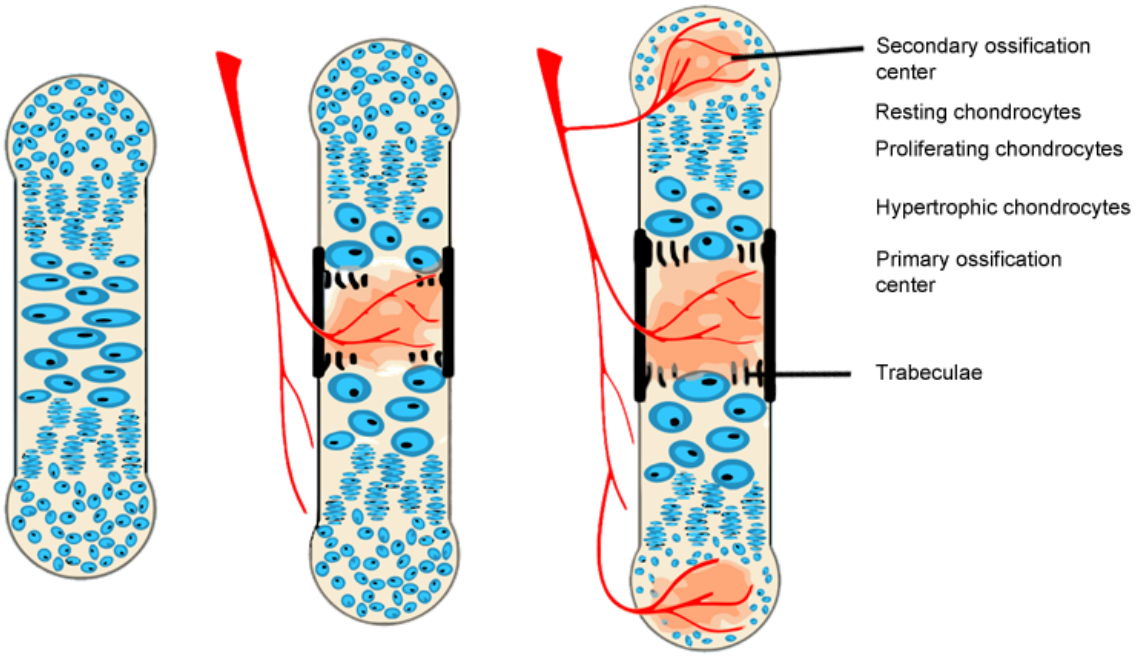


Figure 1-3 Endochondral ossification.

During endochondral ossification, chondrocytes form a model of the later bones and form zones of resting, proliferating, and hypertrophic chondrocytes. Hypertrophic cartilage is invaded by blood vessels and ultimately replaced by bone tissue and bone marrow in the primary ossification center. Postnatally, a similar process results in the formation of the secondary ossification center, and the growth plate forms between primary and secondary ossification center. In humans, the growth plates close and the primary and secondary ossification centers fuse at the end of puberty.

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1.3 Genetic Origins of Brachydactyly

Brachydactyly is a medical condition in which digit bones are shortened relative to other long bones in the body (Stricker et al., 2011). It can be an isolated heritable trait, but is also seen in association with other phenotypes in various malformation syndromes (Temtamy et al., 2008). The similarities between ATR-X syndrome and other brachydactyly syndromes suggest ATRX may control one or several pathways required for proper development of the skeleton and specifically the hands and feet.

1.3.1 Forms of Brachydactyly

Forms of brachydactyly are classified into ten main types: four types of brachydactyly type A (Subdivided into BDA1, BDA2, BDA3, BDA4), brachydactyly types B, C, D, E, Sugarman type brachydactyly and Kirner deformity (Temtamy et al., 2008). The type A brachydactylies are typified by shortening of the middle phalanges, with subtypes defining specific clinical descriptions (Temtamy et al., 2008).

Brachydactyly type A1 has been recapitulated in mice using the BDA1 mouse model, which is carries a point mutation affecting the efficacy of Indian hedgehog (IHH). IHH is a secreted ligand which functions in a graded fashion with its receptor patched [Reviewed in (Varjosalo et al., 2008)]. The BDA mutation alters the range of IHH influence, as well as its capacity to signal in the growing cartilage templates of the distal digits, specifically phalange 2 (Gao et al., 2009). *IHH* mutations in humans lead to both brachydactyly and short stature, as well as generalized skeletal deformities and occasional developmental delay (Kirkpatrick et al., 2003; McCready et al., 2002).

Similar to the type A1 brachydactyly seen in the BDA1 mouse and human cases of *IHH* mutations, Brachydactyly type B is characterized by absence or hypoplasia of all distal phalanges (Temtamy et al., 2008). This form of brachydactyly can be due to mutations in *ROR2*, such as in *ROR2*-related Robinow syndrome, which is associated with growth retardation, limb shortening and brachydactyly in humans, and also genital abnormalities and developmental delay in some cases (Afzal et al., 2000; Wadia et al., 1978). *ROR2* encodes a tyrosine kinase receptor and can affect chondrogenesis by indirectly inhibiting

GDF5 (TGF-beta superfamily member Growth/differentiation factor 5) signaling via canonical and non-canonical WNT signaling (Maeda et al., 2012; Sammar et al., 2004).

Defects in GDF5 signaling also lead to defects in the hand and foot. Mutations in *Gdf5* in mice alter bone length and patterning of the feet, with disorganization of the phalanges (Storm et al., 1994). Over-expression of wild-type or mutant GDF5 in developing chick limbs influences BMP signaling and causes joint fusions (Seemann et al., 2005). Humans heterozygous for *GDF5* mutations present with segmentation defects of the digits, underdevelopment of phalangeal bones, and in some cases, short stature (Everman et al., 2002).

Brachydactyly type C manifests as shortening of proximal hand bones, characteristically all the metacarpals with greatest shortening in the thumb (Burgess, 2001). This condition is rare, and associated with *GDF5* mutations much like Brachydactyly type B (Seo et al., 2013). Brachydactyly type D is a milder form of hand deformity, consisting of abnormally short thumbs with otherwise normal hands (Gray et al., 1984). This syndrome is associated with mutations in the human *HOXD13* gene, which is also associated with the more severe Brachydactyly type E (Johnson et al., 2003). Brachydactyly type E comprises of shortening of one or more metacarpals or metatarsals (Johnson et al., 2003). Genetic syndromes such as Rubinstein-Taybi, Saethre-Chotzen, and Turner syndrome can also present with brachydactyly type D or E (Johnson et al., 2003). Many of these syndromes involve genes are highly conserved with roles in development, and demonstrate that many defects in development can manifest with specific deformities of the hands and feet.

1.4 Genetic Origins of Dwarfism and Skeletal Deformities

Dwarfism and skeletal deformities are medical conditions that can originate from genetic disorders affecting the skeleton. Many of these syndromes originate from defects in regulation of growth plate chondrocytes by transcription factors. One of the most important factors is the sex-determining region Y-box 9 (SOX9), along with SOX family of transcription factors SOX5 and SOX6, which are required to drive induction of

cartilage from precursor cells (Ikeda et al., 2004). These proteins commit mesenchymal cells towards chondrocytes, and promote expression of critical matrix genes such as collagen types II, IX, and XI, and aggrecan (Han et al., 2008). Insufficiency *SOX9* leads to campomelic dysplasia, which presents as skeletal malformations including cleft palate and short stature (Kwok et al., 1995).

Other short stature syndromes, such as Léri-Weill dyschondrosteosis and Langer mesomelic dysplasia are caused by loss-of-function in the human SHOX (short stature homeobox) gene (Belin et al., 1998; Clement-Jones et al., 2000; Shears et al., 1998; Zinn et al., 2002). Many of these mutations affect the transcriptional activity of SHOX and subsequently the normal cell cycle within the endochondral growth plate (Sabherwal et al., 2004; Schneider et al., 2005).

The human *SHOX2* gene shares evolutionary conservation with mouse *Og12x* (*Shox2*), although rodents do not demonstrate any homologous genes to human *SHOX* (Clement-Jones et al., 2000). Human *SHOX2* and mouse *Shox2* possess similar functions in transcriptional gene repression in vitro (Liu et al., 2011; Rao et al., 1997). Both the mouse and human versions of *SHOX2* are located on autosomal chromosome 3, while human *SHOX* is located on the pseudoautosomal region of the sex chromosomes (Blaschke et al., 1998; Rao et al., 1997; Semina et al., 1998). Mouse models of short stature syndromes, generated by conditional deletion of *Shox2* in the limbs, demonstrate shortened proximal limb bones (Cobb et al., 2006; Yu et al., 2007).

Loss of SHOX2 in mice leads to defects in chondrocyte hypertrophy due to reduced levels of RUNX2 (Cobb et al., 2006). Similar growth plate defects and long-bone shortening due to failed progression of endochondral ossification are seen when *Runx2* is deleted in cartilage of developing mice (Chen et al., 2011). However, unlike *Shox2* mutants which demonstrate shortening of specific stylopod elements, *Runx2* and *Runx3* loss of function in mice affects all endochondral bones (Yoshida et al., 2004).

Establishment and longitudinal expansion of the growth plate also requires proper function of myocyte enhancer factor 2 (MEF2), which is involved in many facets of normal development (McKinsey et al., 2002; Potthoff et al., 2007). MEF2C and MEF2D

affect upstream regulation of *Runx2* and influence hypertrophic differentiation in mouse endochondral bones (Arnold et al., 2007). MEF2C levels are upregulated during chondrocyte differentiation and required for *collagen X* promoter activity (James et al., 2005; Stanton et al., 2004). MEF2C is also required for expression of the distal-less homeobox proteins, *Dlx5* and the related *Dlx6*, which are required for craniofacial and skeletal development (Verzi et al., 2007). Mutations in *DLX* genes are associated with split-hand/split-foot malformation and defects in axial limb outgrowth (Robledo et al., 2002). Similar to SHOX, DLX5 and DLX6 are required for proper growth and hypertrophy of the cartilage growth plate (Bendall et al., 2003; Chin et al., 2007; Ferrari et al., 2002; Hsu et al., 2006); therefore, the endochondral growth plate is under tight genetic regulation, and genetic perturbations can cause dwarfisms and skeletal deformities.

1.4.1 Chromatin Proteins Involved in Skeletal Development

Alterations in the function of chromatin-remodeling proteins can lead to severe skeletal malformations (Alvarez-Saavedra et al., 2010; Young et al., 2005). This correlation holds true for ATR-X syndrome, which is associated with loss of function of a SWI/SNF chromatin remodeler and frequently presents with skeletal deformities (Gibbons et al., 2000a). Although there are limited genotype-phenotype correlations between specific mutations in *ATRX* and skeletal defects, severe disease conditions appear to be related to mutations in the conserved functional domains of the protein (Gibbons et al., 2008). These phenotypes have a high degree of overlap with many other syndromes related to common defects of chromatin structure or modification. Dysregulated expression of *Mecp2* in skeletal tissue of mice produces severe malformations, including protrusion of the sternum and kyphosis (Alvarez-Saavedra et al., 2010). Animals over-expressing *Mecp2* in skeletal tissues have shortened limbs and incomplete ossification, possibly due to deregulation of *Runx2* (Alvarez-Saavedra et al., 2010).

Rubinstein-Taybi syndrome (RTS), also known as broad thumb-hallux syndrome, is a mental retardation syndrome with skeletal abnormalities including short stature and deformities of the hands and feet (Rubinstein Jh, 1963). RTS bears pathologic similarities to ATR-X syndrome, such as dysmorphic facial features and microcephaly, and can be

caused by mutations in *CREBBP* (Bartsch et al., 2010; Lopez-Atalaya et al., 2012). CREBBP is a cAMP response element-binding protein (CREB)-binding protein that can acetylate histones H2B and H2A (Lopez-Atalaya et al., 2012). Post-translational modifications to histones can affect their charge and function, leading to significant changes in both chromatin structure and gene transcription [Reviewed in (Struhl, 1998)]. Haploinsufficiency of *Crebbp* in mice leads to defects in osteoclastogenesis and reduced trabecular number, as well as patterning defects, delayed ossification, and brachydactyly (Tanaka et al., 1997).

Another rare syndrome, Brachydactyly Mental Retardation Syndrome (BDMR), is caused by deficiency of *HDAC4* (Williams et al., 2010). HDAC4 enzymatically deacetylates nucleosomal histones (Grozinger et al., 1999). This syndrome, like many other syndromes associated with chromatin-affecting proteins, presents with intellectual disabilities and developmental delays, as well as craniofacial and skeletal abnormalities including brachydactyly type E (Williams et al., 2010). In mice, *Hdac4* is expressed in prehypertrophic chondrocytes during development and regulates chondrocyte hypertrophy through inhibition of *Runx2* (Vega et al., 2004). Mice lacking *Hdac4* display dwarfism with premature ossification of developing bone and unrestrained transcriptional activation of *Mef2* and *Runx2* (Vega et al., 2004).

Compound mouse mutants, lacking both *Hdac4* and *Mef2c,* have a rescue of the ossification defects indicating an important interaction between these factors (Arnold et al., 2007). This direct function of HDACs regulating *MEF2* activity has also been demonstrated in myoblasts (Lu et al., 2000a; Lu et al., 2000b; Zhang et al., 2002). Additionally, HDAC4 has been shown to interact directly with ATRX by yeast two-hybrid assay (Wang et al., 2005), suggesting again that a common epigenetic theme may be involved in chromatin remodeling and skeletal development.

Similarly to Brachydactyly Mental Retardation Syndrome, Cornelia de Lange syndrome (CDLS) is a syndrome with craniofacial dysmorphia, hirsutism, missing or shortened fingers, gastroesophageal dysfunction, growth retardation, neurodevelopmental delay, and other physiological anomalies. This syndrome is caused by functional mutations in

genes encoding members of the cohesin complex, including NIPBL, SMC3, and SMC1A (Deardorff et al., 2007; Krantz et al., 2004; Tonkin et al., 2004). Mice hemizygous for *Nipbl* have decreased body size, delayed embryonic ossification of both endochondral and intramembranous bones, and reduced bone length (Kawauchi et al., 2009). Cohesin subunits involved in CDLS interact with both MeCP2 and ATRX in the mouse brain in-vivo (Kernohan et al., 2010). Together, ATRX, MeCP2, and cohesin can co-localize to imprinted regions such as *H19/Igf2* and *Gtl2/Dlk1*, lending further evidence to the theory that ATRX localizes to specific epigenetic environments (Kernohan et al., 2010). These studies demonstrate an important requirement for chromatin remodeling, and in specific SWI/SNF function, for proper initiation and gene expression in the development of the skeleton.

Dominant negative mutations in the SWI/SNF subunit BRG1 reduce expression of the important skeletal transcription factor *Runx2* (Young et al., 2005). *Brg1* is expressed in cells of the developing mouse skeleton and in bone-forming osteoblasts (Young et al., 2005). Dominant negative mutants of BRG1 SWI/SNF function block induction of Alkaline Phosphatase (APase), a marker of osteoblast differentiation (Young et al., 2005). This model demonstrates a direct requirement of chromatin remodeling complexes in RUNX2-dependent skeletal gene expression.

1.4.2 Indirect Regulation of Bone Development

Many of the defects described in the aforementioned models are due to direct influences of genes within the skeleton; however, the skeleton exists as part of a highly regulated system of growth and development within the context of the entire body and must also be regulated by more central and indirect methods. One important axis involved in determining bone size and bone mineral density is the insulin-like growth factor (IGF)-I axis [Reviewed in (Mohan et al., 2012)]. Levels of circulating IGF-1 are sensed by the epiphyseal cartilage and controlled by neuroendocrine pathways in the brain, which determines overall growth, body size, and lifespan (Hoshi et al., 2004; Kappeler et al., 2008). This feedback system involves the thyroid, brain and skeleton, which regulate endocrine homeostasis and maturation of the epiphyseal growth plate (Wang et al., 2010). IGF-I derived from either the liver or bone can regulate longitudinal bone growth and

contribute to proper growth plate development via growth hormone (GH) and thyroid hormones (Ohlsson et al., 2009). Mice with reduced IGF-1 levels in the liver and serum, including mice lacking ATRX in the developing forebrain, show defects in skeletal growth and development, with reductions in trabecular number and mineral density (Mohan et al., 2012; Watson et al., 2013). Similarly, development and remodeling of bone and cartilage can also be affected by levels of thyroid hormone (Wojcicka et al., 2012). Mutation of the thyroid receptor in mice causes delayed ossification during development and increased mineralization later in life (Bassett et al., 2007; O'Shea et al., 2005). Other models with defects in thyroid signaling leading to hypothyroidism have growth retardation and defects in endochondral ossification (Bassett et al., 2008; Gauthier et al., 2001). The thyroid also influences expression of calcitonin, which affects calcium homeostasis and bone mineral density during growth and aging (Huebner et al., 2006). Therefore, while endochondral bone formation is a highly regulated process in its own right, overall body size and maturation of bone is controlled centrally by both the brain and thyroid. Bone development is controlled both directly and indirectly, and any pathologies must be considered to have both direct and indirect causes.

1.5 Osteoarthritis

Osteoarthritis (OA) is a common form of arthritis, frequently affecting the hips, knees, and other freely moving or weight bearing joints (Felson et al., 2000; Radin et al., 1991). It is a complex degenerative joint disease with a growing number of identified biomechanical and systemic causes, and no known cure (Aigner et al., 2007; Aspden, 2008; Aspden, 2011; Felson et al., 2000; Goldring et al., 2009; Wang et al., 2011). Primary OA currently affects 12% of adults aged 25–74 in the United States, with similar incidences in the Canadian population (Lawrence et al., 2008; O'Donnell et al., 2011). This incidence is only expected to increase with the aging population (Kopec et al., 2008; Lawrence et al., 2008; Van Manen et al., 2012).

Onset and progression of osteoarthritis are dependent on the status of cartilage matrix loss in the joint, and associated with disease phenotypes of stiffness, pain, and changes to the underlying bone of the joint (Altman et al., 1986). Although osteoarthritis is frequently associated with increased age, it can also originate from mechanical factors

such as injury or obesity and systemic factors such as genetics or diet (Felson et al., 2000; Ryder et al., 2008). These disease origins can be described in two groups; as primary (idiopathic with no known origin) or secondary (post-traumatic) (Altman et al., 1986). There is emerging evidence of important roles for chromatin remodeling in disease and maintenance of articular cartilage, in addition to an already important role in development and normal growth. Recent studies have identified important genetic associations with osteoarthritis, including variants of *DOT1L*, an evolutionarily conserved histone methyltransferase and *SIRT1*, a histone deacetylase (Castaño Betancourt et al., 2012; Dvir-Ginzberg et al., 2013; Fujita et al., 2011; Gagarina et al., 2010).

Chondrocytes embedded within the articular cartilage of the joint produce an extracellular matrix (ECM) of collagens, proteoglycans, and other important macromolecules to maintain cartilage integrity (Buckwalter et al., 2005). When degeneration of the cartilage matrix exceeds the capacity of the chondrocytes to repair the cartilage matrix, there is anabolism/catabolism imbalance, cartilage degeneration and dysfunction associated with osteoarthritis. (Buckwalter et al., 2005; Goldring et al., 2009).

Regulation of chondrocyte gene expression can therefore control the balance between cartilage anabolism and catabolism, and ultimately cartilage homeostasis and degeneration. For example, *MMP13* (matrix metalloproteinase 13) is a collagenase up-regulated in cases of osteoarthritis (Mort et al., 2001). It plays a catabolic role in the progression of cartilage degeneration and cleaves collagens, gelatin, and aggrecan (Fosang et al., 1996; Wang et al., 2011). In arthritic joints, MMP13 is upregulated, resulting in the accelerated loss of the collagen matrix and ultimately the erosion of the articular surface (Glasson et al., 2010). *Mmp13* over expression in mice results in spontaneous degeneration of the articular cartilage by accelerating type II collagen and aggrecan degradation (Neuhold et al., 2001). Conversely, loss-of-function studies have shown that MMP13 loss slows the progression of osteoarthritis after meniscal injury to the knees (Little et al., 2009).

Aggrecan is another extracellular matrix protein responsible for the compressive resistance properties of cartilage. It is a proteoglycan with many charged, highly sulfated sugars able to hold and release large quantities of water. Degradation of aggrecan into fragments by specific MMPs and aggrecanase (ADAMTS) cleavage is common in normal aging but accelerated in diseases such as OA (Pollard et al., 2008). Examining the quantity of aggrecan fragments in synovial fluid and serum, and by immunohistochemistry, is an effective method of detecting cartilage breakdown and thus the progression of OA (Solursh et al., 1975). Similar to aggrecan, collagen 2 is an extracellular matrix protein responsible for the compressive resistance and tensile strength of cartilage. Collagen 2 is cleaved by a number of MMPs including MMP13.

1.6 Thesis Overview

This thesis presents the characterization of skeletal defects in mice with conditional deletion of ATRX in cartilage, forelimb, and osteoblasts. Examining the phenotypes associated with specific ATRX loss allows us to determine a molecular role for ATRX in skeletal development and suggest possible treatments for ATR-X syndrome.

1.6.1 Rationale and Hypothesis

Many developmental disorders have associated skeletal phenotypes. Given the importance of epigenetic regulation in the proper formation of the skeleton, it can be predicted that these disorders may be indicative of a common pathway or a centralized role of epigenetic regulation in the development of the skeleton. I hypothesized that the specific skeletal defects observed in ATR-X syndrome are due to a direct role of ATRX in the development of the skeleton. Additionally, loss of ATRX in skeletal tissues may confer a susceptibility to skeletal pathologies later in life, including osteoarthritis.

1.6.2 Study One - Loss of *Atrx* in the Developing Chondrocyte

Chondrocytes determine skeletal element size, so I hypothesized that dwarfism and other skeletal defects may be due to a role of ARTX in cartilage. I tested this hypothesis by examining the outcome of cartilage-specific deletion of *Atrx* in mice. Using the Cre-loxP system, I generated conditionally ATRX-deficient mice and characterized their skeletons

during development and early life. My findings demonstrate that loss of ATRX, specifically in chondrocytes, induces only minor skeletal defects, and does not affect growth plate morphology or overall bone growth (Solomon et al., 2009).

1.6.3 Study Two - *Atrx* in the Developing Limb

Given the high frequency of skeletal abnormalities reported in ATR-X syndrome patients, especially in hands and feet, I hypothesized ATRX has a direct role in the developing limb. Given the results of Study 1, which showed no function of ATRX in the developing cartilage, I deleted ATRX earlier in development during establishment of the limb. I found that conditional deletion of *Atrx* in limb bud mesenchyme causes a specific and significant shortening of the distal phalanges, which is similar to brachydactyly type A1 and brachydactyly type B in humans. I associated this phenotype with increased DNA damage and cell death in the developing limb bud (Solomon et al., 2013, in revision).

1.6.4 Study Three - Loss of *Atrx* Does Not Confer Susceptibility to Osteoarthritis

While there have been various developmental studies on ATRX, none have examined the role of ATRX in cartilage maintenance and aging. As ATRX is expressed in chondrocytes, it could be a potential epigenetic regulator of specific genes involved in healthy cartilage maintenance. Mice lacking ATRX in the forebrain and pituitary have premature aging phenotypes, linking ATRX to aging and potentially aging-associated diseases (Watson et al., 2013). This study examined the role of ATRX in three models of skeletal development and assessed the onset and progression of osteoarthritis using Osteoarthritis Research Society International (OARSI) histopathology guidelines and molecular markers of osteoarthritis. I found that mice lacking ATRX in cartilage, bone, or forelimb mesenchyme did not demonstrate increased susceptibility to osteoarthritis in adulthood. I also characterised the function of ATRX-deficient osteoblasts and found that loss of ATRX does not affect the ability of osteoblasts to mineralise in vitro or in vivo.

1.7 References

Aapola, U., Shibuya, K., Scott, H.S., Ollila, J., Vihinen, M., Heino, M., Shintani, A., Kawasaki, K., Minoshima, S., Krohn, K., *et al.* (2000). Isolation and Initial Characterization of a Novel Zinc Finger Gene, DNMT3L, on 21q22.3, Related to the Cytosine-5- Methyltransferase 3 Gene Family. Genomics *65*, 293-298.

Aasland, R., Gibson, T.J., and Stewart, A.F. (1995). The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends in Biochemical Sciences *20*, 56-59.

Abidi, F., Schwartz, C.E., Carpenter, N.J., Villard, L., Fontés, M., and Curtis, M. (1999). Carpenter-Waziri syndrome results from a mutation in XNP. The American Journal of Human Genetics *85*, 249-251.

Abidi, F.E., Cardoso, C., Lossi, A.M., Lowry, R.B., Depetris, D., Mattei, M.G., Lubs, H.A., Stevenson, R.E., Fontes, M., Chudley, A.E., *et al.* (2005). Mutation in the 5' alternatively spliced region of the XNP/ATR-X gene causes Chudley-Lowry syndrome. European Journal of Human Genetics *13*, 176-183.

Afzal, A.R., Rajab, A., Fenske, C.D., Oldridge, M., Elanko, N., Ternes-Pereira, E., Tüysüz, B., Murday, V.A., Patton, M.A., Wilkie, A.O.M., *et al.* (2000). Recessive Robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of ROR2. Nature Genetics *25*, 419-422.

Aigner, T., Haag, J., Martin, J., and Buckwalter, J. (2007). Osteoarthritis: aging of matrix and cells--going for a remedy. Current Drug Targets *8*, 325-331.

Allard, S., Masson, J.-Y., and Côté, J. (2004). Chromatin remodeling and the maintenance of genome integrity. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression *1677*, 158-164.

Altman, R., Asch, E., Bloch, D., Bole, G., Borenstein, D., Brandt, K., Christy, W., Cooke, T.D., Greenwald, R., Hochberg, M., *et al.* (1986). Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis & Rheumatism *29*, 1039-1049.

Alvarez-Saavedra, M., Carrasco, L., Sura-Trueba, S., Demarchi Aiello, V., Walz, K., Neto, J.X., and Young, J.I. (2010). Elevated expression of MeCP2 in cardiac and skeletal tissues is detrimental for normal development. Human Molecular Genetics *19*, 2177-2190.

Amir, R.E., Van Den Veyver, I.B., Wan, M., Tran, C.Q., Francke, U., and Zoghbi, H.Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nature Genetics *23*, 185-188.

Argentaro, A., Yang, J.C., Chapman, L., Kowalczyk, M.S., Gibbons, R.J., Higgs, D.R., Neuhaus, D., and Rhodes, D. (2007). Structural consequences of disease-causing mutations in the ATRX-DNMT3-DNMT3L (ADD) domain of the chromatin-associated protein ATRX. Proceedings of the National Academy of Sciences *104*, 11939-11944.

Arnold, M.A., Kim, Y., Czubryt, M.P., Phan, D., McAnally, J., Qi, X., Shelton, J.M., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2007). MEF2C Transcription Factor Controls Chondrocyte Hypertrophy and Bone Development. Developmental cell *12*, 377-389.

Aspden, R.M. (2008). Osteoarthritis: a problem of growth not decay? Rheumatology *47*, 1452-1460.

Aspden, R.M. (2011). Obesity punches above its weight in osteoarthritis. Nature Reviews Rheumatology *7*, 65-68.

Bagheri-Fam, S., Argentaro, A., Svingen, T., Combes, A.N., Sinclair, A.H., Koopman, P., and Harley, V.R. (2011). Defective survival of proliferating Sertoli cells and androgen receptor function in a mouse model of the ATR-X syndrome. Human Molecular Genetics *20*, 2213-2224.

Baker, Steven A., Chen, L., Wilkins, Angela D., Yu, P., Lichtarge, O., and Zoghbi, Huda Y. (2013). An AT-Hook Domain in MeCP2 Determines the Clinical Course of Rett Syndrome and Related Disorders. Cell *152*, 984-996.

Banerjee, C., Javed, A., Choi, J.Y., Green, J., Rosen, V., van Wijnen, A.J., Stein, J.L., Lian, J.B., and Stein, G.S. (2001). Differential regulation of the two principal Runx2/Cbfa1 n-terminal isoforms in response to bone morphogenetic protein-2 during development of the osteoblast phenotype. Endocrinology *142*, 4026-4039.

Barresi, V., Ragusa, A., Fichera, M., Musso, N., Castiglia, L., Rappazzo, G., Travali, S., Mattina, T., Romano, C., Cocchi, G.*, et al.* (2010). Decreased expression of GRAF1/OPHN-1-L in the X-linked alpha thalassemia mental retardation syndrome. BMC Med Genomics *3*, 28.

Bartsch, O., Kress, W., Kempf, O., Lechno, S., Haaf, T., and Zechner, U. (2010). Inheritance and variable expression in Rubinstein-Taybi syndrome. The American Journal of Human Genetics *152A*, 2254-2261.

Bassett, J.H.D., Nordström, K., Boyde, A., Howell, P.G.T., Kelly, S., Vennström, B., and Williams, G.R. (2007). Thyroid Status during Skeletal Development Determines Adult Bone Structure and Mineralization. Molecular Endocrinology *21*, 1893-1904.

Bassett, J.H.D., Williams, A.J., Murphy, E., Boyde, A., Howell, P.G.T., Swinhoe, R., Archanco, M., Flamant, F., Samarut, J., Costagliola, S.*, et al.* (2008). A lack of thyroid hormones rather than excess thyrotropin causes abnormal skeletal development in hypothyroidism. Molecular Endocrinology *22*, 501-512.

Baumann, C., and De La Fuente, R. (2009). ATRX marks the inactive X chromosome (Xi) in somatic cells and during imprinted X chromosome inactivation in trophoblast stem cells. Chromosoma *118*, 209-222.

Baumann, C., Viveiros, M.M., and De La Fuente, R. (2010). Loss of maternal ATRX results in centromere instability and aneuploidy in the mammalian oocyte and pre-implantation embryo. PLoS Genetics *6*.

Belin, V., Cusin, V., Viot, G., Girlich, D., Toutain, A., Moncla, A., Vekemans, M., Le Merrer, M., Munnich, A., and Cormier-Daire, V. (1998). SHOX mutations in dyschondrosteosis (Leri-Weill syndrome). Nature Genetics *19*, 67-69.

Bendall, A.J., Hu, G., Levi, G., and Abate-Shen, C. (2003). Dlx5 regulates chondrocyte differentiation at multiple stages. International Journal of Developmental Biology *47*, 335-344.

Bérubé, N.G., Mangelsdorf, M., Jagla, M., Vanderluit, J., Garrick, D., Gibbons, R.J., Higgs, D.R., Slack, R.S., and Picketts, D.J. (2005). The chromatin-remodeling protein ATRX is critical for neuronal survival during corticogenesis. Journal of Clinical Investigation *115*, 258-267.

Berube, N.G., Smeenk, C.A., and Picketts, D.J. (2000). Cell cycle-dependent phosphorylation of the ATRX protein correlates with changes in nuclear matrix and chromatin association. Human Molecular Genetics *9*, 539-547.

Blaschke, R.J., Monaghan, A.P., Schiller, S., Schechinger, B., Rao, E., Padilla-Nash, H., Ried, T., and Rappold, G.A. (1998). SHOT, a SHOX-related homeobox gene, is implicated in craniofacial, brain, heart, and limb development. Proceedings of the National Academy of Sciences *95*, 2406-2411.

Breur, G.J., Vanenkevort, B.A., Farnum, C.E., and Wilsman, N.J. (1991). Linear relationship between the volume of hypertrophic chondrocytes and the rate of longitudinal bone growth in growth plates. Journal of Orthopaedic Research *9*, 348-359.

Buckwalter, J.A., Mankin, H.J., and Grodzinsky, A.J. (2005). Articular cartilage and osteoarthritis. Instructional course lectures *54*, 465-480.

Burgess, R.C. (2001). Brachydactyly type C. Journal of Hand Surgery *26*, 31-39.

Cardoso, C., Lutz, Y., Mignon, C., Compe, E., Depetris, D., Mattei, M.G., Fontes, M., and Colleaux, L. (2000). ATR-X mutations cause impaired nuclear location and altered DNA binding properties of the XNP/ATR-X protein. Journal of Medical Genetics *37*, 746-751.

Cardoso, C., Timsit, S., Villard, L., Khrestchatisky, M., Fontès, M., and Colleaux, L. (1998). Specific Interaction between the XNP ATR-X Gene Product and the SET Domain of the Human EZH2 Protein. Human Molecular Genetics *7*, 679-684.

Carlson, M., and Laurent, B.C. (1994). The SNF/SWI family of global transcriptional activators. Current Opinion in Cell Biology *6*, 396-402.

Castaño Betancourt, M.C., Cailotto, F., Kerkhof, H.J., Cornelis, F.M.F., Doherty, S.A., Hart, D.J., Hofman, A., Luyten, F.P., Maciewicz, R.A., Mangino, M.*, et al.* (2012). Genome-wide association and functional studies identify the DOT1L gene to be involved in cartilage thickness and hip osteoarthritis. Proceedings of the National Academy of Sciences *109*, 8218-8223.

Cesare, A.J., and Reddel, R.R. (2010). Alternative lengthening of telomeres: models, mechanisms and implications. Nature Reviews Genetics *11*, 319-330.

Chai, B., Huang, J., Cairns, B.R., and Laurent, B.C. (2005). Distinct roles for the RSC and Swi/Snf ATP-dependent chromatin remodelers in DNA double-strand break repair. Genes & Development *19*, 1656-1661.

Chen, H., Ghori-Javed, F.Y., Rashid, H., Serra, R., Gutierrez, S.E., and Javed, A. (2011). Chondrocyte-specific regulatory activity of Runx2 is essential for survival and skeletal development. Cells Tissues Organs *194*, 161-165.

Chin, H.-J., Fisher, M.C., Li, Y., Ferrari, D., Wang, C.-K.L., Lichtler, A.C., Dealy, C.N., and Kosher, R.A. (2007). Studies on the role of Dlx5 in regulation of chondrocyte differentiation during endochondral ossification in the developing mouse limb. Development, Growth & Differentiation *49*, 515-521.

Clapier, C.R., and Cairns, B.R. (2009). The Biology of Chromatin Remodeling Complexes. Annual Review of Biochemistry *78*, 273-304.

Clement-Jones, M., Schiller, S., Rao, E., Blaschke, R.J., Zuniga, A., Zeller, R., Robson, S.C., Binder, G., Glass, I., Strachan, T.*, et al.* (2000). The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome. Human Molecular Genetics *9*, 695-702.

Cobb, J., Dierich, A., Huss-Garcia, Y., and Duboule, D. (2006). A mouse model for human short-stature syndromes identifies Shox2 as an upstream regulator of Runx2 during long-bone development. Proceedings of the National Academy of Sciences of the United States of America *103*, 4511-4515.

Conte, D., Huh, M., Goodall, E., Delorme, M., Parks, R.J., and Picketts, D.J. (2012). Loss of Atrx Sensitizes Cells to DNA Damaging Agents through p53-Mediated Death Pathways. PLoS ONE *7*, e52167.

Cote, J., Quinn, J., Workman, J.L., and Peterson, C.L. (1994). Stimulation of GAL4 derivative binding to nucleosomal DNA by the yeast SWI/SNF complex. Science *265*, 53-60.

Craig, F.M., Bentley, G., and Archer, C.W. (1987). The spatial and temporal pattern of collagens I and II and keratan sulphate in the developing chick metatarsophalangeal joint. Development *99*, 383-391.

De La Fuente, R., Viveiros, M.M., Wigglesworth, K., and Eppig, J.J. (2004). ATRX, a member of the SNF2 family of helicase/ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes. Developmental Biology *272*, 1-14.

Deardorff, M.A., Kaur, M., Yaeger, D., Rampuria, A., Korolev, S., Pie, J., Gil-Rodríguez, C., Arnedo, M., Loeys, B., Kline, A.D.*, et al.* (2007). Mutations in Cohesin Complex Members SMC3 and SMC1A Cause a Mild Variant of Cornelia de Lange Syndrome with Predominant Mental Retardation. The American Journal of Human Genetics *80*, 485-494.

Dhayalan, A., Tamas, R., Bock, I., Tattermusch, A., Dimitrova, E., Kudithipudi, S., Ragozin, S., and Jeltsch, A. (2011). The ATRX-ADD domain binds to H3 tail peptides and reads the combined methylation state of K4 and K9. Human Molecular Genetics *20*, 2195-2203.

Dotti, M.T., Orrico, A., De Stefano, N., Battisti, C., Sicurelli, F., Severi, S., Lam, C.W., Galli, L., Sorrentino, V., and Federico, A. (2002). A Rett syndrome MECP2 mutation that causes mental retardation in men. Neurology *58*, 226-230.

Drané, P., Ouararhni, K., Depaux, A., Shuaib, M., and Hamiche, A. (2010). The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. Genes & Development *24*, 1253-1265.

Ducy, P., Schinke, T., and Karsenty, G. (2000). The Osteoblast: A Sophisticated Fibroblast under Central Surveillance. Science *289*, 1501-1504.

Dvir-Ginzberg, M., and Steinmeyer, J. (2013). Towards elucidating the role of SirT1 in osteoarthritis. Frontiers in Bioscience *18*, 343-355.

Eisen, J.A., Sweder, K.S., and Hanawalt, P.C. (1995). Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. Nucleic Acids Research *23*, 2715-2723.

Everman, D.B., Bartels, C.F., Yang, Y., Yanamandra, N., Goodman, F.R., Mendoza-Londono, J.R., Savarirayan, R., White, S.M., Graham, J.M., Gale, R.P.*, et al.* (2002). The mutational spectrum of brachydactyly type C. The American Journal of Human Genetics *112*, 291-296.

Felson, D.T., Lawrence, R.C., Dieppe, P.A., Hirsch, R., Helmick, C.G., Jordan, J.M., Kington, R.S., Lane, N.E., Nevitt, M.C., Zhang, Y.*, et al.* (2000). Osteoarthritis: new insights. Part 1: the disease and its risk factors. Annals of Internal Medicine *133*, 635-646.

Ferrari, D., and Kosher, R.A. (2002). Dlx5 Is a Positive Regulator of Chondrocyte Differentiation during Endochondral Ossification. Developmental Biology *252*, 257-270.

Flanagan, J.F., and Peterson, C.L. (1999). A role for the yeast SWI/SNF complex in DNA replication. Nucleic Acids Research *27*, 2022-2028.

Flaus, A., Martin, D.M.A., Barton, G.J., and Owen-Hughes, T. (2006). Identification of multiple distinct Snf2 subfamilies with conserved structural motifs. Nucleic Acids Research *34*, 2887-2905.

Fosang, A.J., Last, K., Knäuper, V., Murphy, G., and Neame, P.J. (1996). Degradation of cartilage aggrecan by collagenase-3 (MMP-13). FEBS Letters *380*, 17-20.

Francis-West, P.H., Parish, J., Lee, K., and Archer, C.W. (1999). BMP/GDF-signalling interactions during synovial joint development. Cell and Tissue Research *296*, 111-119.

Fujita, N., Matsushita, T., Ishida, K., Kubo, S., Matsumoto, T., Takayama, K., Kurosaka, M., and Kuroda, R. (2011). Potential involvement of SIRT1 in the pathogenesis of osteoarthritis through the modulation of chondrocyte gene expressions. Journal of Orthopaedic Research *29*, 511-515.

Gagarina, V., Gabay, O., Dvir-Ginzberg, M., Lee, E.J., Brady, J.K., Quon, M.J., and Hall, D.J. (2010). SirT1 enhances survival of human osteoarthritic chondrocytes by repressing protein tyrosine phosphatase 1B and activating the insulin-like growth factor receptor pathway. Arthritis & Rheumatism *62*, 1383-1392.

Gao, B., Hu, J., Stricker, S., Cheung, M., Ma, G., Law, K.F., Witte, F., Briscoe, J., Mundlos, S., He, L.*, et al.* (2009). A mutation in Ihh that causes digit abnormalities alters its signalling capacity and range. Nature *458*, 1196-1200.

Garcia-Cao, M., O'Sullivan, R., Peters, A.H., Jenuwein, T., and Blasco, M.A. (2004). Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. Nature Genetics *36*, 94-99.

Garrick, D., Samara, V., McDowell, T.L., Smith, A.J.H., Dobbie, L., Higgs, D.R., and Gibbons, R.J. (2004). A conserved truncated isoform of the ATR-X syndrome protein lacking the SWI/SNF-homology domain. Gene *326*, 23-34.

Garrick, D., Sharpe, J.A., Arkell, R., Dobbie, L., Smith, A.J., Wood, W.G., Higgs, D.R., and Gibbons, R.J. (2006). Loss of Atrx affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues. PLoS Genetics *2*, e58.

Gauthier, K., Plateroti, M., Harvey, C.B., Williams, G.R., Weiss, R.E., Refetoff, S., Willott, J.F., Sundin, V., Roux, J.P., Malaval, L.*, et al.* (2001). Genetic analysis reveals different functions for the products of the thyroid hormone receptor α locus. Molecular and Cellular Biology *21*, 4748-4760.

Gibbons, R.J. (2006). Alpha thalassaemia-mental retardation, X linked. Orphanet Journal of Rare Diseases *1*.

