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Regulation of Imprinted Genes in the brain by the ATRX Chromatin Remodeling Protein

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(Thesis format: Integrated Article)

by

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Abstract

Alpha-thalassemia mental retardation, X-linked (ATRX) is a SWI/SNF-like chromatin remodeling protein, enriched at heterochromatic regions of the genome. Disruption of *ATRX* in humans causes a neurodevelopmental disorder known as ATR-X Syndrome, and has been linked to paediatric neuronal cancers, suggesting an important role for ATRX in the regulation of chromatin structure in the developing brain. At the outset of this study direct ATRX target genes had not yet been identified. This thesis identifies imprinted genes as targets of ATRX in the developing brain, and explores the mechanism of ATRX regulation at these sites, using the *H19/Igf2* imprinted domain as a model. My findings indicate that in the forebrain ATRX localizes to the maternal allele of the *H19* imprinting control region (*H19* ICR) with methyl CpG binding protein 2 (MeCP2), CCCTC-binding factor (CTCF) and Cohesin, three important regulators of chromatin structure. ATRX is recruited by MeCP2 to the *H19* ICR, where it then governs the profile of post-translational histone modifications and nucleosome occupancy to maintain CTCF and Cohesin binding. CTCF and Cohesin are essential constituents of the *cis* and *trans* chromatin interactions that regulate the expression of imprinted genes. Loss of either ATRX or MeCP2 disrupts *cis* chromosomal interactions across *H19/Igf2*. A role for ATRX in *cis* at several imprinted genes is supported by its ability to bind directly to many imprinted domains. Taken together, these findings indicate that ATRX can regulate the expression of target genes in the brain by altering nucleosome positioning to control local chromatin interactions.

Keywords

ATRX, MeCP2, CTCF, Cohesin, higher-order chromatin structure, chromatin remodeling, imprinted gene network, brain development

Co-Authorship Statement

I participated in the design and execution of all experiments presented in this thesis with the following exceptions:

In chapter two, co-immunoprecipitation experiments in Figure 2-1 and Supplementary Figure 2-7, as well as the identification of the ATRX, MeCP2 and Cohesin bound region in Figure 2-2B, and allelic histone ChIP in Supplementary Figure 2-10 were conducted by Yan Jiang. For statistical purposes some chromatin immunoprecipitation (ChIP) reactions in Figure 2-6B,C were also conducted by Yan Jiang (6 of 16). Methylation analysis in Figure 2-3A, and Supplementary Figure 2-9, as well as semi-quantitative expression in Figure 2-4A was conducted by Deanna Tremblay. Figure 2-4D-F allelic expression analysis was conducted by Anne Bonvisutto. Statistical analysis on ChIP data was conducted by Dr. Andrew Fernandez. Some mouse husbandry was conducted by Yan Jiang. Portions of chapter two were written by Dr. Nathalie Bérubé.

In chapter three, 4C bioinformatics analysis was conducted by Dr. Gregory Gloor, embryonic stem cell ChIP sequencing analysis was conducted by Michael Levy and mouse husbandry was conducted by Yan Jiang.

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fortunate to have such a large support system, you are always my biggest champions, and each and every one of you holds a special place in my heart.

Abbreviations

Abbreviation	Meaning
µL:	Microliter
^{o}C:	Degrees Celsius
3C:	Chromosome conformation capture
3D:	Three dimensional
4C:	Circular chromosome conformation capture
5C:	Chromosome conformation capture carbon copy
6C:	Combined chromosome conformation capture ChIP cloning
ADD:	ATRX-DNMT3-DNMT3L
ALT:	Alternative lengthening of telomeres
ANOVA:	Analysis of variance
ATP:	Adenosine tri-phosphate
ATRX:	Alpha-thalassemia mental retardation, X-linked
ATR-X:	Alpha-thalassemia mental retardation, X-linked Syndrome
BAC:	Bacterial artificial chromosome
BDNF:	Brain-derived neurotrophic factor
bp:	Base pairs
BRG1:	Brahma-related gene 1
Calcr:	Calcitonin receptor

CAST:	*Mus musculus castaneus*
CCD:	Centrally conserved domain
cDNA:	Complementary DNA
Cdkn1c:	Cyclin-dependent kinase inhibitor 1C
CdLS:	Cornelia de Lange Syndrome
ChIA-PET:	Chromatin interaction analysis by paired-end tag sequencing
ChIP:	Chromatin immunoprecipitation
Copg2:	Coatomer subunit gamma-2
CTCF:	CCCTC-binding factor
DAPI:	4',6-diamidino-2-phenylindole
DAXX:	Death associated protein 6
DIG:	Digoxigenin
Dlk1:	Delta-like homolog 1
DMEM:	Dulbecco's Modified Eagle Medium
DMR:	Differentially methylated region
DNA:	Deoxyribonucleic acid
DNAse:	Deoxyribonuclease
DNMT:	DNA Methyltransferase
DTT:	Dithiothreitol
E13.5:	Embryonic day 13.5