Gibbons, R.J., Bachoo, S., Picketts, D.J., Aftimos, S., Asenbauer, B., Bergoffen, J., Berry, S.A., Dahl, N., Fryer, A., Keppler, K.*, et al.* (1997). Mutations in transcriptional regulator ATRX establish the functional significance of a PHD-like domain. Nature Genetics *17*, 146-148.

Gibbons, R.J., Brueton, L., Buckle, V.J., Burn, J., Clayton-Smith, J., Davison, B.C.C., Gardner, R.J.M., Homfray, T., Kearney, L., Kingston, H.M.*, et al.* (1995a). Clinical and hematologic aspects of the X-linked α-thalassemia/mental retardation syndrome (ATR-X). The American Journal of Human Genetics *55*, 288-299.

Gibbons, R.J., and Higgs, D.R. (2000a). Molecular-clinical spectrum of the ATR-X syndrome. The American Journal of Human Genetics *97*, 204-212.

Gibbons, R.J., McDowell, T.J., Raman, S., O'Rourke, D.M., Garrick, D., Ayyub, H., and Higgs, D.R. (2000b). Mutations in ATRX, encoding a SWI/SNF-like protein,cause diverse changes in the pattern of DNA methylation. Nature Genetics *24*, 361-371.

Gibbons, R.J., Picketts, D.J., Villard, L., and Higgs, D.R. (1995b). Mutations in a putative global transcriptional regulator cause X-linked mental retardation with alpha-thalassemia (ATR-X syndrome). Cell *80*, 837-845.

Gibbons, R.J., Suthers, G.K., Wilkie, A.O., Buckle, V.J., and Higgs, D.R. (1992). X-linked alpha-thalassemia/mental retardation (ATR-X) syndrome: Localization to Xq12-q21.31 by X inactivation and linkage analysis. The American Journal of Human Genetics *51*, 1136-1149.

Gibbons, R.J., Wada, T., Fisher, C.A., Malik, N., Mitson, M.J., Steensma, D.P., Fryer, A., Goudie, D.R., Krantz, I.D., and Traeger-Synodinos, J. (2008). Mutations in the chromatin-associated protein ATRX. Human Mutation *29*, 796-802.

Glasson, S.S., Chambers, M.G., Van Den Berg, W.B., and Little, C.B. (2010). The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis and Cartilage *18, Supplement 3*, S17-S23.

Goldberg, A.D., Banaszynski, L.A., Noh, K.-M., Lewis, P.W., Elsaesser, S.J., Stadler, S., Dewell, S., Law, M., Guo, X., Li, X.*, et al.* (2010). Distinct Factors Control Histone Variant H3.3 Localization at Specific Genomic Regions. Cell *140*, 678-691.

Goldring, M., and Marcu, K. (2009). Cartilage homeostasis in health and rheumatic diseases. Arthritis Research & Therapy *11*, 224.

Gray, E., and Hurt, V.K. (1984). Inheritance of brachydactyly type D. Journal of Heredity *75*, 297-299.

Grozinger, C.M., Hassig, C.A., and Schreiber, S.L. (1999). Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proceedings of the National Academy of Sciences of the United States of America *96*, 4868-4873.

Hall, B.K., and Miyake, T. (1995). Divide, accumulate, differentiate: cell condensation in skeletal development revisited. International Journal of Developmental Biology *39*, 881-893.

Han, Y., and Lefebvre, V. (2008). L-Sox5 and Sox6 Drive Expression of the Aggrecan Gene in Cartilage by Securing Binding of Sox9 to a Far-Upstream Enhancer. Molecular and Cellular Biology *28*, 4999-5013.

Havas, K., Flaus, A., Phelan, M., Kingston, R., Wade, P.A., Lilley, D.M., and Owen-Hughes, T. (2000). Generation of superhelical torsion by ATP-dependent chromatin remodeling activities. Cell *103*, 1133-1142.

Heaphy, C.M., de Wilde, R.F., Jiao, Y., Klein, A.P., Edil, B.H., Shi, C., Bettegowda, C., Rodriguez, F.J., Eberhart, C.G., Hebbar, S.*, et al.* (2011). Altered Telomeres in Tumors with ATRX and DAXX Mutations. Science *333*, 425.

Higgs, D.R., and Weatherall, D.J. (2009). The Alpha Thalassaemias. Cellular and Molecular Life Sciences *66*, 1154-1162.

Hoess, R.H., Ziese, M., and Sternberg, N. (1982). P1 site-specific recombination: nucleotide sequence of the recombining sites. Proceedings of the National Academy of Sciences *79*, 3398-3402.

Hoshi, K., Ogata, N., Shimoaka, T., Terauchi, Y., Kadowaki, T., Kenmotsu, S.-I., Chung, U.-I., Ozawa, H., Nakamura, K., and Kawaguchi, H. (2004). Deficiency of Insulin Receptor Substrate-1 Impairs Skeletal Growth Through Early Closure of Epiphyseal Cartilage. Journal of Bone and Mineral Research *19*, 214-223.

Hsu, S.-h.C., Noamani, B., Abernethy, D.E., Zhu, H., Levi, G., and Bendall, A.J. (2006). Dlx5- and Dlx6-mediated chondrogenesis: Differential domain requirements for a conserved function. Mechanisms of Development *123*, 819-830.

Huebner, A.K., Schinke, T., Priemel, M., Schilling, S., Schilling, A.F., Emeson, R.B., Rueger, J.M., and Amling, M. (2006). Calcitonin Deficiency in Mice Progressively Results in High Bone Turnover. Journal of Bone and Mineral Research *21*, 1924-1934.

Huh, M.S., Price O'Dea, T., Ouazia, D., McKay, B.C., Parise, G., Parks, R.J., Rudnicki, M.A., and Picketts, D.J. (2012). Compromised genomic integrity impedes muscle growth after Atrx inactivation. Journal of Clinical Investigation *122*, 4412-4423.

Hunziker, E.B. (1994). Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. Microscopy Research and Technique *28*, 505-519.

Ikeda, T., Kamekura, S., Mabuchi, A., Kou, I., Seki, S., Takato, T., Nakamura, K., Kawaguchi, H., Ikegawa, S., and Chung, U.-i. (2004). The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. Arthritis & Rheumatism *50*, 3561-3573.

Ishov, A.M., Vladimirova, O.V., and Maul, G.G. (2004). Heterochromatin and ND10 are cell-cycle regulated and phosphorylation-dependent alternate nuclear sites of the transcription repressor Daxx and SWI/SNF protein ATRX. Journal of Cell Science *117*, 3807-3820.

James, C.G., Appleton, C.T.G., Ulici, V., Underhill, T.M., and Beier, F. (2005). Microarray Analyses of Gene Expression during Chondrocyte Differentiation Identifies Novel Regulators of Hypertrophy. Molecular Biology of the Cell *16*, 5316-5333.

Johnson, D., Kan, S.H., Oldridge, M., Trembath, R.C., Roche, P., Esnouf, R.M., Giele, H., and Wilkie, A.O. (2003). Missense mutations in the homeodomain of HOXD13 are associated with brachydactyly types D and E. The American Journal of Human Genetics *72*, 984-997.

Kappeler, L., Filho, C.D.M., Dupont, J., Leneuve, P., Cervera, P., Périn, L., Loudes, C., Blaise, A., Klein, R., Epelbaum, J.*, et al.* (2008). Brain IGF-1 Receptors Control Mammalian Growth and Lifespan through a Neuroendocrine Mechanism. PLoS Biology *6*, e254.

Kawauchi, S., Calof, A.L., Santos, R., Lopez-Burks, M.E., Young, C.M., Hoang, M.P., Chua, A., Lao, T., Lechner, M.S., Daniel, J.A.*, et al.* (2009). Multiple Organ System Defects and Transcriptional Dysregulation in the Nipbl $^{+/-}$ Mouse, a Model of Cornelia de Lange Syndrome. PLoS Genetics *5*, e1000650.

Kernohan, K.D., Jiang, Y., Tremblay, D.C., Bonvissuto, A.C., Eubanks, J.H., Mann, M.R.W., and Bérubé, N.G. (2010). ATRX Partners with Cohesin and MeCP2 and Contributes to Developmental Silencing of Imprinted Genes in the Brain. Developmental cell *18*, 191-202.

Kirkpatrick, T.J., Au, K.S., Mastrobattista, J.M., McCready, M.E., Bulman, D.E., and Northrup, H. (2003). Identification of a mutation in the Indian Hedgehog (IHH) gene causing brachydactyly type A1 and evidence for a third locus. Journal of Medical Genetics *40*, 42-44.

Kopec, J.A., Rahman, M.M., Sayre, E.C., Cibere, J., Flanagan, W.M., Aghajanian, J., Anis, A.H., Jordan, J.M., and Badley, E.M. (2008). Trends in physician-diagnosed osteoarthritis incidence in an administrative database in British Columbia, Canada, 1996–1997 through 2003–2004. Arthritis Care & Research *59*, 929-934.

Krantz, I.D., McCallum, J., DeScipio, C., Kaur, M., Gillis, L.A., Yaeger, D., Jukofsky, L., Wasserman, N., Bottani, A., Morris, C.A.*, et al.* (2004). Cornelia de Lange syndrome is caused by mutations in NIPBL, the human homolog of Drosophila melanogaster Nipped-B. Nature Genetics *36*, 631-635.

Kronenberg, H.M. (2003). Developmental regulation of the growth plate. Nature *423*, 332-336.

Kwok, C., Weller, P.A., Guioli, S., Foster, J.W., Mansour, S., Zuffardi, O., Punnett, H.H., Dominguez-Steglich, M.A., Brook, J.D., Young, I.D.*, et al.* (1995). Mutations in SOX9, the gene responsible for Campomelic dysplasia and autosomal sex reversal. The American Journal of Human Genetics *57*, 1028-1036.

Law, M.J., Lower, K.M., Voon, H.P.J., Hughes, J.R., Garrick, D., Viprakasit, V., Mitson, M., De Gobbi, M., Marra, M., Morris, A.*, et al.* (2010). ATR-X Syndrome Protein Targets Tandem Repeats and Influences Allele-Specific Expression in a Size-Dependent Manner. Cell *143*, 367-378.

Lawrence, R.C., Felson, D.T., Helmick, C.G., Arnold, L.M., Choi, H., Deyo, R.A., Gabriel, S., Hirsch, R., Hochberg, M.C., Hunder, G.G.*, et al.* (2008). Estimates of the prevalence of arthritis and other rheumatic conditions in the United States: Part II. Arthritis & Rheumatism *58*, 26-35.

Le Douarin, B., Nielsen, A.L., Garnier, J.M., Ichinose, H., Jeanmougin, F., Losson, R., and Chambon, P. (1996). A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. The EMBO Journal *15*, 6701-6715.

Lechner, M.S., Schultz, D.C., Negorev, D., Maul, G.G., and Rauscher Iii, F.J. (2005). The mammalian heterochromatin protein 1 binds diverse nuclear proteins through a common motif that targets the chromoshadow domain. Biochemical and Biophysical Research Communications *331*, 929-937.

Levy, M.A., Fernandes, A.D., Tremblay, D.C., Seah, C., and Berube, N.G. (2008). The SWI/SNF protein ATRX co-regulates pseudoautosomal genes that have translocated to autosomes in the mouse genome. BMC Genomics *9*, 468.

Little, C.B., Barai, A., Burkhardt, D., Smith, S.M., Fosang, A.J., Werb, Z., Shah, M., and Thompson, E.W. (2009). Matrix metalloproteinase 13–deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development. Arthritis & Rheumatism *60*, 3723-3733.

Liu, H., Chen, C.-H., Espinoza-Lewis, R.A., Jiao, Z., Sheu, I., Hu, X., Lin, M., Zhang, Y., and Chen, Y. (2011). Functional Redundancy between Human SHOX and Mouse Shox2 Genes in the Regulation of Sinoatrial Node Formation and Pacemaking Function. Journal of Biological Chemistry *286*, 17029-17038.

Lopez-Atalaya, J.P., Gervasini, C., Mottadelli, F., Spena, S., Piccione, M., Scarano, G., Selicorni, A., Barco, A., and Larizza, L. (2012). Histone acetylation deficits in lymphoblastoid cell lines from patients with Rubinstein-Taybi syndrome. Journal of Medical Genetics *49*, 66-74.

Lossi, A.M., Millan, J.M., Villard, L., Orellana, C., Cardoso, C., Prieto, F., Fontes, M., and Martinez, F. (1999). Mutation of the XNP/ATR-X gene in a family with severe mental retardation, spastic paraplegia and skewed pattern of X inactivation: demonstration that the mutation is involved in the inactivation bias. The American Journal of Human Genetics *65*, 558-562.

Lovejoy, C.A., Li, W., Reisenweber, S., Thongthip, S., Bruno, J., de Lange, T., De, S., Petrini, J.H.J., Sung, P.A., Jasin, M.*, et al.* (2012). Loss of ATRX, Genome Instability, and an Altered DNA Damage Response Are Hallmarks of the Alternative Lengthening of Telomeres Pathway. PLoS Genetics *8*, e1002772.

Lu, J., McKinsey, T.A., Nicol, R.L., and Olson, E.N. (2000a). Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. Proceedings of the National Academy of Sciences *97*, 4070-4075.

Lu, J., McKinsey, T.A., Zhang, C.-L., and Olson, E.N. (2000b). Regulation of Skeletal Myogenesis by Association of the MEF2 Transcription Factor with Class II Histone Deacetylases. Molecular Cell *6*, 233-244.

Maeda, K., Kobayashi, Y., Udagawa, N., Uehara, S., Ishihara, A., Mizoguchi, T., Kikuchi, Y., Takada, I., Kato, S., Kani, S.*, et al.* (2012). Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. Nature Medicine *18*, 405-412.

Mahmoudi, T., and Verrijzer, C.P. (2001). Chromatin silencing and activation by Polycomb and trithorax group proteins. Oncogene *20*, 3055-3066.

McCready, M.E., Sweeney, E., Fryer, A.E., Donnai, D., Baig, A., Racacho, L., Warman, M.L., Hunter, A.G., and Bulman, D.E. (2002). A novel mutation in the IHH gene causes brachydactyly type A1: a 95-year-old mystery resolved. Human Genetics *111*, 368-375.

McDowell, T.L., Gibbons, R.J., Sutherland, H., O'Rourke, D.M., Bickmore, W.A., Pombo, A., Turley, H., Gatter, K., Picketts, D.J., Buckle, V.J.*, et al.* (1999). Localization of a putative transcriptional regulator (ATRX) at pericentromeric heterochromatin and the short arms of acrocentric chromosomes. Proceedings of the National Academy of Sciences *96*, 13983-13988.

McKinsey, T.A., Zhang, C.L., and Olson, E.N. (2002). MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends in Biochemical Sciences *27*, 40-47.

Medina, C.F., Mazerolle, C., Wang, Y., Bérubé, N.G., Coupland, S., Gibbons, R.J., Wallace, V.A., and Picketts, D.J. (2009). Altered visual function and interneuron survival in Atrx knockout mice: inference for the human syndrome. Human Molecular Genetics *18*, 966-977.

Mitson, M., Kelley, L.A., Sternberg, M.J., Higgs, D.R., and Gibbons, R.J. (2011a). Functional significance of mutations in the Snf2 domain of ATRX. Human Molecular Genetics.

Mitson, M., Kelley, L.A., Sternberg, M.J.E., Higgs, D.R., and Gibbons, R.J. (2011b). Functional significance of mutations in the Snf2 domain of ATRX. Human Molecular Genetics *20*, 2603-2610.

Mohan, S., and Kesavan, C. (2012). Role of Insulin-like Growth Factor-1 in the Regulation of Skeletal Growth. Current Osteoporosis Reports *10*, 178-186.

Mort, J.S., and Billington, C.J. (2001). Articular cartilage and changes in arthritis: matrix degradation. Arthritis Research & Therapy *3*, 337-341.

Nan, X., Hou, J., Maclean, A., Nasir, J., Lafuente, M.J., Shu, X., Kriaucionis, S., and Bird, A. (2007). Interaction between chromatin proteins MECP2 and ATRX is disrupted by mutations that cause inherited mental retardation. Proceedings of the National Academy of Sciences *104*, 2709-2714.

Neuhold, L.A., Killar, L., Zhao, W., Sung, M.-L.A., Warner, L., Kulik, J., Turner, J., Wu, W., Billinghurst, C., Meijers, T.*, et al.* (2001). Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. Journal of Clinical Investigation *107*, 35-44.

Niswander, L., Tickle, C., Vogel, A., Booth, I., and Martin, G.R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. Cell *75*, 579-587.

Noonan, K.J., Hunziker, E.B., Nessler, J., and Buckwalter, J.A. (1998). Changes in cell, matrix compartment, and fibrillar collagen volumes between growth-plate zones. Journal of Orthopaedic Research *16*, 500-508.

O'Donnell, S., Lagace, C., McRae, L., and Bancej, C. (2011). Life with arthritis in Canada: a personal and public health challenge. Chronic Diseases and Injuries in Canada *31*, 135-136.

O'Shea, P.J., Bassett, J.H.D., Sriskantharajah, S., Ying, H., Cheng, S.-y., and Williams, G.R. (2005). Contrasting Skeletal Phenotypes in Mice with an Identical Mutation Targeted to Thyroid Hormone Receptor α1 or β. Molecular Endocrinology *19*, 3045-3059.

Ohlsson, C., Mohan, S., Sjogren, K., Tivesten, A., Isgaard, J., Isaksson, O., Jansson, J.O., and Svensson, J. (2009). The role of liver-derived insulin-like growth factor-I. Endocrine Reviews *30*, 494-535.

Ota, S., Zhou, Z.-Q., Keene, D.R., Knoepfler, P., and Hurlin, P.J. (2007). Activities of N-Myc in the developing limb link control of skeletal size with digit separation. Development *134*, 1583-1592.

Peterson, C.L., and Workman, J.L. (2000). Promoter targeting and chromatin remodeling by the SWI/SNF complex. Current Opinion in Genetics & Development *10*, 187-192.

Picketts, D.J., Higgs, D.R., Bachoo, S., Blake, D.J., Quarrell, O.W., and Gibbons, R.J. (1996). ATRX encodes a novel member of the SNF2 family of proteins: mutations point to a common mechanism underlying the ATR-X syndrome. Human Molecular Genetics *5*, 1899-1907.

Picketts, D.J., Tastan, A.O., Higgs, D.R., and Gibbons, R.J. (1998). Comparison of the human and murine ATRX gene identifies highly conserved, functionally important domains. Mammalian Genome *9*, 400-403.

Pollard, T.C.B., Gwilym, S.E., and Carr, A.J. (2008). The assessment of early osteoarthritis. Journal of Bone & Joint Surgery, British Volume *90-B*, 411-421.

Potthoff, M.J., and Olson, E.N. (2007). MEF2: a central regulator of diverse developmental programs. Development *134*, 4131-4140.

Radin, E.L., Burr, D.B., Caterson, B., Fyhrie, D., Brown, T.D., and Boyd, R.D. (1991). Mechanical determinants of osteoarthrosis. Seminars in Arthritis and Rheumatism *21*, 12-21.

Rao, E., Weiss, B., Fukami, M., Rump, A., Niesler, B., Mertz, A., Muroya, K., Binder, G., Kirsch, S., Winkelmann, M.*, et al.* (1997). Pseudoautosomal deletions encompassing a novel homeobox gene cause growth failure in idiopathic short stature and Turner syndrome. Nature Genetics *16*, 54-63.

Reddel, R.R. (2000). The role of senescence and immortalization in carcinogenesis. Carcinogenesis *21*, 477-484.

Riddle, R.D., Johnson, R.L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. Cell *75*, 1401-1416.

Ritchie, K., Seah, C., Moulin, J., Isaac, C., Dick, F., and Bérubé, N.G. (2008). Loss of ATRX leads to chromosome cohesion and congression defects. The Journal of Cell Biology *180*, 315-324.

Robey, P.G., Fedarko, N.S., Hefferan, T.E., Bianco, P., Vetter, U.K., Grzesik, W., Friedenstein, A., Van der Pluijm, G., Mintz, K.P., Young, M.F.*, et al.* (1993). Structure and molecular regulation of bone matrix proteins. Journal of Bone and Mineral Research *8 Suppl 2*, S483-487.

Robledo, R.F., Rajan, L., Li, X., and Lufkin, T. (2002). The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. Genes & Development *16*, 1089-1101.

Rubinstein Jh, T.H. (1963). Broad thumbs and toes and facial abnormalities: A possible mental retardation syndrome. American Journal of Diseases of Children *105*, 588-608.

Ryder, J.J., Garrison, K., Song, F., Hooper, L., Skinner, J., Loke, Y., Loughlin, J., Higgins, J.P.T., and MacGregor, A.J. (2008). Genetic associations in peripheral joint osteoarthritis and spinal degenerative disease: a systematic review. Annals of the Rheumatic Diseases *67*, 584-591.

Sabherwal, N., Schneider, K.U., Blaschke, R.J., Marchini, A., and Rappold, G. (2004). Impairment of SHOX nuclear localization as a cause for Léri-Weill syndrome. Journal of Cell Science *117*, 3041-3048.

Saha, A., Wittmeyer, J., and Cairns, B.R. (2006). Chromatin remodelling: the industrial revolution of DNA around histones. Nature Reviews Molecular Cell Biology *7*, 437-447.

Sammar, M., Stricker, S., Schwabe, G.C., Sieber, C., Hartung, A., Hanke, M., Oishi, I., Pohl, J., Minami, Y., Sebald, W.*, et al.* (2004). Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2. Genes to Cells *9*, 1227-1238.

Sanz-Ezquerro, J.J., and Tickle, C. (2003). Fgf Signaling Controls the Number of Phalanges and Tip Formation in Developing Digits. Current Biology *13*, 1830-1836.

Sauer, B. (1987). Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. Molecular and Cellular Biology *7*, 2087-2096.

Schneider, K.U., Marchini, A., Sabherwal, N., Röth, R., Niesler, B., Marttila, T., Blaschke, R.J., Lawson, M., Dumic, M., and Rappold, G. (2005). Alteration of DNA binding, dimerization, and nuclear translocation of SHOX homeodomain mutations identified in idiopathic short stature and Leri-Weill dyschondrosteosis. Human Mutation *26*, 44-52.

Schwartzentruber, J., Korshunov, A., Liu, X.Y., Jones, D.T., Pfaff, E., Jacob, K., Sturm, D., Fontebasso, A.M., Quang, D.A., Tonjes, M.*, et al.* (2012). Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. Nature *482*, 226-231.

Seah, C., Levy, M.A., Jiang, Y., Mokhtarzada, S., Higgs, D.R., Gibbons, R.J., and Berube, N.G. (2008). Neuronal death resulting from targeted disruption of the Snf2 protein ATRX is mediated by p53. Journal of Neuroscience *28*, 12570-12580.

Seemann, P., Schwappacher, R., Kjaer, K.W., Krakow, D., Lehmann, K., Dawson, K., Stricker, S., Pohl, J., Ploger, F., Staub, E.*, et al.* (2005). Activating and deactivating mutations in the receptor interaction site of GDF5 cause symphalangism or brachydactyly type A2. Journal of Clinical Investigation *115*, 2373-2381.

Semina, E.V., Reiter, R.S., and Murray, J.C. (1998). A New Human Homeobox Gene OGI2X is a Member of the Most Conserved Homeobox Gene Family and is Expressed During Heart Development in Mouse. Human Molecular Genetics *7*, 415-422.

Sen, D., and Gilbert, W. (1988). Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis. Nature *334*, 364-366.

Seo, S.H., Park, M.J., Kim, S.H., Kim, O.H., Park, S., Cho, S.I., Park, S.S., and Seong, M.W. (2013). Identification of a GDF5 mutation in a Korean patient with brachydactyly type C without foot involvement. Annals of Laboratory Medicine *33*, 150-152.

Shah, P.P., Zheng, X., Epshtein, A., Carey, J.N., Bishop, D.K., and Klein, H.L. (2010). Swi2/Snf2-Related Translocases Prevent Accumulation of Toxic Rad51 Complexes during Mitotic Growth. Molecular Cell *39*, 862-872.

Shears, D.J., Vassal, H.J., Goodman, F.R., Palmer, R.W., Reardon, W., Superti-Furga, A., Scambler, P.J., and Winter, R.M. (1998). Mutation and deletion of the pseudoautosomal gene SHOX cause Leri-Weill dyschondrosteosis. Nature Genetics *19*, 70-73.

Solinger, J.A., Kiianitsa, K., and Heyer, W.-D. (2002). Rad54, a Swi2/Snf2-like Recombinational Repair Protein, Disassembles Rad51:dsDNA Filaments. Molecular Cell *10*, 1175-1188.

Solursh, M., and Reiter, R.S. (1975). Determination of limb bud chondrocytes during a transient block of the cell cycle. Cell Differentiation *4*, 131-137.

Stanton, L.A., Sabari, S., Sampaio, A.V., Underhill, T.M., and Beier, F. (2004). p38 MAP kinase signalling is required for hypertrophic chondrocyte differentiation. The Journal of Biochemistry *378*, 53–62.

Sternberg, N., and Hamilton, D. (1981). Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. Journal of Molecular Biology *150*, 467-486.

Stevenson, R.E., Abidi, F., Schwartz, C.E., Lubs, H.A., and Holmes, L.B. (2000). Holmes-Gang syndrome is allelic with XLMR-hypotonic face syndrome. The American Journal of Human Genetics *94*, 383-385.

Storm, E.E., Huynh, T.V., Copeland, N.G., Jenkins, N.A., Kingsley, D.M., and Lee, S.J. (1994). Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. Nature *368*, 639-643.

Stricker, S., and Mundlos, S. (2011). Mechanisms of digit formation: Human malformation syndromes tell the story. Developmental Dynamics *240*, 990-1004.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes & Development *12*, 599-606.

Tanaka, Y., Naruse, I., Maekawa, T., Masuya, H., Shiroishi, T., and Ishii, S. (1997). Abnormal skeletal patterning in embryos lacking a single Cbp allele: a partial similarity with Rubinstein-Taybi syndrome. Proceedings of the National Academy of Sciences *94*, 10215-10220.

Tang, J., Wu, S., Liu, H., Stratt, R., Barak, O.G., Shiekhattar, R., Picketts, D.J., and Yang, X. (2004). A Novel Transcription Regulatory Complex Containing Death Domain-

associated Protein and the ATR-X Syndrome Protein. Journal of Biological Chemistry *279*, 20369-20377.

Temtamy, S., and Aglan, M. (2008). Brachydactyly. Orphanet Journal of Rare Diseases *3*, 15.

Tickle, C. (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. Nature *289*, 295-298.

Tickle, C. (2006). Making digit patterns in the vertebrate limb. Nature Reviews Molecular Cell Biology *7*, 45-53.

Tonkin, E.T., Wang, T.J., Lisgo, S., Bamshad, M.J., and Strachan, T. (2004). NIPBL, encoding a homolog of fungal Scc2-type sister chromatid cohesion proteins and fly Nipped-B, is mutated in Cornelia de Lange syndrome. Nature Genetics *36*, 636-641.

Van Manen, M.D., Nace, J., and Mont, M.A. (2012). Management of primary knee osteoarthritis and indications for total knee arthroplasty for general practitioners. The Journal of the American Osteopathic Association *112*, 709-715.

Varjosalo, M., and Taipale, J. (2008). Hedgehog: functions and mechanisms. Genes & Development *22*, 2454-2472.

Vega, R.B., Matsuda, K., Oh, J., Barbosa, A.C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J.M., Richardson, J.A.*, et al.* (2004). Histone Deacetylase 4 Controls Chondrocyte Hypertrophy during Skeletogenesis. Cell *119*, 555-566.

Verzi, M.P., Agarwal, P., Brown, C., McCulley, D.J., Schwarz, J.J., and Black, B.L. (2007). The Transcription Factor MEF2C Is Required for Craniofacial Development. Developmental cell *12*, 645-652.

Villard, L., Fontes, M., Ades, L.C., and Gecz, J. (2000). Identification of a mutation in the XNP/ATR-X gene in a family reported as Smith-Fineman-Myers syndrome. The American Journal of Human Genetics *91*, 83-85.

Villard, L., Gecz, J., Mattei, J.F., Fontes, M., Saugier-Veber, P., Munnich, A., and Lyonnet, S. (1996). XNP mutation in a large family with Juberg-Marsidi syndrome. Nature Genetics *12*, 359-360.

Villard, L., Lossi, A.M., Cardoso, C., Proud, V., Chiaroni, P., Colleaux, L., Schwartz, C., and Fontes, M. (1997). Determination of the genomic structure of the XNP/ATRX gene encoding a potential zinc finger helicase. Genomics *43*, 149-155.

Wadia, R.S., Shirole, D.B., and Dikshit, M.S. (1978). Recessively inherited costovertebral segmentation defect with mesomelia and peculiar facies (Covesdem syndrome): A new genetic entity? Journal of Medical Genetics *15*, 123-127.

Wang, A.H., Grégoire, S., Zika, E., Xiao, L., Li, C.S., Li, H., Wright, K.L., Ting, J.P., and Yang, X.-J. (2005). Identification of the Ankyrin Repeat Proteins ANKRA and RFXANK as Novel Partners of Class IIa Histone Deacetylases. Journal of Biological Chemistry *280*, 29117-29127.

Wang, L., Shao, Y.Y., and Ballock, R.T. (2010). Thyroid hormone-mediated growth and differentiation of growth plate chondrocytes involves IGF-1 modulation of beta-catenin signaling. Journal of Bone and Mineral Research *25*, 1138-1146.

Wang, M., Shen, J., Jin, H., Im, H.-J., Sandy, J., and Chen, D. (2011). Recent progress in understanding molecular mechanisms of cartilage degeneration during osteoarthritis. Annals of the New York Academy of Sciences *1240*, 61-69.

Watson, L.A., Solomon, L.A., Li, J.R., Jiang, Y., Edwards, M., Shin-Ya, K., Beier, F., and Berube, N.G. (2013). Atrx deficiency induces telomere dysfunction, endocrine defects, and reduced life span. Journal of Clinical Investigation *123*, 2049–2063.

Williams, S.R., Aldred, M.A., Der Kaloustian, V.M., Halal, F., Gowans, G., McLeod, D.R., Zondag, S., Toriello, H.V., Magenis, R.E., and Elsea, S.H. (2010). Haploinsufficiency of HDAC4 Causes Brachydactyly Mental Retardation Syndrome, with Brachydactyly Type E, Developmental Delays, and Behavioral Problems. The American Journal of Human Genetics *87*, 219-228.

Wojcicka, A., Bassett, J.H.D., and Williams, G.R. (2012). Mechanisms of action of thyroid hormones in the skeleton. Biochimica et Biophysica Acta (BBA) - General Subjects.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. Journal of Theoretical Biology *25*, 1-47.

Wong, L.H., McGhie, J.D., Sim, M., Anderson, M.A., Ahn, S., Hannan, R.D., George, A.J., Morgan, K.A., Mann, J.R., and Choo, K.H.A. (2010). ATRX interacts with H3.3 in maintaining telomere structural integrity in pluripotent embryonic stem cells. Genome Research *20*, 351-360.

Wong, L.H., Ren, H., Williams, E., McGhie, J., Ahn, S., Sim, M., Tam, A., Earle, E., Anderson, M.A., Mann, J.*, et al.* (2009). Histone H3.3 incorporation provides a unique and functionally essential telomeric chromatin in embryonic stem cells. Genome Research *19*, 404-414.

Xie, S., Wang, Z., Okano, M., Nogami, M., Li, Y., He, W.-W., Okumura, K., and Li, E. (1999). Cloning, expression and chromosome locations of the human DNMT3 gene family. Gene *236*, 87-95.

Xue, Y., Gibbons, R., Yan, Z., Yang, D., McDowell, T.L., Sechi, S., Qin, J., Zhou, S., Higgs, D., and Wang, W. (2003). The ATRX syndrome protein forms a chromatin-remodeling complex with Daxx and localizes in promyelocytic leukemia nuclear bodies. Proceedings of the National Academy of Sciences *100*, 10635-10640.

Yoshida, C.A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K.-i., Yamana, K., Zanma, A., Takada, K., Ito, Y., *et al.* (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. Genes & Development *18*, 952-963.

Young, D.W., Pratap, J., Javed, A., Weiner, B., Yasuyuki Ohkawa, Wijnen, A.v., Montecino, M., Stein, G.S., Stein, J.L., Imbalzano, A.N., *et al.* (2005). SWI/SNF chromatin remodeling complex is obligatory for BMP2-induced, Runx2-dependent skeletal gene expression that controls osteoblast differentiation. Journal of Cellular Biochemistry *94*, 720-730.

Yu, L., Liu, H., Yan, M., Yang, J., Long, F., Muneoka, K., and Chen, Y. (2007). Shox2 is required for chondrocyte proliferation and maturation in proximal limb skeleton. Developmental Biology *306*, 549-559.

Zeller, R. (2004). It Takes Time to Make a Pinky: Unexpected Insights into How SHH Patterns Vertebrate Digits. Science Signaling *2004*, pe53-.

Zhang, C.L., McKinsey, T.A., Chang, S., Antos, C.L., Hill, J.A., and Olson, E.N. (2002). Class II Histone Deacetylases Act as Signal-Responsive Repressors of Cardiac Hypertrophy. Cell *110*, 479-488.

Zhang, J., Zhao, J., Jiang, W.J., Shan, X.W., Yang, X.M., and Gao, J.G. (2012). Conditional gene manipulation: Cre-ating a new biological era. Journal of Zhejiang University Science B *13*, 511-524.

Zhu, J., Nakamura, E., Nguyen, M.-T., Bao, X., Akiyama, H., and Mackem, S. (2008). Uncoupling Sonic Hedgehog Control of Pattern and Expansion of the Developing Limb Bud. Developmental cell *14*, 624-632.

Zinn, A.R., Wei, F., Zhang, L., Elder, F.F., Scott, C.I., Marttila, P., and Ross, J.L. (2002). Complete SHOX deficiency causes Langer mesomelic dysplasia. The American Journal of Human Genetics *110*, 158-163.

Chapter 2

2 Loss of *Atrx* in Chondrocytes has Minimal Effects on Skeletal Development

Mutations in the human ATRX gene are known to cause developmental defects, including skeletal deformities and dwarfism. ATRX encodes a chromatin remodeling protein, however the role of ATRX in skeletal development is currently unknown. We induced conditional *Atrx* deletion in mouse cartilage using the Cre-loxP system, with Cre expression driven by the collagen II (Col2a1) promoter. Growth rate, body size and weight, and long bone length did not differ in *Atrx* (Col2) cKO mice compared to control littermates. Histological analyses of the growth plate did not reveal any differences between control and mutant mice. Expression patterns of *Sox9*, a transcription factor required for cartilage morphogenesis, and p57, a marker of cell cycle arrest and hypertrophic chondrocyte differentiation, was unaffected. However, loss of ATRX in cartilage led to a delay in the ossification of the hips in some mice. We also observed hindlimb polydactily in one out of 61 mutants. In this chapter I demonstrate that ATRX is not directly required for development or growth of cartilage in the mouse, suggesting that the short stature in ATR-X patients is caused by defects in cartilage-extrinsic mechanisms.

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2.1 Introduction

ATR-X syndrome (Alpha-Thalassemia/Mental Retardation, X-linked) is a human disorder caused by mutations in the *ATRX* gene (Gibbons et al., 1995a; Gibbons et al., 1995b). Clinical manifestations include severe psychomotor and mental retardation, characteristic facial features, urogenital abnormalities, skeletal deformities and α-thalassemia (Gibbons et al., 1995a). Over 200 male ATR-X syndrome cases have been reported (Gibbons et al., 2008), and female carriers are unaffected due to the skewed pattern of X-inactivation (Gibbons et al., 2000). ATR-X syndrome patients display a

wide range of skeletal abnormalities, and 66% of patients show dwarfism (Gibbons et al., 2000). About half of ATR-X patients have spinal deformities such as kyphosis or scoliosis (Gibbons et al., 1995a). Delayed bone age is characteristic of most cases studied by thorough radiological investigation (Gibbons et al., 1995a). For some patients, these skeletal abnormalities are apparent at birth, for others they manifest later in life, during the pubertal growth spurt (Gibbons et al., 2000). Other skeletal deformities commonly seen in ATR-X patients include clinodactyly, brachydactyly, tapering of the fingers, overlapping digits, and 40% of patients have foot deformities (Gibbons et al., 1995a). A single case of bifid thumb has been reported (Gibbons et al., 2000). Despite a broad characterization of the variety of physical and mental phenotypes, very few genotype/phenotype correlations have been established, and none are associated with the severity of skeletal deformities (Badens et al., 2006). The molecular and genetic basis of these phenotypes are therefore unknown and it is unclear if these defects are due to a direct role of ATRX in the skeleton. Global knockout of *Atrx* using a ubiquitous *GATA*-Cre system leads to placental defects and embryonic lethality (Garrick et al., 2006). Conditional ablation of *Atrx* in the forebrain causes p53-dependent apoptotic cell death during embryogenesis, resulting in a smaller brain at birth (Bérubé et al., 2005; Seah et al., 2008a), while *Atrx* deficiency in the retina induces specific loss of interneurons (Medina et al., 2009).

The ATRX protein contains a domain displaying high homology to SNF2 (Sucrose Non-Fermenting 2) proteins, suggesting a role as a chromatin remodeling protein (Picketts et al., 1996). The SNF family of proteins is involved in transcriptional regulation, maintenance of chromosome stability during mitosis and processing of DNA damage (Eisen et al., 1995). ATR-X patient mutations are generally hypomorphic and do not cause chromosomal instability (Gibbons et al., 2000). However, depletion of ATRX in mammalian cells leads to defects in chromosome cohesion and mitotic progression (Ritchie et al., 2008). In addition to its role during mitosis, ATRX appears to be involved in the regulation of gene expression (Seah et al., 2008b).

The development of long bones occurs through endochondral ossification, a highly regulated multi-step process initiated when pluripotent mesenchymal cells aggregate to form the beginning of a cartilage model (DeLise et al., 2000). Under the control of several transcription factors, such as *Sox9*, *Sox5*, and *Sox6* (Yamaguchi et al., 2000) (Akiyama et al., 2002), mesenchymal cells differentiate into chondroblasts, which produce large quantities of type II collagen (ColII) (Hoffman et al., 2003). Chondroblasts mature to form chondrocytes, which undergo rapid proliferation along the longitudinal axis of the future bone, forming the cartilage growth plate (Kronenberg, 2003). The differentiated chondrocytes in the center of the cartilage model undergo hypertrophy, increasing in cell size and secreting type X collagen (ColX) (Kronenberg, 2003). The hypertrophic chondrocytes encased in calcified ECM secrete vascular endothelial growth factor (VEGF), which recruits blood vessels bringing osteoblasts and osteoclasts to form the primary ossification center; and eventually undergo apoptosis (Bobick et al., 2008). Chondrocytes on either end of this primary ossification center continue to proliferate, enter hypertrophy and undergo apoptosis, thus allowing the bone to grow longitudinally. Finally, woven bone is laid down in the area of apoptotic chondrocytes and is remodeled by osteoblasts and osteoclasts to form lamellar bone (Ducy et al., 2000).

To examine whether skeletal defects in ATR-X patients could be due to a requirement for ATRX in cartilage development, we examined the outcome of cartilage-specific inactivation of ATRX in mice, using the Cre-loxP system. Our findings demonstrate that loss of ATRX specifically in chondrocytes induces minor skeletal defects, but does not affect growth plate morphology and bone growth.

2.2 Materials and Methods

2.2.1 Histology and Immunohistochemistry

Histology and immunohistochemistry procedures were performed as described (Wang et al., 2007) with minor modifications. Sections were incubated in 3% H_2O_2 for 15 min at room temperature, followed by boiling for 2 min and incubation for 20 min at 97°C in 10 mM sodium citrate (pH 6.0). Paraffin sections were incubated with 5% goat serum for 30 min, and subsequently with primary antibodies (ATRX D-19) (Santa Cruz) at a dilution

of 1:50 overnight at 4°C. Secondary goat anti-Rabbit HRP conjugated antibody (Santa Cruz) at 1:200 dilution was used to recognize the primary antibodies. After washing, the horseradish peroxidase (HRP) conjugated polymer complex was visualized by incubation for 2 to 10 min with 3,3"-diaminobenzidine (DAB) substrate-chromogen (DAKO). Sections were counterstained with methyl green, washed, and mounted.

All images were taken at room temperature with a Retiga EX camera (Leeds Precision Instruments, Inc.) connected to a DMRA2 microscope (Leica). Image analysis was performed using Openlab 4.0.4 software *(*Improvision).

Growth plate morphology was analyzed by Safranin-O stain on P0.5 and P21 long bone sections. Sections were dewaxed, stained in haematoxylin followed by staining in fast green and safranin-O. Proportions of resting, proliferating and hypertrophic cells were determined using Openlab 4.0.4 software *(*Improvision) from at least three different mice.