EKLF:	Erythroid kruppel-like factor
Ercc3:	Excision repair cross-complementing rodent repair deficiency, complementation group 3
ES:	Embryonic stem
EZH2:	Enhancer of zeste homolog 2
FBS:	Fetal bovine serum
FISH:	Fluorescent in-situ hybridization
FOG1:	Friend of GATA protein 1
FoxG1:	Forkhead box G1
Gabrb3:	Gamma-aminobutyric acid A receptor, beta 3
GATA1:	GATA binding protein 1
Gnas:	Guanine nucleotide binding protein, alpha stimulating
Gtl2:	Gene trap locus 2
H3Ac:	Acetylated histone H3
H3K9MTase:	Histone 3 lysine 9 methyltransferase
H4Ac:	Acetylated histone H4
HDAC:	Histone deacetylase
Hi-C:	Global 3C interactions
IAP:	Intracisternal A-particle

ICF:	Roberts, Rubinstein-Taybi and Immunodeficiency, Chromosome instability and Facial anomalies Syndrome
ICR:	Imprinting control region
Igf2:	Insulin-like growth factor 2
IgG:	Immunoglobulin G
IgH:	Immunoglobulin heavy chain
IGN:	Imprinted gene network
IgY:	Immunoglobulin Y
Ins:	Insulin
kb:	Kilobase
KCl:	Potassium chloride
kDa:	Kilodalton
M:	Molar
mM:	Millimolar
MAR:	Matrix-attachment region
Mash2:	Mammalian achaete scute homologue 2
MBD:	Methyl binding domain
MeCP2:	Methyl CpG binding protein 2
meH3K9:	Methylated histone 3 lysine 9
meH3K37:	Methylated histone 3 lysine 27
meH4K20:	Methylated histone 4 lysine 20

$MgCl_2$:	Magnesium chloride
MHC:	Major histocompatibility complex
mL:	Milliliter
mRNA:	Messenger RNA
mSin3a:	Mammalian *saccharomyces cerevisiae* general transcriptional repressor 3a
NaCl:	Sodium chloride
ncRNA:	Non-coding RNA
Ndn:	Non-synaptic diffusion neurotransmission
NIPBL:	Nipped-B-like protein
NL1:	Nuclear LIM interactor
NP40:	Nonyl phenoxypolyethoxylethanol
P0.5:	Postnatal day 0.5
P17:	Postnatal day 17
PAGE:	Polyacrylamide gel electrophoresis
PBS:	Phosphate buffered saline
PCH:	Pericentromeric heterochromatin
PCR:	Polymerase chain reaction
PDS5:	Precocious dissociation of sisters 5
Peg3:	Paternally expressed gene 3

Peg10:	Paternally expressed gene 10
PHD:	Plant homeo domain
PK:	Proteinase K
Rad21:	Radiation mutant 21
Rad54:	Radiation mutant 54
RasGrf1:	Ras protein-specific guanine nucleotide-releasing factor 1
rDNA:	Ribosomal DNA
RNA:	Ribonucleic acid
RNAi:	Ribonucleic acid interference
RNase:	Ribonuclease
RSC:	Chromatin structure remodeling
RTT:	Rett Syndrome
SA1:	Stromal antigen 1
SA2:	Stromal antigen 2
SDS:	Sodium dodecyl sulfate
Ski:	Sloan-Kettering Institute
SMC:	Structural maintenance of chromosome
SNF2:	Sucrose non-fermenting 2
Snrpn:	Small nuclear ribonucleoprotein polypeptide N
SSC:	Saline-sodium citrate

SWI/SNF:	Switch/sucrose non-fermenting
TH2:	T helper type 2
TRD:	Transcriptional repression domain
Tris:	Tris(hydroxymethyl)aminomethane
Tris-HCl:	Tris(hydroxymethyl)aminomethane-hydrogen chloride
TX-100:	TritonX-100
U:	Units
UV:	Ultraviolet
Ubea3:	Ubiquitin-protein ligase E3A
Usp29:	Ubiquitin specific peptidase 29
Walpl:	Wings apart-like 1
XPB:	Xeroderma pigmentosum B
YY1:	Yin Yang 1
Zac1:	Zinc finger associated 1

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Chapter 1

Portions of Chapter 1 are reproduced with permission from (Kernohan and Bérubé, 2010) (Appendix A)