2.2.2 Immunofluorescence of Cultured Primary Chondrocytes

Primary chondrocytes were prepared from long bones of E15.5 mouse embryos (James et al., 2007). Briefly, long bones (tibia, femur, humerus) were dissected, rinsed in PBS and incubated at 37°C for 20 min in trypsin-EDTA followed by digestion with 2 mg/ml collagenase P at 37°C for 2 h in Dulbecco's Modified Eagles Medium (DMEM) with 10% FBS. The cell suspension was filtered through a 70 μm cell strainer (Falcon), washed, counted and plated. For ATRX immunofluorescence, cells were grown on glass coverslips and fixed for 15 min in 4% paraformaldehyde, then blocked with 5% goat serum for 30 min at room temperature. Cells were then incubated with ATRX antibody at a 1:300 dilution (H-300, Santa Cruz) and mouse anti-alpha tubulin at a 1:10,000 dilution (Sigma) followed by FITC-conjugated anti-rabbit and Alexa 594-conjugated anti-mouse secondary antibodies at 1:200 (Invitrogen). Slides were mounted in media containing DAPI (Vectashield) and images were acquired on a Leica DMI 6000b automated inverted microscope.

2.2.3 Mouse Breeding and Genotyping

Ethics Statement: All procedures involving animals were approved by the University of Western Ontario Animal Care and Use Committee, Protocol 2007-045.

Mice were exposed to a 12-hour light–dark cycle and fed tap water and regular chow ad libitum. Mice conditionally deficient in ATRX were generated by crossing of $Atrx^{LoxP}$ females (129sv background) (Garrick et al., 2006) with heterozygous *Col2a1-Cre* knock-in male mice (Terpstra et al., 2003). The resultant conditionally Atrx-deficient offspring from this cross are referred to as *Atrx* (Col2) cKO mice in this text. For developmental studies, midday of the day of vaginal plug discovery was considered E0.5. At scheduled times pregnant females were sacrificed by CO_2.

PCR genotyping was performed from ear biopsy DNA for the presence of the *Cre* transgene as previously described (Terpstra et al., 2003). Genotyping of embryonic and newborn mice was performed using PCR of DNA isolated from skin biopsies. PCR amplification was performed to detect the *Atrx* floxed alleles as previously described (Seah et al., 2008a), the *Cre* transgene (Terpstra et al., 2003), as well as the sex determining region Y (*Sry*) gene to identify male mice. A 1.5 kb fragment of *neo* gene within the floxed allele of *Atrx* was identified with one set of primers (5'-GATCGGCCATTGAACAAGAT-3' and 5'-ATA GGT CGG CGG TTC AT-3') whereas the other set (5'-CCC GAG TAT CTG GAA GAC AG-3' and 5'-ATA GGT CGG CGG TTC AT-3') amplified a 600 bp fragment of wild type. Primers (5'-CCT GGA AAA TGC TTC TGT CC-3') and (5'-CAG GGT GTT ATA AAC AAT CCC-3') amplified a 300 bp fragment of the *cre* gene, whereas the other set (5'-GCA GGT GGA AAA GCC TTA CA-3') and (5'-AAG CTT TGC TGG TTT TG GA -3') amplified a 250 bp fragment of *Sry*. PCR conditions were as follows: 95°C for 3 min (95°C for 30 s, 55°C for 45s, and 72°C for 1 min) x 36, 72°C for 10 min for *Cre* and *Sry*, 95°C for 3 min (95°C for 30 s, 55°C for 1 min, and 72°C for 5 min) x 36, 72°C for 10 min for *Atrx*.

2.2.4 Skeletal Stains and Measurements

Live mice were weighed at P0, P7 and P21. Whole body length was measured using a ruler after sacrifice. For Alizarin Red/Alcian Blue staining, mouse carcasses were skinned and eviscerated, then fixed overnight in 95% ethanol followed by overnight fixation in acetone. Whole skeletons were placed in staining solution for 7-10 days

(0.05% Alizarin Red, 0.015% Alcian Blue, 5% acetic acid in 70% ethanol) (Wang et al., 2007). Skeletons were then cleared in 2% KOH. Images of stained bones were obtained with an Olympus SP-57OUZ. Limb bones and skulls from four different littermate pairs were measured using a dissecting microscope with a ruler.

2.2.5 RT-PCR

Five micrograms of total RNA obtained from *Atrx* (Col2) cKO and littermate control chondrocytes and control brains was reverse-transcribed using Omniscript RT kit (Qiagen) and used for PCR amplification with the following *Atrx*-specific primers: 17F (5' –AGA ACC GTT AGT GCA GGT TCA-3') for exon 17 and 20a (5'-ACC ACC ATC TTC TTG CCA TC -3') for exon 20. Conditions for amplification were as follows: 95°C for 3 min (95°C for 30 s, 56°C for 30s, and 72°C for 1 min) x 32, 72°C for 10 min.

2.2.6 Western Blot Analysis

Primary rib cartilage was isolated from newborn mice. Ribs were dissociated using collagenase P and strained through a 70 μm nylon filter to remove ossified tissue, followed by 3 days in culture over 1.5% agarose in PBS to specifically select for chondrocytes (Beier et al., 1999). All tissues were lysed with MITO buffer (20mM HEPES pH 7.5, 1 mM EDTA pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$ and 1 protease inhibitor cocktail tablet [Complete mini, EDTA-free; Roche]), followed by treatment and lysis of nuclei with nuclear buffers without and with salt, respectively (20mM HEPES pH 7.9, 450 mM NaCl (omitted for no salt) 1.5 mM $MgCl_2$, 0.2 mM EDTA pH 8.0 and 1 protease inhibitor cocktail tablet). Nuclear buffer without salt was used for swelling nuclei, nuclear buffer with salt used for 25 min nuclear lysis on ice. Extracts were quantified using the BCA protein assay (Sigma-Aldrich). Protein (5 μg) was resolved on a 6 % SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were probed with rabbit α-ATRX H300 (Santa Cruz Biotechnology, Inc.) followed by the appropriate horseradish peroxidase–conjugated secondary antibody (1:5,000; GE Healthcare). After washing, the membrane was incubated in ECL before exposure using a ChemiImager 5500 (Alpha Innotech). The

membrane was reprobed with mouse anti–beta-actin (1:10,000; Sigma-Aldrich) as a loading control.

2.3 Results

2.3.1 *Atrx* is Expressed in Chondrocytes

Expression of *Atrx* in chondrocytes was assessed using in vitro and in vivo models. Immunohistochemistry of embryonic, newborn (P0.5), and three week-old wild type mice demonstrated that *Atrx* is expressed throughout the cartilage growth plate. Nuclear staining of Atrx was apparent in all cartilage cells (Figure 2-1A). Primary chondrocytes in monolayer culture were stained for Atrx by immunofluorescence. In interphase, we observed a punctate staining pattern within the nucleus that corresponded to bodies stained intensely with DAPI (Figure 2-1B, left panel). Alpha-tubulin (red) was used to visualize the location of the cytoplasm and to identify microtubules during cell division. During metaphase, ATRX was present exclusively at the edges of the aligned chromosomes, similar to the localization at pericentromeric heterochromatin reported in other cell types (Figure 2-1B, right panel) (Bérubé et al., 2000; Ritchie et al., 2008). Western blots of primary cultured chondrocytes suggest that full-length *Atrx* was expressed in cartilage (Figure 2-2B).

2.3.2 Loss of Atrx in Mouse Chondrocytes does Not Affect Viability or Growth

We utilized the Cre-LoxP system to generate mice with cartilage-specific inactivation of the *Atrx* gene. Female mice previously engineered with loxP sites flanking exon 18 of *Atrx* (Bérubé et al., 2005) were mated with male mice expressing *Cre* recombinase under the control of the mouse collagen II (*Col2a1*) promoter (Terpstra et al., 2003). Reverse transcriptase PCR (RT-PCR) analysis of *Atrx* transcripts in cultured primary chondrocytes confirmed a strong reduction in wild type *Atrx* mRNA and the presence of low levels of a shorter transcript resulting from the recombination event in mutant cartilage (Figure 2-2A).

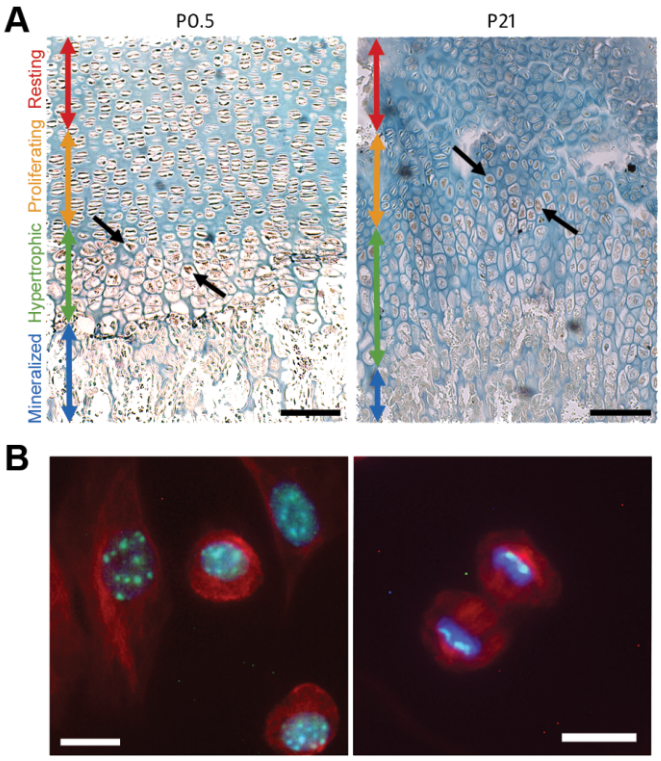


Figure 2-1 Expression and localization of ATRX in growth plate chondrocytes.

(A) Immunostaining of Atrx in humerus growth plates of P0.5 and P21 mice. Atrx is seen in the nuclei of resting, proliferating and early hypertrophic cells in the growth plates (arrows). Scale bar: 100 μm. Regions of the growth plate are identified as resting (red arrow), proliferating (yellow), hypertrophic (green) and mineralized (blue). (B) Immunofluorescence detection of Atrx in primary mouse chondrocytes isolated from E15.5 long bones. Merged image of Atrx (green), DAPI (blue) and alpha-tubulin (red) reveals a punctate Atrx staining pattern restricted to the nucleus during interphase (left panel) and specific localization to condensed chromatin during mitosis (right panel). Scale bar: 10 μm

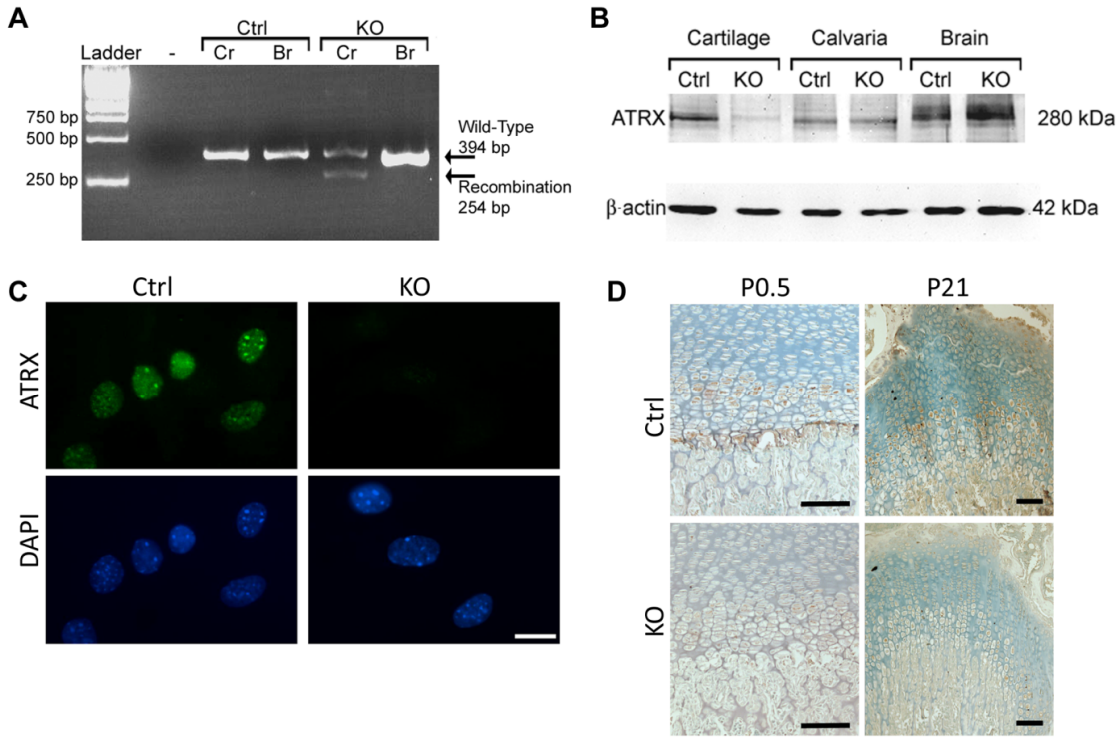


Figure 2-2 Levels of ATRX are decreased in the cartilage of *Atrx*(Col2) cKO mice.

(A) PCR analysis of cDNA from RNA isolated from newborn rib cartilage (Cr) or brain (Br) from *Atrx*(Col2) cKO males (KO) and control littermates (Ctrl). Amplification was performed with primers flanking the loxP sites, in exons 17 and 20. The expected amplicon of 394bp was obtained in control and an additional amplicon of 254 bp was obtained in cKO cartilage caused by the recombination event. The levels of the recombined product are low, as described previously (Bérubé et al., 2005; Garrick et al., 2006), suggesting that this product is unstable . (B) Immunoblotting for Atrx from proteins isolated from newborn control and KO rib chondrocytes, calvariae and brain tissue. Atrx expression is greatly reduced in KO chondrocytes, but unaffected in calvariae and brain of cKO animals. Beta-actin was used as a loading control. (C) Immunofluorescence detection of Atrx in control and *Atrx*(Col2) cKO primary chondrocytes isolated from newborn long bones shows the loss of Atrx protein in KO chondrocytes. Scale bar: 10 μm. (D) Immunohistochemistry on growth plates from P0.5 and P21 mice. Atrx staining is reduced in cKO growth plates. Scale bar: 100 μm.

These results confirm previous reports that the mRNA species generated by the recombination event is unstable (Bérubé et al., 2005; Garrick et al., 2006). Levels of Atrx protein in chondrocytes from ribs of newborn mice were assessed by immunoblotting. Atrx protein amounts were substantially decreased in *Atrx*(Col2) cKO chondrocytes (Figure 2-2B). In contrast, ATRX protein levels in brain and bone (calvariae) were unchanged in mutant mice, validating the tissue specific activity of the Col2a1 Cre (Figure 2-2B). Loss of ATRX protein was further confirmed by immunofluorescence of cultured chondrocytes isolated from *Atrx*(Col2) cKO and control littermate mice (Figure 2-2C) and by immunohistochemistry on sections of *Atrx*(Col2) cKO and control growth plates (Figure 2-2D). These data showed that our breeding scheme resulted in specific and efficient loss of ATRX protein in cartilage.

Atrx(Col2) cKO mice were obtained at expected Mendelian ratios, indicating that loss of ATRX in cartilage does not result in embryonic or neonatal lethality (59 control males, 61 cKO males out of 29 litters). In addition, *Atrx*(Col2) cKO mice showed no significant difference in growth when weighed from birth to weaning and again at one year of age (Figure 2-3A), or in body length at three weeks of age compared to wild-type littermates (Ctrl: 6.30 cm STD: 0.21, cKO: 6.17 cm STD: 0.38, $p>0.05$). Body length at 6 and 12 months was also unaffected (data not shown). *Atrx*(Col2) cKO males were capable of mating and producing offspring when backcrossed to floxed females to produce second-generation tissue-specific knockout mice.

Skeletal preparations from newborn and weanling (21 day old) *Atrx*(Col2) cKO mice showed no change in skeletal morphology, bone length or extent of ossification, as determined by the ratio of Alizarin red to Alcian blue staining in the long bones (Figure 2-3B,C). Length of tibia, femur, radius, ulna, and humerus were measured, as well as the length and width of the skulls. Average measurements obtained from four independent littermate pairs revealed no significant changes in the length of long bone elements between control and mutant mice (Figure 2-3D).

2.3.3 Growth Plate Morphology is Not Affected by the Loss of Atrx

Histochemical staining was conducted on paraffin sections of the growth plates of *Atrx*(Col2) cKO and control mice to examine proportions of resting, proliferative and hypertrophic zones. No difference in growth plate architecture or chondrocyte

A

	Average Weight (g)
P0.5 Ctrl	1.38 +/- 0.17
P0.5 KO	1.49 +/- 0.12
P10 Ctrl	3.72 +/- 0.86
P10 KO	3.81 +/- 0.77
P21 Ctrl	8.86 +/- 2.54
P21 KO	8.77 +/- 2.09
1 yr Ctrl	27.85 +/- 1.91
1 yr KO	28.45 +/- 2.47

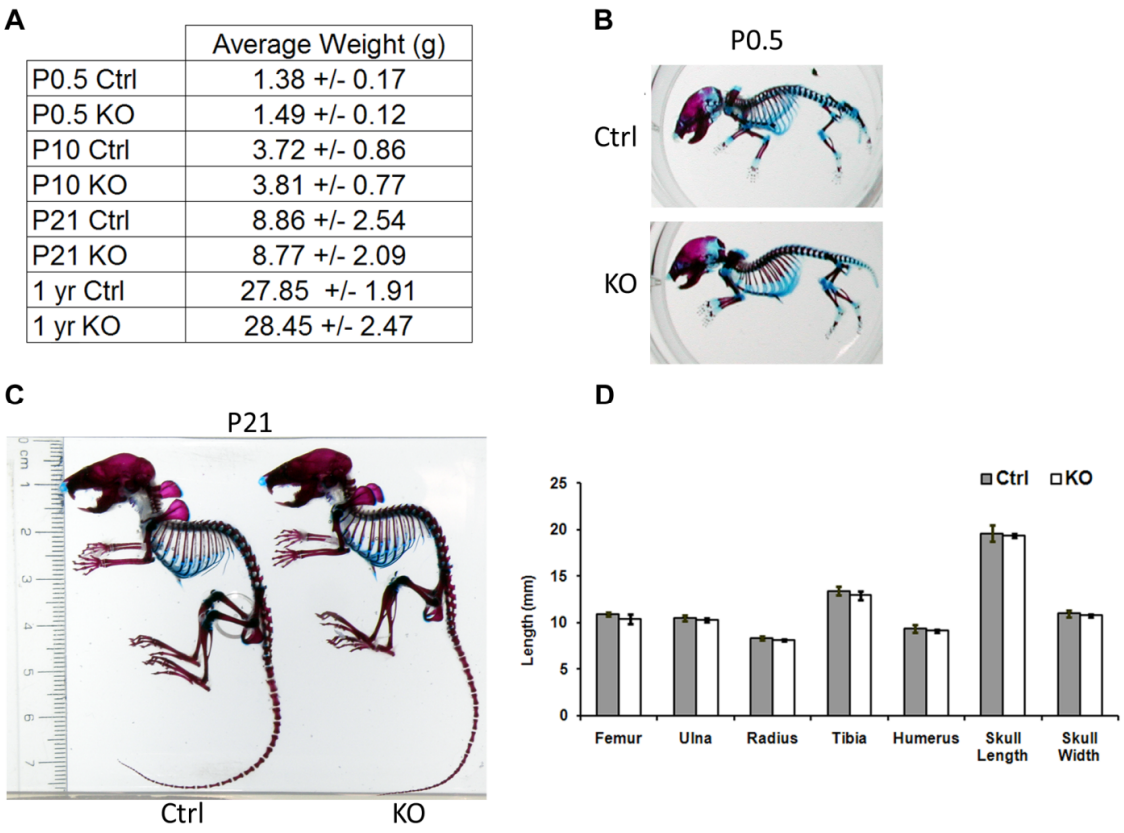


Figure 2-3 Effects of ATRX loss-of-function on growth and development of the skeleton.

(A) Weight measurements from control and cKO mice. No differences were seen in the growth of *Atrx*(Col2) cKO mice compared to control littermates, and weight gain was normal at all stages of development. N>5 for all timepoints (B, C) Skeletal stains of newborn and p21 control and cKO mice showing cartilage (blue) and bone (red) proportions. Extent of limb ossification and skeletal proportions between control and mutant pups were unaffected at birth and at three weeks (D) Long bone lengths and skull proportions were measured from four control/cKO littermate pairs at P21, and found to be unaffected by Atrx loss. N = 4, error bars = SD.

morphology was detected between genotypes at P0.5 or P21 (Figure 2-4A). In agreement with these data, no significant differences were seen in the length of any growth plate zone in three independent litters (Figure 2-4B). The early chondrogenic marker *Sox9* was expressed in resting and proliferating chondrocytes of control and cKO sections, demonstrating that loss of *Atrx* in growth plates had no effect on *Sox9* expression (Figure 2-4C). To confirm that differentiation was unaffected in mutant chondrocytes, long bone sections were stained for p57, a cyclin-dependent protein kinase inhibitor that is expressed by prehypertrophic, postmitotic chondrocytes (Zhang et al., 1997). The pattern of p57-positive cells in the mutant growth plates at P0.5 and P21 (Figure 2-4D) was not different between control and mutant mice, suggesting that loss of ATRX in the cartilage growth plate does not affect terminal differentiation of chondrocytes. In addition, the expression of other cartilage markers such as ColX, ColII, or aggrecan was not altered at P0.5 or at P21, indicating that chondrocyte differentiation was unaffected by loss of ATRX expression in cartilage.

2.3.4 Conditional Loss of *Atrx* in the Mouse Skeleton Causes Minor Ossification Defects

Although no overall changes in skeletal size or proportions were seen in the *Atrx*(Col2) cKO mice, minor delays in development were seen in some mice. Half (2 of 4) of the mutants examined by skeletal staining at day 21 displayed a delay in ossification of the union between the pubis and the ischium (Figure 2-5A). In those mutant mice where ossification was complete by day 21, the site of union was uneven with a spur-like bone projection. Both phenotypes suggest a mild defect in the ossification of the hip in the *Atrx*(Col2) cKO mice.

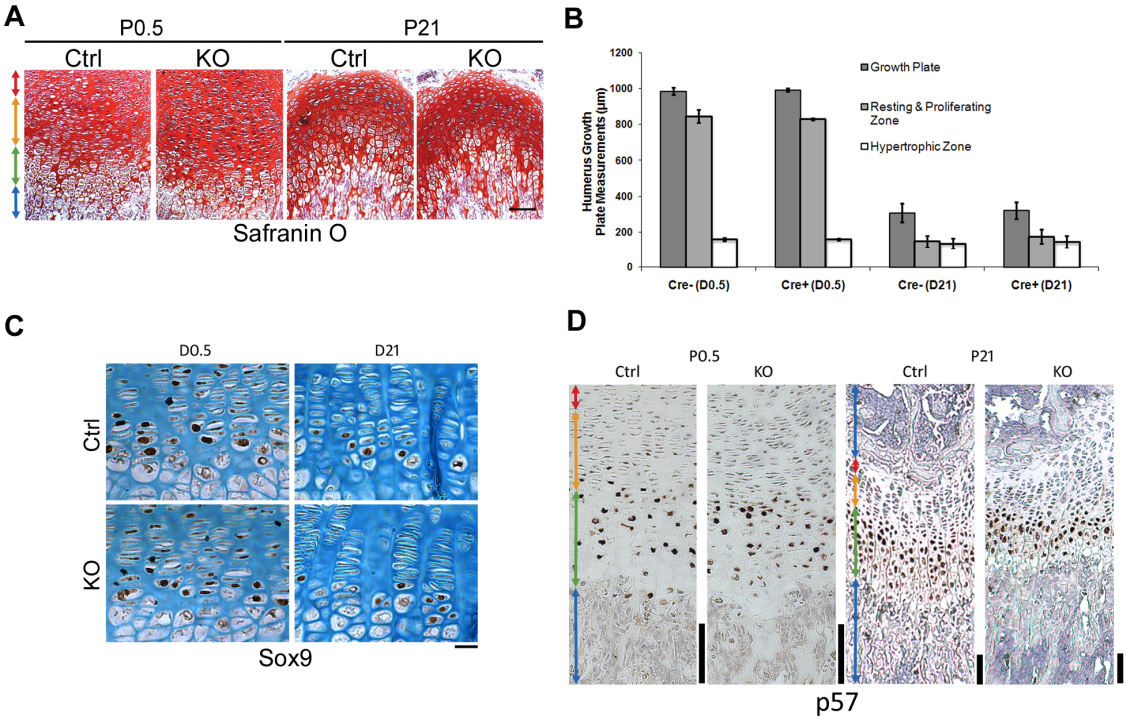


Figure 2-4 ATRX-null growth plates are indistinguishable from controls.

(A) Growth plate sections from control and *Atrx*(Col2) cKO mice at P0.5 and P21 were stained with safranin-O/Fast green, demonstrating unaltered growth plate architecture and morphology in mutant mice. Scale bar: 100 µm. (B) No significant difference in the length of the resting, proliferating or hypertrophic zones could be detected between genotypes at birth or weaning (N=3 littermate pairs; two-tailed T-test). Error bars depict standard error of the mean (SEM). (C) Expression of *Sox9*, an early chondrocyte differentiation marker expressed in resting and proliferating chondrocytes, is not altered in the absence of Atrx at P0.5 or P21. Scale bar: 50 µm. (D) Immunohistochemistry for the differentiation marker p57 reveals that *Atrx* loss has no effect on the proportion of chondrocytes reaching terminal differentiation. Scale bar: 100 µm.

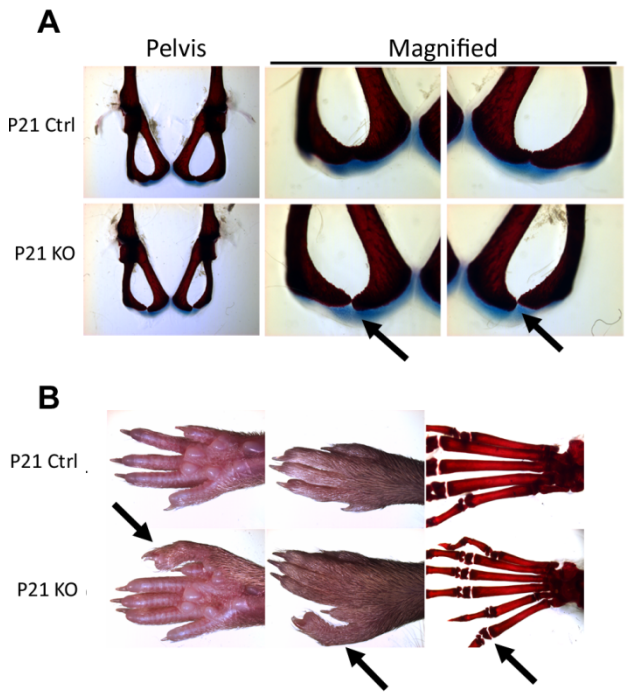


Figure 2-5 Minor defects observed in *Atrx*(Col2) cKO mice.

(A) Pelvis phenotype of P21 control and *Atrx*(Col2) cKO mice. Arrows indicate the site of delayed ossification in mutant mice. (B) Hind limb of mutant mouse with one additional digit, before and after skeletal preparation to stain for cartilage (blue) and bone (red). The additional digit is indicated by an arrow.

A second phenotype observed in one of the 61 *Atrx*(Col2) cKO mice was an extra digit in a hind foot (Figure 2-5B). This digit resembled an additional toe, similar to the fourth toe, rather than a bifurcation of the fifth toe. The toe appeared normal, and in skeletal preparations showed no gross differences from other digits.

2.4 Discussion

In this study, we show that loss of the Atrx protein specifically in cartilage of mice does not cause a major defect in skeletal growth or development. Although ATRX loss in the cartilage of mice was confirmed by immunohistochemistry, immunofluorescence and western blot analyses, we show that long bones from *Atrx*(Col2) cKO mice do not differ from controls in length or growth-plate morphology, and that these mice achieve the same adult length and weight as control littermates. Furthermore, mutant mice are viable and breed normally, with no defects seen in second-generation knockouts. Whole skeletal stains for ossified bone and cartilage were used to characterize the entire skeleton of *Atrx*(Col2) cKO mice. Qualitative and quantitative study of the control and *Atrx*(Col2) cKO skeletons showed no overall changes in cartilage proportions and lengths of individual elements.

The minor defects observed in our mouse model suggest a mild effect from ATRX deficiency that may manifest only occasionally. This is not unexpected, as the limb and digit phenotypes observed in ATR-X syndrome patients are also highly variable. In a study of sixty-five unrelated patients, one case of bifid thumb was observed (Badens et al., 2006). The varied hand and foot deformities in some ATR-X patients suggest that the effect on limb development may differ between individuals, even those with identical mutations (Badens et al., 2006). Thorough radiologic investigations of ATR-X patients have shown that the most common phenotypic abnormalities were delayed bone age and coxa valga (Gibbons, 2006). The delay in hip ossification seen in our mice may be due to a similar delay in bone age.

Previous studies have shown that loss of ATRX in other tissues in the mouse causes severe and fatal defects (Bérubé et al., 2005; Garrick et al., 2006; Medina et al., 2009). Loss of ATRX in the mouse forebrain has been shown to result in widespread hypocellularity in the hippocampus and neocortex, as well as an overall reduction in forebrain size. These mice exhibit reduced weight and perinatal lethality, demonstrating the importance of ATRX in the brain (Bérubé et al., 2005). Similarly, loss of ATRX in the 16-cell morula stage has revealed a specific and essential role for ATRX in the formation of the extraembryonic trophoblast in mice. Mutant mice showed a dramatic reduction in mitotic cells and early embryonic death at E9.5 (Garrick et al., 2006). A third model examining the role of ATRX in the developing retina showed that ATRX loss-of-function leads to loss of interneurons, specifically, amacrine and horizontal cells. These mice demonstrated a defect in interneuron differentiation and survival, which is associated with functional deficits that may be similar to the subset of ATR-X patients with visual anomalies (Medina et al., 2009).

This is the first study examining the specific loss of ATRX in the skeletal system, and surprisingly we observed only minor abnormalities. Since these effects are much less severe than in all other cell types examined, despite marked expression of ATRX in wild type chondrocytes, our data suggest cell type-specific requirement for ATRX function in cell survival and differentiation.

One assumption in our model is that destabilization and subsequent breakdown of the Atrx mRNA is an accurate model of the hypomorphic gene expression seen in ATRX patients. While our model deletes only the long isoform of ATRX and retains the short isoform (ATRXt), it is unlikely that ATRXt has equivalent functions to the full length protein, as it lacks the functional SWI/SNF domain (Garrick et al., 2004). In addition, the same floxed Atrx mouse line has been used by us and others to successfully conduct loss of function studies in other organs, such as the brain (Bérubé et al., 2005; Seah et al., 2008a) or eye (Medina et al., 2009). Since loss of ATRX does not affect cartilage development in mice, it seems likely that the skeletal defects in ATR-X patients are not due to direct effects of the mutant proteins in chondrocytes.

We have used the cartilage-specific collagen II promoter driving *cre* to inactivate the *Atrx* gene. However, there remains a possibility that some of the limb phenotypes seen in ATR-X patients (e.g. patterning defects affecting the digits) are due to an earlier effect, such as a function in formation or outgrowth of the limb bud or in formation of the initial mesenchymal cell template. Since loss of ATRX occurs later in development in our model, we cannot rule out such an earlier function for the protein. Use of an early limb bud-specific promoter, such as the Prx1 limb enhancer (Logan et al., 2002), to direct Atrx inactivation could answer this outstanding question. Additionally, a later deficiency may also contribute to the observed patient skeletal abnormalities. For example, defects in osteoblasts could contribute to delayed bone age seen in patients or reduced overall growth. *Atrx* deletion specifically in osteoblasts could be induced by using the a1(I)-collagen promoter to drive *cre* expression (Dacquin et al., 2002). These experiments are discussed within Chapter 4 of this thesis.

An alternative explanation for the surprising lack of skeletal defects in our mice is that the skeletal defects observed in patients might be secondary to abnormalities in other organs, such as neuroendocrine defects stemming from ATRX dysfunction in the nervous system (Ballock et al., 2003). There is much new evidence demonstrating that bone growth can be regulated centrally via cytokines, hormones and transcription factors, including the hormone leptin (Takeda, 2005). Experiments using leptin directly on bone cells have shown no effect on bone remodeling, however intracerebroventricular leptin infusion into leptin-deficient mice leads to an effect on bone mass via the nervous system (Takeda, 2005). Similarly, hematopoietic systems within the bone marrow have been shown to be influenced by the neurohormone melanin and catecholamines (Maestroni, 2000). Both factors also have hematopoietic roles, and are present in substantial amounts in the bone marrow, supporting the idea that neural and neuroendocrine factors have a direct effect on the bone microenvironment. Together, these studies suggest that cell defects in the hypothalamus (in the case of leptin) or the pineal gland (in the case of melatonin) may have a central influence on the development and homeostasis of the skeleton.

Importantly, it appears likely that the large variety of skeletal and growth abnormalities in ATRX patients does not have one common cellular origin; instead it appears that some defects (e.g. digit malformations) could be due to alterations in the early patterning of the limb, others (such as delayed bone age) could be caused by defects in osteoblasts (or possibly osteoclasts) and a third group (such as growth retardation) could be caused by abnormal neuroendocrine signaling. These possibilities will require examination as alternative mechanisms for the pathogenesis of ATR-X syndrome.

2.5 References

Akiyama, H., Chaboissier, M.-C., Martin, J.F., Schedl, A., and Crombrugghe, B.d. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes & Development *16*, 2813-2828

Badens, C., Lacoste, C., Philip, N., Martini, N., Courrier, S., Giuliano, F., Verloes, A., Munnich, A., Leheup, B., Burglen, L.*, et al.* (2006). Mutations in PHD-like domain of the ATRX gene correlate with severe psychomotor impairment and severe urogenital abnormalities in patients with ATRX syndrome. Clin Genet *70*, 57-62.

Ballock, R.T., and O'Keefe, R.J. (2003). Physiology and pathophysiology of the growth plate. Birth Defects Research Part C: Embryo Today: Reviews *69*, 123-143.

Beier, F., Lee, R.J., Taylor, A.C., Pestell, R.G., and LuValle, P. (1999). Identification of the cyclin D1 gene as a target of activating transcription factor 2 in chondrocytes. Proceedings of the National Academy of Sciences *96*, 1433-1438.

Bérubé, N.G., Mangelsdorf, M., Jagla, M., Vanderluit, J., Garrick, D., Gibbons, R.J., Higgs, D.R., Slack, R.S., and Picketts, D.J. (2005). The chromatin-remodeling protein ATRX is critical for neuronal survival during corticogenesis. Journal of Clinical Investigation *115*, 258-267.

Bérubé, N.G., Smeenk, C.A., and Picketts, D.J. (2000). Cell cycle-dependent phosphorylation of the ATRX protein correlates with changes in nuclear matrix and chromatin association Human Molecular Genetics *9*, 539-547.

Bobick, B.E., and Kulyk, W.M. (2008). Regulation of cartilage formation and maturation by Mitogen-Activated Protein Kinase signaling. Birth Defects Research Part C: Embryo Today: Reviews *84*, 131-154.

Dacquin, R., Starbuck, M., Schinke, T., and Karsenty, G. (2002). Mouse alpha1(I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast. Developmental Dynamics *224*, 245-251

DeLise, A.M., Fischer, L., and Tuan, R.S. (2000). Cellular interactions and signaling in cartilage development Osteoarthritis and Cartilage *8*, 309-334.

Ducy, P., Schinke, T., and Karsenty, G. (2000). The Osteoblast: A Sophisticated Fibroblast under Central Surveillance. Science *289*, 1501 - 1504.

Eisen, J.A., Sweder, K.S., and Hanawalt, P.C. (1995). Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. Nucleic Acids Research *23*, 2715-2723.

Garrick, D., Samara, V., McDowell, T.L., Smith, A.J.H., Dobbie, L., Higgs, D.R., and Gibbons, R.J. (2004). A conserved truncated isoform of the ATR-X syndrome protein lacking the SWI/SNF-homology domain. Gene *326*, 23-34.

Garrick, D., Sharpe, J.A., Arkell, R., Dobbie, L., Smith, A.J.H., Wood, W.G., Higgs, D.R., and Gibbons, R.J. (2006). Loss of Atrx Affects Trophoblast Development and the Pattern of X-Inactivation in Extraembryonic Tissues. PLoS Genet *2*, e58.

Gibbons, R.J. (2006). Alpha thalassaemia-mental retardation, X linked. Orphanet Journal of Rare Diseases *1*.

Gibbons, R.J., Brueton, L., Buckle, V.J., Burn, J., Clayton-Smith, J., Davison, B.C.C., Gardner, R.J.M., Homfray, T., Kearney, L., Kingston, H.M.*, et al.* (1995a). Clinical and hematologic aspects of the X-linked α-thalassemia/mental retardation syndrome (ATR-X). The American Journal of Human Genetics *55*, 288-299.

Gibbons, R.J., and Higgs, D.R. (2000). Molecular-clinical spectrum of the ATR-X syndrome. The American Journal of Human Genetics *97*, 204-212.

Gibbons, R.J., Picketts, D.J., Villard, L., and Higgs, D.R. (1995b). Mutations in a putative global transcriptional regulator cause X-linked mental retardation with alpha-thalassemia (ATR-X syndrome). Cell *80*, 837-845.

Gibbons, R.J., Wada, T., Fisher, C.A., Malik, N., Mitson, M.J., Steensma, D.P., Fryer, A., Goudie, D.R., Krantz, I.D., and Traeger-Synodinos, J. (2008). Mutations in the chromatin-associated protein ATRX. Hum Mutat *29*, 796-802.

Hoffman, L.M., Weston, A.D., and Underhill, T.M. (2003). Molecular mechanisms regulating chondroblast differentiation. Journal of Bone & Joint Surgery *85-A*, 124-132.

James, C., Ulici, V., Tuckermann, J., Underhill, T.M., and Beier, F. (2007). Expression profiling of Dexamethasone-treated primary chondrocytes identifies targets of glucocorticoid signalling in endochondral bone development. BMC Genomics *8*, 205.

Kronenberg, H.M. (2003). Developmental regulation of the growth plate. Nature *423*, 332-336.

Logan, M., Martin, J.F., Nagy, A., Lobe, C., .Olson, E.N., and Tabin, C.J. (2002). Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. Genesis *33*, 77–80.

Maestroni, G.J.M. (2000). Neurohormones and Catecholamines as Functional Components of the Bone Marrow Microenvironment. Annals of the New York Academy of Sciences *917*, 29-37.

Medina, C.F., Mazerolle, C., Wang, Y., Bérubé, N.G., Coupland, S., Gibbons, R.J., Wallace, V.A., and Picketts, D.J. (2009). Altered visual function and interneuron survival

in Atrx knockout mice: inference for the human syndrome. Human Molecular Genetics *18*, 966-977.

Picketts, D.J., Higgs, D.R., Bachoo, S., Blake, D.J., Quarrell, O.W., and Gibbons, R.J. (1996). ATRX encodes a novel member of the SNF2 family of proteins: mutations point to a common mechanism underlying the ATR-X syndrome. Human Molecular Genetics *5*, 1899-1907.

Ritchie, K., Seah, C., Moulin, J., Isaac, C., Dick, F., and Bérubé, N.G. (2008). Loss of ATRX leads to chromosome cohesion and congression defects. The Journal of Cell Biology *180*, 315-324.

Seah, C., Levy, M.A., Jiang, Y., Mokhtarzada, S., Higgs, D.R., Gibbons, R.J., and Bérubé, N.G. (2008a). Neuronal death resulting from targeted disruption of the Snf2 protein ATRX is mediated by p53. J Neurosci *28*, 12570-12580.

Seah, C., Levy, M.A., Jiang, Y., Mokhtarzada, S., Higgs, D.R., Gibbons, R.J., and Bérubé, N.G. (2008b). Neuronal death resulting from targeted disruption of the Snf2 protein ATRX is mediated by p53. J Neurosci *28*, 12570-12580.

Solomon, L.A., Li, J.R., Berube, N.G., and Beier, F. (2009). Loss of ATRX in chondrocytes has minimal effects on skeletal development. PLoS ONE *4*, e7106.

Takeda, S. (2005). Central control of bone remodeling. Biochemical and Biophysical Research Communications *328*, 697-699.

Terpstra, L., Prud'homme, J., Arabian, A., Takeda, S., Karsenty, G., Dedhar, S., and St-Arnaud, R. (2003). Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes. Journal of Cell Biology *162*, 139–148.

Wang, G., Woods, A., Agoston, H., Ulici, V., Glogauer, M., and Beier, F. (2007). Genetic ablation of Rac1 in cartilage results in chondrodysplasia Developmental Biology *306*, 612-623

Yamaguchi, A., Komori, T., and Suda, T. (2000). Regulation of Osteoblast Differentiation Mediated by Bone Morphogenetic Proteins, Hedgehogs, and Cbfa1. Endocrinology Reviews *21*, 393-411.

Zhang, P., Liegeois, N.J., Wong, C., Finegold, M., Hou, H., Thompson, J.C., Silverman, A., Harper, J.W., DePinho, R.A., and Elledge, S.J. (1997). Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. Nature *387*, 151-158.