1 General Introduction

As our understanding of the genome evolves, we are continually discovering the complex and dynamic nature of its regulation. In addition to the encoded DNA sequence, we now know that epigenetic regulatory mechanisms can dictate and refine gene expression. Imprinted genes are a distinct class of epigenetically regulated genes that are preferentially expressed from one parental allele. A number of these genes are crucial for placental function and embryonic growth in mice and humans(Lefebvre, 2012; Piedrahita, 2011). Disruption of imprinted genes is also associated with several neurodevelopmental disorders, although the role and regulation of genomic imprinting in the brain remains largely unresolved(Kernohan and Bérubé, 2010). Mutations in alpha-thalassemia mental retardation, X-linked (*ATRX*), encoding the ATRX chromatin remodeling protein, cause a neurological syndrome known as ATR-X Syndrome(Gibbons et al., 1995b). Studies in mice have confirmed a requirement for ATRX in brain development(Bérubé et al., 2005); however, direct gene expression targets of ATRX in the brain have not yet been identified. The work presented herein reveals that ATRX regulates the expression of imprinted genes during the developmental switch from a highly proliferative to a post-mitotic state occurring in the neonatal brain. The *H19/Igf2* imprinted domain was used as a model to elucidate the mechanisms by which ATRX regulates gene expression.

1.1 The ATRX Gene and Protein

1.1.1 The ATRX Gene and Protein

The Alpha-Thalassemia mental Retardation X-linked (*ATRX*) gene contains 36 exons and spans over 300 kb of genomic DNA on the X chromosome(Picketts et al., 1996). *ATRX* is translated into two protein isoforms, the full length 280 kDa ATRX protein, and a truncated 200 kDa protein known as ATRXt(Garrick et al., 2004) (Figure 1-1). ATRXt is

generated from the alternative splicing of exon 11, which results in the use of an alternative polyA signal(Garrick et al., 2004). ATRX and ATRXt are highly conserved between mouse and human and their abundance differs throughout development, suggesting each may have important biological functions(Garrick et al., 2004; Gecz et al., 1994; Picketts et al., 1998).

The ATRX protein has two main conserved domains: an ADD domain and a SWI/SNF domain, joined by a large flexible linker region(Picketts et al., 1998) (Figure 1-1). The ATRX-DNMT3-DNMT3L (ADD) domain, named for its homology to the DNMT3 family of DNA methyltransferases, is located at the N-terminus and is comprised of a plant homeo domain (PHD)-like zinc-finger, a GATA-like zinc finger, and an alpha-helical region(Aapola et al., 2000; Argentaro et al., 2007; Xie et al., 1999). Together these motifs are responsible for ATRX's ability to associate with DNA and other proteins (Argentaro et al., 2007; Cardoso et al., 2000; Dhayalan et al., 2011; Wong et al., 2010). The switch/sucrose non-fermenting (SWI/SNF) domain is located at the C-terminus and contains seven highly conserved collinear helicase motifs(Flaus et al., 2006), which confer ATRX's ATPase activity(Tang et al., 2004). Amino acid sequence alignment has shown that ATRX's SWI/SNF domain is similar to Rad54, a DNA translocase, and to other SWI/SNF proteins that function in complexes utilizing the energy of ATP to translocate along the chromatin fiber(Flaus et al., 2006). SWI/SNF protein translocation modifies the histone-DNA interface in a process known as chromatin remodeling, which functions to regulate chromatin structure and gene expression(Reviewed in (Clapier and Cairns, 2009; Euskirchen et al., 2012; Hargreaves and Crabtree, 2011; Kasten et al., 2011)) (discussed in section 1.4.1). *In vitro* biochemical studies have confirmed that ATRX is a DNA translocase(Mitson et al., 2011; Xue et al., 2003), and that its ATPase activity is dependent on the presence of nucleosomes(Tang et al., 2004). Importantly, ATRXt lacks the SWI/SNF domain and therefore likely does not function as an ATPase or chromatin remodeler(Garrick et al., 2004). Aside from this, little is known about the structure or function of ATRXt.