Chapter 3

3 Targeted Loss of the ATR-X Syndrome Protein in the Limb Mesenchyme of Mice Causes Brachydactyly

ATR-X syndrome is a rare genetic disorder caused by mutations in the *ATRX* gene. Affected individuals are cognitively impaired and display a variety of developmental abnormalities, including skeletal deformities. To investigate the function of ATRX during skeletal development, we selectively deleted the gene in the developing forelimb mesenchyme of mice. The absence of ATRX in the limb mesenchyme resulted in shorter digits, or brachydactyly, a defect also observed in a subset of ATR-X patients. This phenotype persisted until adulthood, causing reduced grip strength and altered gait in mutant mice. Examination of the embryonic ATRX-null forelimbs revealed a significant increase in apoptotic cell death, which could explain the reduced digit length. In addition, staining for the DNA-damage markers γ-H2AX and 53BP1 demonstrated a significant increase in the number of cells with DNA damage in the embryonic ATRX-null forepaw. Strikingly, only one large bright DNA damage event was observed per nucleus in proliferating cells. These large γ-H2AX foci were located in close proximity to the nuclear lamina and remained largely unresolved after cell differentiation. In addition, ATRX-depleted forelimb mesenchymal cells did not exhibit hypersensitivity to DNA fork-stalling compounds, suggesting that the nature as well as the response to DNA damage incurred by loss of ATRX in the developing limb fundamentally differs from other tissues. Our data suggest that DNA damage-induced apoptosis is a novel cellular mechanism underlying brachydactyly that might be relevant to additional skeletal syndromes.

3.1 Introduction

Alpha-Thalassemia Mental Retardation Syndrome, X-linked (ATR-X [MIM 301040]) is a rare genetic disorder caused by mutations to the *ATRX* gene (Gibbons et al., 1995b). Manifestations of the disease include intellectual disabilities, severe developmental delay, facial dimorphisms, urogenital abnormalities, and skeletal deformities (Gibbons et al.,

1995a; Gibbons et al., 2000a). The latter include brachydactyly, clinodactyly, tapering of the fingers, overlapping digits, and foot deformities. Approximately two-thirds of patients have short stature (Gibbons et al., 1995a; Gibbons et al., 2000a). This X-linked syndrome predominantly affects males whereas carrier females are mostly asymptomatic, presumably due to skewed X-inactivation (Gibbons et al., 1995a; Wada et al., 2005). Many ATR-X patients have α-thalassemia caused by impaired production of the α-globin gene leading to unstable tetramers of β-globin chains, or HbH inclusions, in the blood (Gibbons et al., 2000b; Gibbons et al., 1995b).

The ATRX protein contains two highly conserved domains where the majority of disease-causing mutations are located. A plant homeodomain-type (PHD-type) zinc finger motif, also called the ADD domain, mediates binding to histone H3 trimethylated at lysine 9 and unmethylated at lysine 4 (Dhayalan et al., 2011; Otani et al., 2009; Wong et al., 2010). ATRX also contains a Sucrose Non Fermenting 2 (SNF2)-type DNA-dependent ATPase domain in the C-terminal portion of the protein (Gibbons et al., 1995a; Picketts et al., 1996). SNF2 proteins are a family of helicase-like proteins that can hydrolyse adenosine triphosphate (ATP) to remodel chromatin or help repair DNA damage (Eisen et al., 1995).

The ATRX protein interacts with the heterochromatin protein 1 alpha (HP1α) and the Fas death domain-associated protein (DAXX), proteins located at heterochromatin and promyelocytic leukemia (PML) nuclear bodies (Xue et al., 2003). The DAXX/ATRX complex deposits the histone variant H3.3 at telomeres and at pericentromeric heterochromatin and can modulate transcription from these highly repetitive genomic regions (Drané et al., 2010; Lewis et al., 2010).

Emerging evidence indicates that ATRX is required to maintain genomic integrity. Depletion of ATRX in human somatic cells by RNA interference caused mitotic defects including chromosome cohesion, congression and segregation defects (Ritchie et al., 2008). In the mouse, conditional inactivation of *Atrx* in forebrain, muscle and Sertoli cells was reported to induce cell death (Bagheri-Fam et al., 2011; Bérubé et al., 2005; Garrick et al., 2006; Huh et al., 2012). Surprisingly, we showed that deletion of *Atrx* in

chondrocytes did not result in increased cell death, demonstrating that the outcome of ATRX deficiency differs across cell types (Solomon et al., 2009). In mouse embryonic stem cells, ATRX depletion results in reduced histone H3.3 deposition at telomeres and in telomere-dysfunction phenotypes (Wong et al., 2010). Our group and others have shown that increased instability at telomeres associated with ATM activation occurs upon ATRX inactivation (Huh et al., 2012; Watson et al., 2013). We demonstrated that combined deletion of the *p53* and *Atrx* genes in the developing nervous system abolishes embryonic cell death, leading to an accumulation of neurons with DNA damage. Thus, loss of ATRX causes DNA damage, which triggers p53-dependent apoptotic cell death (Seah et al., 2008; Watson et al., 2013). ATRX-deficient cells are hypersensitive to fork-stalling agents, but not to gamma irradiation, suggesting that loss of ATRX specifically promotes DNA replication stress (Leung et al., 2013; Watson et al., 2013). This is supported by the co-localization of DNA damage foci with the replication marker PCNA in ATRX-null cells (Huh et al., 2012; Watson et al., 2013). Leung et al. demonstrated that ATRX is recruited to sites of DNA damage with the MRN complex and promotes restart of stalled forks (Leung et al., 2013). Finally, we were able to show that neuroprogenitors lacking ATRX accumulate more DNA damage and display reduced survival upon treatment with telomestatin, a G-quadruplex ligand, suggesting that replication stress induced by ATRX deficiency is linked to G-quadruplex stability (Watson et al., 2013).

Given the high frequency of skeletal abnormalities reported in ATR-X syndrome patients, especially in hands and feet, we hypothesized that ATRX may protect cells in the developing limbs from endogenous DNA replication damage or that it might control pathways required for proper development of the skeleton in the distal limbs. We find that conditional deletion of *Atrx* in limb bud mesenchyme causes a specific and significant shortening of the distal phalanges. Embryonic ATRX-null limb bud cells display one large γ-H2AX/53BP1-positive focus adjacent to the nuclear membrane in each nucleus that persists upon cell differentiation. While the majority of cells seem to be able to differentiate despite unrepaired DNA damage, we detected an increase in apoptotic cell death that might explain the reduced digit length in the *Atrx*(Prx1) cKO mice. Our findings suggest that apoptosis in response to DNA damage is a novel mechanism giving rise to brachydactyly.

3.2 Materials and Methods

3.2.1 Mouse Husbandry, Genotyping and Tissue preparation

Mice were housed with a 12-hour light-dark cycle, and fed tap water and regular chow ad libitum. Mice conditionally deficient for Atrx in the early limb bud mesenchyme were generated using the 129SV $Atrx^{loxP}$ mice described previously(Bérubé et al., 2005; Garrick et al., 2006). $Atrx^{loxP}$ mice were mated to mice expressing transgenic Cre recombinase under the control of the *Prx1* promoter (Prrx1-cre)(Logan et al., 2002) to generate $Atrx^{loxP/Prx1Cre+}$ animals lacking ATRX in the developing forelimb. Tg(Prrx1-cre)1Cjt mice were obtained from Jackson Laboratories. For embryonic time points, noon of the day after mating was considered to be E0.5. Genotyping for *Atrx*, *Cre*, and Y chromosome was determined by PCR as previously described (Bérubé et al., 2005; Seah et al., 2008). Embryonic tissue collected for histology was fixed in PFA overnight at 4°C overnight, then equilibrated in 30% sucrose and embedded in VWR clear frozen section compound over liquid nitrogen. Forelimbs were sectioned at 5 µm and stored at -80°C. All procedures involving animals were conducted in accordance with the regulations of the Animals for Research Act of the Province of Ontario and approved by the University of Western Ontario Animal Care and Use Committee, Protocol 2007-045.

3.2.2 Quantification of Skeletal Defects

Embryonic forepaws were isolated and dehydrated in 95% ethanol for 24 h, followed by acetone for 24 h. Paws were then stained with 0.015% alcian blue, 0.05% alizarin red, and 5% acetic acid in 70% ethanol as described(Wang et al., 2007). The stained paws were cleared in glycerol/1% KOH (1:1) and stored in glycerol/ethanol (1:1). Adult skeletons were cleaned and stained as described, then cleared in 2% KOH and stored in glycerol/ethanol (1:1). Embryonic forepaws were cleared and imaged using a Nikon SMZ1500 dissecting microscope with a Photometrics (Tucson, AZ) Coolsnap camera using ImageMaster 5.0 software. Adult phalanges were microdissected and imaged as described. Digit length was measured in ImageJ and analysed using Graphpad from at least three independent litters at each age. Means were compared using a student's T-test.

3.2.3 Microcomputed Tomography

Mice were euthanized at P21, forelimbs were dissected and fixed in paraformaldehyde overnight followed by immobilisation in agarose. Whole forepaws were scanned on a GE Locus scanner at 80 kV and 0.08 mA with a 0.013 mm^3 voxel resolution with 900 slices per scan. Scans were analysed using Microview 3D visualization and analysis software (MicroView, Version 2.1.2, GE Healthcare Biosciences), as described (Ulici et al., 2009). Whole forepaw isosurface images were generated by applying a threshold of 932 HU, surface quality factor of 1.00 and a surface decimation factor of 30.

3.2.4 Western blot Analysis

Embryonic forelimbs were dissected in PBS into RIPA buffer and homogenised using a fine-gauge needle. SDS-PAGE sample buffer (5 µl) was added to samples, followed by boiling for 5 min. Samples were separated by gradient SDS-polyacrylamide gel electrophoresis (Biorad) and transferred to nitrocellulose membranes (Amersham). Membranes were blocked in 2% BSA and incubated with primary antibodies in accordance with the instructions from the primary antibody supplier. Representative blots from three independent experiments are shown.

3.2.5 Proliferation and Cell Death Assays

Proliferating cells were visualized using antibodies for Ki67. Frozen sections were rehydrated and antigens retrieved using 0.1 M sodium citrate. Sections were permeabilized for one hour with blocking buffer containing 5% goat serum and 0.1% tween-20 in phosphate-buffered saline, followed by overnight incubation with primary antibodies mouse anti-gamma H2AX (Abcam) and either rabbit anti-53BP1 (Cell signalling) or rabbit-antiKi67 (Abcam) at a concentration of 1:300 in blocking buffer. Antibodies were detected using fluorescently conjugated secondary antibodies, FITC goat anti-rabbit (Biosource) and Alexa-594 goat anti-mouse (Invitrogen) at dilutions of 1:200. Slides were mounted in media containing DAPI (Vectashield) and confocal images were acquired on an Olympus FluoView TV1000 coupled to the IX81 Motorized Inverted System Microscope (IX2 Series). Damage was quantified by a blinded observer within a fixed area of 10,000 μm^2 for digit tips and 5,000 μm^2 for digit rays. Counts were

averaged from three independent litters at each time point. To assess apoptosis, 5 μm sections from E13.5 and E15.5 mice were used for the TUNEL assay using the Roche In Situ Cell Death Detection Kit, Fluorescein according to manufacturer's instructions. Briefly, frozen sections were hydrated in PBS and treated with cold citrate for 2 min. Slides were washed and incubated in the enzyme/label solution mix for 1h at 37^{o}C, then mounted with DAPI-containing mounting media (Vectashield). Sections were imaged on a Leica DMRA2 automated inverted microscope. Signal was quantified from sections using a fixed area over each digit by a blinded observer. Counts were averaged from at least three independent litters.

Activated caspase 3 was visualised in sections using rabbit anti-cleaved caspase-3 (Cell Signaling, 1:400 dilution). Cryosections were rehydrated in 1XPBS for 5 min and subjected to antigen retrieval by heating 0.1M sodium citrate ph6 to 95 degrees. Sections were permeabilized with 1X PBS/0.3% Triton X for 5 min and incubated with primary antibody diluted in 1XPBS/0.3% Triton-X overnight at 4 degrees. Sections were washed 3X with 1X PBS/0.3% Triton X for 5 min each and incubated with goat anti-rabbit Alexa 488 (1:800 dilution) diluted in 1XPBS/0.3% Triton-X for 1h at RT. Sections were washed 2X with 1X PBS/0.3% Triton X for 5 min each, incubated with 1μg/μl DAPI/1XPBS for 5 min, and washed 3X with 1X PBS/0.3% Triton X for 5 min each. Slides were then mounted using SlowFade Gold (Invitrogen) and imaged using an inverted fluorescence microscope (Leica, DMI6000b).

Immunofluorescence assays were conducted for γ-H2AX and the nuclear envelope marker Lamin-B as described previously (Kuo et al., 2008; Vidaković et al., 2005). Nuclear lamina was detected using a goat anti-LaminB antibody (Abcam) at a concentration of 1:300 while DNA double strand breaks were visualized with mouse anti-γ-H2AX (Abcam) at a concentration of 1:200 in blocking buffer and detected with Alexa-594 donkey anti-mouse (Invitrogen) and FITC donkey anti-goat (Invitrogen) at a concentration of 1:200. Three-dimensional images of nuclei were collected on an Olympus FluoView TV1000 coupled to the IX81 Motorized Inverted System Microscope (IX2 Series) in image stacks captures at 0.25 μm Z-sections and deconvoluted in

Volocity (PerkinElmer). Measurements were taken from the center of the γ-H2AX focus to the closest nuclear periphery for 100 individual nuclei.

3.2.6 Hydroxyurea Treatment and Assessment of DNA Damage and Survival

Primary forelimb mesenchyme was prepared using distal forelimb tissue dissected from E13.5 embryos. Cells were dissociated with collagenase at 37^{o}C for one hour and plated in DMEM/F12 media supplemented with 10% FBS, 0.25% pen/strep and 0.25% l-glutamate (Gibco). Forelimb mesenchyme cultures were treated with increasing doses of HU (0.05 to 1 mM) for 24 hours, recovered for 24 or 48 hours, and cell viability measured by MTT assay as described (Halawani et al., 2004). Briefly, media containing HU was removed and replaced with fresh media for 24 or 48 h, followed by replacement with media containing 0.5 mg/ml MTT for 4 h. Product was stabilized in DMSO and quantified on a spectrophotometer at 595 nm.

3.2.7 Gait and Grip Strength Analyses

Behavioural assays were performed at the behavioural core facility at the Robarts Research institute (London, ON). Control and mutant mice at P21 and one year of age freely ambulated on an illuminated glass platform, and movements were recorded with a high speed camera using the Noldus CatWalk system. After an initial training period, at least three uninterrupted runs were collected from each animal. Each run was analysed using Catwalk 7.1 software for classifying foot contacts. Run data for each animal were averaged, and then averages calculated within each genotype. Means were analyzed for statistical differences between genotypes using a student's t -test. Mouse forelimb grip strength was assessed using a digital force gauge as described previously (Meyer et al., 1979). Briefly, the animal was grasped by the scruff of the neck in one hand, and the base of the tail in the other. Measurements were recorded on the meter as the subject was allowed to grip, and then was pulled away from, the grip bar. Total peak force was determined from an average of five measurements taken from each animal, and the means of each genotype were compared. Statistical analysis of the means was conducted using a two-tailed student's t-test.

3.3 Results

3.3.1 Generation of Mice Lacking *Atrx* Specifically in the Limb Mesenchyme

We utilized the Cre-LoxP system to generate mice lacking the *Atrx* gene in early limb bud mesenchyme. Homozygous floxed $Atrx^{loxP}$ female mice (Bérubé et al., 2005) were mated with male Tg(Prx1-cre)1Cjt mice expressing Cre recombinase under the control of the *Prx1* promoter that drives recombination in early limb bud mesenchyme (Logan et al., 2002). Since *Atrx* is located on the X chromosome, males resulting from this cross carry one copy of the *Atrx* gene that contains the loxP sites. Cre-positive males are conditionally ATRX-null and are referred to as *Atrx*(Prx1) cKO. All animals in this study were from the first generation of this cross.

Reverse transcriptase PCR (RT-PCR) analysis of *Atrx* expression in embryonic day 16.5 forepaws shows a reduction in wild type *Atrx* mRNA and the presence of low levels of a shorter transcript resulting from the recombination of exon 18 in mutant limb mesenchyme. The amount of this truncated RNA was greatly reduced, confirming that the RNA is unstable and is equivalent to a null mutation (Figure 3-1A), as we have shown previously (Bérubé et al., 2005). ATRX protein was detected at high levels in the control, but was absent in the cartilage and pre-cartilaginous condensations of the *Atrx*(Prx1) cKO mice (Figure 3-1B,C). ATRX protein is retained in the nucleus of epithelial cells of the limb, confirming that *Cre* activity is indeed limited to the limb mesenchyme when expressed under the control of the *Prx1* promoter. Mutant *Atrx*(Prx1) cKO mice were born at normal Mendelian ratios (Figure 3-1D) and had a normal birth weight (Figure 3-S1). They lived beyond the age of one year and were fertile.

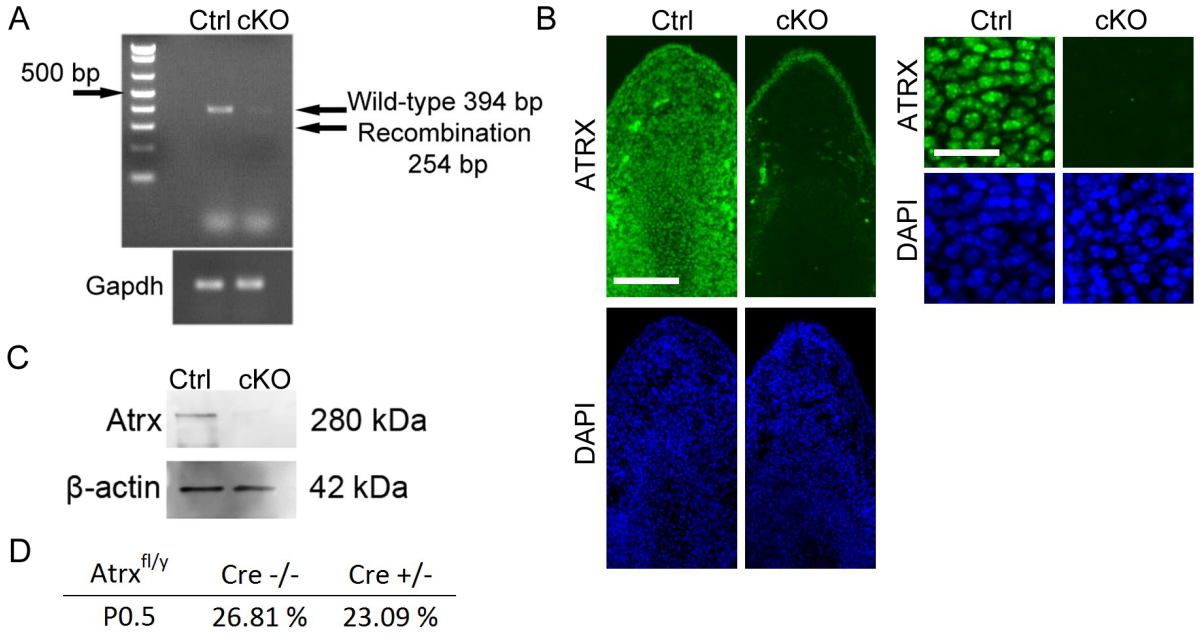


Figure 3-1 *Atrx* is deleted in the forelimbs of *Atrx*(Prx1) cKO mice

(A) RT-PCR analysis of RNA isolated from embryonic forelimbs of $Atrx^{fl/yPrxcre+}$ males *Atrx*(Prx1) cKO (KO) and control littermates (Ctrl). Amplification was performed with primers flanking the loxP sites, in introns 17 and 20. Recombined RNA in *Atrx*(Prx1) cKO is unstable and degraded. (B) Cryosections of E15.5 forelimb tissue were stained for ATRX protein. *Atrx*(Prx1) cKO mice lack ATRX in the nucleus of all mesenchyme tissues. Scale bar: 200 μm (representational high-resolution image: 50 μm) (C) Immunoblot of proteins isolated from E15.5 forelimbs shows loss of ATRX protein in *Atrx*(Prx1) cKO mice. (D) *Atrx*(Prx1) cKO mice are born at normal Mendelian ratios, with no associated lethality.

3.3.2 Mice lacking *Atrx* in the Forelimb Mesenchyme Develop Brachydactyly

To evaluate the phenotypes of *Atrx*(Prx1) cKO mice, forelimbs were collected and patterns of cartilage and mineralised bone were examined at various embryonic times by alcian blue/alizarin red staining (respectively). Embryonic forepaws showed no difference between control and mutant mice at embryonic day 14.5 (Figure 3-2A). However, at E15.5, we observed that the condensation of the anlage for phalange 2 was reduced in size (Figure 3-2B). A more severe delay was seen at E16.5. The paws in controls had four cartilage condensations and establishment of phalange 3, while these condensations were significantly shortened or absent in the Atrx mutant littermates (Figure 3-2C). At this stage of embryonic development, the *Atrx*(Prx1) cKO embryos displayed a significant shortening of total digit length, with smaller ossified elements in the distal phalanges. This phenotype is consistent with brachydactyly in ATR-X syndrome (Gibbons, 2006).

Whole skeletal preparations were obtained from newborn, weanling (P21), and adult mice. Distal phalanges in *Atrx*(Prx1) cKO mice displayed reduced or absent mineralisation at birth and were significantly shorter than the phalanges of control littermates (Figure 3-3A). This phenotype was evaluated at P21, and we found that the shortening of the distal phalanges persisted even after complete mineralisation of the paw (Figure 3-3B). Proximal phalanges (metacarpals and phalange 1) were not affected, whereas the distal phalanges (phalanges 2 and 3) are significantly shorter in all digits. Micro-computed tomography analysis of P21 forepaws was performed and showed that the shape of the fingers and claws is altered. Fingers displayed abnormal flexation, while mineralization and cortical thickness are not affected (Figure 3-3C). Adult *Atrx*(Prx1) cKO mice still exhibit brachydactyly at one year of age (Figure 3-3D), indicating that the early phenotypes do not simply represent a delay in development but rather a permanent phenotype. The proximal bones of mutant forelimbs (humerus, radius, ulna) did not show any abnormalities at any investigated age.

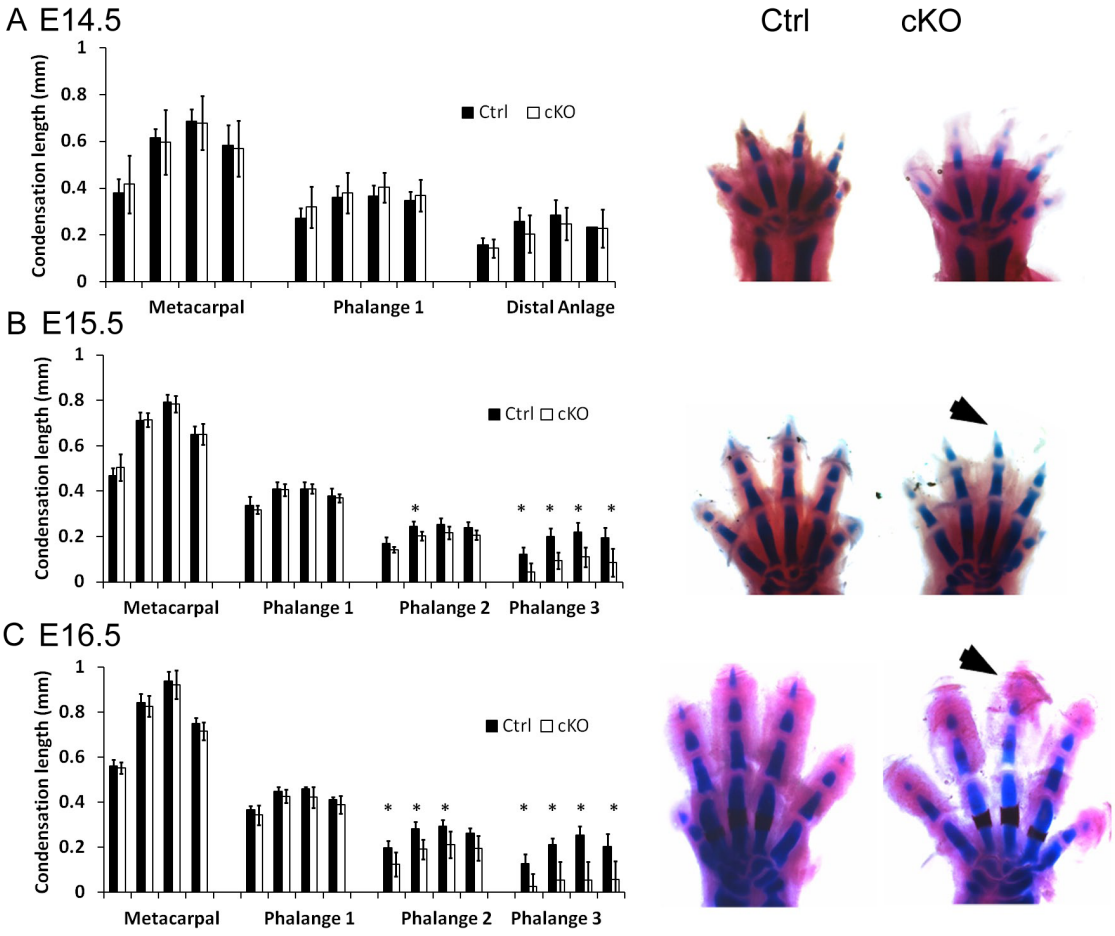


Figure 3-2 *Atrx*(Prx1) cKO digit defects appear at embryonic day 15.5

(A) Phalange length measurements from embryonic day 14.5 *Atrx*$^{fl/yPrxcre-}$ (CTRL) and *Atrx*(Prx1) cKO (cKO) mice. No differences are seen in the length of cartilaginous condensations in the forelimb. (B) Phalange length measurements at embryonic day 15.5. cKO mice exhibit shortening of terminal phalanges. (C) Phalange length measurements from embryonic day 16.5. All distal phalanges are significantly shortened in mutant mice. * = $p < 0.05$, N=3. Arrow indicates shortened phalange.

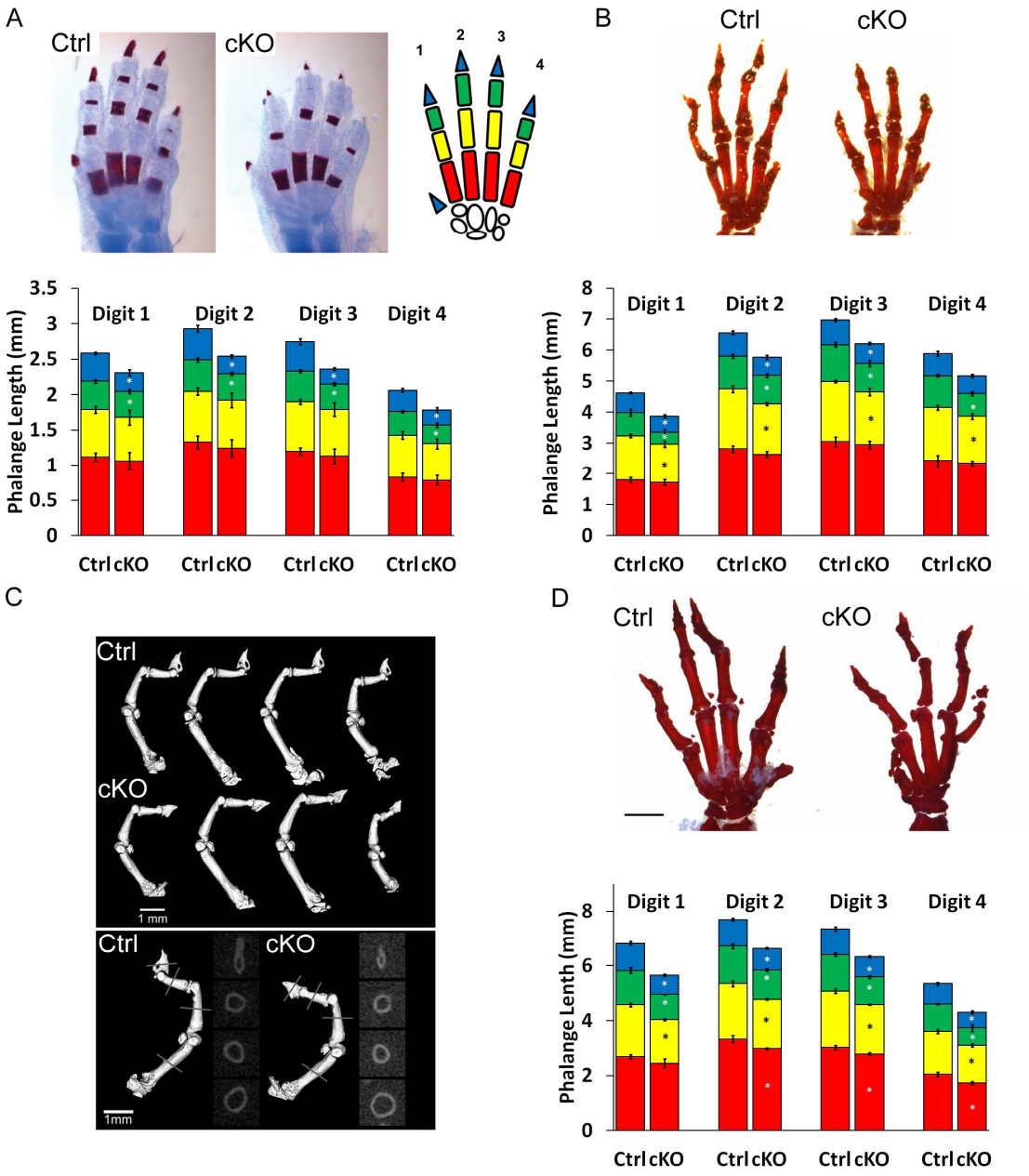


Figure 3-3 *Atrx*(Prx1) cKO mice show brachydactyly

(A) Skeletal stains of control (Ctrl) and *Atrx*(Prx1) cKO (KO) newborn (P0.5) forelimbs. Cartilage is stained in blue, mineralised tissue is stained in red. Proximal bones (metacarpals and the first phalange) were not affected in the mutant, however distal phalanges were significantly shortened and lacked mineralisation. * = $p < 0.05$, N=3 (B) Skeletal stains of control (Ctrl) and *Atrx*(Prx1) cKO (KO) weanling (P21) forelimbs. Significant shortening is observed in all phalanges, excluding the metacarpals * = $p < 0.05$, N=3. (C) MicroCT isosurfaces of P21 digits. *Atrx*(Prx1) cKO digits lack flexation of the terminal phalanges, and have short, malformed claws. Cortical thickness was unaffected. Scale bar: 1 mm (D) Skeletal stains of control (Ctrl) and *Atrx*(Prx1) cKO

(KO) adult (1 year old) forelimbs. Mineralised tissue is stained in red. Significant shortening is observed in all phalanges. N=3 for all time points, * = $p < 0.05$.

3.3.3 Cell Death is Increased in the *Atrx*(Prx1) cKO Embryonic Limb Bud Mesenchyme.

The observation that *Atrx*(Prx1) cKO mice have shorter digits suggests a reduction in cell numbers during embryogenesis, which could be explained by reduced proliferation or increased cell death. Proliferation was assessed using Ki67 staining (a marker of proliferation). Quantification of Ki67-positive cells in the distal portions of control and *Atrx*(Prx1) cKO digits showed that cell proliferation is not different between genotypes at E13.5 and E15.5, which represent times before and after the appearance of the phenotype, respectively (Figure 3-4).

We next assessed the level of apoptosis by TUNEL assay and staining for activated caspase 3 at E13.5. As expected, we observed clusters of apoptotic cells in the interdigital mesenchyme in both genotypes. We also observed an increase in the number of apoptotic cells in the mutant compared to control digits. At E13.5, there was a 6.9-fold increase in apoptotic cells in the *Atrx*(Prx1) cKO ($p=0.038$, N=4), and a 4.8-fold increase in the number of cells containing activated caspase 3 ($p=0.004$, N=4) (Figure 3-5). At E15.5 there was a significant 3.8-fold increase in apoptotic cells in the *Atrx(*Prx1) cKO ($p=0.028$, N=3)(Figure 3-S2).

3.3.4 Increased Level of γ-H2AX foci in Atrx-null Embryonic Limb Bud Mesenchyme and Chondrocytes.

As we and others previously reported increased levels of DNA damage upon loss of *Atrx* (Huh et al., 2012; Watson et al., 2013), we examined *Atrx*(Prx1) cKO forelimbs for evidence of DNA damage. We stained embryonic limb cryosections from E12.5 to E17.5 with an antibody against phosphorylated histone 2A family member X (γ-H2AX), a marker of DNA double strand breaks. Confocal imaging and quantification at E13.5 and E15.5 showed a significant increase in the number of cells harbouring γ-H2AX foci in the forelimb mesenchyme of *Atrx*(Prx1) cKO mice (Figure 3-6A,B) and cartilaginous digit rays (Figure 3-S3). To confirm this result, we co-stained cryosections with 53BP1, a DNA damage response protein that promptly re-localizes to damaged sites. We observed a higher number of 53BP1 foci in mutant forepaws that largely overlapped with the γ-H2AX foci (Figure 3-6C). In the limb buds of heterozygous female embryos, *Atrx* is

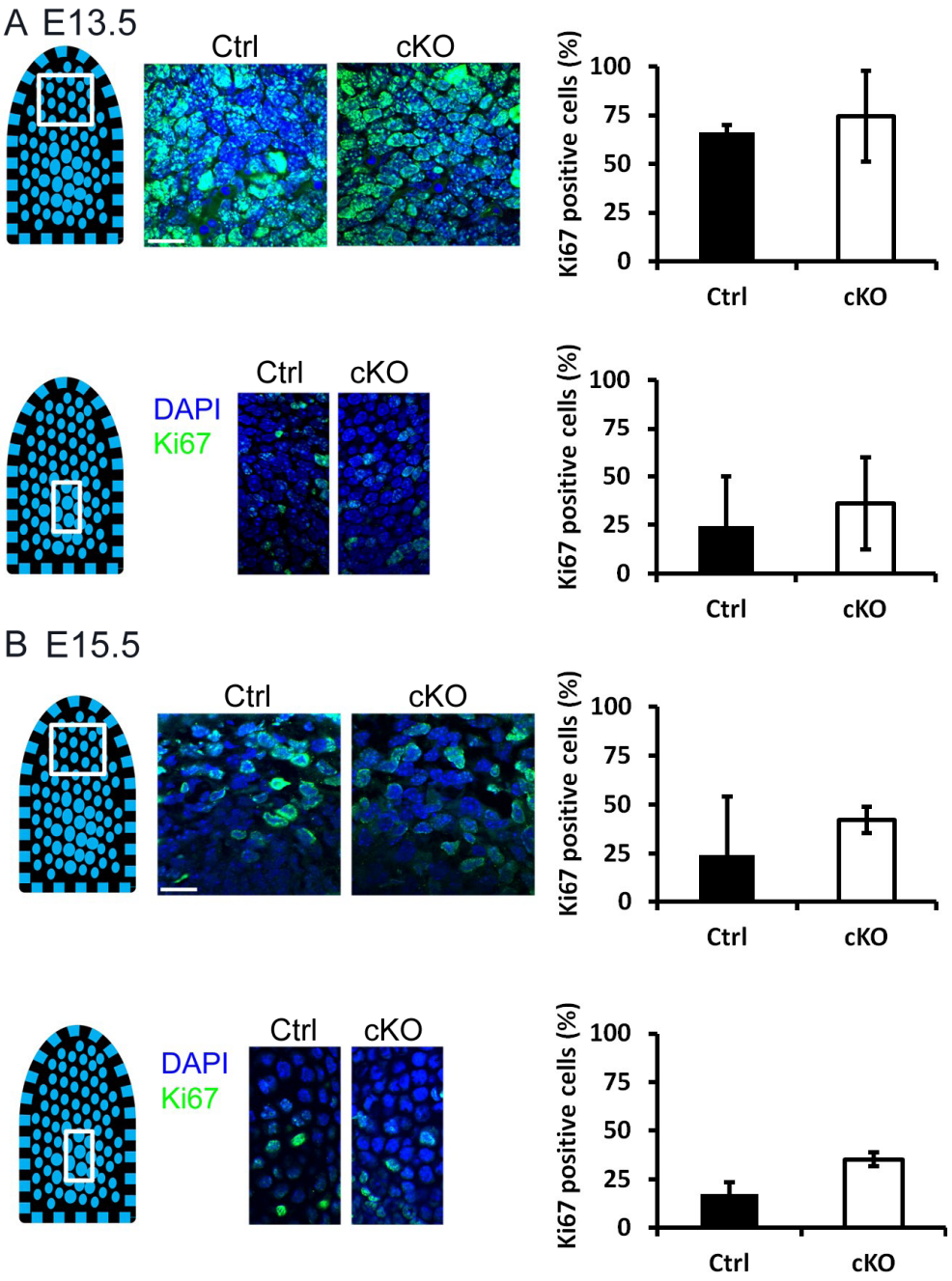


Figure 3-4 Proliferation is unchanged in the *Atrx*(Prx1) cKO embryonic limb bud.

Immunofluorescent stains for the S-phase marker Ki67 in embryonic day 13.5 (A) and 15.5 (B) forelimb cryosections. More Ki67+ cells are present in the highly proliferative distal tip as compared to the differentiated digit ray, but no significant change is observed between genotypes. Scale bar: 20 μm, Error bars = SD, N = 4.

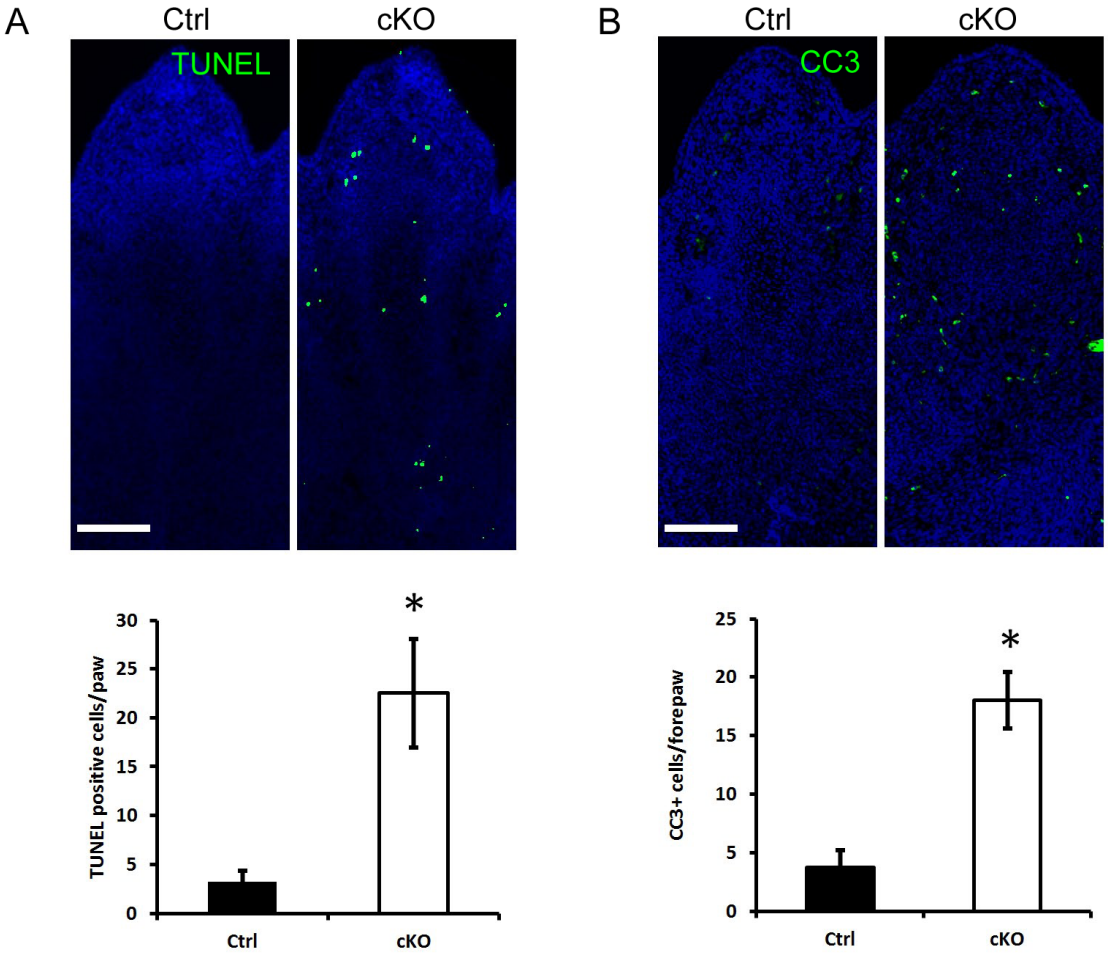


Figure 3-5 Cell death is increased in the *Atrx*(Prx1) cKO embryonic limb bud.

Fluorescent TUNEL and activated caspase 3 stains of frozen sections for embryonic day 13.5 forelimbs. (A) At E13.5, more TUNEL positive nuclei are observed per digit tip in mutant forelimb sections (N=4). (B) *Atrx*(Prx1) cKO embryonic limb buds contain significantly more cells containing activated caspase 3 (N=4). Scale bar: 200 μm Error bars = SEM. * $p < 0.05$ (students T-test)

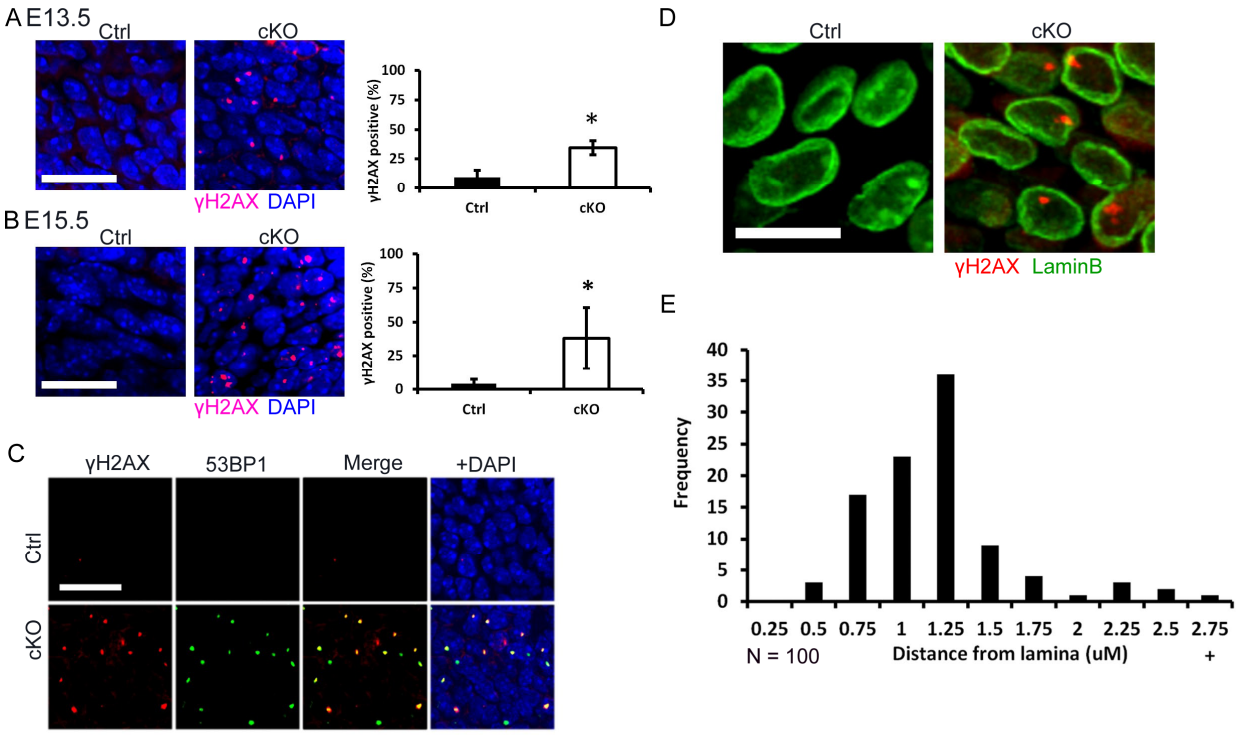


Figure 3-6 ATRX-deficient limb buds have increased levels of DNA double strand breaks

(A) Immunofluorescent stains for the phosphorylated histone gamma-H2A.X (γ-H2AX) in embryonic day 13.5 forelimb sections. γ-H2AX signal frequently appears in the mutant nuclei as a single bright focus which is absent in the control. Expression of γ-H2AX was similar between distal phalange tips and differentiated digit rays. Scale bar: 50 μm. N =4 (B) Immunofluorescent stains for γ-H2AX in embryonic day 15.5 forelimb section demonstrating persistence of the γ-H2AX foci within the nucleus of *Atrx*(Prx1) cKO cells. Scale bar: 50 μm. N =4 (C) Immunofluorescent stains for γ-H2AX and 53BP1 in embryonic day 13.5 forelimb sections. Sites of γ-H2AX signal co-localise with 53BP1 in all parts of the forelimb. Scale bar: 50 μm (D) Immunofluorescent staining for Lamin B and γ-H2AX, demonstrating large DNA damage foci located in close proximity to the nuclear lamina. Scale bar: 10 μm (E) Quantification of distance between the center of the bright γ-H2AX focus and the nearest nuclear lamina.

Error bars = SD, * $p < 0.05$ Student's T-test

deleted in approximately 50% of cells, as expected from a random pattern of X-inactivation. Forelimb tissue of heterozygote female mice at E13.5 had γ-H2AX foci in 17% of cells (Figure 3-S4), but less than 2% of ATRX+ cells contained DNA damage foci. These findings suggest that DNA damage incurred is due to a cell-autonomous effect of Atrx loss.

Surprisingly, the γ-H2AX staining in *Atrx*(Prx1) cKO cells appeared as one discrete, bright focus in the nucleus (Figure 3-6D). Moreover, staining of the sections with an antibody that recognizes lamin B demonstrates that the large majority of the DNA damage foci are located in close proximity to the nuclear lamina, which was confirmed by measurements of distance of the foci to the nuclear lamina using confocal microscopy (Figure 3-6D, E). Interestingly, no increase in γ-H2AX staining was observed in male embryos when the *Atrx* gene was inactivated using the cartilage-specific Col2-Cre driver line (Figure 3-S5).

3.3.5 Atrx-deficient Forelimb Mesenchyme cells are not Hypersensitive to Hydroxyurea Treatment.

Control and *Atrx*(Prx1) cKO embryonic forelimbs were cultured and treated with increasing concentrations of the fork-stalling compound hydroxyurea (HU) for 24 h to determine whether ATRX-deficient limb bud cells are more sensitive to replication stress, as has been reported for other cell types (Leung et al., 2013; Seah et al., 2008; Watson et al., 2013). 24 or 48 h after end of treatment, cell viability was assessed by MTT assays. γ-H2AX staining increased in a dose-dependent manner in response to HU treatment. However, we observed that cells of all genotypes showed a similar reduction in viability in response to HU (Figure 3-S6).

3.3.6 Reduced Forelimb Function and Shorter Stride Length in *Atrx* cKO Mice

To determine whether the brachydactyly phenotype observed in the *Atrx*(Prx1) cKO (Figure 3-7A) had any functional consequence, we evaluated various parameters of forelimb function and gait. We found that the maximal grip strength values of adult *Atrx*(Prx1) cKO mice were significantly lower than those of control littermates

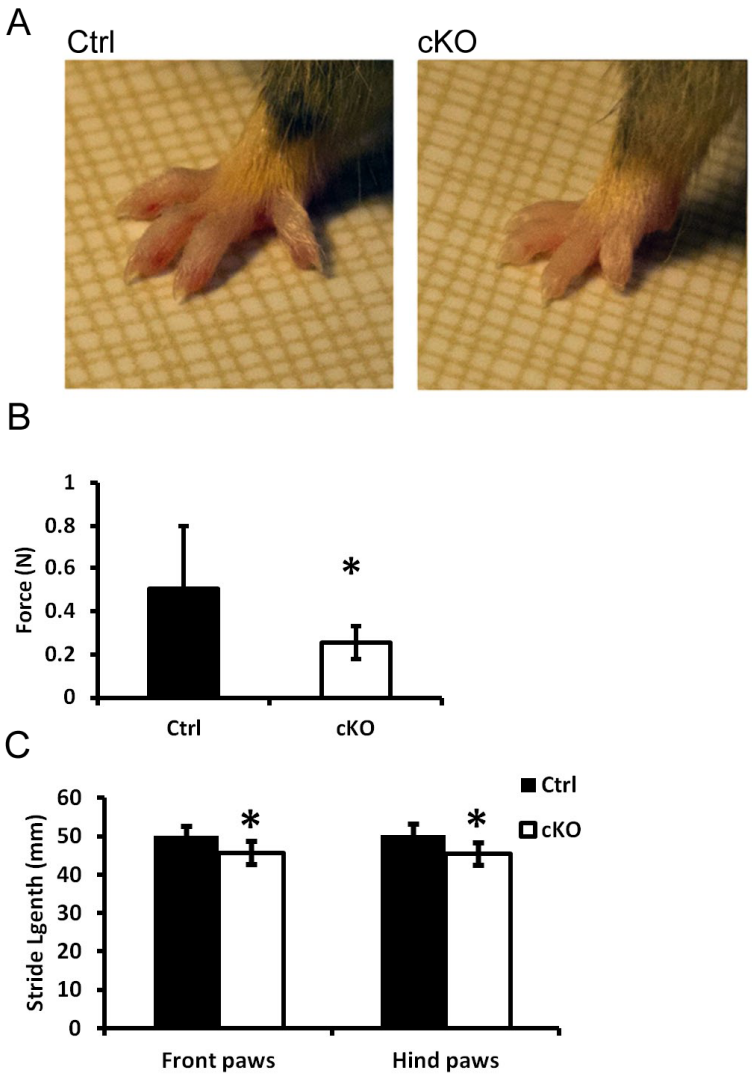


Figure 3-7 Reduced forelimb function at one year of age in *Atrx*(Prx1) cKO mice

(A) Characteristic forelimb appearance of Ctrl and *Atrx*(Prx1) cKO mice showing shortened fingers and a smaller paw. (B) Forelimb grip strength of control and mutant adult mice. Mutant mice exert significantly less force with their forelimbs before releasing the bar. Error bars = SD, N=5 $p < 0.05$, Student's T-test (C) Quantification of mouse gait parameters. Stride length of the fore- and hind-paws is shorter in *Atrx*(Prx1) cKO mice. Error bars = SD, N=5 $p < 0.05$, Student's T-test

(Figure 3-7B), reflecting a loss of forelimb function that coincides with shortening of the phalanges. Next, we utilized the Catwalk system to analyse gait parameters in freely ambulating mice. The spatial relationship between paw prints was used to determine stride length and walking pattern. Forelimb stride length was reduced in *Atrx*(Prx1) cKO mice compared to control littermates (45.65 mm ± 3.06 mm vs 50.09 mm ± 2.64 mm, $p < 0.05$,N=5 pairs Students T-test). Hind limb stride length was also reduced (45.45 mm ± 2.94 mm vs 50.23 mm ± 2.93 mm, $p < 0.05$, N=5 pairs Students T-test), despite the absence of any morphological changes in the hind limbs of mutant mice (Figure 3-7C). Conversely, the regularity index of footfall patterns did not vary between genotypes, indicating that mutants had a normal walking pattern and showed no instability or impairment in gait (Figure 3-S7A,B). Additionally, there was no significant variation in base of support or swing duration when walking (Figure 3-S7C,D).

3.4 Discussion

This study shows that loss of ATRX in early limb mesenchyme leads to brachydactyly and smaller digits, associated with DNA damage and apoptosis. Specifically, brachydactyly occurred as shortening of the distal phalanges of the forelimb, beginning at E15.5. *Atrx* has previously been deleted in the early developing cartilage without phenotypic consequence, raising questions about the cell types responsible for the skeletal abnormalities in ATR-X patients (Solomon et al., 2009). We demonstrate here that loss of ATRX in mesenchymal precursor cells prior to differentiation into cartilage gives rise to one skeletal phenotype observed in ATR-X syndrome patients. Furthermore, our model suggests that apoptosis in response to DNA damage is a new molecular and cellular mechanism responsible for brachydactyly. This is distinct from other forms of brachydactyly, many of which are due to mutations in key developmental signalling genes, such as components of the hedgehog, Wnt or TGFbeta family pathways or Hox genes (Gao et al., 2009; Minami et al., 2010).

This model, unlike loss of ATRX in developing cartilage (Solomon et al., 2009), deletes *Atrx* early in limb development and leads to a more severe phenotype. This suggests that ATRX function is more critical in mesenchymal precursor cells than in differentiated chondrocytes. In support of this model, we did not observe any DNA damage in our

cartilage-specific ATRX-deficient mice. In our experiments, the distal portions of the digit are first to show significant shortening, and during post-natal development the affected mice do not catch up to littermates.

One question addressed by this study is whether loss-of-function of ATRX has a direct effect in the developing skeleton. The observed brachydactyly is likely to be a direct effect of ATRX in the limb bud, rather than a secondary effect of ATRX loss in another tissue, as this model uses a cre-lox system specific for the early forelimb. The hind limbs were not affected in the mutant mice, which was expected since Prx-cre activity is limited in the hind limb compared to the forelimb (Logan et al., 2002). Our findings demonstrate that not only does loss of ATRX in the forelimb mesenchyme have direct effects on skeletal development, but it also has wide-reaching effects on the physiology of the adult animal. Adult mutant mice retain shortening on the distal phalanges, and have visibly smaller paws than control littermates. Furthermore, the digits in the paws are not flexed normally, and have reduced function. These mice have weaker grip strength due to the smaller digits, an indication that the shorter fingers are more difficult to bend and have limited strength. The reduced function is also observed in the gait of these animals, in which they maintain a normal pattern of walking but take significantly shorter steps.

Prior to the appearance of the phalange shortening, mutant mice display widespread DNA double-strand breaks in limb bud tissue as identified by increased γ-H2AX staining. Previous studies have shown that ATRX is required for maintaining structural integrity of telomeres and depletion of ATRX leads to telomere instability and fusions (Watson et al., 2013; Wong et al., 2010). However, in contrast to other models of ATRX deficiency, the DNA damage seen in limb bud cells in our study exhibit two specific characteristics. First, DNA damage occurs as one discrete dot in the nucleus, close to the nuclear periphery. This single focus is unusual, as γH2AX-postive DNA damage has a more speckled or diffuse appearance in other ATRX-null tissues (Huh et al., 2012; Watson et al., 2013; Wong et al., 2010). It is not clear at this point whether the signal we observe is from one chromosomal site or from an amalgamation of several damaged sites at the nuclear lamina. It has been shown in budding yeast that numerous domains containing DSBs can merge into one aggregate in the nucleus. In this manner, persistent DSBs and

telomeres can be sequestered to the periphery of the nucleus, a common location for dangerous and damaged DNA elements in the genome (Aten et al., 2004; Oza et al., 2009; Schober et al., 2009; Soutoglou et al., 2007). Large DNA damage foci have also been observed in pig skin cells following gamma irradiation: the damage appears initially at multiple sites but after 70 days can be seen as one or a few large nuclear foci (Ahmed et al., 2012). In mouse embryonic fibroblasts, the inner nuclear envelope proteins SUN1 and SUN2 have been shown to be important in DNA damage response and possibly play a role in nonhomologous end joining repair (Lei et al., 2012). Mammalian nuclear lamins themselves are also important in nuclear organization and cell-cycle response to DNA damage (Johnson et al., 2004). Thus, persistent DSBs within the nuclei of ATRX-depleted forelimb cells might be transported to the nuclear periphery, either for sequestering from the rest of the genome, repair or activation of cell cycle arrest.

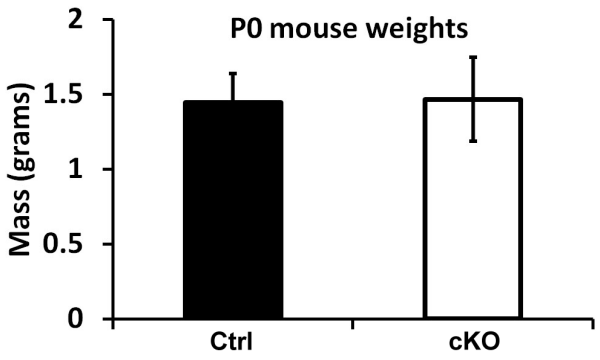
A second unusual feature of ATRX-deficient limb bud cells is that they are not hypersensitive to HU treatment while ATRX deficiency in neuroprogenitors and other cells leads to increased sensitivity to replication stress-inducing drugs (Conte et al., 2012; Watson et al., 2013). In the developing forelimb, the most affected cells at the distal end of the digit are highly proliferative and contribute to lengthening of the digits. Thus, one could have expected these cells to be very sensitive to replication stress, but our data show that loss of ATRX does not increase the sensitivity of these cells to HU treatment. In addition, the level of cell death observed in mutant digits is markedly lower than that seen in the ATRX null brain (Huh et al., 2012; Leung et al., 2013; Watson et al., 2013). The specific connection between loss of ATRX and DNA damage in limb bud cells therefore remains to be explored.

Our studies add to recent publications implicating epigenetic regulators in brachydactyly and skeletal development. Heterozygote mutations in the *HDAC4* gene, encoding a histone deacetylase, have been identified as a cause of brachydactyly type E, and translocation of a gene encoding a long non-coding RNA results in brachydactyly type E as well (Maass et al., 2012; Williams et al., 2010). Maybe most closely related to our studies, loss of the single-stranded DNA binding protein OBFC2B leads to growth delay and skeletal abnormalities due to accumulation of γ-H2AX and apoptosis in the pre-

cartilage condensations (Feldhahn et al., 2012). While OBFC2A, a homologue, is required in many other cell types (Feldhahn et al., 2012), it appears that the skeletal lineage has developed specific methods of dealing with increased levels of DNA damage during development. A genetic assault, such as the loss of ATRX, is still hazardous in the developing forelimb, but not to the same degree as in the CNS. Thus, our results suggest that the role of ATRX varies between cell types, or alternatively that different cell types utilize distinct mechanisms to maintain genomic integrity.

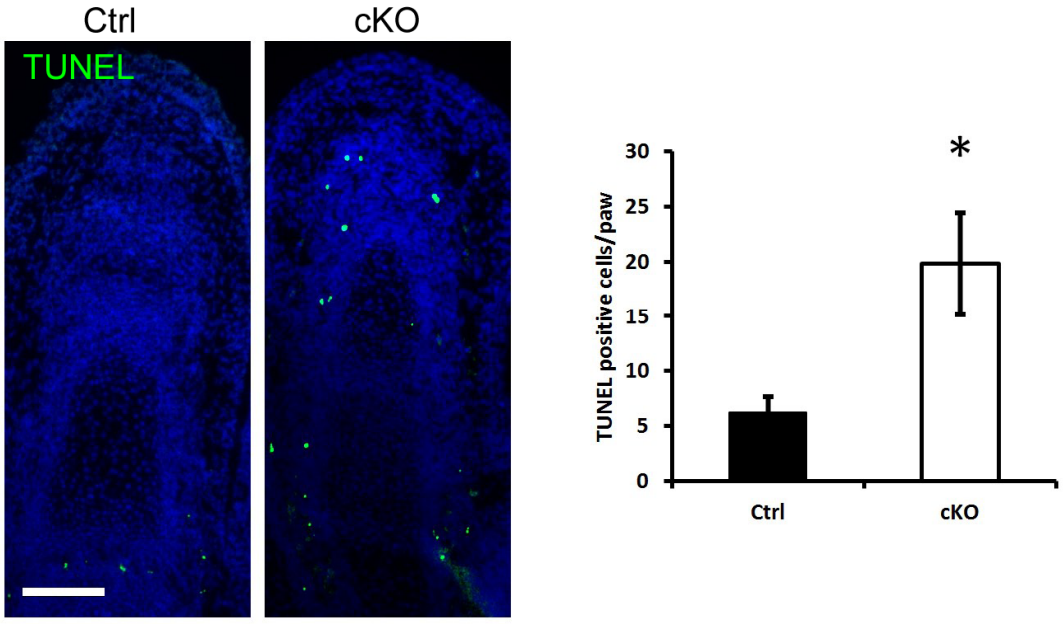
In summary, by using tissue-specific deletion of *Atrx*, we have recapitulated part of the skeletal phenotype of ATR-X syndrome and found a direct role for ATRX in preventing DNA damage and maintaining cell survival in the highly proliferative developing digit mesenchyme. Our data suggest that DNA damage-induced apoptosis of limb mesenchymal cells can result in brachydactyly, a mechanism that might be functionally relevant to additional brachydactylies and other skeletal dysplasias.

3.5 Supplementary Figures



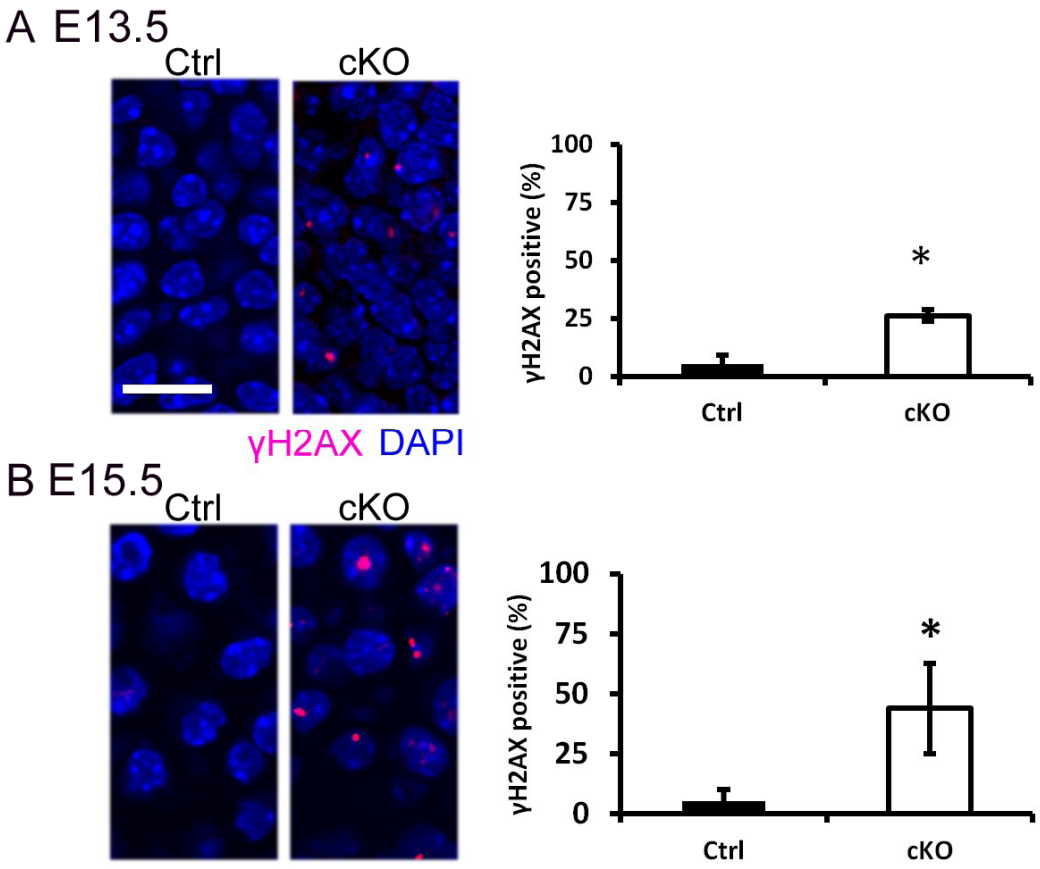
Supplementary Figure 3-1 *Atrx(*Prx1) cKO mice are born at normal weight.

No significant difference was observed between the weight of mutant and control littermates at birth. N = 5, Error bars = SD.



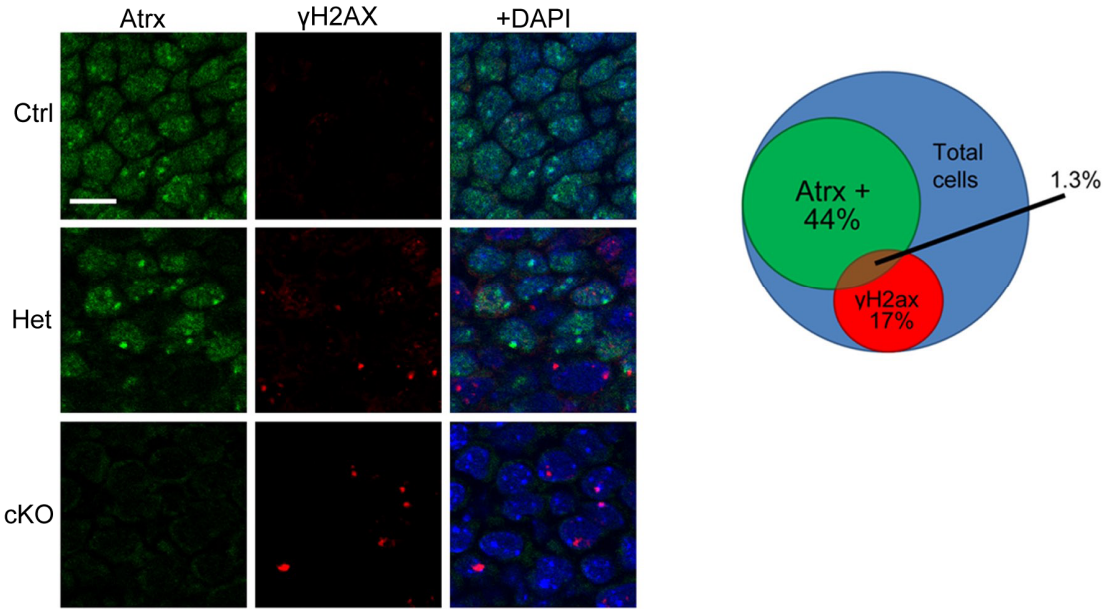
Supplementary Figure 3-2 ATRX-deficient limb buds have increased levels of DNA double strand breaks at E15.5

At E15.5 there is significantly more apoptotic nuclei per digit in the *Atrx*(Prx1) cKO Scale bar: 200 μm. N=3, * = $p < 0.05$ by Student's T-test,. Error bars = SEM.



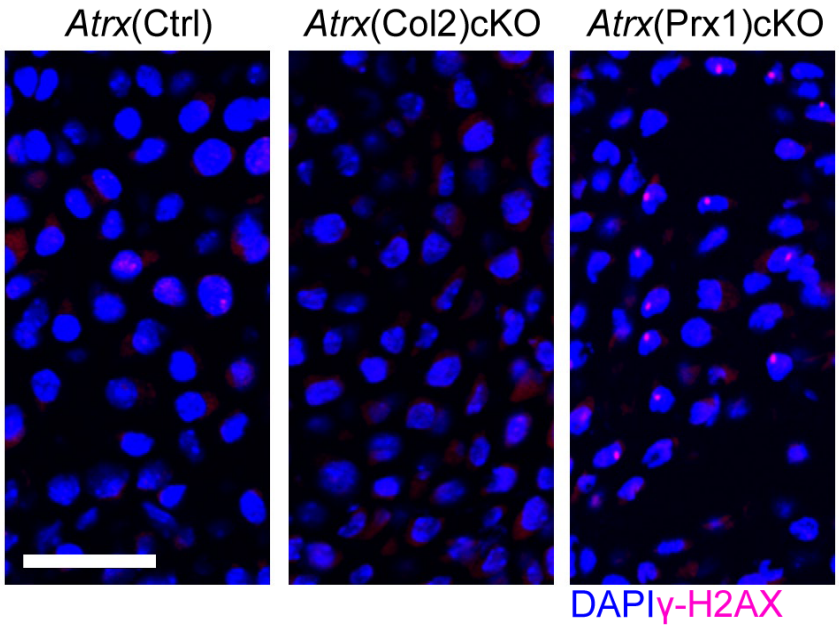
Supplementary Figure 3-3 ATRX-deficient digit rays have increased levels of DNA double strand breaks

Confocal imaging and quantification at (A) E13.5 and (B) E15.5 showed a significant increase in the number of cells harbouring γ-H2AX foci in the cartilaginous digit rays. Scale bar: 50 μm. N=4, * = $p < 0.05$ by Student's T-test,. Error bars = SD.



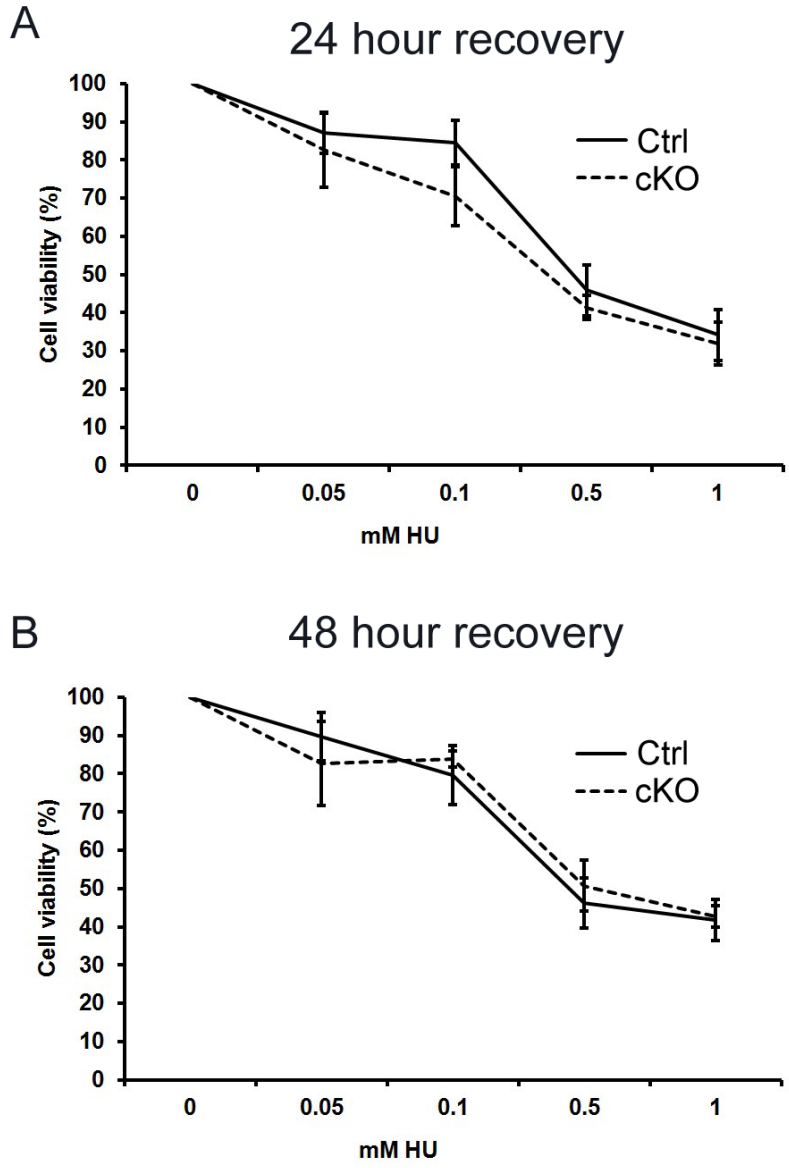
Supplementary Figure 3-4 Heterozygous female *Atrx*(Prx1) cKO female mice demonstrate DNA damage foci preferentially in ATRX-depleted cells

DNA damage is associated with ATRX-null cells in heterozygote female mice. Representative images of immunofluorescence staining of cells for ATRX (green) and γ-H2AX (red). Scale bar: 10 μm. Approximately half (44%) of the cells retained ATRX, but almost all the cells containing γ-H2AX foci were ATRX negative.



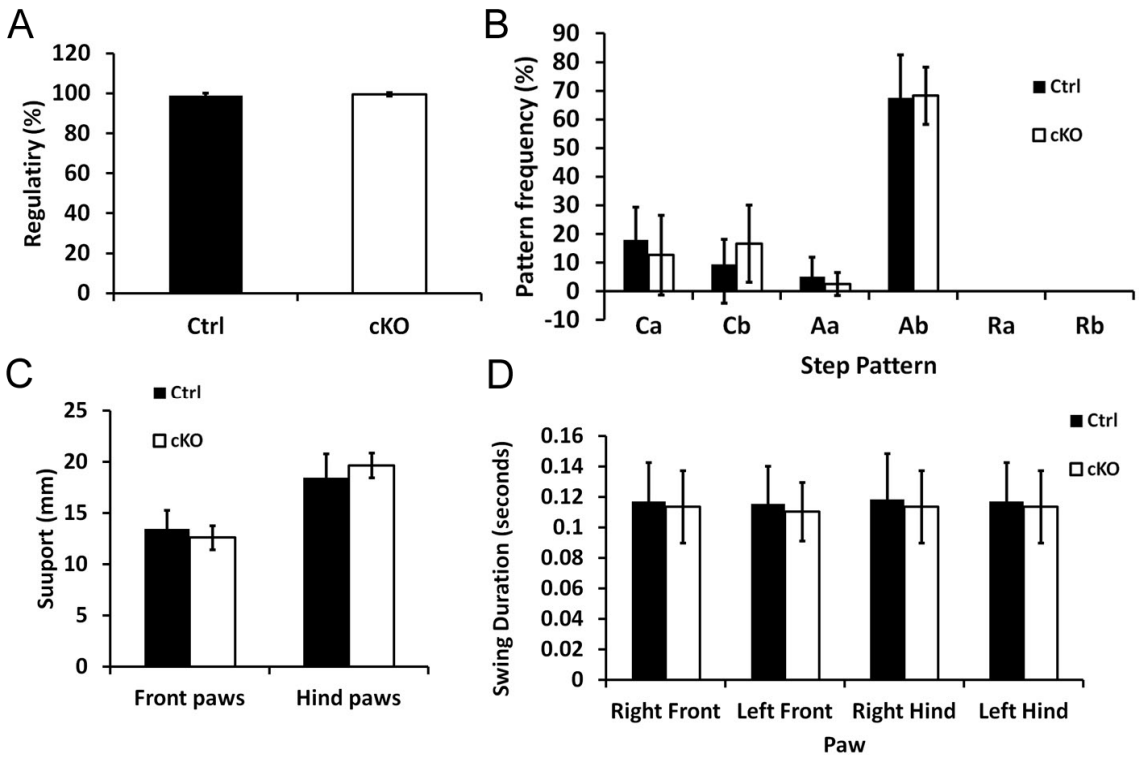
Supplementary Figure 3-5 γ-H2AX nuclear foci are not present in the nuclei of chondrocytes in cartilage-specific ATRX-deficient mice.

Ctrl and *Atrx(*Col2) cKO forelimb chondrocytes do not demonstrate large nuclear γ-H2AX nuclear foci, unlike *Atrx(*Prx1) cKO mice. Scale bar: 50 μm



Supplementary Figure 3-6 ATRX-deficient forelimb cells are not hypersensitive to hydroxyurea treatment.

Cultured cells from Ctrl and *Atrx(*Prx1) cKO embryonic forelimbs were treated with increasing concentrations of the fork-stalling compound hydroxyurea (HU) for 24h and allowed to recover for 1 day (A) or 2 days (B). Cells of all genotypes show similarly reduced viability after HU treatment, and ATRX-null cells did not display increased loss of viability.



Supplementary Figure 3-7 Gait parameters of instability and impairment are unaffected in *Atrx*(Prx1) cKO mice.

(A) Regularity index of mouse gait was unchanged between genotypes. (B) Step patterns of Ctrl and *Atrx*(Prx1) cKO mice, demonstrating that both mice mainly used a normal trotting gait (Ab pattern, in which diagonal legs move together and in phase). (C) Base of support between limbs was unaffected between genotypes. (D) All paws demonstrated a similar swing speed, indicating that each foot was in the air for a similar amount of time and no specific foot was being favoured. No difference in swing speed was observed between genotypes. Error Bars = SD, N = 5

3.6 References

Ahmed, E.A., Agay, D., Schrock, G., Drouet, M., Meineke, V., and Scherthan, H. (2012). Persistent DNA Damage after High Dose In Vivo Gamma Exposure of Minipig Skin. PLoS ONE *7*, e39521.

Aten, J.A., Stap, J., Krawczyk, P.M., van Oven, C.H., Hoebe, R.A., Essers, J., and Kanaar, R. (2004). Dynamics of DNA Double-Strand Breaks Revealed by Clustering of Damaged Chromosome Domains. Science *303*, 92-95.

Bagheri-Fam, S., Argentaro, A., Svingen, T., Combes, A.N., Sinclair, A.H., Koopman, P., and Harley, V.R. (2011). Defective survival of proliferating Sertoli cells and androgen receptor function in a mouse model of the ATR-X syndrome. Human Molecular Genetics *20*, 2213-2224.

Bérubé, N.G., Mangelsdorf, M., Jagla, M., Vanderluit, J., Garrick, D., Gibbons, R.J., Higgs, D.R., Slack, R.S., and Picketts, D.J. (2005). The chromatin-remodeling protein ATRX is critical for neuronal survival during corticogenesis. Journal of Clinical Investigation *115*, 258-267.

Conte, D., Huh, M., Goodall, E., Delorme, M., Parks, R.J., and Picketts, D.J. (2012). Loss of Atrx Sensitizes Cells to DNA Damaging Agents through p53-Mediated Death Pathways. PLoS ONE *7*, e52167.

Dhayalan, A., Tamas, R., Bock, I., Tattermusch, A., Dimitrova, E., Kudithipudi, S., Ragozin, S., and Jeltsch, A. (2011). The ATRX-ADD domain binds to H3 tail peptides and reads the combined methylation state of K4 and K9. Human Molecular Genetics *20*, 2195-2203.

Drané, P., Ouararhni, K., Depaux, A., Shuaib, M., and Hamiche, A. (2010). The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. Genes & Development *24*, 1253-1265.

Eisen, J.A., Sweder, K.S., and Hanawalt, P.C. (1995). Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. Nucleic Acids Research *23*, 2715-2723.

Feldhahn, N., Ferretti, E., Robbiani, D.F., Callen, E., Deroubaix, S., Selleri, L., Nussenzweig, A., and Nussenzweig, M.C. (2012). The hSSB1 orthologue Obfc2b is essential for skeletogenesis but dispensable for the DNA damage response in vivo. The EMBO Journal *31*, 4045-4056.

Gao, B., Hu, J., Stricker, S., Cheung, M., Ma, G., Law, K.F., Witte, F., Briscoe, J., Mundlos, S., He, L.*, et al.* (2009). A mutation in Ihh that causes digit abnormalities alters its signalling capacity and range. Nature *458*, 1196-1200.

Garrick, D., Sharpe, J.A., Arkell, R., Dobbie, L., Smith, A.J., Wood, W.G., Higgs, D.R., and Gibbons, R.J. (2006). Loss of Atrx affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues. PLoS Genetics *2*, e58.

Gibbons, R.J. (2006). Alpha thalassaemia-mental retardation, X linked. Orphanet Journal of Rare Diseases *1*.

Gibbons, R.J., Brueton, L., Buckle, V.J., Burn, J., Clayton-Smith, J., Davison, B.C.C., Gardner, R.J.M., Homfray, T., Kearney, L., Kingston, H.M.*, et al.* (1995a). Clinical and hematologic aspects of the X-linked α-thalassemia/mental retardation syndrome (ATR-X). The American Journal of Human Genetics *55*, 288-299.

Gibbons, R.J., and Higgs, D.R. (2000a). Molecular-clinical spectrum of the ATR-X syndrome. The American Journal of Human Genetics *97*, 204-212.

Gibbons, R.J., McDowell, T.J., Raman, S., O'Rourke, D.M., Garrick, D., Ayyub, H., and Higgs, D.R. (2000b). Mutations in ATRX, encoding a SWI/SNF-like protein,cause diverse changes in the pattern of DNA methylation. Nature Genetics *24*, 361-371.

Gibbons, R.J., Picketts, D.J., Villard, L., and Higgs, D.R. (1995b). Mutations in a putative global transcriptional regulator cause X-linked mental retardation with alpha-thalassemia (ATR-X syndrome). Cell *80*, 837-845.

Halawani, D., Mondeh, R., Stanton, L.A., and Beier, F. (2004). p38 MAP kinase signaling is necessary for rat chondrosarcoma cell proliferation. Oncogene *23*, 3726-3731.

Huh, M.S., Price O'Dea, T., Ouazia, D., McKay, B.C., Parise, G., Parks, R.J., Rudnicki, M.A., and Picketts, D.J. (2012). Compromised genomic integrity impedes muscle growth after Atrx inactivation. Journal of Clinical Investigation *122*, 4412-4423.

Johnson, B.R., Nitta, R.T., Frock, R.L., Mounkes, L., Barbie, D.A., Stewart, C.L., Harlow, E., and Kennedy, B.K. (2004). A-type lamins regulate retinoblastoma protein function by promoting subnuclear localization and preventing proteasomal degradation. Proceedings of the National Academy of Sciences of the United States of America *101*, 9677-9682.

Kuo, L.J., and Yang, L.-X. (2008). γ-H2AX - A Novel Biomarker for DNA Double-strand Breaks. In Vivo *22*, 305-309.

Lei, K., Zhu, X., Xu, R., Shao, C., Xu, T., Zhuang, Y., and Han, M. (2012). Inner Nuclear Envelope Proteins SUN1 and SUN2 Play a Prominent Role in the DNA Damage Response. Current Biology *22*, 1609-1615.

Leung, J.W.-C., Ghosal, G., Wang, W., Shen, X., Wang, J., Li, L., and Chen, J. (2013). Alpha thalassemia/mental retardation syndrome X-linked gene product ATRX is required for proper replication restart and cellular resistance to replication stress. Journal of Biological Chemistry *288*, 6342-6350.

Lewis, P.W., Elsaesser, S.J., Noh, K.-M., Stadler, S.C., and Allis, C.D. (2010). Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. Proceedings of the National Academy of Sciences *107*, 14075-14080.

Logan, M., Martin, J.F., Nagy, A., Lobe, C., Olson, E.N., and Tabin, C.J. (2002). Expression of Cre recombinase in the developing mouse limb bud driven by a Prxl enhancer. genesis *33*, 77-80.