Early immunofluorescence studies demonstrated that ATRX is a nuclear protein that localizes largely to pericentromeric heterochromatin (PCH) and ribosomal DNA, both of which are repetitive heterochromatic regions(McDowell et al., 1999). More recently, genome-wide chromatin immunoprecipitation (ChIP) studies have shown that in addition to these sites, ATRX is also enriched at GC-rich regions and telomeric repeats(Law et al., 2010). ATRX may target heterochromatin by recognizing specific post-translational histone modifications, including the presence of H3K9me3 and absence of H3K4me2 and H3K4me3(Dhayalan et al., 2011; Eustermann et al., 2011; Lewis et al., 2010; Wong et al., 2010). Additionally, ATRX can be recruited to these sites by other proteins. For example, it was found that ATRX functions with the death domain associated protein DAXX at telomeres(Lewis et al., 2010). When the G-rich telomeric repeat sequences are single stranded, as occurs during DNA replication or transcription, they are predicted to form physical DNA structures called G-quadruplexes(Bifi et al., 2013). It was proposed that DAXX recruits ATRX to G-quadruplex-DNA where it translocates along the chromatin fibre to help insert H3.3 and resolve these DNA structures(Goldberg et al. 2010; Law et al., 2010; Lewis et al., 2010; Wong et al., 2010). This relationship with DAXX is likely one of many partnerships ATRX forms throughout the genome, as interactions have already been described with a number of other proteins, including the heterochromatin associated protein HP1α(Lechner et al., 2005), the polycomb group protein EZH2(Cardoso et al., 1998), and the methyl CpG-binding protein MeCP2(Nan et al., 2007). The function and dynamics of these partnerships throughout the genome are not yet known.

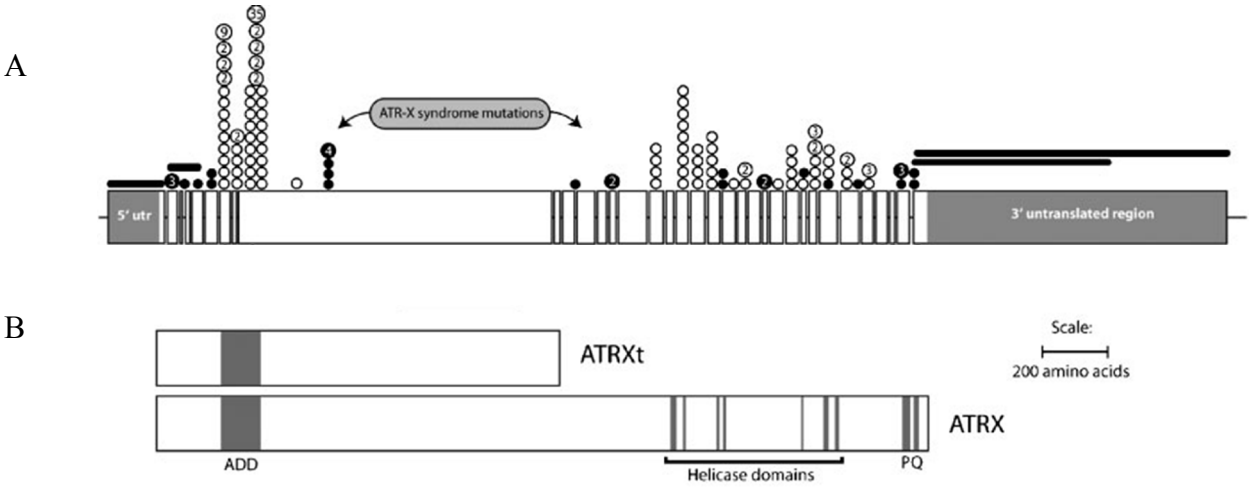

Figure 1-1. The ATRX gene and protein

(A) Schematic of the ATRX gene. Boxes mark exons, while horizontal lines represent introns. Marks above the gene symbolize ATR-X Syndrome mutation sites: filled circles represent truncating mutations, open circles signify missense mutations or small deletions which maintain the open reading frame, horizontal lines indicate deletion mutations, and recurrent mutations are illustrated by larger circles with the number of families indicated. Overall, the spectrum of ATR-X mutations highlights the ADD and SWI/SNF helicase domains as chiefly affected. (B) Schematic of the ATRX and ATRXt proteins displays the organization of the ADD and SWI/SNF domains and the truncation site for ATRXt.

Reproduced with permission from: Gibbons et al. (2008) Human Mutation 29(6):796-802. (Appendix B)