Maass, P.G., Rump, A., Schulz, H., Stricker, S., Schulze, L., Platzer, K., Aydin, A., Tinschert, S., Goldring, M.B., Luft, F.C.*, et al.* (2012). A misplaced lncRNA causes brachydactyly in humans. Journal of Clinical Investigation *122*, 3990-4002.

Meyer, O.A., Tilson, H.A., Byrd, W.C., and Riley, M.T. (1979). A method for the routine assessment of fore- and hindlimb grip strength of rats and mice. Neurobehavioral Toxicology and Teratology *1*, 233-236.

Minami, Y., Oishi, I., Endo, M., and Nishita, M. (2010). Ror-family receptor tyrosine kinases in noncanonical Wnt signaling: Their implications in developmental morphogenesis and human diseases. Developmental Dynamics *239*, 1-15.

Otani, J., Nankumo, T., Arita, K., Inamoto, S., Ariyoshi, M., and Shirakawa, M. (2009). Structural basis for recognition of H3K4 methylation status by the DNA methyltransferase 3A ATRX-DNMT3-DNMT3L domain. EMBO Reports *10*, 1235-1241.

Oza, P., Jaspersen, S.L., Miele, A., Dekker, J., and Peterson, C.L. (2009). Mechanisms that regulate localization of a DNA double-strand break to the nuclear periphery. Genes & Development *23*, 912-927.

Picketts, D.J., Higgs, D.R., Bachoo, S., Blake, D.J., Quarrell, O.W., and Gibbons, R.J. (1996). ATRX encodes a novel member of the SNF2 family of proteins: mutations point to a common mechanism underlying the ATR-X syndrome. Human Molecular Genetics *5*, 1899-1907.

Ritchie, K., Seah, C., Moulin, J., Isaac, C., Dick, F., and Bérubé, N.G. (2008). Loss of ATRX leads to chromosome cohesion and congression defects. The Journal of Cell Biology *180*, 315-324.

Schober, H., Ferreira, H., Kalck, V., Gehlen, L.R., and Gasser, S.M. (2009). Yeast telomerase and the SUN domain protein Mps3 anchor telomeres and repress subtelomeric recombination. Genes & Development *23*, 928-938.

Seah, C., Levy, M.A., Jiang, Y., Mokhtarzada, S., Higgs, D.R., Gibbons, R.J., and Berube, N.G. (2008). Neuronal death resulting from targeted disruption of the Snf2 protein ATRX is mediated by p53. Journal of Neuroscience *28*, 12570-12580.

Solomon, L.A., Li, J.R., Berube, N.G., and Beier, F. (2009). Loss of ATRX in chondrocytes has minimal effects on skeletal development. PLoS ONE *4*, e7106.

Soutoglou, E., Dorn, J.F., Sengupta, K., Jasin, M., Nussenzweig, A., Ried, T., Danuser, G., and Misteli, T. (2007). Positional stability of single double-strand breaks in mammalian cells. Nature Cell Biology *9*, 675-682.

Ulici, V., Hoenselaar, K.D., Agoston, H., McErlain, D.D., Umoh, J., Chakrabarti, S., Holdsworth, D.W., and Beier, F. (2009). The role of Akt1 in terminal stages of endochondral bone formation: Angiogenesis and ossification. Bone *45*, 1133-1145.

Vidaković, M., Koester, M., Goetze, S., Winkelmann, S., Klar, M., Poznanović, G., and Bode, J. (2005). Co-localization of PARP-1 and lamin B in the nuclear architecture: A halo-fluorescence- and confocal-microscopy study. Journal of Cellular Biochemistry *96*, 555-568.

Wada, T., Sugie, H., Fukushima, Y., and Saitoh, S. (2005). Non-skewed X-inactivation may cause mental retardation in a female carrier of X-linked α-thalassemia/mental retardation syndrome (ATR-X): X-inactivation study of nine female carriers of ATR-X. The American Journal of Human Genetics *138A*, 18-20.

Wang, G., Woods, A., Agoston, H., Ulici, V., Glogauer, M., and Beier, F. (2007). Genetic ablation of Rac1 in cartilage results in chondrodysplasia Developmental Biology *306*, 612-623

Watson, L.A., Solomon, L.A., Li, J.R., Jiang, Y., Edwards, M., Shin-Ya, K., Beier, F., and Berube, N.G. (2013). Atrx deficiency induces telomere dysfunction, endocrine defects, and reduced life span. Journal of Clinical Investigation *123*, 2049–2063.

Williams, S.R., Aldred, M.A., Der Kaloustian, V.M., Halal, F., Gowans, G., McLeod, D.R., Zondag, S., Toriello, H.V., Magenis, R.E., and Elsea, S.H. (2010). Haploinsufficiency of HDAC4 Causes Brachydactyly Mental Retardation Syndrome, with Brachydactyly Type E, Developmental Delays, and Behavioral Problems. The American Journal of Human Genetics *87*, 219-228.

Wong, L.H., McGhie, J.D., Sim, M., Anderson, M.A., Ahn, S., Hannan, R.D., George, A.J., Morgan, K.A., Mann, J.R., and Choo, K.H.A. (2010). ATRX interacts with H3.3 in maintaining telomere structural integrity in pluripotent embryonic stem cells. Genome Research *20*, 351-360.

Xue, Y., Gibbons, R., Yan, Z., Yang, D., McDowell, T.L., Sechi, S., Qin, J., Zhou, S., Higgs, D., and Wang, W. (2003). The ATRX syndrome protein forms a chromatin-remodeling complex with Daxx and localizes in promyelocytic leukemia nuclear bodies. Proceedings of the National Academy of Sciences *100*, 10635-10640.

Chapter 4

4 Loss of Atrx Does Not Confer Susceptibility to Osteoarthritis

The chromatin remodelling protein ATRX is associated with the rare genetic disorder ATR-X syndrome. This syndrome includes developmental delay, cognitive impairment and a variety of skeletal deformities. *Atrx* plays a role in several basic chromatin-mediated cellular events including DNA replication, telomere stability, gene transcription, and chromosome congression and cohesion during cell division. We have used a loss-of-function approach to directly investigate the role of Atrx in the adult skeleton in three different models of selective *Atrx* loss. We specifically targeted deletion of *Atrx* to the forelimb mesenchyme, to cartilage and to bone-forming osteoblasts. We previously demonstrated that loss of Atrx in forelimb mesenchyme causes brachydactyly while deletion in chondrocytes had minimal effects during development. We now show that targeted deletion of *Atrx* in osteoblasts causes reduced body size but does not recapitulate most of the skeletal phenotypes seen in ATR-X syndrome patients. In adult mice from all three models, we find that joints lacking Atrx are not more susceptible to osteoarthritis, as determined by OARSI scoring and immunohistochemistry. These results indicate that while Atrx plays limited roles during early stages of skeletal development, deficiency of the protein in adult tissues does not confer susceptibility to osteoarthritis.

4.1 Introduction

Osteoarthritis (OA) is a degenerative joint disease to which there is no cure. It is characterized by the degeneration of articular cartilage and changes in other joint tissues including subchondral bone and synovium. Cartilage is maintained by a balance of both anabolic and catabolic activities. OA occurs when these processes are in disequilibrium and catabolism outweighs anabolic repair (Goldring et al., 2009). Osteoarthritis can be triggered by many factors including diet, injury, strain and genetic abnormalities (Aigner et al., 2007; Aspden, 2008; Aspden, 2011; Goldring et al., 2009; Wang et al., 2011). However, the molecular mechanisms driving disease onset and progression are incompletely understood.

Alterations in epigenetic mechanisms affecting gene expression have been previously reported in articular chondrocytes (Aspden, 2008). A recent study has linked genetic variants of *DOT1L*, an evolutionarily conserved histone methyltransferase required for chondrogenic differentiation, to increased susceptibility to osteoarthritis (Castaño Betancourt et al., 2012). Alterations in expression of histone acetylases and deacetylases has also been associated with arthritis (Huber et al., 2007). The ageing-related gene *SirT1* is of particular interest, as it codes for a protein capable of deacetylating histones and other proteins (Dvir-Ginzberg et al., 2013). Expression of SirT1 in chondrocytes is associated with increased survival and down-regulation of the proapoptotic protein PTP1B associated with OA (Gagarina et al., 2010). Age-related diseases, as well as normal aging, are frequently influenced by changes in chromatin structure, leading to deleterious effects on cell and tissue function (Sedivy et al., 2008).

Hypomorphic mutations causing dysfunction of the ATRX chromatin remodeling protein can lead to various skeletal deformities, including dwarfism, spine deformities and malformation of the hands and feet (Gibbons et al., 1995a; Gibbons et al., 2000). These defects occur in conjunction with developmental delay, psychomotor and mental retardation, distinct facial features, urogenital abnormalities and α-thalassemia (Gibbons et al., 1995a). Radiological analysis in a few cases has shown that individuals with *ATRX* mutations show generalised retardation in skeletal maturation (Gibbons et al., 1995a). ATRX contains two conserved domains where the majority of disease-related mutations are located. The N-terminus contains a plant homeodomain-type (PHD-type) zinc finger that acts as a histone reader module by mediating binding to specific post-translational modifications on histone H3 (Dhayalan et al., 2011; Otani et al., 2009; Wong et al., 2010). Towards the C-terminus, ATRX contains a Sucrose Non Fermenting 2 (SNF2)-type DNA-dependent ATPase domain that functions to remodel chromatin (Eisen et al., 1995; Gibbons et al., 1995b; Picketts et al., 1996). The functional full-length protein has been shown to play an important role in chromosomal integrity, maintaining organization and sister chromatid cohesion during cell division (Ritchie et al., 2008).

While there have been various developmental studies that show severe effects of tissue-specific *Atrx* deficiency (Bérubé et al., 2005; Garrick et al., 2006; Huh et al., 2012;

Medina et al., 2009) cartilage-specific inactivation of the *Atrx* gene in mice does not result in major phenotypes. (Solomon et al., 2009). On the other hand, loss of *Atrx* in the forebrain and pituitary of mice leads to features of aging including low bone mass, loss of subcutaneous fat, and clouded ocular lenses (Watson et al., 2013), prompting us to ask whether Atrx is important in aging skeletal elements and joints. Since Atrx is highly expressed in chondrocytes (Solomon et al., 2009), it could be a potential epigenetic regulator of specific genes involved in healthy cartilage maintenance and its loss may lead to diseases such as osteoarthritis. This study examined the role of ATRX in three models of *Atrx* deficiency and assessed the onset and progression of osteoarthritis using Osteoarthritis Research Society International (OARSI) histopathology guidelines and molecular markers of OA.

4.2 Materials and Methods

4.2.1 Animals

All animals studies were approved by the Council on Animal Care at the University of Western Ontario, protocol 2007-045.

The cartilage-specific *Atrx* depleted Atrx (Col2) cKO ($Atrx^{Col2}$) mice were generated as described previously (Solomon et al., 2009). Both wild-type and $Atrx^{Col2}$ mice were maintained until the age of two years. Bone-specific Atrx depleted mice were generated using an osteoblast-specific cre mouse (Liu et al., 2004) to generate Atrx(Col1) cKO ($Atrx^{Col1}$) mice. Forelimb specific deletion of Atrx was generated as described previously using the forelimb-specific Prx1-cre to generate Atrx (Prx1) cKO mice ($Atrx^{Prx}$) (Logan et al., 2002; Solomon et al., 2013).

PCR genotyping from ear tissue for *cre* and *Atrx* as described previously (Solomon et al., 2009). $Atrx^{floxed}$ and *Cre* alleles were confirmed as previously described (Seah et al., 2008; Terpstra et al., 2003). A 1.5 kb fragment containing the neo cassette within the *Atrx* floxed allele was identified with one set of primers (5′-GATCGGCCATTGAACAAGAT-3′ and 5′-ATA GGT CGG CGG TTC AT-3′) whereas the wild-type allele was detected with another set (5′-CCC GAG TAT CTG GAA GAC AG-3′and 5′-ATA GGT CGG CGG TTC AT-3′). Primers (5′-CCT GGA AAA TGC TTC

TGT CC-3′) and (5′-CAG GGT GTT ATA AAC AAT CCC-3′) were used to amplify a common 300 base pair fragment for all three versions of the *cre* gene. PCR conditions were as follows: 95°C for 3 min (95°C for 30 s, 55°C for 45 s, and 72°C for 1 min) × 36, 72°C for 10 min for Cre , 95°C for 3 min (95°C for 30 s, 55°C for 1 min, and 72°C for 5 min) × 36, 72°C for 10 min for *Atrx*.

4.2.2 Histological Staining of Bone and Joint Sections

Limbs were dissected from *Atrx*-depleted mice upon sacrifice and processed for histological analyses by Picrosirius Red, safranin-O and immunohistochemical staining as described (Appleton et al., 2007; Wang et al., 2007; Yan et al., 2010). For histological analyses, specimens were fixed in 4% paraformaldehyde, decalcified with 5% EDTA, paraffin embedded and sectioned at 5 μm. For immunohistochemistry, samples were examined in dewaxed paraffin–embedded sections. Following dehydration, sections were blocked in hydrogen peroxide followed by 5% goat serum, and incubated with a polyclonal rabbit antibodies to MMP13 (Santa Cruz), aggrecan fragments (MMP cleaved, N-terminus FFGVG neoepitope, Millipore), Atrx (Prestige Antibodies, Sigma-Aldrich) or type II collagen (Santa Cruz) according to manufacturer's instructions. Following overnight incubation at 4°C sections were incubated with HRP–conjugated secondary goat antibody against rabbit IgG and detected by staining with DAB (Dako). To quantify trabecular area, sections were stained for one hour in 0.1% Picrosirius Red. Using imageJ software, an area of interest (AOI) was set from the chondro-osseous junction to 200 μm below the growth plate in the trabecular bone area of the mineralised zone, as described previously (Watson et al., 2013).

4.2.3 Histological End-Stage Analysis

Sections from a minimum of three independent litters were stained with Safranin-O and fast green, and graded according to the Osteoarthritis Research Society International (OARSI) scoring system (Glasson et al., 2010). OA scores were assigned by two blinded observers and total score was calculated by averaging the grade and stage values for each slide (a minimum score of 0 represents no OA degradation and a score of 6 represents the

maximum degree of OA). Maximal OARSI grade was determined for each joint based on the surface displaying the highest OARSI score in each section.

4.2.4 Skeletal Stains and Measurements

Live mice were weighed at P0 and P21. Euthanized animals were skinned and eviscerated, fixed overnight in 95% ethanol followed by overnight fixation in acetone for Alizarin Red/Alcian Blue staining. Whole skeletons were stained for 7–10 days (0.05% Alizarin Red, 0.015% Alcian Blue, 5% acetic acid in 70% ethanol) (Wang et al., 2007). Skeletons were cleared in 2% w/w KOH. Bones were imaged on an Olympus SP-57OUZ camera. Limb bones and skulls from at least three independent littermate pairs were measured using a dissecting microscope with a ruler.

4.2.5 Microcomputed Tomography

Male $Atrx^{Col1}$ and control littermate mice were sacrificed at weaning (21 days of age), skinned and eviscerated, followed by fixation in formalin. Whole bodies were imaged using a scanner (eXplore Locus MicroCT; GE Healthcare) at 120 kV and 20 mA, with a 0.154 mm3 voxel resolution with a total of 900 slices per scan. Bone mineral density (BMD), cortical thickness and trabecular numbers were calculated using the Microview 3D visualization and analysis software (MicroView, Version 2.1.2, GE Healthcare Biosciences) (Ulici et al., 2009).

4.2.6 Osteoblast Isolation and Differentiation

Calvarial cells were isolated from 8-10 day old $Atrx^{Col1}$ mice using sequential collagenase digestion, as previously described (Bellows et al., 1989; Panupinthu et al., 2008). Briefly, calvaria from control and mutant mice were dissected, scraped clean of mesenchyme by micro dissection, and digested through three changes of collagenase. Pooled cells from the latter two changes of collagenase were plated on six-well plates at a density of 1.5×10^4 cells/cm^2 in culture medium. After three days, cells were transferred to high density micromass cultures in 24-well plates, with each well containing 5×10^4 cells in a 150-µl drop of culture medium. After 12 hours, culture medium was added to the micromass

cultures. Culture media was supplemented with 50 μg/ml ascorbic acid and 2 mM β-glycerophosphate, and cultures were maintained for up to 4 weeks.

4.2.7 Analyses of Alkaline Phosphatase Activity and Mineral Deposition

Calvarial micromass cultures were fixed in 4% PFA overnight at 4°C. Alkaline phosphatase activity was assayed using a mixture of 1.25 mM Fast Red B salt in 0.1 M Tris-HCl, pH 8, and 0.25 mM naphthol AS-MX phosphate, protected from light for one hour. Mineralised nodules were visualised following ALP staining using 2.5% silver nitrate solution for 1 h with exposure to light.

4.3 Results

4.3.1 Skeletal Phenotypes of Adult *Atrx*^{col2} Mice

To determine if *Atrx* played a direct role in skeletal development, mice with cartilage-specific inactivation of *Atrx* were generated utilizing the Cre-LoxP system. Female mice previously engineered with *LoxP* sites flanking exon 18 of *Atrx* (Bérubé et al., 2005) were crossed with male mice expressing Cre recombinase under the control of the mouse collagen II (*Col2a1*) promoter (Terpstra et al., 2003). Cre-positive male offspring lacked Atrx in the developing cartilage but developed normally with only minor defects in skeletogenesis (Solomon et al., 2009). *Atrx*^{Col2} mice were born at normal Mendelian ratios and did not demonstrate defects in skeletal size or growth plate morphology at birth or weaning (Solomon et al., 2009).

To examine if embryonic depletion of *Atrx* in the developing cartilage contributes to reduced cartilage integrity later in life, two-year old *Atrx*^{Col2} mice were examined for signs of osteoarthritis and other skeletal defects. No significant weight difference was observed between control and mutant mice at two years of age (Ctrl: 36.57 ± 6.38 grams, *Atrx*^{Col2}: 31.56 ± 8.51 grams). Paraffin sections of knee joints from control and mutant *Atrx*^{Col2} mice were assessed for osteoarthritis using the OARSI histopathology guidelines (Glasson et al., 2010) on tissue sections stained for cartilage proteoglycan with safranin-O. In both genotypes, cartilage appeared relatively healthy with only minor degeneration.

Maximal OARSI score of the knee joint was not different in controls compared to *Atrx*^{Col2} mice. Control mice had a maximal knee score 1.70 and mutant mice had a maximal score of 1.53 (p= 0.89) (Figure 4-1A). *Atrx*^{Col2} mice did not show a significant increase in fibrillation and fissuring of the articular surfaces. Absence of ATRX protein in articular cartilage was confirmed by immunohistochemistry using a rabbit anti-ATRX antibody (Prestige Antibodies, Sigma-Aldrich) on knee joint sections (Figure 4-1B).

4.3.2 Mmp13, Aggrecan Fragment and Type II Collagen Levels are Normal in *Atrx*^{col2} Mice

Several molecular changes have been associated with OA. MMP13 (matrix metalloproteinase 13) plays a catabolic role in the progression of cartilage degeneration and cleaves collagens, gelatin, and aggrecan (Fosang et al., 1996; Wang et al., 2011). Aggrecan is an extracellular matrix protein and its degradation into fragments is accelerated in OA (Pollard et al., 2008). Finally, type II collagen is cleaved by a number of MMPs including MMP13. In arthritic joints, loss of the collagen matrix is accelerated, leading to erosion of the articular surface (Glasson et al., 2010).

Using immunohistochemistry, we detected no visible difference in MMP13 expression in tibia articular cartilage between control and mutant mice. MMP13 expression was quantified as a percentage of MMP13 positive cells to total cells in 1000 pixel wide sections of articular cartilage. Statistical analysis confirmed that there was no significant difference in MMP13 levels between mutants and control littermates (35.7% vs. 39.3% respectively, p= 0.451) (Supplementary Figure 4-1A,B). Tibial aggrecan fragment levels was also not altered between control and *Atrx*^{Col2} mice as revealed by IHC analysis. Control and knockout mice were not significantly different in the percentage of aggrecan fragment-positive cells versus total cells in articular cartilage(34.02% vs. 22.87%, p= 0.074) (Supplementary Figure 4-1C, D). Upon immunostaining for Collagen II, we observed that the mean thickness of the collagen II-positive zone of the articular cartilage was not significantly different when comparing the tibiae of control and mutant mice (87.993 μm vs. 90.065 μm, p= 0.837). There was also no significant difference in the

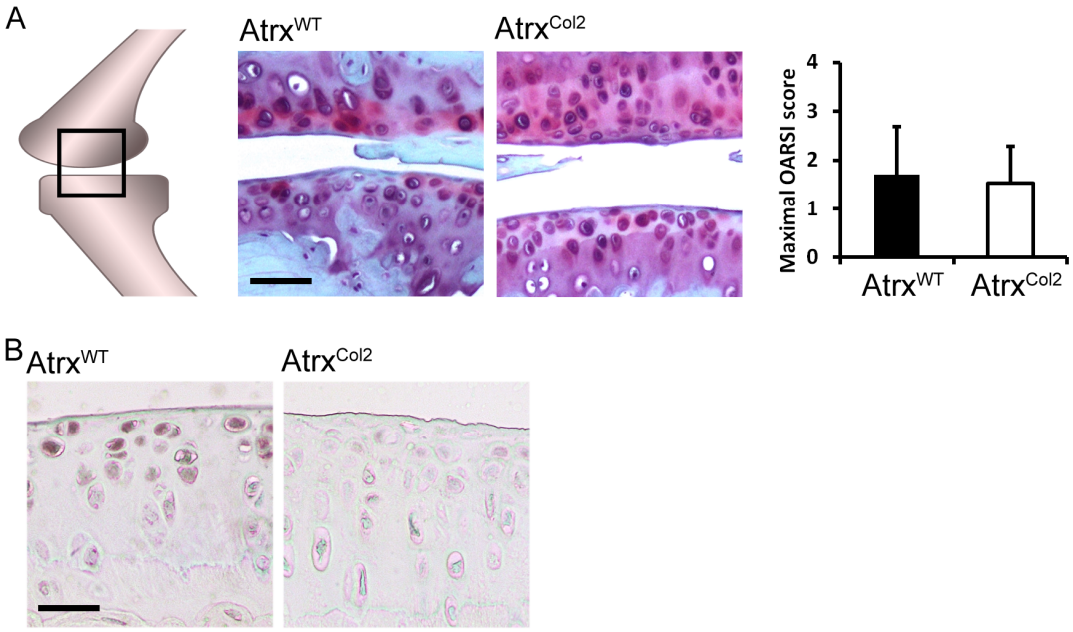


Figure 4-1 Characterisation of joint morphology in two year-old *Atrx^{Col2}* mice.

A) Knee sections were stained with safranin-O to detect the presence of proteoglycans. Minor fibrillation and fissuring of the tibial and femoral articular surfaces was observed in both genotypes, demonstrating mild osteoarthritis in both genotypes. OARSI scoring confirmed the absence of significant differences between genotypes. Sections Scale bar = 96 µm. B) Immunohistological staining of articular cartilage, demonstrating that ATRX protein is present in normal aged articular cartilage, but absent from the nuclei of mutant chondrocytes. Scale bar = 48 µm.

thickness of collagen II positive articular cartilage between the femurs of controls and mutant mice (82.080 μm vs. 81.142 μm, p= 0.923)(Supplementary Figure 4-1E,F).

4.3.3 Loss of ATRX During Forelimb Development does Not Lead to Forelimb Osteoarthritis

Atrx^{Prx1} mice were previously characterised and display brachydactyly and abnormal gait (Solomon et al., 2013). We now examined the joints to determine if structural variations in the limb might alter kinematics and thus lead to joint disease. Forelimb and hindlimb joints from *Atrx*^{Prx1} mice were examined for osteoarthritis at two years of age (Figure 4-2). Although the forelimbs of *Atrx*^{Prx1} are derived from ATRX-depleted mesenchyme, no difference was seen in the articular cartilage of the forelimb joint (Figure 4-2A). Control animals had a mean score of 1.32 and mutants had a mean score of 1.50 (p= 0.82). Similar to other models of ATRX loss in the skeleton, *Atrx*^{Prx1} mice do not demonstrate knee osteoarthritis, with a maximal OA score of 2.18 in mutant knees and 1.78 in control littermates (p=0.69) (Figure 4-2B). Loss of ATRX in articular cartilage derived from ATRX-deficient forelimb mesenchyme was confirmed by staining with IHC (Figure 4-2C).

4.3.4 *Atrx*^{col1} Mice Exhibit Reduced Growth Without Growth Plate Abnormalities

To examine the role of ATRX in bone-forming osteoblasts, the osteoblast-specific Col1a1-Cre driver line was utilized (Liu et al., 2004). *Atrx*^{Col1} mice were born at normal Mendelian ratios, showing that loss of *Atrx* in the osteoblast does not lead to embryonic or perinatal lethality (Figure 4-3A). In the neonatal period *Atrx*^{Col1} mice exhibited reduced growth compared to their control littermates, and at weaning they were smaller and had shorter limbs (Figure 4-3B). Bone length measurements of the appendicular skeleton showed that the forelimb long bones of *Atrx*^{Col1} mice (ulna, radius, humerus) are significantly shorter than those of controls, while the shortening of femur and tibia was not statistically significant (Figure 4-3C).

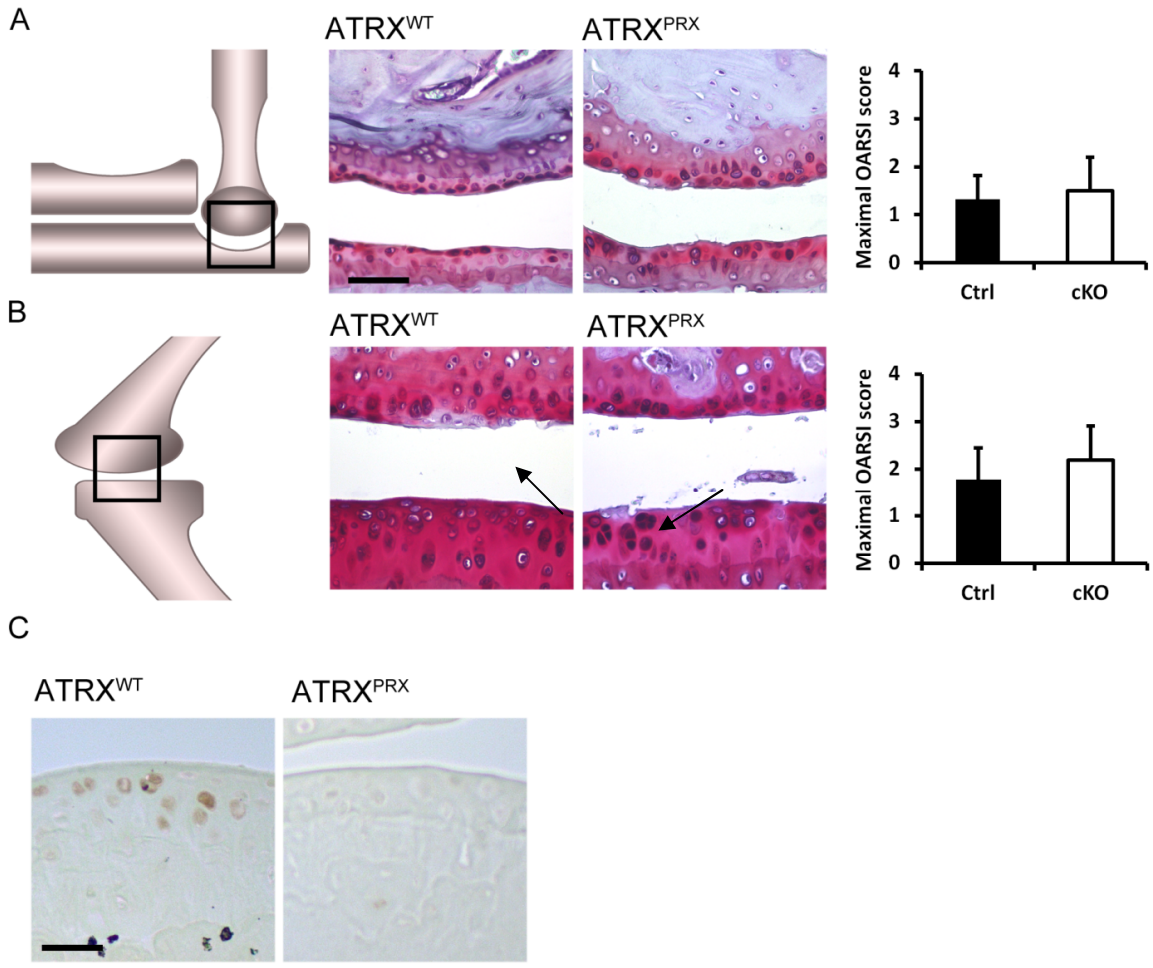


Figure 4-2 Characterisation of joint osteoarthritis in *Atrx*^{Prx1} mice.

A) Knee and elbow sections were stained with safranin-O to detect the presence of proteoglycans. No change was observed in the articular surfaces was observed in k of control or mutant mice, and no difference was seen between genotypes. Scale bar = 96 µm. B) Integrity of the knee was similar in control and mutant mice, with minor fibrillation and fissuring of the articular surface (Arrows). C) Immunohistological staining of articular cartilage from the elbows of mice, demonstrating that ATRX protein is present in normal aged articular cartilage, but absent from the nuclei of chondrocytes derived from ATRX deficient forelimb mesenchyme. Scale bar = 48 µm.

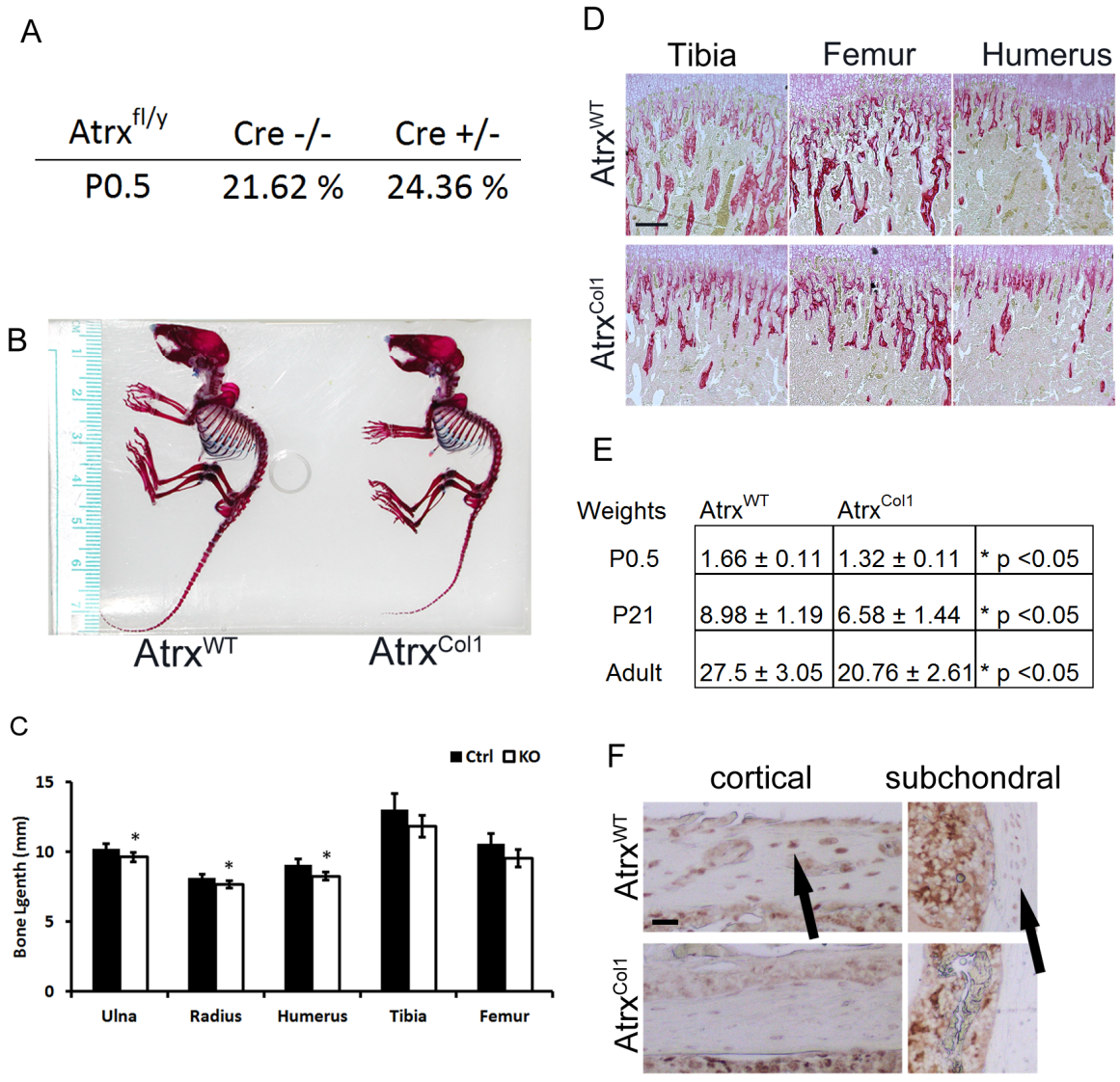


Figure 4-3 *Atrx*^{Col1} mice exhibit reduced growth without growth plate abnormalities

A) Mice lacking Atrx in osteoblasts are born at normal Mendelian ratios. B) Overall skeletal morphology of *Atrx*^{Col1} examined by alican blue/alizarin red stain reveals reduced overall size and shortened limbs. C) Longitudinal measurements of the bones of the axial skeleton demonstrate that all bones in the mutant are shortened, and forelimb bones show significant shortening compared to controls N=5 (* p<0.05) Error bars = SD. D) Mineralised trabecular bone area below the growth plate at weaning is not different between genotypes upon visualization of sections stained with picrosirius red staining. Scale bar = 192 µm. E) *Atrx*^{Col1} mice are smaller by body weight compared to littermates at birth, weaning and adulthood. F) IHC staining for Atrx protein demonstrates reduced Atrx in *Atrx*^{Col1} cortical and subchondral bone. Scale bar = 48 µm.

Histological analysis of long bones by picrosirius red staining did not show any changes in growth plate morphology or trabecular density between genotypes (Figure 4-3D, and Supplementary Figures 4-2, 4-3). Additionally, establishment of the secondary ossification center was not delayed in long bones of 10 day old mutant mice, as determined by safranin-O staining (not shown). At one year of age, *Atrx*^{Col1} mice remained smaller than control littermates and did not catch up in weight (Figure 4-3E). Loss of ATRX in bone was confirmed by IHC staining with rabbit anti-ATRX (Prestige Antibodies, Sigma Aldrich) in cortical and subchondral bones (Figure 4-3F).

4.3.5 *Atrx*^{col1} Mice Have Normal Mineralisation and Circulating IGF-1

Micro-computed tomography (Micro-CT) analysis was performed at weaning and showed no changes in endochondral or intramembranous ossifications. Tibias appeared to have no gross morphological differences (Figure 4A) and trabecular bone mineral density (BMD) was unchanged between genotypes (Figure 4B) (N = 3, $p > 0.05$). Trabecular number was also not significantly changed when analysed by Micro-CT (Figure 4C).

IGF-1 promotes longitudinal bone growth and reduced IGF-1 is associated with limb shortening (Wang et al., 1999). To determine a possible endocrine component to the growth restriction in *Atrx*^{Col1} mice, levels of circulating IGF-1 were measured by ELISA. This analysis revealed no significant difference in IGF-1 levels between control and mutant mice (207.433 ± 35.4 ng/ml vs. KO: 252.010 ± 56.8 ng/ml, respectively $p = 0.17$).

To further investigate the role of ATRX in osteogenesis, we compared mineralisation in calvarial cells from control and *Atrx*^{Col1} mice. Unlike long bones, which form through a cartilage intermediate, calvarial bone forms by intramembranous ossification and does not contain cartilage. Examination of skeletal preparations from 21 day old mutant mice revealed no differences in calvarial structure or suture formation (Figure 5A). Mutant skulls appeared smaller than those of littermates, but were not significantly smaller by length ($p=0.15$) or width ($p=0.14$) (Figure 5B). To analyze mineralisation by ATRX-depleted osteoblasts, calvarial cells in 3D micromass culture were examined.

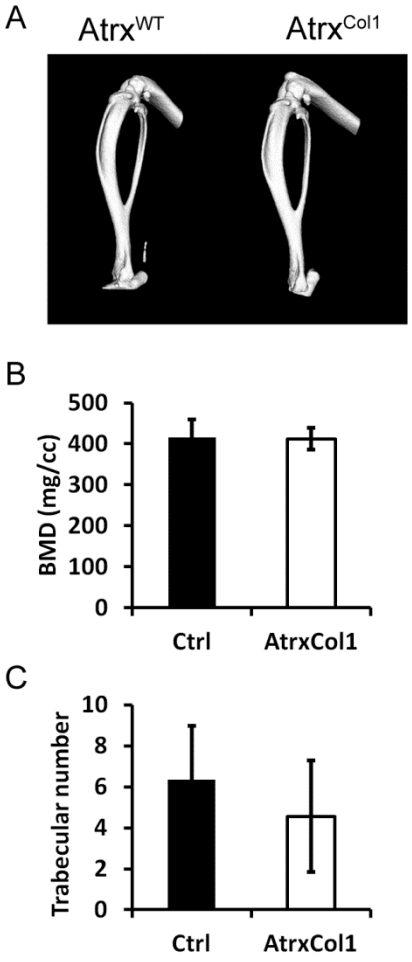


Figure 4-4 *Atrx*^{Col1} mice have normal mineralisation

A) Representative tibias from weaned *Atrx*^{Col1} showing that there was no gross morphological differences in the long bones between genotypes. B) No difference in bone mineral density ($p = 0.90$) was observed between *Atrx*^{Col1} mice and controls. Error Bars - SD, N=3 C) Trabecular number was unchanged between *Atrx*^{Col1} mice compared with controls ($p = 0.46$). Micro-CT data were obtained from hind legs using MicroView 3D software . Error Bars - SD, N=3

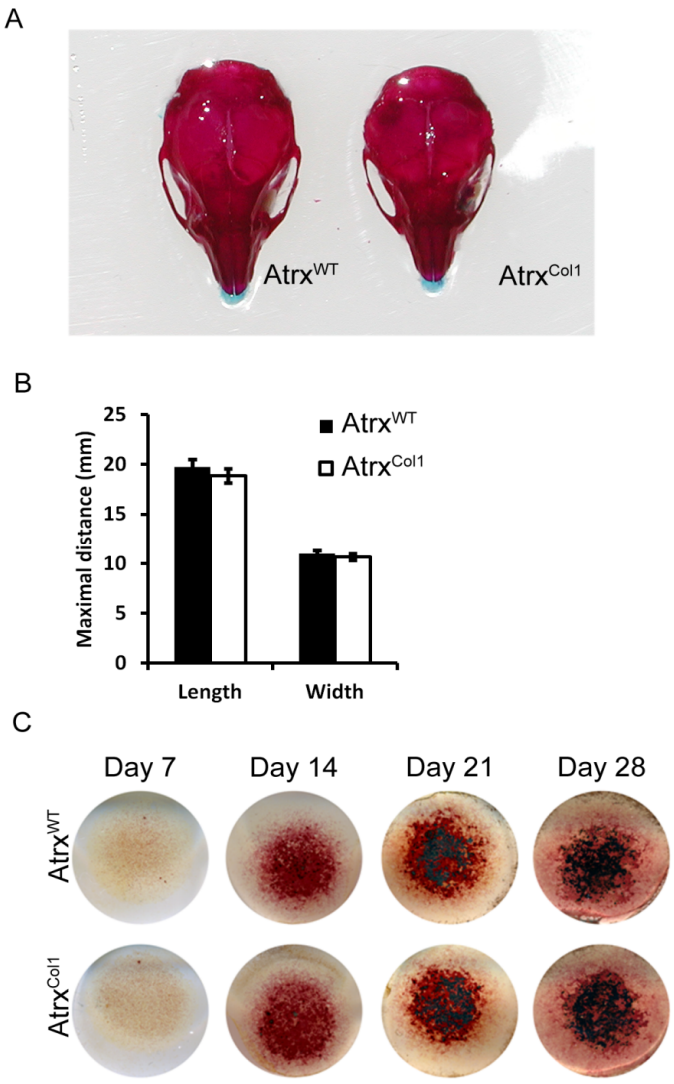


Figure 4-5 Ossification is not impeded or delayed by *Atrx* depletion in osteoblasts.

A) Ossification of the skull is normal in $Atrx^{Col1}$ mice, and no changes are seen in suture formation. B) Skull length and width are not significantly affected in $Atrx^{Col1}$ mice compared to controls. N=3, Error bars = SD C) Mineralisation of high-density osteoblast cultures. Control and Atrx-null osteoblasts show mineralised nodules (black) after two weeks in osteogenic media, and mineralise at similar rates at three and four weeks.

Osteogenic media was supplemented with β-glycerophosphate and ascorbic acid for a four-week time course. The extent of differentiation and formation of mineralised nodules was assayed by alkaline phosphatase and von Kossa staining. While cultures increased mineralization over the time course as expected, no difference in APase staining was observed between genotypes at any time over the four-week culture (Figure 4-5C).