1.1.2 Mutations in the ATRX Gene Cause X-linked Mental Retardation

ATR-X Syndrome is a neurodevelopmental disorder associated with moderate-to-severe cognitive deficits, lack of speech development, microcephaly (reduced brain size), seizures, facial dysmorphisms, and genital, skeletal, and urogenital abnormalities(Gibbons et al., 1995a; Gibbons et al., 1995b; Gibbons et al., 1992). Patients are also commonly afflicted with alpha-thalassemia, a type of anaemia resulting from reduced alpha-globin expression in red blood cells(Gibbons et al., 1995a; Gibbons et al., 1995b; Gibbons et al., 1992). Signs of ATR-X Syndrome are evident at birth and affect multiple organ systems, but most notably the central nervous system(Gibbons et al., 1995a; Gibbons et al., 1995b; Gibbons et al., 1992). This rare syndrome predominantly affects males and is a consequence of inherited mutations in the *ATRX* gene(Gibbons et al., 1995b; Gibbons et al., 1992). Females who inherit *ATRX* mutations are unaffected due to a skewed pattern of X-inactivation(Gibbons et al., 1992). Currently approximately 200 ATR-X patients have been identified, carrying a total of 113 different *ATRX* mutations, all of which lie within the ADD domain (49%), the SWI/SNF domain (30%), or are truncating (21%)(Gibbons et al., 2008) (Figure 1-1). A number of mutations within the SWI/SNF domain have been demonstrated to compromise ATPase and DNA translocase activity(Mitson et al., 2011), while those in the ADD domain impair ATRX's DNA and/or protein binding capacity(Cardoso et al., 2000). The distribution of disease mutations indicate that alterations in other regions of the protein are either undisruptive or lethal. To date all ATR-X mutations studied function as hypomorphic alleles, and no patients have been documented with a complete lack of ATRX protein, suggesting that ATRX-null mutations are not compatible with life(Gibbons et al., 2008). While the limited cohort of ATR-X patients largely precludes studies employing patient samples, analysis of erythroid cells have highlighted a link between ATRX and DNA methylation(Gibbons et al., 2000). It was found that ribosomal DNA repeats, Y chromosome repeats, and subtelomeric repeats were abnormally methylated in erythroid cells from ATR-X patients(Gibbons et al., 2000). Whether this phenomenon occurs elsewhere in the genome and contributes to the ATR-X phenotype remains unknown.

1.1.3 ATRX is Required for Mouse Brain Development

Several mouse models have been created in an effort to understand the developmental role of ATRX and the various pathologies seen in ATR-X Syndrome patients(Bagheri-Fam et al., 2011; Bérubé et al., 2002; Bérubé et al., 2005; Garrick et al., 2006; Medina et al., 2009; Nogami et al., 2011; Seah et al., 2008; Shioda et al., 2011; Solomon et al., 2009). However, these do not necessarily replicate the human syndrome, which results from hypomorphic mutations rather than complete loss of ATRX(Gibbons and Higgs, 2000). To date, only one mouse model has been created which recapitulates patient mutations; this model lacks exon 2 as seen in patients with more mild forms of mental retardation(Nogami et al., 2011). Brain morphology in the ATRX-exon2 mutant model was largely normal; however, dendritic spine formation was abnormal in the medial prefrontal cortex, and hippocampal functioning was altered(Nogami et al., 2011). Other models have focused on effects of removing the entire ATRX protein. Ablation of full-length ATRX starting at the 8–16 cell stage (using *GATA1*- driven expression of Cre Recombinase) causes embryonic lethality due to defective formation of the extra-embryonic trophoblast and abnormal imprinted X-inactivation in the placenta(Garrick et al., 2006). To circumvent this lethality and study the effects of ATRX loss of function during the development of the central nervous system, a system was utilized where Cre recombinase is conditionally expressed under the control of the forkhead box G1 (*Foxg1*) promoter(Hebert and McConnell, 2000). Mating the $Foxg1Cre^{+/-}$ males to $Atrx^{loxP}$ heterozygous females generates male mice with a conditional loss of the full length ATRX protein in the forebrain beginning at embryonic day 8.5 (E8.5) (referred to as ATRX-null and ATRX-KO hereafter)(Bérubé et al., 2005). ATRXt protein levels are unaffected due to the placement of loxP sites following the alternatively spliced exon(Bérubé et al., 2005). ATRX-null mice are smaller than littermate controls, display reduced forebrain size, and most die in the neonatal period of unknown causes(Bérubé et al., 2005). Overall, ATRX-null mice provide a valuable tool to study the effects of ATRX on various processes throughout neuronal development, including gene transcription.

1.1.4 ATRX in Replication and Mitosis

While ATRX's classification as a chromatin remodeling protein and its effect on alpha-globin expression has led to a focus on gene regulation, a second role for ATRX has also emerged. ATRX is highly enriched at PCH of condensed mitotic chromosomes and becomes hyperphosphorylated during mitosis, suggesting it may also play a role during this phase of the cell cycle(Bérubé et al., 2000; McDowell et al., 1999). RNAi depletion of ATRX in cultured human cells followed by live video analysis uncovered many mitotic abnormalities, including defects in chromosome congression and cohesion, abnormal spindle morphology, and binucleated cells(Ritchie et al., 2008). Similar effects were also reported during meiosis in ATRX-deficient mouse oocytes(Baumann et al., 2010; De La Fuente et al., 2004). Moreover, analysis of the ATRX-null developing forebrain revealed evidence of mitotic defects and highlighted a concomitant increase in DNA damage foci(Ritchie et al., 2008; Watson et al., 2013). It was found that DNA damage occurred mainly at the replication fork, and resulted in increased p53-dependent cell death(Bérubé et al., 2005; Seah et al., 2008; Watson et al., 2013). Interestingly, the combined loss of ATRX and p53 provided only a partial recovery in postnatal brain size, suggesting there are multiple ATRX-dependent pathways necessary to achieve proper neuronal development. The identification and characterization of ATRX functions and potential transcriptional targets will likely provide insight into the neurodevelopmental roles of the ATRX protein.

1.2 The MeCP2 Gene and Protein

1.2.1 MeCP2

The methyl CpG binding protein 2 (*MeCP2)* gene encodes a nuclear methyl CpG binding protein that can bind directly to chromatin(Lewis et al., 1992; Meehan et al., 1992). This 53 kDa protein has three main functional domains: a methyl binding domain(Nan et al., 1993), nuclear localization signal(Kudo, 1998; Nan et al., 1996) and a transcriptional repression domain(Jones et al., 1998; Nan et al., 1998) (Figure 1-2A). Identification of these domains led to the hypothesis that MeCP2 functions as a global transcriptional repressor by binding methylated DNA and recruiting histone deacetylases (HDACs)(Nan et al., 1998). However, expression profiling studies have shown that the loss of MeCP2

results in only subtle changes in gene expression and do not support a role as a classical transcriptional repressor(Jordan et al., 2007; Nuber et al., 2005; Tudor et al., 2002; Urdinguio et al., 2008). Emerging studies have shown that MeCP2 can associate with both methylated and unmethylated DNA(Hansen et al., 2010; Yasui et al., 2007), and is frequently found within both active and inactive genes(Yasui et al., 2007), further complicating the questions surrounding the function of MeCP2. ChIP profiles have demonstrated that MeCP2 is enriched at specific sites(Yasui et al., 2007; Yasui et al., 2013), where it may play a role in regulating higher-order chromatin structure and organization(Horike et al., 2005; Yasui et al., 2007). However, it also appears to coat large regions of chromosomes where it competes with histone H1 to bind internucleosomal regions and may compact nucleosomal arrays(Nikitina et al., 2007; Skene et al., 2010). Further studies are needed before any conclusions can be drawn on the localization patterns and functions of MeCP2 throughout the genome. It is plausible that MeCP2 has multiple binding patterns and functions, depending on its genomic environment and/or interaction partners.

MeCP2 has been shown to interact with a number of other proteins, including the corepressors mSin3a and cSki(Kokura et al., 2001; Nan et al., 1998), heterochromatin associated proteins HP1(Agarwal et al., 2007), DNMT1(Kokura et al., 2001) and H3K9MTase(Fuks et al., 2003b), as well as a number of transcriptional regulators including YY1(Forlani et al., 2010), and ATRX(Nan et al., 2007). The MeCP2-ATRX interaction was first reported in an *in vitro* yeast-two-hybrid screen for MeCP2-interactors, and it was subsequently demonstrated that these proteins co-localize at PCH(Baker et al., 2013; Nan et al., 2007). Importantly, loss of ATRX had no effect on MeCP2 enrichment, while mutation or loss of MeCP2 abrogated ATRX localization to PCH(Baker et al., 2013; Nan et al., 2007). The interaction domains between ATRX and MeCP2 were also mapped, demonstrating that a region overlapping the methyl binding domain of MeCP2 interacts with the SWI/SNF domain of ATRX(Baker et al., 2013; Nan et al., 2007) (Figure 1-2B). Whether ATRX and MeCP2 co-localize elsewhere in the cell, and the dynamics and function of this partnership have not yet been investigated.