4.3.6 Loss of Atrx in Bone Does Not Lead to Osteoarthritis

To determine if loss of ATRX in osteoblasts would lead to joint damage and osteoarthritis, joints of *Atrx*^{Col1} mice were examined for osteoarthritis at one year of age (Figure 4-6). *Atrx*^{Col1} mice expressed the same amount of ATRX in articular cartilage as control littermates, as expected with this Cre driver line (data not shown). Maximal OARSI scores of osteoarthritis in the elbows of *Atrx*^{Col1} mice were low in both genotypes; control animals had a mean score of 0.63 ± 0.60 and mutants had a mean score of 0.67 ± 0.70 (p= 0.92) (Figure 4-6A). Knee osteoarthritis scores were slightly higher, with control animals having an averaged maximal score of 1.46 for any quadrant of frontal knee sections and ATRX-deficient animals having a score of 0.67 (Figure 4-6B). These values were not significantly different (p=0.20).

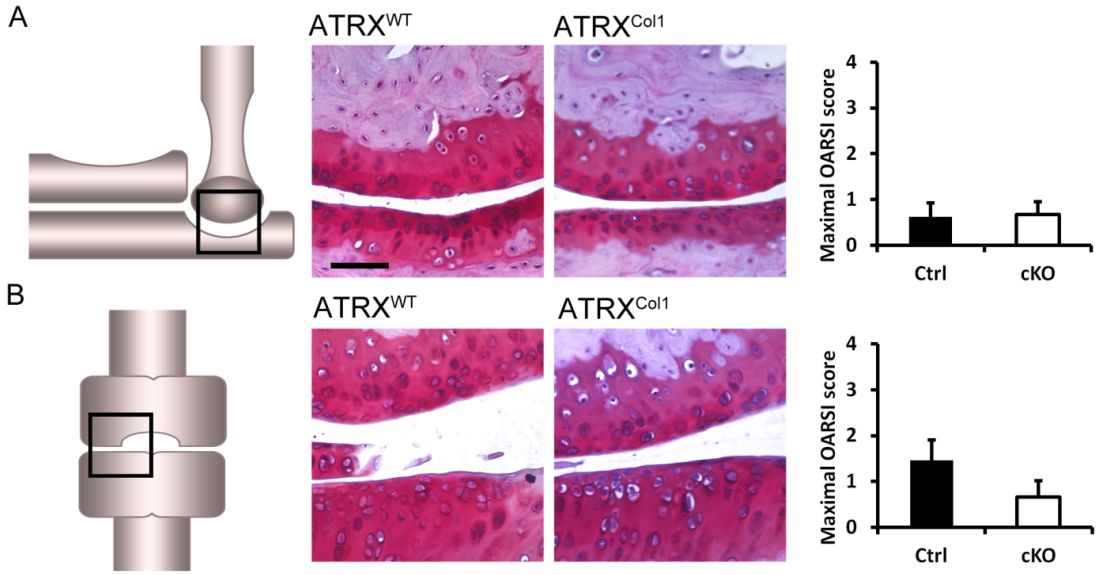


Figure 4-6 Characterisation of joint osteoarthritis in *Atrx*^{Col1} mice.

A) Knee and elbow sections were stained with safranin-O to detect the presence of proteoglycans. Fibrillation or fissuring of the articular surfaces was not observed in elbows of control or mutant mice, demonstrating very mild OA in both genotypes. Scale bar = 96 µm. B) Joint integrity of the medial surface of the knee was similar in control and mutant mice. N = 4, Error bars = SD

4.4 Discussion

Loss-of-function mutations in the human *ATRX* gene are associated with skeletal defects and high incidence of dwarfism. Mouse models of ATR-X syndrome have similar phenotypes, and demonstrate premature aging (Solomon et al., 2013; Watson et al., 2013). We report that aged mice lacking *Atrx* in skeletal tissues ($Atrx^{Col2}$, $Atrx^{Col1}$, and $Atrx^{Prx1}$) show very limited skeletal defects, with no associated increase in osteoarthritis.

$Atrx^{Col2}$ mice exhibit minimal skeletal defects during development (Solomon et al., 2009), so it was not completely surprising that cartilage integrity was maintained during normal aging. Adult $Atrx^{Col2}$ mice displayed minor fibrillation and loss of proteoglycan in the articular cartilage, but at equal levels as aged-matched control littermates. Thus, given the relative stability of *Atrx*-null articular cartilage, *Atrx* does not appear to be required for structural maintenance of articular cartilage.

No phenotype was observed in the $Atrx^{Prx1}$ mouse, despite biomechanical differences observed between genotypes leading to stride alterations (Solomon et al., 2013). $Atrx^{Prx1}$ mice have shortened forelimb digits and reduced forelimb function, but have no alterations in the hindlimb.

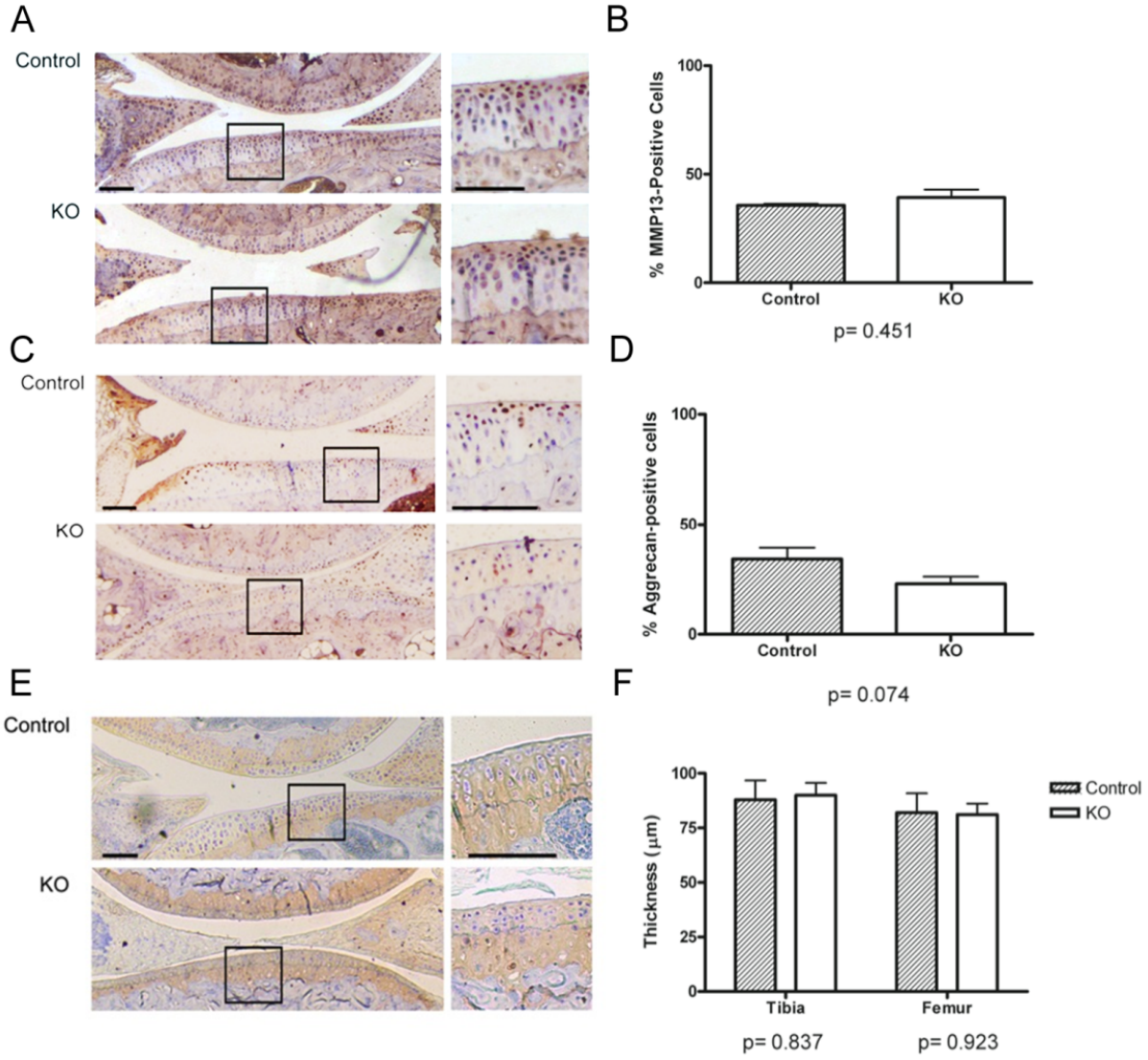
In the present study, we found that loss of *Atrx* in osteoblasts causes mild but significant shortening of the limbs. Given the similar, but more severe, dwarfism observed when *Atrx* is deleted in the developing forebrain and anterior pituitary (Watson et al., 2013), it is possible that this dwarfism is due to an indirect effect, because of unspecific Cre expression. The possibility of an indirect effect is supported by the fact that ex-vivo cultures of ATRX-null osteoblasts mineralise normally. Evidence suggests that the Col1a1-cre used in this study is capable of some LoxP recombination in the brain, so there remains a possibility that part of the dwarfism phenotype may be due to leaky *Atrx* recombination in the brain (Scheller et al., 2011). On the other hand, normal circulating IGF1 levels suggest that reduced skeletal growth is due to disruption of a bone-intrinsic role of *Atrx*.

In previous reports, a direct or indirect role for ATRX in the development of the skeleton has been characterised (Solomon et al., 2009; Solomon et al., 2013; Watson et al., 2013). Alterations in the expression of other chromatin-remodelling proteins in the skeleton can lead to severe malformations (Alvarez-Saavedra et al., 2010; Young et al., 2005). For example, dominant negative mutations in the SWI/SNF subunit *BRG1* reduce levels of the transcription factor RUNX2, an important regulator of osteogenesis (Young et al., 2005). However, no equivalent changes were observed in the expression of *Runx2* or other osteogenic genes in *Atrx*-null calvaria (data not shown). Osteoblast precursors isolated from $Atrx^{Col1}$ mice matured in high density micromass culture and formed mineral at the same rate as cells from control littermates. Finally, trabecular density was unaffected when examined histologically, as well as by Micro-CT analysis. Therefore, we conclude that loss of *Atrx* in osteoblasts does not impede their ability to mineralise tissue.

When *Atrx* is lost in the developing limb bud, DNA damage is observed as an accumulation of y-H2AX-positive nuclei in forelimb cells (Solomon et al., 2013). This single focus co-localises with DNA damage response factor 53BP1 at the nuclear lamina, and forelimbs lacking *Atrx* have higher incidence of TUNEL staining (Solomon et al., 2013). However, loss of *Atrx* in this model caused only a slight increase in apoptotic cells compared to other tissues such as brain, trophoblast, muscle and Sertoli cells (Bagheri-Fam et al., 2011; Bérubé et al., 2005; Garrick et al., 2006; Huh et al., 2012). Thus, our findings suggest that the protective effect of ATRX against DNA damage and replicative stress is tissue-specific, and that skeletal tissues are more resistant to the effects of *Atrx* loss.

ATRX deletion in various skeletal lineages confers only mild, tissue-specific defects and does not fully recapitulate the skeletal phenotypes of ATR-X syndrome. Furthermore, there is no increased incidence of joint osteoarthritis and no difference in the progression of skeletal pathologies in adulthood. Although *ATRX* mutations are associated with skeletal defects in humans, the underlying cause is likely indirect due to reduced function of ATRX in other tissues (Watson et al., 2013). In conclusion, ATRX is important in the developing skeleton prior to chondrocyte differentiation and during mineralisation, but is not important for maintenance of adult skeletal tissues.

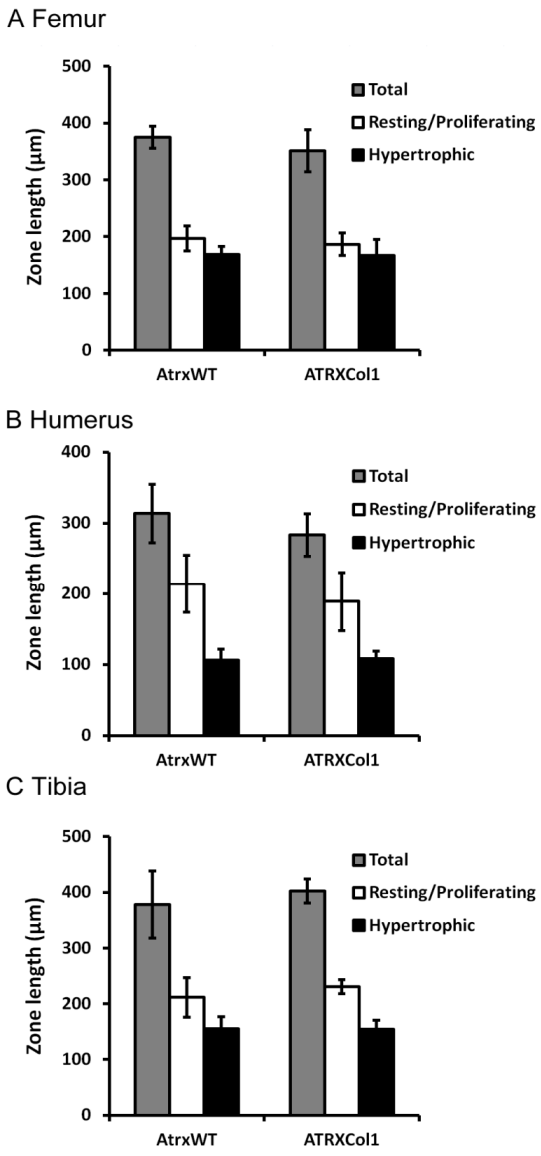
4.5 Supplementary Figures



Supplementary Figure 4-1 MMP13, Aggrecan fragment and type II collagen levels are normal in *Atrx*^{Col2} mice.

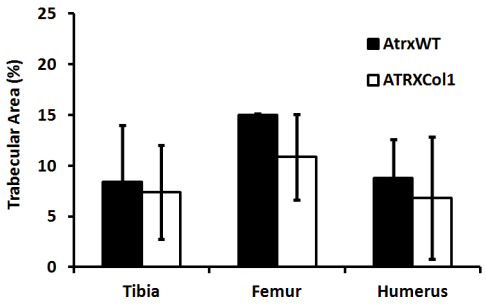
(A) MMP13 expression is similar in the articular cartilage of *Atrx*^{Col2} mice compared to controls. Representational immunohistochemical stains of articular cartilage in *Atrx* mutant and control mice performed on paraffin sections. Scale bar = 200 µm. (B). MMP13 expression was not significantly changed in *Atrx*^{Col2} mice. Analysis was performed by counting the percentage of MMP13-positive cells in paraffin sections using representational subsets of tibial articular cartilage. The average percentage of positive cells per articular surface was 35.7 ± 0.67% for controls and 39.3 ± 14.2% positive cells for mutants(n= 8). Control and knockout sections were not significantly different (p>

0.05). (C) Tibial aggrecan fragmentation is unchanged in *Atrx*^{Col2} mice. Representational stains for aggrecan fragments in control and knockout mice on 5 μm paraffin sections. Scale bar = 200μm. (D) Tibial aggrecan fragmentation in unchanged in *Atrx*^{Col2} mice. The average number of aggrecan fragment-positive cells in controls was 34.02 ± 5.92% (Mean ± SEM) (n= 5). The mean percentage of positive cells in knockouts was 22.8 ± 10.65% (n= 8). Mean percentages of aggrecan fragment-positive cells between controls and knockout tibiae were not significantly different (p> 0.05). (E) The type II collagen - positive zone in articular cartilage of knees of control and *Atrx*^{Col2} mice. Immunohistochemistry stains on paraffin knee sections for type II collagen. Scale bar = 200 μm. (F) The thickness of the type II collagen positive zone in the articular cartilage of the tibia and femur is not different in *Atrx*^{Col2} mice. Three measurements of the type II collagen-positive zone in the tibia and femur were taken. The average tibial thickness of the collagen 2-positive zone in controls vs. knockout mice was 87.993 ± 8.827 μm vs. 90.065 ± 5.529 μm (Mean ± SEM) (n= 5). The average femoral thickness of the type II collagen–positive zone in controls and knockout mice was 82.080 ± 8.820 μm vs. 81.142 ± 5.084 μm (n= 8). Neither the tibiae nor femurs of control and knockout mice showed any significant differences (p> 0.05).



Supplementary Figure 4-2 Growth plate measurements in *Atrx*^{Col1} mice.

No significant difference was seen in the length of the resting, proliferating or hypertrophic zones in long bones in *Atrx*^{Col1} or Control littermates at weaning (N = 3 littermate pairs; two-tailed T-test). Error bars = SD.



Supplementary Figure 4-3 Trabecular area quantification in *Atrx*^{Col1} mice.

Quantification of the area of mineralised trabecular area shows that mineralisation is unaffected in *Atrx*^{Col1} mice. No difference in trabecular area below the growth plates in the tibia, femur or humerus between *Atrx*^{Col1} mice and controls (N=3), Error bars = SD

4.6 References

Aigner, T., Haag, J., Martin, J., and Buckwalter, J. (2007). Osteoarthritis: aging of matrix and cells--going for a remedy. Current Drug Targets *8*, 325-331.

Alvarez-Saavedra, M., Carrasco, L., Sura-Trueba, S., Demarchi Aiello, V., Walz, K., Neto, J.X., and Young, J.I. (2010). Elevated expression of MeCP2 in cardiac and skeletal tissues is detrimental for normal development. Human Molecular Genetics *19*, 2177-2190.

Appleton, C.T., McErlain, D., Pitelka, V., Schwartz, N., Bernier, S., Henry, J., Holdsworth, D., and Beier, F. (2007). Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis. Arthritis Research & Therapy *9*, R13.

Aspden, R.M. (2008). Osteoarthritis: a problem of growth not decay? Rheumatology *47*, 1452-1460.

Aspden, R.M. (2011). Obesity punches above its weight in osteoarthritis. Nature Reviews Rheumatology *7*, 65-68.

Bagheri-Fam, S., Argentaro, A., Svingen, T., Combes, A.N., Sinclair, A.H., Koopman, P., and Harley, V.R. (2011). Defective survival of proliferating Sertoli cells and androgen receptor function in a mouse model of the ATR-X syndrome. Human Molecular Genetics *20*, 2213-2224.

Bellows, C.G., and Aubin, J.E. (1989). Determination of numbers of osteoprogenitors present in isolated fetal rat calvaria cells in vitro. Developmental Biology *133*, 8-13.

Bérubé, N.G., Mangelsdorf, M., Jagla, M., Vanderluit, J., Garrick, D., Gibbons, R.J., Higgs, D.R., Slack, R.S., and Picketts, D.J. (2005). The chromatin-remodeling protein ATRX is critical for neuronal survival during corticogenesis. Journal of Clinical Investigation *115*, 258-267.

Castaño Betancourt, M.C., Cailotto, F., Kerkhof, H.J., Cornelis, F.M.F., Doherty, S.A., Hart, D.J., Hofman, A., Luyten, F.P., Maciewicz, R.A., Mangino, M.*, et al.* (2012). Genome-wide association and functional studies identify the DOT1L gene to be involved in cartilage thickness and hip osteoarthritis. Proceedings of the National Academy of Sciences *109*, 8218-8223.

Dhayalan, A., Tamas, R., Bock, I., Tattermusch, A., Dimitrova, E., Kudithipudi, S., Ragozin, S., and Jeltsch, A. (2011). The ATRX-ADD domain binds to H3 tail peptides and reads the combined methylation state of K4 and K9. Human Molecular Genetics *20*, 2195-2203.

Dvir-Ginzberg, M., and Steinmeyer, J. (2013). Towards elucidating the role of SirT1 in osteoarthritis. Frontiers in Bioscience *18*, 343-355.

Eisen, J.A., Sweder, K.S., and Hanawalt, P.C. (1995). Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. Nucleic Acids Research *23*, 2715-2723.

Fosang, A.J., Last, K., Knäuper, V., Murphy, G., and Neame, P.J. (1996). Degradation of cartilage aggrecan by collagenase-3 (MMP-13). FEBS Letters *380*, 17-20.

Gagarina, V., Gabay, O., Dvir-Ginzberg, M., Lee, E.J., Brady, J.K., Quon, M.J., and Hall, D.J. (2010). SirT1 enhances survival of human osteoarthritic chondrocytes by repressing protein tyrosine phosphatase 1B and activating the insulin-like growth factor receptor pathway. Arthritis & Rheumatism *62*, 1383-1392.

Garrick, D., Sharpe, J.A., Arkell, R., Dobbie, L., Smith, A.J., Wood, W.G., Higgs, D.R., and Gibbons, R.J. (2006). Loss of Atrx affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues. PLoS Genetics *2*, e58.

Gibbons, R.J., Brueton, L., Buckle, V.J., Burn, J., Clayton-Smith, J., Davison, B.C.C., Gardner, R.J.M., Homfray, T., Kearney, L., Kingston, H.M.*, et al.* (1995a). Clinical and hematologic aspects of the X-linked α-thalassemia/mental retardation syndrome (ATR-X). The American Journal of Human Genetics *55*, 288-299.

Gibbons, R.J., and Higgs, D.R. (2000). Molecular-clinical spectrum of the ATR-X syndrome. The American Journal of Human Genetics *97*, 204-212.

Gibbons, R.J., Picketts, D.J., Villard, L., and Higgs, D.R. (1995b). Mutations in a putative global transcriptional regulator cause X-linked mental retardation with alpha-thalassemia (ATR-X syndrome). Cell *80*, 837-845.

Glasson, S.S., Chambers, M.G., Van Den Berg, W.B., and Little, C.B. (2010). The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis and Cartilage *18, Supplement 3*, S17-S23.

Goldring, M., and Marcu, K. (2009). Cartilage homeostasis in health and rheumatic diseases. Arthritis Research & Therapy *11*, 224.

Huber, L.C., Brock, M., Hemmatazad, H., Giger, O.T., Moritz, F., Trenkmann, M., Distler, J.H.W., Gay, R.E., Kolling, C., Moch, H.*, et al.* (2007). Histone deacetylase/acetylase activity in total synovial tissue derived from rheumatoid arthritis and osteoarthritis patients. Arthritis & Rheumatism *56*, 1087-1093.

Huh, M.S., Price O'Dea, T., Ouazia, D., McKay, B.C., Parise, G., Parks, R.J., Rudnicki, M.A., and Picketts, D.J. (2012). Compromised genomic integrity impedes muscle growth after Atrx inactivation. Journal of Clinical Investigation *122*, 4412-4423.

Liu, F., Woitge, H.W., Braut, A., Kronenberg, M.S., Lichtler, A.C., Mina, M., and Kream, B.E. (2004). Expression and activity of osteoblast-targeted Cre recombinase transgenes in murine skeletal tissues. International Journal of Developmental Biology *48*, 645-653.

Logan, M., Martin, J.F., Nagy, A., Lobe, C., Olson, E.N., and Tabin, C.J. (2002). Expression of Cre recombinase in the developing mouse limb bud driven by a Prxl enhancer. genesis *33*, 77-80.

Medina, C.F., Mazerolle, C., Wang, Y., Bérubé, N.G., Coupland, S., Gibbons, R.J., Wallace, V.A., and Picketts, D.J. (2009). Altered visual function and interneuron survival in Atrx knockout mice: inference for the human syndrome. Human Molecular Genetics *18*, 966-977.

Otani, J., Nankumo, T., Arita, K., Inamoto, S., Ariyoshi, M., and Shirakawa, M. (2009). Structural basis for recognition of H3K4 methylation status by the DNA methyltransferase 3A ATRX-DNMT3-DNMT3L domain. EMBO Reports *10*, 1235-1241.

Panupinthu, N., Rogers, J.T., Zhao, L., Solano-Flores, L.P., Possmayer, F., Sims, S.M., and Dixon, S.J. (2008). P2X7 receptors on osteoblasts couple to production of lysophosphatidic acid: a signaling axis promoting osteogenesis The Journal of Cell Biology *181*, 859-871.

Picketts, D.J., Higgs, D.R., Bachoo, S., Blake, D.J., Quarrell, O.W., and Gibbons, R.J. (1996). ATRX encodes a novel member of the SNF2 family of proteins: mutations point to a common mechanism underlying the ATR-X syndrome. Human Molecular Genetics *5*, 1899-1907.

Pollard, T.C.B., Gwilym, S.E., and Carr, A.J. (2008). The assessment of early osteoarthritis. Journal of Bone & Joint Surgery, British Volume *90-B*, 411-421.

Ritchie, K., Seah, C., Moulin, J., Isaac, C., Dick, F., and Bérubé, N.G. (2008). Loss of ATRX leads to chromosome cohesion and congression defects. The Journal of Cell Biology *180*, 315-324.

Scheller, E.L., Leinninger, G.M., Hankenson, K.D., Myers, J.M.G., and Krebsbach, P.H. (2011). Ectopic Expression of Col2.3 and Col3.6 Promoters in the Brain and Association with Leptin Signaling. Cells Tissues Organs *194*, 268-273.

Seah, C., Levy, M.A., Jiang, Y., Mokhtarzada, S., Higgs, D.R., Gibbons, R.J., and Berube, N.G. (2008). Neuronal death resulting from targeted disruption of the Snf2 protein ATRX is mediated by p53. Journal of Neuroscience *28*, 12570-12580.

Sedivy, J.M., Banumathy, G., and Adams, P.D. (2008). Aging by epigenetics—A consequence of chromatin damage? Experimental Cell Research *314*, 1909-1917.

Solomon, L.A., Li, J.R., Berube, N.G., and Beier, F. (2009). Loss of ATRX in chondrocytes has minimal effects on skeletal development. PLoS ONE *4*, e7106.

Solomon, L.A., Russell, B.A., Watson, L.A., Beier, F., and Berube, N.G. (2013). Targeted loss of the ATR-X syndrome protein in the limb mesenchyme of mice causes brachydactyly.

Terpstra, L., Prud'homme, J., Arabian, A., Takeda, S., Karsenty, G., Dedhar, S., and St-Arnaud, R. (2003). Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes. Journal of Cell Biology *162*, 139–148.

Ulici, V., Hoenselaar, K.D., Agoston, H., McErlain, D.D., Umoh, J., Chakrabarti, S., Holdsworth, D.W., and Beier, F. (2009). The role of Akt1 in terminal stages of endochondral bone formation: Angiogenesis and ossification. Bone *45*, 1133-1145.

Wang, G., Woods, A., Agoston, H., Ulici, V., Glogauer, M., and Beier, F. (2007). Genetic ablation of Rac1 in cartilage results in chondrodysplasia Developmental Biology *306*, 612-623

Wang, J., Zhou, J., and Bondy, C.A. (1999). Igf1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. The FASEB Journal *13*, 1985-1990.

Wang, M., Shen, J., Jin, H., Im, H.-J., Sandy, J., and Chen, D. (2011). Recent progress in understanding molecular mechanisms of cartilage degeneration during osteoarthritis. Annals of the New York Academy of Sciences *1240*, 61-69.

Watson, L.A., Solomon, L.A., Li, J.R., Jiang, Y., Edwards, M., Shin-Ya, K., Beier, F., and Berube, N.G. (2013). Atrx deficiency induces telomere dysfunction, endocrine defects, and reduced life span. Journal of Clinical Investigation *123*, 2049–2063.

Wong, L.H., McGhie, J.D., Sim, M., Anderson, M.A., Ahn, S., Hannan, R.D., George, A.J., Morgan, K.A., Mann, J.R., and Choo, K.H.A. (2010). ATRX interacts with H3.3 in maintaining telomere structural integrity in pluripotent embryonic stem cells. Genome Research *20*, 351-360.

Yan, Q., Feng, Q., and Beier, F. (2010). Endothelial nitric oxide synthase deficiency in mice results in reduced chondrocyte proliferation and endochondral bone growth. Arthritis & Rheumatism *62*, 2013-2022.

Young, D.W., Pratap, J., Javed, A., Weiner, B., Yasuyuki Ohkawa, Wijnen, A.v., Montecino, M., Stein, G.S., Stein, J.L., Imbalzano, A.N.*, et al.* (2005). SWI/SNF chromatin remodeling complex is obligatory for BMP2-induced, Runx2-dependent skeletal gene expression that controls osteoblast differentiation. Journal of Cellular Biochemistry *94*, 720-730.

Chapter 5

5 General Discussion

This thesis presents data on the role of ATRX in the development and disease of the skeleton. In this thesis I generated three models of ATRX depletion in the mouse skeleton, and describe physiological and molecular consequences of ATRX loss during development. Mutations in the human *ATRX* gene are associated with skeletal deformities, so I anticipated that these mouse mutants would demonstrate similar skeletal defects (Gibbons et al., 2000). Additionally, there exists a large body of evidence demonstrating important roles for chromatin-modifying enzymes, particularly those with histone modifying or SWI/SNF activity, in normal development of the skeleton (Alvarez-Saavedra et al., 2010; Arnold et al., 2007; Hug, 2004; Jensen et al., 2008; Shapiro et al., 2010; Sun et al., 2009a; Sun et al., 2009b; Vega et al., 2004).

Surprisingly, the only ATR-X phenotype recapitulated by these mouse models was a shortening of the distal phalanges when *Atrx* is deleted in the developing forelimb mesenchyme. Mice lacking ATRX in the cartilage or osteoblasts had only minor defects, with limited similarities to ATR-X syndrome. In contrast, a mouse model of ATR-X syndrome in which *Atrx* is deleted in the forebrain and pituitary demonstrates severe skeletal defects including growth restriction, low bone mineral density, and reduced trabecular area (Watson et al., 2013). Collectively, these findings establish that many of the skeletal phenotypes observed in ATR-X syndrome may not be due to a direct effect of ATRX loss-of-function in the skeleton, but secondary to defects in the brain or endocrine system.

Interestingly, these studies revealed a particular and important property of skeletal tissue to resist DNA damage, or the cell death associated with DNA damage. DNA damage and genome instability is observed in other tissues when *Atrx* is deleted, which frequently results in widespread apoptosis, suggesting skeletal tissues may have some method of abrogating this outcome (Baumann et al., 2010; Lovejoy et al., 2012; Ritchie et al., 2008; Watson et al., 2013).

5.1 Thesis Summary

In chapter two, I observed that loss of ATRX in the cartilage of mice does not lead to skeletal defects, and conclude that the skeletal deformities in ATR-X syndrome must arise from cartilage-extrinsic mechanisms. In chapter three, I described a role for ATRX in maintaining genomic integrity in the mesenchyme of developing forelimb of the mouse, and demonstrate a digit malformation phenotype similar to ATR-X syndrome when ATRX is lost. I correlated the DNA double-strand breaks and associated apoptotic cell death to loss of proliferating digit cells and shortening of the distal phalanges. In chapter four I report evidence that loss of ATRX, despite its association with genome instability and premature aging, does not confer susceptibility of osteoarthritis when lost in skeletal tissues. By examining joint disease phenotypes in three conditional knockout models, I concluded that there is no direct role for ATRX in the skeleton in the establishment of osteoarthritis. Additionally, I quantified mineralisation on a mouse lacking ATRX in osteoblasts, and conclude that ATRX is not required for osteoblast function.

5.2 Contributions to Current State of Knowledge Regarding ATRX in the Skeleton

5.2.1 ATRX in Developing Cartilage

In Chapter two, I tested the hypothesis that mice lacking ATRX in developing cartilage will demonstrate dwarfism and other skeletal defects similar to those observed in humans with ATR-X syndrome. I quantified the skeletal phenotypes of mice that lack *Atrx* expression in cartilage beginning with the onset of cartilage differentiation from mesenchyme. These mice showed that loss of ATRX in chondrocytes has minimal effects on skeletal development, and that mice lacking ATRX in cartilage were viable and were fertile in adulthood. ATRX loss was confirmed at the level of both mRNA and protein, and shown to have no effect on the expression of important cartilage growth plate genes such as *Sox9* and *p57*. Conditionally ATRX-deficient mice had normal growth plate morphology, with no defects in the ratios of resting, proliferating, or hypertrophic cells at birth or at weaning. These animals demonstrated only a mild delay in the ossification of

the pelvic girdle, and have none of the associated skeletal phenotypes of ATR-X syndrome. I concluded that skeletal phenotypes of ATR-X syndrome are likely due to secondary effects from tissues like the brain or anterior pituitary.

5.2.2 ATRX in Developing Forelimb

In Chapter three, I investigated the development of the appendicular skeleton from ATRX-deficient mesenchyme. I reported that mice conditionally lacking ATRX in the forelimb mesenchyme demonstrate specific brachydactyly, characterised by a shortening of the cartilage anlage during embryonic development and subsequent shortening of the ossified digit at, and following, birth. While the shortening of distal phalanges persists into adulthood, there are limited disease phenotypes in this model. These animals demonstrate reduced forelimb function, quantified as a significant decrease in forepaw grip strength. Adult animals have a mild alteration in gait which manifests as shorter strides, but do not show any major interlimb coordination defects. The brachydactyly phenotype correlated with increased levels of the DNA-damage marker γ-H2AX, which appeared as one bright focus within the nucleus of ATRX-deficient embryonic forelimb mesenchyme and chondrocytes. This γ-H2AX focus was often located at the periphery of the nucleus, and using 3D confocal microscopy, I determined that this focus of damaged DNA was frequently located within one micron of the nuclear lamina. I attribute the digit shortening to an increase in TUNEL-positive apoptotic cell death in proliferating distal tip of the digit. I propose that some of the hand deformities observed in ATR-X syndrome may be due to similar cell death in the developing digit, and predict the cell death is related to the DNA damage associated with loss of ATRX.

5.2.3 ATRX in Developing Bone

In Chapter four, I describe a model of ATRX-deficiency in mineralising osteoblasts, which leads to mild dwarfism but does not cause defects in bone mineral formation during development or in vitro. Although these mice have shortened limbs, this phenotype does not fully recapitulate the variety of skeletal deformities seen in human cases of ATRX depletion. These animals do not show premature aging or the defects in

bone mineral density and trabecular number seen in mice lacking ATRX in the forebrain and anterior pituitary.

5.2.4 Secondary Effects of ATRX on the Skeleton

There are many reasons to expect a skeletal phenotype in a model of ATRX-deficiency. Firstly, mutations to *ATRX* are associated with a wide variety of severe pathologic skeletal phenotypes in humans. These include short stature, spinal deformities, clubbed foot, tapered or conical fingers, and delayed bone age (Gibbons et al., 2000). Secondly, genes with similar epigenetic properties to *ATRX* such as *MeCP2, HDAC4* and *NIPBL* are also associated with skeletal deformities in humans and defective skeletal development in related mouse models (Alvarez-Saavedra et al., 2010; Kawauchi et al., 2009; Shapiro et al., 2010; Tonkin et al., 2004). Some of these gene products can interact directly with ATRX and affect gene expression (Baker et al., 2013; Kernohan et al., 2010; Nan et al., 2007). Thirdly, there are important roles for proteins with similar functions to ATRX in skeletogenesis, including SWI/SNF chromatin remodeling, histone modifications, and genomic integrity (Williams et al., 2010; Young et al., 2005).

It was unexpected that mouse models lacking ATRX in cartilage, bone, or forelimb mesenchyme displayed relatively minor phenotypes. I propose that the skeletal phenotypes and dwarfism seen in ATR-X syndrome are more likely due to secondary effects. These effects were later demonstrated succinctly in a forebrain and pituitary-specific *Atrx*-deletion mouse displaying dwarfism, reduced bone mass, and premature aging (Watson et al., 2013). In this model of ATR-X syndrome, *Atrx* is deleted in the embryonic forebrain starting at E8.5. These mice are born small, and before three weeks of age, display defective and degenerate skeletal phenotypes (Bérubé et al., 2005; Watson et al., 2013). In addition to significant dwarfism, these animals have shortened growth plates, reduced bone density, and reduced trabecular number (Watson et al., 2013). It is crucial to note that these mice lacking ATRX in the forebrain and anterior pituitary have severe endocrine defects, including defects in the TH/IGF1 axis. In this model of ATRX-deficiency, the skeletal defects are attributed to a secondary, systemic defect where skeletal development is affected indirectly.

There are other indirect effects that can influence both skeletal development and growth. Normal regulation of skeletal growth and mineralisation requires sex steroids, including androgens and androgen receptors, as well as estrogens and estrogen receptors [Reviewed in (Callewaert et al., 2010)]. Defects in estrogen sensing in the male mouse leads to decreased radial and longitudinal bone growth, associated with decreased serum levels of IGF-I (Vidal et al., 2000). Skeletal defects are seen in androgen-resistant testicular feminized males, which have low trabecular bone mineral density (Vandenput et al., 2004). These phenotypes are usually seen post-natally, in late pubertal, or adult stages (Vidal et al., 2000). This is similar to the forebrain and pituitary conditional *Atrx* deletion mouse, which has normal growth plate morphology and trabecular number early in life, but later develops decreased bone density and other skeletal phenotypes (Watson et al., 2013). In cases of ATR-X syndrome, individuals frequently present with hypogonadisms and in some cases even sex reversal (Gibbons et al., 1995; Ion et al., 1996). These phenotypes are often correlated with delayed or arrested puberty, and manifestation of growth retardation at the time of the pubertal growth spurt (Gibbons, 2006). In these cases, levels of sex steroids and their receptors would be affected, and lead to skeletal defects without a direct effect on gene expression within the cartilage and bone. Thus, it can be concluded that the most ideal model for studying ATRX in skeletal development would be a mouse lacking ATRX in the brain and other indirect systems, rather than removing ATRX directly in the skeleton.

Compared with mouse models of steroid and endocrine defects, mice lacking ATRX in the cartilage, bone, or forelimb demonstrate slight and limited phenotypes. It can be predicted that a model combining both direct and indirect skeletal roles of ATRX may modulate the subtle skeletal phenotypes, such as brachydactyly, towards more severe defects. My data suggest that some of the skeletal defects are treatable via hormonal methods. In cases where circulating levels of hormones are affected, patient skeletal size could be influenced by treatment with growth hormone or drugs to increase endogenous production of growth hormones.

When considering developmental phenotypes caused by other chromatin-modifying proteins, such as MECP2 and HDAC4, it is important to consider that ATRX does not

always affect expression of genes directly. Complexes containing ATRX modulate genome structure and integrity, and have both tissue-specific and chromosome-specific functions. ATRX can affect expression of wide-ranging genetic factors, like imprinting loci and silencing whole chromosomes (Baumann et al., 2009; Kernohan et al., 2010). Additionally, the role of ATRX can be influenced by other factors including variable number tandem repeats (VNTRs), which can vary between individuals (Law et al., 2010). There are very few genotype/phenotype correlations in ATRX syndrome, and even patients with the same mutation may demonstrate different pathological phenotypes [Reviewed in (Gibbons, 2006)]; therefore, it is important to consider the inherently variable nature of ATRX on gene expression when looking at functional phenotypes.

5.3 ATRX and Genome Integrity in the Limb Bud Mesenchyme

The major objective of this thesis was to determine the role for ATRX in skeletal development and recapitulate the skeletal defects observed in ATR-X syndrome using a mouse model. In utilising a tissue-specific conditional knockout model, the role for ATRX in developing skeletal tissues was identified. Seemingly, the most significant and specific phenotype seen in these animals did not reveal any new roles for ATRX but rather confirmed the importance of ATRX in genome integrity and prevention of DNA damage.

It remains unknown if the cell death observed in conditionally ATRX-deficient forelimb is a direct consequence of the DNA damage. In other models, mouse *Atrx* deletion and loss of ATRX is associated with cell death and hypocellularity (Bérubé et al., 2005; Huh et al., 2012). In ATRX-deficient neuroprogenitors, γ-H2AX positive DNA damage accumulates leading to activation of ATM and p53 followed by cell death (Watson et al., 2013). p53 activation has not been demonstrated in ATRX-deficient forelimbs, but it can be expected that protein quantification for the ATM-p53 pathway in this tissue would demonstrate lower activation compared to neuroprogenitors.

The defect in the limbs of these animals was observed only in the distal digit tip and not the whole limb. The digit tip is the last portion of the limb to develop, with the final

phalange not being specified until embryonic day 15.5 in the mouse, six days after the initial development of the limb bud and three days after the metacarpals. Throughout the development of the limb, the entire structure is laid down by distal outgrowth of progenitor cells from the progress zone [Reviewed in (Tickle, 2003)]. Proximal limb bones, as well as the more proximal digit bones such as the metacarpals, differentiate earlier than those distal; therefore, I expect that distal tissues of the ATRX-deficient limb bud spend more replicative cycles as mesenchymal cells before finally differentiating into cartilage. Given the requirement for ATRX observed in mesenchymal cells, but not seen in cartilage, it can be expected that the most distal portions of the digit would retain the most DNA damage.

A growing body of evidence has correlated loss of ATRX with both genome instability and altered DNA damage response (Leung et al., 2013; Lovejoy et al., 2012; Ritchie et al., 2008; Watson et al., 2013). When ATRX is depleted in HeLa cells, chromatids demonstrate defective chromosome dynamics and abnormal mitosis (Ritchie et al., 2008). ATRX-depleted colorectal cancer lines show increased replication stress and have increased DNA damage following release from thymidine block (Leung et al., 2013). Defective double-strand break repair and telomere dysfunction have been observed in ATRX- or DAXX -deficient cancer cell lines (Lovejoy et al., 2012). Endogenous and replicative DNA damage is also observed in ATRX-deficient neuroprogenitors, demonstrating an important requirement for ATRX in preventing the accumulation of DNA damage (Watson et al., 2013). Accumulation of DNA double-strand breaks was also observed in ATRX-deficient forelimb mesenchyme cells, but unlike neuroprogenitors, these cells do not have reduced viability when challenged with additional replicative stress. HU treatment of ATRX-null forelimb mesenchyme cells does not lead to a decrease in viability, suggesting a difference in stress response compared to other cell types.