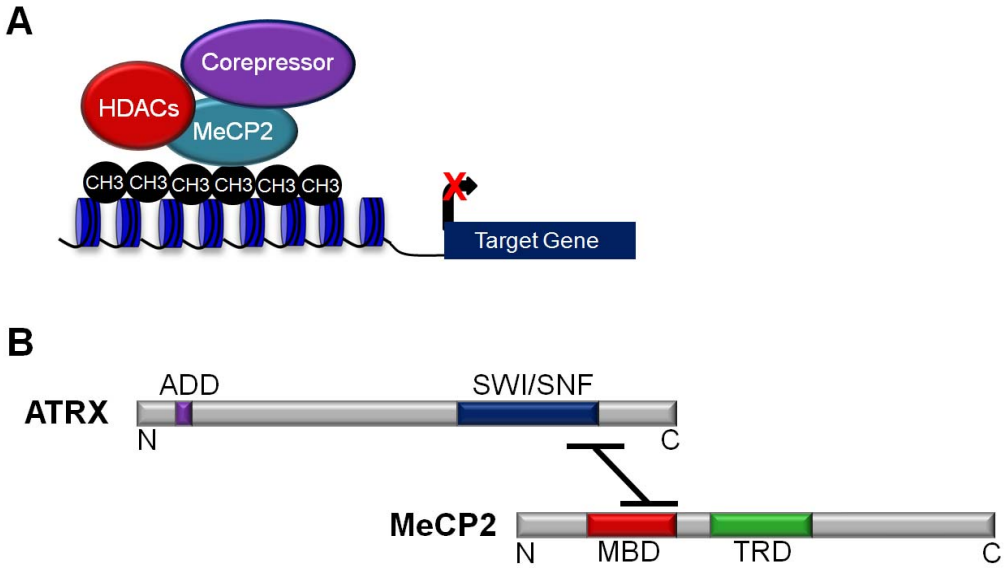

Figure 1-2. MeCP2 function and interaction with ATRX

(A) The canonical role of MeCP2 in gene regulation. MeCP2 binds to methylated DNA, along with HDACs and corepressor proteins to repress target genes. (B) Protein structure and interaction of ATRX and MeCP2. Deletion analysis has shown that the helicase domain of ATRX binds within the methyl binding domain of MeCP2. MBD, methyl binding domain; TRD, transcriptional repression domain

1.2.2 MeCP2 Mutations in Humans and Mice Cause Rett Syndrome

Rett Syndrome (RTT) is an autism-spectrum disorder associated with severe and progressive neurological abnormalities(Hagberg et al., 1983). Over 95% of RTT cases are due to spontaneous mutations in *MeCP2*, a gene located on the X chromosome at Xq28(Amir et al., 1999). In males, MeCP2 mutations on the single X chromosome lead to neonatal encephalopathy and infant mortality(Kankirawatana et al., 2006; Schule et al., 2008). In RTT females, X chromosome inactivation results in only half the cells expressing the mutant MeCP2, while the other half express the normal protein. Girls affected with RTT develop apparently normal until the emergence of overt phenotypes at 6–18 months of age(Armstrong, 2002; Shahbazian and Zoghbi, 2001). RTT phenotypes include postnatal microcephaly, ataxia, gait apraxia, loss of language, seizures, and respiratory dysfunction(Armstrong, 2002; Shahbazian and Zoghbi, 2001). The MeCP2 protein is ubiquitously expressed, though it is most abundant in the mature brain(Balmer et al., 2003; Kishi and Macklis, 2004). This expression profile is likely responsible for the predominantly postnatal neurological RTT phenotype, though few reports have analyzed a role for MeCP2 prior to the onset of symptoms.

A number of mouse models of RTT have been generated utilizing global Cre-mediated *Mecp2* deletions or truncations(Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002). These mutant mice exhibit many aspects of the disorder, including a postnatal onset of symptoms such as motor impairment, tremors, breathing abnormalities, and limb stereotypies(Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002). Interestingly, several groups have been able to partially rescue these murine RTT phenotypes by re-expressing MeCP2 through various means(Giacometti et al., 2007; Guy et al., 2007; Luikenhuis et al., 2004; Tropea et al., 2009). These studies demonstrated that neuronal defects induced by MeCP2 deficiency might be reversible. In addition to aiding in the development of RTT treatments, RTT mouse models provide a system to study the role(s) of MeCP2 throughout development.

1.2.3 MeCP2 and Gene Regulation

Expression profiling studies conducted in RTT humans and mice have uncovered many MeCP2 regulated genes, including several involved in brain development (e.g. brain derived neurotrophic factor (*Bdnf*)(Zhou et al., 2006b), and the GABRB3 receptor (*Gabrb3*)(Samaco et al., 2005)), and a number of imprinted genes(Horike et al., 2005; Samaco et al., 2005). The links between MeCP2 and imprinted gene expression are numerous, but have been the subject of controversy(LaSalle, 2007). Some features of RTT are reminiscent of Angelman Syndrome, an imprinting disorder affecting genes within human 15q11–13, including *UBE3A*(Jedele, 2007*)*. While two independent studies found MeCP2 deficiency in humans and mice decreased *UBE3A/Ube3a* expression(Makedonski et al., 2005; Samaco et al., 2005), these outcomes could not be reproduced by others(Jordan and Francke, 2006). Second, MeCP2 was found to bind within the 6qA1 imprinted domain and govern the expression of the four imprinted genes: *Dlx5, Sgce, Peg10* and *Calcr*(Horike et al., 2005). It was suggested that loss of MeCP2 altered 6qA1 chromatin structure, at least in the region surrounding *Dlx5*(Horike et al., 2005); however, MeCP2 regulation of *Dlx5* has also been disputed(Miyano et al., 2008). Finally, *in vitro* studies have shown that MeCP2 can bind to the imprinting control region (ICR) within the *H19/Igf2* domain and repress transcription(Drewell et al., 2002), but allele-specific or *in vivo* binding has not yet been analyzed. Overall, these lines of evidence suggest a complex link between MeCP2 and imprinted genes that requires further study.