In my model of ATRX-deficient forelimb development, I demonstrate that loss of ATRX leads to increased DNA damage, which associates as a single focus at the nuclear lamina. Although this damage does not appear to be due to replicative stress, it demonstrates an increased susceptibility to cell death in the developing digit. This DNA damage is

significantly increased in mutants compared to controls, and is seen predominantly in the ATRX-negative cells of mosaic heterozygote females. Although this DNA damage is not due to replicative stress, the DNA double strand breaks could still activate the p53/ATM pathway within the forelimb cells. This theory could be examined further through standard tests of genome stability, and protein analysis for phosphorylation of ATM/ATR and other checkpoint proteins. Although preliminary studies has shown no difference in the cell cycle profiles of normal and ATRX-null forelimb cells, the rate of progression through each part of the cell cycle is unknown. Additionally, the status of the telomeres in ATRX-deficient forelimbs has not been studied, but telomere FISH may reveal an increase in telomere dysfunction–induced foci (TIFs). Finally, characterisation of the single large DNA damage focus in ATRX-depleted cells may reveal characteristic genome segregation and repair pathways that are activated.

The forelimb deletion model demonstrates a direct correlation between loss of *Atrx* expression and increased incidence of DNA damage in forelimb tissue. A similar mechanism is expected to be at work in the distal hindlimb, and a similar distal phalange shortening phenotype would be expected if *Atrx* was deleted in the rear limb bud. My model did not demonstrate hind limb phenotypes, as expression of the Prx-Cre is limited in the hindlimb compared to the forelimb during early development (Logan et al., 2002). My work demonstrates that ATRX is required for normal development of the distal forelimb, and that skeletal phenotypes observed in ATR-X patients may be due, in part, to a direct role for ATRX in genomic stability in the developing limb.

5.4 Susceptibility of Skeletal Tissue to DNA Damage

One clear question raised by my study is how some cell types are susceptible to DNA damage when ATRX is lost, while others are not. Research has shown there is a high requirement for ATRX for genome stability and survival in the neuronal cells (Watson et al., 2013), and no requirement for normal growth of cartilage cells (Solomon et al., 2009). Between these two extremes, proliferative tissues like forelimb mesenchyme have limited requirement for ATRX, or only a temporal requirement. There are two possible reasons for this discrepancy; either ATRX is not required for genome stability in certain tissues,

or these tissues are better equipped to deal with genome instability caused by ATRX-deficiency.

To date, studies have detected ATRX in all mouse tissues examined, and ATRX is absolutely required during very early embryonic and placental development (Garrick et al., 2006); however, when ATRX is lost in more differentiated tissues, the requirement for ATRX appears to be diminished. One possible distinction is that certain tissues, especially skeletal tissues, are more tolerant to DNA damage and less likely to undergo p53-dependant cell death observed in other models of ATRX loss (Bagheri-Fam et al., 2011; Huh et al., 2012; Seah et al., 2008; Solomon et al., 2013). Tissues demonstrating the highest levels of DNA damage and p53 response when ATRX is lost tend to be rapidly proliferating (Seah et al., 2008; Watson et al., 2013). In the case of the developing forelimb, the most affected cells are located in the highly proliferative zone at the distal tip of the digit which contributes to digit lengthening. These proliferating cells would be expected to have a sensitivity to replicative stress, but our data demonstrate that HU treatment in vitro does not reduce viability. This means that forelimb mesenchyme cells are resistant to specific drugs that induce replication fork stalling, unlike other ATRX-deficient tissues which have shown a sensitivity to this treatment; therefore, loss of ATRX sensitises certain cell types to conditions that cause DNA damage and viability loss, but not all cell types.

Certain tissues express proteins that can directly compete with p53 for promoter binding and prevent cell death (Qian et al., 2012). Senescence marker *Dec1* (Differentiated embryo chondrocyte expressed gene 1) is expressed in response to p53 activation, and can allow cells to 'choose' between senescence or cell death based on cellular context and type of DNA damage (Qian et al., 2012). Expression of *Dec1* in different cell types may lead to difference responses to DNA damage. Similar to our study, loss of the single-stranded DNA binding protein OBFC2B in mice leads to delayed growth and skeletal abnormalities due to accumulation of γ-H2AX and apoptosis in the pre-cartilage condensations (Feldhahn et al., 2012). While OBFC2A, a homologue, is required in many other cell types, OBFC2B is only required in skeletogenesis (Feldhahn et al., 2012).

Thus, it appears that the skeletal lineage has developed specific methods of dealing with increased levels of DNA damage during development.

The developmental requirement for ATRX in cell viability varies by cell type. At the earliest stages of development, ATRX is required for normal mitosis and genome stability in the ooctye (Baumann et al., 2010; De La Fuente et al., 2004). Complete ablation of ATRX in the developing embryo is lethal due to defects in the trophectoderm (Garrick et al., 2006). Loss of ATRX in the developing brain leads to cell death and hypocellularity, with loss of cells in the dentate gyrus, pyramidal cells of upper cortical layers, and GABAergic interneurons (Bérubé et al., 2005; Seah et al., 2008). ATRX is also required in the developing retina, where loss of ATRX is also associated with cell death of specific neurons. (Medina et al., 2009).

In contrast, some models of ATRX deficiency have mild phenotypes and limited death or hypocellularity. Mice lacking ATRX in developing muscle demonstrate delayed muscle growth and deficient muscle regeneration (Huh et al., 2012); however, these animals are only 20% reduced in size at three weeks of age, and as adults have no significant difference in body weight (Huh et al., 2012). Similarly, mice lacking ATRX in the developing cartilage show no change in adult bodyweight, in addition to the absence of cell population changes in the growth plate or articular cartilage. Mice lacking ATRX in bone have mild dwarfism, but no defects in patterning, and appear healthy.

I have shown that loss of ATRX in the limb chondrocyte precursors leads to increased DNA damage, but loss of ATRX following chondrogenic differentiation does not have the same effect. This is particularly important considering the highly proliferative cartilage growth plate, which does not appear to have hypocellularity or decreased proliferation when ATRX is deleted (Solomon et al., 2009); therefore, we can conclude that skeletal tissues either have reduced susceptibility to DNA damage when ATRX is lost, or increased capacity to repair damage. In either scenario, loss of ATRX directly in developing skeletal tissues does not lead to the wide variety of disease phenotypes associated with ATR-X syndrome.

5.5 Limitations of Research and Suggestions for Future Studies

5.5.1 Limitations of the Mouse Model

ATRX is present within the nucleus of normal chondrocytes during development, but there remains a possibility that there is a compensatory mechanism within the cartilage when *Atrx* is deleted. Although there are no direct homologues to *Atrx* in the mouse genome, *Atrx* has homology with members of the Rad54-like subfamily of SNF2 proteins (Flaus et al., 2006). Deletion of other SNF2 members in cartilage, such as *Etl1*, have demonstrated limited penetrance and suggested compensation from other SNF2 family members (Schoor et al., 1999). By western blot, ATRX shows much higher abundance in the brain than in cartilage or bone, indicating it may have a reduced requirement in these tissues (Solomon et al., 2009).

One shortcoming of our model is the assumption that destabilisation of the ATRX RNA by deleting exon 18 recapitulates the effect of disease-causing mutations in humans. A truncated isoform of human ATRX, called ATRXt, is produced from a shortened transcript generated by alternative splicing at exons six and seven, leading to incorporation of an alternate poly(A) signal (Garrick et al., 2004). This truncated version is also observed in mouse, producing a ~200 kDa product as opposed to the larger ~280 full-length ATRX (Garrick et al., 2004; Picketts et al., 1998). ATRXt retains the N-terminal ADD domain, but lacks the C-terminal SWI/SNF domain which has been shown to carry some of the disease-causing mutations (Garrick et al., 2004). Use of the Cre-LoxP system, with the $Atrx^{flox/flox}$ mouse, generates an animal that lacks full-length ATRX, but retains normal *Atrxt* expression (Bérubé et al., 2005). The function of this shorter transcript remains unknown. Future investigations into the role of ATRX in skeletal tissue may benefit from generation of new conditional deletion model which includes loss of the shortened isoform in additional to full-length ATRX.

It is important to note that in many models utilising this conditional deletion model, severe and sometimes lethal phenotypes are still observed even with the retention of the shortened isoform (Bérubé et al., 2005; Garrick et al., 2006; Watson et al., 2013).

Additionally, retention of some ATRX function in mouse may be beneficial in replicating ATR-X syndrome, as all human cases are hypomorphs and not complete deletions (Gibbons et al., 2008).

To further elucidate the specific role of ATRX, it may be beneficial to replicate specific patient mutations and therefore investigate specific genotype/phenotype correlations. Mouse studies have characterised a milder variant of ATRX-deficiency, in which exon 2 of *Atrx* was mutated (Nogami et al., 2010). Unlike complete loss-of-function models, these mice survive and reproduce normally, but demonstrate deficiency in fear conditioning and dysfunction of αCaMKII and GluR1 (Nogami et al., 2010). Identifying mutations most frequently associated with skeletal defects, and then replicating these mutations in a mouse model, would be a more specific tool in studying the direct role for ATRX in skeletal development.

One particular limitation in the limb-specific deletion model is the specificity of the Prx-Cre. While the Prx-1 promoter drives cre expression in the developing limb mesenchyme, it does not drive deletion of floxed genes in the ectoderm including the apical ectodermal ridge (Nohno et al., 1993); therefore, our model demonstrates the importance of ATRX in cells of mesenchymal origin, but not those of ectodermal origin. The AER communicates with the rest of the developing limb, and modulation of Wnt/β–catenin signalling between the AER and limb mesenchyme leads to digit abnormalities in mice (Villacorte et al., 2010). To examine the specific role of ATRX in the AER, a different cre line such as the Keratin 5 (K5) Cre mouse line would be required (Tarutani et al., 1997). A double-knockout, utilising both the mesenchyme and epithelial deletion of ATRX, would be expected to demonstrate a more severe phenotype. If ATRX is deleted in the AER, the expected DNA-damage and potential cell death in the AER would decrease proliferative signalling to the limb mesenchyme and lead to more severe defects. To study the role of ATRX in the entire skeleton, it would be useful to delete ATRX in the condensed mesenchyme as well as perichondrial cells. This model can be achieved using the Dermo1-cre driver, where cre is expressed in condensed mesodermal tissues leading to deletion of floxed alleles in both chondrocytes and osteoblasts (Yu et al., 2003). This

model would provide a combined phenotype of ATRX loss in both bone and chondrocyte, without the need for two different cre driver lines.

5.5.2 Limitations of Culture Systems

In characterising the animal models in this thesis, various ex-vivo systems were used to characterise ATRX-deficient cells. Primary chondrocytes from ribs were cultured over agarose to enrich ATRX-deficient cartilage for Western blot analysis. Primary osteoblasts were cultured in high density micromass cultures to induce, compare, and quantify mineralisation between genotypes. Forelimb mesenchymal cells were cultured in monolayer for drug treatment and viability quantification. In all these cases, there are specific pitfalls and limitations that must be recognised when interpreting results from these studies.

When isolating primary animal cells, tissues are subjected to enzymatic digestion which can alter gene expression (Hayman et al., 2006). In the case of cultured chondrocytes, culturing over agar is preferred to monolayer culture (Horwitz et al., 1970). Chondrocytes cultured in monolayer flatten out and become more fibroblast-like, while chondrocytes cultured over agar retain their phenotype and provide a method to differentiating chondrocytes from other connective tissue cells (Horwitz et al., 1970). All efforts were made to use a culture system that would replicate normal chondrocytes and therefore produce a representative sample of cartilage.

Similarly, cultured osteoblasts from calvaria bone were used to examine ex-vivo mineralisation of ATRX-deficient osteoblasts. Although primary isolates of osteoblasts were expanded in monolayer, subsequent long-term culture was conducted in a high-density micromass system. This model increases the amount of cell-cell contact and is more physiologically similar to an in vivo model of mineralisation (Gerber et al., 2002). While this system exists outside of the influence of the endocrine system, it is still sufficient for studying direct functions of gene loss of osteoblasts.

Treatment with HU was conducted on monolayer cultures of forelimb mesenchyme cells to determine sensitivity of ATRX-depleted forelimb cells to exogenous replicative stress.

When examining stress in culture system, it is important to recognise that cell culture systems experience increased oxidative stress of 'culture shock' and are not always representative of the cell's environment in vivo [Reviewed in (Halliwell, 2003)]. Staining for DNA damage marker γ-H2AX increased with HU treatments in both controls and mutants, and even without any HU treatment DNA damage was observed in control cells; however, there was no difference in cell viability between mutant and appropriate controls. The cell culture model still provides many advantages to an in vivo animal model, particularly in regard to drug treatments and cell cycle control.

5.5.3 Limitations of the DNA Damage Study and Future Directions

The induction of the DNA damage observed in the forelimb is also poorly understood. Using HU treatments, it was demonstrated that ATRX-deficient forelimb mesenchyme cells are not more susceptible to exogenous replicative stress; however, other models of replicative stress remain to be characterised, including cross-linking with mitromycin C. There are other forms of stress in the developing limb, which have not been investigated. Cells exposed to γ-irradiation have induction of double strand breaks in both proliferative cells and post-mitotic cells, and show activation of the DNA damage response. It is unknown if there is a requirement for ATRX in repair of exogenous DNA damage in forelimb tissue, although it is not required for repair of irradiation–induced DSBs in neuroprogenitors (Watson et al., 2013). Overall, this work suggests that while the role for ATRX in preventing and reversing DNA damage is not understood, a highly resilient tissue such as the developing skeleton is not the ideal model for molecular analysis of ATRX function.

Another topic of future study is the status of the IHH pathway in ATRX-deficient forelimbs. The digit shortening phenotype observed in these animals resembles Brachydactyly type A1, which is observed in the BDA mouse harbouring a point mutation in *Ihh* (Gao et al., 2009). This mutation abrogates signaling of IHH through its receptor PTCH and leads to distal digit shortening consistent with the human disorder (Gao et al., 2009; Guo et al., 2010). Although there is no current role known for ATRX in regulating members of the IHH pathway, the similarities in the phenotype are unmistakable. Preliminary unpublished results from our lab have not shown changes in

the expression of *Ihh* in ATRX-deficient forelimbs, but a correlation between ATRX and expression of other brachydactyly related genes remains to be investigated.

One remaining question in this study is the identity of the single DNA damage focus at the periphery of the nucleus of ATRX-depleted cells. γ-H2AX positive DNA double strand breaks have been observed in various models of ATRX deletion, but the damage is observed as speckles throughout the entire nucleus (Leung et al., 2013; Watson et al., 2013). The forelimb-deletion model is the only model with accumulation of DNA damage to a single, specific site. It is clear from the presence of the focus in female, heterozygote cells that the focus cannot be the Y-chromosome; however, the localisation to the nuclear lamina may offer clues to the identity, and in fact purpose, of this focus. Recent research in yeast has demonstrated that the nuclear periphery may play a role as a subcompartment for dangerous DNA elements to be sequestered and possibly repaired (Gartenberg, 2009). In mammalian cells, the inner nuclear envelope proteins SUN1 and SUN2 have been shown to be important in DNA damage response and even play a possible role in nonhomologous end joining repair (Lei et al., 2012). Disregulation of nuclear envelope proteins has been associated with DNA damage, progeroid, and aging phenotypes (Capell et al., 2006; Chen et al., 2012). These phenotypes are observed in models of ATR-X syndrome in which ATRX is deleted in the developing forebrain and anterior pituitary of mice (Watson et al., 2013). Although it can be predicted that localisation to the nuclear envelope is an important part of the cell response to ATRX depletion, the identity of the complex localising to the damaged sequences remains unknown. Chromatin immunoprecipitations for γ-H2AX on chromatin isolated from various ATRX-deficient cells may be useful in studying protein-DNA interactions at the site of DNA damage. Furthermore, chromatin immunoprecipitation techniques in conjunction with next-generation sequencing (ChIP-Seq) can offer a different perspective on the DNA-damage focus. By unbiased sequencing of all binding sites of γ-H2AX in ATRX-deficient cells, it may be possible to identify a common element or site of DNA breaks.

5.6 Concluding Remarks

In conclusion, my studies have demonstrated that ATRX has a limited direct role in skeletal development. Mouse models of ATRX loss in the skeleton reveal that cartilage, bone, and forelimb mesenchyme are relatively protected from the apoptotic and hypocellularity phenotypes seen in other ATRX-depletion models.

ATRX is unlikely to have a direct effect on skeletogenesis at the level of the cartilage, as mice lacking ATRX in this tissue are almost completely physically normal. Genetic mutations that directly affect important gene signalling in the growth plate lead to dwarfisms or other skeletal deformities which were not observed in this model. The deformities observed in ATR-X syndrome are not just in the growth plate; every tissue in the body is affected. Taken together, it can be predicted that skeletal phenotypes of ATR-X syndrome are caused by an indirect effect on the skeleton.

Loss of ATRX in the forelimb confers a brachydactyly phenotype, associated with DNA damage in limb cells which has been attributed to the role of ATRX in genomic integrity. In cases where loss of ATRX leads to p53-activated apoptotic cell death, the phenotype can be partially rescued by deleting p53 in the same tissue (Seah et al., 2008). However, in some models of tissue-specific loss of ATRX, there can be DNA damage without activation of cell death, or even loss of ATRX without associated DNA damage. These studies illustrate an important property of the skeletal system, which is that skeletal tissues may have reduced susceptibility to apoptosis related to DNA damage compared to other tissues.

References

Alvarez-Saavedra, M., Carrasco, L., Sura-Trueba, S., Demarchi Aiello, V., Walz, K., Neto, J.X., and Young, J.I. (2010). Elevated expression of MeCP2 in cardiac and skeletal tissues is detrimental for normal development. Human Molecular Genetics *19*, 2177-2190.

Arnold, M.A., Kim, Y., Czubryt, M.P., Phan, D., McAnally, J., Qi, X., Shelton, J.M., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2007). MEF2C Transcription Factor Controls Chondrocyte Hypertrophy and Bone Development. Developmental cell *12*, 377-389.

Bagheri-Fam, S., Argentaro, A., Svingen, T., Combes, A.N., Sinclair, A.H., Koopman, P., and Harley, V.R. (2011). Defective survival of proliferating Sertoli cells and androgen receptor function in a mouse model of the ATR-X syndrome. Human Molecular Genetics *20*, 2213-2224.

Baker, Steven A., Chen, L., Wilkins, Angela D., Yu, P., Lichtarge, O., and Zoghbi, Huda Y. (2013). An AT-Hook Domain in MeCP2 Determines the Clinical Course of Rett Syndrome and Related Disorders. Cell *152*, 984-996.

Baumann, C., and De La Fuente, R. (2009). ATRX marks the inactive X chromosome (Xi) in somatic cells and during imprinted X chromosome inactivation in trophoblast stem cells. Chromosoma *118*, 209-222.

Baumann, C., Viveiros, M.M., and De La Fuente, R. (2010). Loss of maternal ATRX results in centromere instability and aneuploidy in the mammalian oocyte and pre-implantation embryo. PLoS Genetics *6*.

Bérubé, N.G., Mangelsdorf, M., Jagla, M., Vanderluit, J., Garrick, D., Gibbons, R.J., Higgs, D.R., Slack, R.S., and Picketts, D.J. (2005). The chromatin-remodeling protein ATRX is critical for neuronal survival during corticogenesis. Journal of Clinical Investigation *115*, 258-267.

Callewaert, F., Boonen, S., and Vanderschueren, D. (2010). Sex steroids and the male skeleton: a tale of two hormones. Trends in Endocrinology & Metabolism *21*, 89-95.

Capell, B.C., and Collins, F.S. (2006). Human laminopathies: nuclei gone genetically awry. Nature Reviews Genetics *7*, 940-952.

Chen, C.-Y., Chi, Y.-H., Mutalif, Rafidah A., Starost, Matthew F., Myers, Timothy G., Anderson, Stasia A., Stewart, Colin L., and Jeang, K.-T. (2012). Accumulation of the Inner Nuclear Envelope Protein Sun1 Is Pathogenic in Progeric and Dystrophic Laminopathies. Cell *149*, 565-577.

De La Fuente, R., Viveiros, M.M., Wigglesworth, K., and Eppig, J.J. (2004). ATRX, a member of the SNF2 family of helicase/ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes. Developmental Biology *272*, 1-14.

Feldhahn, N., Ferretti, E., Robbiani, D.F., Callen, E., Deroubaix, S., Selleri, L., Nussenzweig, A., and Nussenzweig, M.C. (2012). The hSSB1 orthologue Obfc2b is essential for skeletogenesis but dispensable for the DNA damage response in vivo. The EMBO Journal *31*, 4045-4056.

Flaus, A., Martin, D.M.A., Barton, G.J., and Owen-Hughes, T. (2006). Identification of multiple distinct Snf2 subfamilies with conserved structural motifs. Nucleic Acids Research *34*, 2887-2905.

Gao, B., Hu, J., Stricker, S., Cheung, M., Ma, G., Law, K.F., Witte, F., Briscoe, J., Mundlos, S., He, L.*, et al.* (2009). A mutation in Ihh that causes digit abnormalities alters its signalling capacity and range. Nature *458*, 1196-1200.

Garrick, D., Samara, V., McDowell, T.L., Smith, A.J.H., Dobbie, L., Higgs, D.R., and Gibbons, R.J. (2004). A conserved truncated isoform of the ATR-X syndrome protein lacking the SWI/SNF-homology domain. Gene *326*, 23-34.

Garrick, D., Sharpe, J.A., Arkell, R., Dobbie, L., Smith, A.J., Wood, W.G., Higgs, D.R., and Gibbons, R.J. (2006). Loss of Atrx affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues. PLoS Genetics *2*, e58.

Gartenberg, M.R. (2009). Life on the edge: telomeres and persistent DNA breaks converge at the nuclear periphery. Genes & Development *23*, 1027-1031.

Gerber, I., and ap Gwynn, I. (2002). Differentiation of rat osteoblast-like cells in monolayer and micromass cultures. European Cells and Materials Journal *3*, 19-30.

Gibbons, R.J. (2006). Alpha thalassaemia-mental retardation, X linked. Orphanet Journal of Rare Diseases *1*.

Gibbons, R.J., Brueton, L., Buckle, V.J., Burn, J., Clayton-Smith, J., Davison, B.C.C., Gardner, R.J.M., Homfray, T., Kearney, L., Kingston, H.M.*, et al.* (1995). Clinical and hematologic aspects of the X-linked α-thalassemia/mental retardation syndrome (ATR-X). The American Journal of Human Genetics *55*, 288-299.

Gibbons, R.J., and Higgs, D.R. (2000). Molecular-clinical spectrum of the ATR-X syndrome. The American Journal of Human Genetics *97*, 204-212.

Gibbons, R.J., Wada, T., Fisher, C.A., Malik, N., Mitson, M.J., Steensma, D.P., Fryer, A., Goudie, D.R., Krantz, I.D., and Traeger-Synodinos, J. (2008). Mutations in the chromatin-associated protein ATRX. Human Mutation *29*, 796-802.

Guo, S., Zhou, J., Gao, B., Hu, J., Wang, H., Meng, J., Zhao, X., Ma, G., Lin, C., Xiao, Y.*, et al.* (2010). Missense mutations in IHH impair Indian Hedgehog signaling in C3H10T1/2 cells: Implications for brachydactyly type A1, and new targets for Hedgehog signaling. Cellular & Molecular Biology Letters *15*, 153-176.

Halliwell, B. (2003). Oxidative stress in cell culture: an under-appreciated problem? FEBS Letters *540*, 3-6.

Hayman, D.M., Blumberg, T.J., Scott, C.C., and Athanasiou, K.A. (2006). The effects of isolation on chondrocyte gene expression. Tissue Engineering *12*, 2573-2581.

Horwitz, A.L., and Dorfman, A. (1970). THE GROWTH OF CARTILAGE CELLS IN SOFT AGAR AND LIQUID SUSPENSION. The Journal of Cell Biology *45*, 434-438.

Hug, B.A. (2004). HDAC4: A corepressor controlling bone development. Cell *119*, 448-449.

Huh, M.S., Price O'Dea, T., Ouazia, D., McKay, B.C., Parise, G., Parks, R.J., Rudnicki, M.A., and Picketts, D.J. (2012). Compromised genomic integrity impedes muscle growth after Atrx inactivation. Journal of Clinical Investigation *122*, 4412-4423.

Ion, A., Telvi, L., Chaussain, J.L., Galacteros, F., Valayer, J., Fellous, M., and McElreavey, K. (1996). A novel mutation in the putative DNA helicase XH2 is responsible for male-to-female sex reversal associated with an atypical form of the ATR-X syndrome. The American Journal of Human Genetics *58*, 1185-1191.

Jensen, E.D., Schroeder, T.M., Bailey, J., Gopalakrishnan, R., and Westendorf, J.J. (2008). Histone deacetylase 7 associates with Runx2 and represses its activity during osteoblast maturation in a deacetylation-independent manner. . Journal of Bone and Mineral Research *23*, 361-372.

Kawauchi, S., Calof, A.L., Santos, R., Lopez-Burks, M.E., Young, C.M., Hoang, M.P., Chua, A., Lao, T., Lechner, M.S., Daniel, J.A.*, et al.* (2009). Multiple Organ System Defects and Transcriptional Dysregulation in the Nipbl $^{+/-}$ Mouse, a Model of Cornelia de Lange Syndrome. PLoS Genetics *5*, e1000650.

Kernohan, K.D., Jiang, Y., Tremblay, D.C., Bonvissuto, A.C., Eubanks, J.H., Mann, M.R.W., and Bérubé, N.G. (2010). ATRX Partners with Cohesin and MeCP2 and Contributes to Developmental Silencing of Imprinted Genes in the Brain. Developmental cell *18*, 191-202.

Law, M.J., Lower, K.M., Voon, H.P.J., Hughes, J.R., Garrick, D., Viprakasit, V., Mitson, M., De Gobbi, M., Marra, M., Morris, A.*, et al.* (2010). ATR-X Syndrome Protein Targets Tandem Repeats and Influences Allele-Specific Expression in a Size-Dependent Manner. Cell *143*, 367-378.

Lei, K., Zhu, X., Xu, R., Shao, C., Xu, T., Zhuang, Y., and Han, M. (2012). Inner Nuclear Envelope Proteins SUN1 and SUN2 Play a Prominent Role in the DNA Damage Response. Current Biology *22*, 1609-1615.

Leung, J.W.-C., Ghosal, G., Wang, W., Shen, X., Wang, J., Li, L., and Chen, J. (2013). Alpha thalassemia/mental retardation syndrome X-linked gene product ATRX is required for proper replication restart and cellular resistance to replication stress. Journal of Biological Chemistry *288*, 6342-6350.

Logan, M., Martin, J.F., Nagy, A., Lobe, C., Olson, E.N., and Tabin, C.J. (2002). Expression of Cre recombinase in the developing mouse limb bud driven by a Prxl enhancer. genesis *33*, 77-80.

Lovejoy, C.A., Li, W., Reisenweber, S., Thongthip, S., Bruno, J., de Lange, T., De, S., Petrini, J.H.J., Sung, P.A., Jasin, M.*, et al.* (2012). Loss of ATRX, Genome Instability, and an Altered DNA Damage Response Are Hallmarks of the Alternative Lengthening of Telomeres Pathway. PLoS Genetics *8*, e1002772.

Medina, C.F., Mazerolle, C., Wang, Y., Bérubé, N.G., Coupland, S., Gibbons, R.J., Wallace, V.A., and Picketts, D.J. (2009). Altered visual function and interneuron survival in Atrx knockout mice: inference for the human syndrome. Human Molecular Genetics *18*, 966-977.

Nan, X., Hou, J., Maclean, A., Nasir, J., Lafuente, M.J., Shu, X., Kriaucionis, S., and Bird, A. (2007). Interaction between chromatin proteins MECP2 and ATRX is disrupted by mutations that cause inherited mental retardation. Proceedings of the National Academy of Sciences *104*, 2709-2714.

Nogami, T., Beppu, H., Tokoro, T., Moriguchi, S., Shioda, N., Fukunaga, K., Ohtsuka, T., Ishii, Y., Sasahara, M., Shimada, Y.*, et al.* (2010). Reduced expression of the ATRX gene, a chromatin-remodeling factor, causes hippocampal dysfunction in mice. Hippocampus, n/a-n/a.

Nohno, T., Koyama, E., Myokai, F., Taniguchi, S., Ohuchi, H., Saito, T., and Noji, S. (1993). A Chicken Homeobox Gene Related to Drosophila paired Is Predominantly Expressed in the Developing Limb. Developmental Biology *158*, 254-264.

Picketts, D.J., Tastan, A.O., Higgs, D.R., and Gibbons, R.J. (1998). Comparison of the human and murine ATRX gene identifies highly conserved, functionally important domains. Mammalian Genome *9*, 400-403.

Qian, Y., Jung, Y.-S., and Chen, X. (2012). Differentiated embryo-chondrocyte expressed gene 1 regulates p53-dependent cell survival versus cell death through macrophage inhibitory cytokine-1. Proceedings of the National Academy of Sciences *109*, 11300-11305.

Ritchie, K., Seah, C., Moulin, J., Isaac, C., Dick, F., and Bérubé, N.G. (2008). Loss of ATRX leads to chromosome cohesion and congression defects. The Journal of Cell Biology *180*, 315-324.

Schoor, M., Schuster-Gossler, K., Roopenian, D., and Gossler, A. (1999). Skeletal dysplasias, growth retardation, reduced postnatal survival, and impaired fertility in mice lacking the SNF2/SWI2 family member ETL1. Mechanisms of Development *85*, 73-83.

Seah, C., Levy, M.A., Jiang, Y., Mokhtarzada, S., Higgs, D.R., Gibbons, R.J., and Berube, N.G. (2008). Neuronal death resulting from targeted disruption of the Snf2 protein ATRX is mediated by p53. Journal of Neuroscience *28*, 12570-12580.

Shapiro, J.R., Bibat, G., Hiremath, G., Blue, M.E., Hundalani, S., Yablonski, T., Kantipuly, A., Rohde, C., Johnston, M., and Naidu, S. (2010). Bone mass in Rett syndrome: association with clinical parameters and MECP2 mutations. Pediatric Research *68*, 446-451.

Solomon, L.A., Li, J.R., Berube, N.G., and Beier, F. (2009). Loss of ATRX in chondrocytes has minimal effects on skeletal development. PLoS ONE *4*, e7106.

Solomon, L.A., Russell, B.A., Watson, L.A., Beier, F., and Berube, N.G. (2013). Targeted loss of the ATR-X syndrome protein in the limb mesenchyme of mice causes brachydactyly.

Sun, F., Chen, Q., Yang, S., Pan, Q., Ma, J., Wan, Y., Chang, C.-H., and Hong, A. (2009a). Remodeling of chromatin structure within the promoter is important for bmp-2-induced fgfr3 expression. Nucleic Acids Research.

Sun, X., Wei, L., Chen, Q., and Terek, R.M. (2009b). HDAC4 Represses Vascular Endothelial Growth Factor Expression in Chondrosarcoma by Modulating RUNX2 Activity. Journal of Biological Chemistry *284*, 21881-21890.

Tarutani, M., Itami, S., Okabe, M., Ikawa, M., Tezuka, T., Yoshikawa, K., Kinoshita, T., and Takeda, J. (1997). Tissue-specific knockout of the mouse Pig-a gene reveals important roles for GPI-anchored proteins in skin□development. Proceedings of the National Academy of Sciences *94*, 7400-7405.

Tickle, C. (2003). Patterning Systems--From One End of the Limb to the Other. Developmental cell *4*, 449-458.

Tonkin, E.T., Wang, T.J., Lisgo, S., Bamshad, M.J., and Strachan, T. (2004). NIPBL, encoding a homolog of fungal Scc2-type sister chromatid cohesion proteins and fly Nipped-B, is mutated in Cornelia de Lange syndrome. Nature Genetics *36*, 636-641.

Vandenput, L., Swinnen, J.V., Boonen, S., Van Herck, E., Erben, R.G., Bouillon, R., and Vanderschueren, D. (2004). Role of the Androgen Receptor in Skeletal Homeostasis: The Androgen-Resistant Testicular Feminized Male Mouse Model. Journal of Bone and Mineral Research *19*, 1462-1470.

Vega, R.B., Matsuda, K., Oh, J., Barbosa, A.C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J.M., Richardson, J.A.*, et al.* (2004). Histone Deacetylase 4 Controls Chondrocyte Hypertrophy during Skeletogenesis. Cell *119*, 555-566.

Vidal, O., Lindberg, M.K., Hollberg, K., Baylink, D.J., Andersson, G., Lubahn, D.B., Mohan, S., Gustafsson, J.-Å., and Ohlsson, C. (2000). Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. Proceedings of the National Academy of Sciences *97*, 5474-5479.

Villacorte, M., Suzuki, K., Hayashi, K., de Sousa Lopes, S.C., Haraguchi, R., Taketo, M.M., Nakagata, N., and Yamada, G. (2010). Antagonistic crosstalk of Wnt/β-catenin/Bmp signaling within the Apical Ectodermal Ridge (AER) regulates interdigit formation. Biochemical and Biophysical Research Communications *391*, 1653-1657.

Watson, L.A., Solomon, L.A., Li, J.R., Jiang, Y., Edwards, M., Shin-Ya, K., Beier, F., and Berube, N.G. (2013). Atrx deficiency induces telomere dysfunction, endocrine defects, and reduced life span. Journal of Clinical Investigation *123*, 2049–2063.

Williams, S.R., Aldred, M.A., Der Kaloustian, V.M., Halal, F., Gowans, G., McLeod, D.R., Zondag, S., Toriello, H.V., Magenis, R.E., and Elsea, S.H. (2010). Haploinsufficiency of HDAC4 Causes Brachydactyly Mental Retardation Syndrome, with Brachydactyly Type E, Developmental Delays, and Behavioral Problems. The American Journal of Human Genetics *87*, 219-228.

Young, D.W., Pratap, J., Javed, A., Weiner, B., Yasuyuki Ohkawa, Wijnen, A.v., Montecino, M., Stein, G.S., Stein, J.L., Imbalzano, A.N.*, et al.* (2005). SWI/SNF chromatin remodeling complex is obligatory for BMP2-induced, Runx2-dependent skeletal gene expression that controls osteoblast differentiation. Journal of Cellular Biochemistry *94*, 720-730.

Yu, K., Xu, J., Liu, Z., Sosic, D., Shao, J., Olson, E.N., Towler, D.A., and Ornitz, D.M. (2003). Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development *130*, 3063-3074.

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>
> Figure 1-3: Solomon, L.A., Bérubé, N.G., Beier, F. (2008). Transcriptional regulators of chondrocyte hypertrophy. Birth Defects Res C Embryo Today. 2008 Jun;84(2):123-30. doi: 10.1002/bdrc.20124.

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> Solomon LA, Li JR, Bérubé NG, Beier F. (2009) Loss of ATRX in chondrocytes has minimal effects on skeletal development. PLoS One. 2009 Sep 23;4(9):e7106. doi: 10.1371/journal.pone.0007106.

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> Solomon LA, Russell RA, Watson LA, Beier F, Bérubé NG (2013) Targeted loss of the ATR-X syndrome protein in the limb mesenchyme of mice causes brachydactyly. Human Molecular Genetics. doi:10.1093/hmg/ddt351

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Appendix F: Statement of Permission for the Use of Animals for Experimental Research.

All animal experimentation was conducted in compliance with the animal use protocol 2007-045 held by Dr. Frank Beier, principal investigator at the Schulich School of Medicine and Dentistry and the department of Physiology and Pharmacology at the University of Western Ontario in London, Ontario, Canada.

Western

2007-045-06::5:

AUP Number: 2007-045-06
AUP Title: Regulation of Endochondral Bone Growth by Hormones
Yearly Renewal Date: 08/01/2012

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-268 has been approved, and will be approved for one year following the above review date.

This AUP number must be indicated when ordering animals for this project.

Animals for other projects may not be ordered under this AUP number.

Purchases of animals other than through this system must be cleared through the ACVS office.
Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Curriculum Vitae

Lauren Solomon

EDUCATION

2007-present **University of Western Ontario, London, ON, Canada**

PhD. Biochemistry (Developmental Biology)

Thesis title: The chromatin remodeling protein Atrx in development and maintenance of mouse skeletal tissues

2002-2007 **Ryerson University, Toronto, ON, Canada**
BSc. Hon (Applied Chemistry and Biology, Co-op)

Thesis title: Allelic Imbalance Analysis of Paediatric Astrocytomas by high-density SNP arrays using whole-genome amplified DNA

PUBLICATIONS

Solomon LA, Russell BA, Watson LA, Beier F, Bérubé NG (2013) Targeted loss of the ATR-X syndrome protein in the limb mesenchyme of mice causes brachydactyly. Human Molecular Genetics. doi:10.1093/hmg/ddt351

Watson A, **Solomon LA**, Li JR, Jiang Y, Edwards M, Shin-Ya K, Beier F, Bérubé NG (2013) Atrx deficiency induces telomere dysfunction, endocrine defects, and reduced life span. *Journal of Clinical Investigation.* Apr 8; doi:10.1172/JCI65634.

Solomon LA, Li JR, Bérubé NG, Beier F (2009) Loss of ATRX in Chondrocytes Has Minimal Effects on Skeletal Development. *PLoS ONE* 4(9): e7106. doi:10.1371/journal.pone.0007106

Solomon LA, Bérubé NG, Beier F (2008) Transcriptional regulators of chondrocyte hypertrophy. *Birth Defects Res C Embryo Today*. Jun 10; 84(2):123-130

Qu HQ, Jacob K, Fatet S, Ge B, Barnett D, Delattre O, Faury D, Montpetit A, **Solomon L**, Hauser P, Garami M, Bognar L, Hansely Z, Mio R, Farmer JP, Albrecht S, Polychronakos C, Hawkins C, Jabado N. (2010) Genome-wide profiling using single-nucleotide polymorphism arrays identifies novel chromosomal imbalances in pediatric glioblastomas. *Neuro Oncol*. Feb;12(2):153-63.

Liang ML, Ma J, Ho M, **Solomon L**, Bouffet E, Rutka JT, Hawkins C (2008). Tyrosine kinase expression in pediatric high grade astrocytoma. *J Neurooncol*. May; 87(3):247-53.

AWARDS AND SCHOLARSHIPS

Suzanne Bernier Memorial Award in Skeletal Biology, 2013

Ontario Graduate Scholarship May 2011, May 2010, May 2009

Ontario Graduate Scholarship for Science and Technology (OGSST) May 2008

Curtis Cadman Graduate Studentship Award 2008, 2009

COMMITTEES

London District Science and Technology Fair, Social Media coordinator

-Volunteer committee member to support, encourage & operate activities that promote the science fair for grades 4-12 in London, Ontario - Since 2011

TEACHING EXPERIENCE

Honors student mentor

-Supervised fourth-year honors students conducting research projects in the lab of Dr. Frank Beier 2008-2013

Teaching Assistant

-Science and Technology in the 20th Century 2007

CONFERENCES AND MEETINGS ATTENDED

London Health Sciences Research Day. London, ON. 2013
"Targeted loss of the ATR-X syndrome protein in the limb mesenchyme of mice causes brachydactyly" - poster presentation

Bone and Cartilage: from Development to Human Diseases. Cold Spring Harbor Asia, China. 2012
"Analysis of defects caused by ATRX deficiency in the mouse forelimb" - poster presentation

Paediatrics Research Day, CHRI, London, ON. 2012
"Analysis of defects caused by ATRX deficiency in the mouse forelimb" - poster presentation

Epigenetics, Eh! London, ON. 2011

London Health Sciences Research Day. London, ON. 2011
" Atrx in Skeletal Development" - poster presentation

Developmental Biology Research Day, London, ON. 2010
" Atrx in Skeletal Development" - platform presentation

Great Lakes Mammalian Development Conference. Toronto, ON. 2010
" Atrx in Skeletal Development" - platform presentation

Gordon Research Conference (Bones and Teeth). University of New England, Maine. 2009
" Loss of ATRX in Chondrocytes has Minimal Effects on Skeletal Development" - poster presentation

Great Lakes Mammalian Development Conference. Toronto, ON. 2009
" The Alpha-thalassemia X-linked Mental Retardation (Atrx) Gene in Skeletal Development. " - poster presentation

Paediatrics Research Day, LHSC, London, ON. 2008
"The Alpha-thalassemia X-linked Mental Retardation (Atrx) Gene in Skeletal Development. " - poster presentation

Great Lakes Mammalian Development Conference. Toronto, ON. 2008

Margaret Moffat Research Day, UWO 2008
"The Alpha-thalassemia X-linked Mental Retardation (ATRX) Gene Affects Development of Bone" - poster presentation