1.3 Genomic Imprinting

1.3.1 Imprinted Genes

Epigenetic regulation encompasses different mechanisms that modify gene expression in a heritable manner without affecting the DNA sequence. The major types of epigenetic modifications include DNA methylation, post-translational histone modifications, non-coding RNAs (ncRNAs), and higher-order chromatin structure. Collectively, these marks alter the environment of the chromatin fiber and the relative accessibility of chromatin remodeling proteins, transcription factors and transcriptional machinery to the DNA. Genomic imprinting is a distinctive form of epigenetic regulation resulting in mono-

allelic, parent-of-origin-dependent gene expression. For example, the *H19* imprinted gene is maternally expressed, while the paternal allele is silent(Bartolomei et al., 1991). At present, there are approximately 150 confirmed imprinted genes identified in the mouse genome(MRC Harwell, 2013). These genes are generally conserved among mammals, and often cluster in large domains which are dispersed throughout the genome(MRC Harwell, 2013).

In the 1980's a number of groups used pronuclear transfer experiments in mice to demonstrate that imprinted genes are essential for growth and development(Cattanach and Kirk, 1985; McGrath and Solter, 1984a; b; Surani et al., 1984). These experiments included the transfer of pronuclei between one-cell stage embryos to create diploid parthenogenetic embryos possessing two oocyte-derived genomes and androgenetic embryos with two sperm-derived genomes(Cattanach and Kirk, 1985; McGrath and Solter, 1984a; b; Surani et al., 1984). Both the parthenogenetic and androgenetic embryos failed to survive beyond early postimplantation development(Cattanach and Kirk, 1985; McGrath and Solter, 1984a; b; Surani et al., 1984). These pre-eminent experiments established that the maternal and paternal genetic contributions are not equivalent, and that both are necessary for the mouse to develop normally(Cattanach and Kirk, 1985; McGrath and Solter, 1984a; b; Surani et al., 1984). We now know that the expression of several imprinted genes begins as early as preimplantation embryogenesis and persists throughout development in a range of tissues(Huntriss et al., 1998; MRC Harwell, 2013; Ohlsson et al., 1994; Tremblay et al., 1997; 2013a; Wu et al., 2013b), although it is markedly higher in the placenta and brain(Wu et al., 2013b). The mechanisms that govern imprinted gene expression are multifaceted and remain a subject of intense investigation.

1.3.2 Imprinting Mechanisms

While each imprinted domain may possess unique regulatory features, imprinted gene expression is generally controlled by DNA methylation, post-translational histone modifications, ncRNAs, and higher-order chromatin structures(Ideraabdullah et al., 2008). A hallmark of imprinted domains is the presence of differentially methylated regions (DMRs), which are CpG-rich regulatory sequences methylated on one parental

allele(Smith and Meissner, 2013). In this context, DNA methylation blocks the binding of activating proteins and transcriptional machinery, and functions as a recognition site for repressive factors(Smith and Meissner, 2013). A subset of DMRs act as imprinting control regions (ICRs) based on evidence that deletion of these sequences ablates imprinting across their respective domains(Spahn and Barlow, 2003). DNA methylation at these sites originates in the germline and is maintained throughout development(Spahn and Barlow, 2003). DMRs are also marked by post-translational histone modifications that track DNA methylation; repressive marks (e.g., meH3K9, meH3K27 and meH4K20) are associated with the silent methylated allele while active marks (e.g., AcH3 and AcH4) are found on the active unmethylated allele(Fournier et al., 2002; Henckel et al., 2009; Umlauf et al., 2004). Histone modifications can alter the biochemical nature of the chromatin fibre and provide a code that is recognized by regulatory proteins(Fischle et al., 2003). The combination of DNA methylation and histone modifications at DMRs is essential to direct proper imprinted gene expression.

Perhaps the least understood feature that governs imprinting involves ncRNAs. Every imprinted cluster discovered to date includes at least one ncRNA, but the mechanisms by which they influence imprinted gene expression are not well understood. Imprinted mRNAs and ncRNAs are always oppositely expressed, suggesting that ncRNAs could repress mRNA genes. Three theories have been put forward to explain the mechanics of this repression: transcript degradation through the RNAi pathway; repression via heterochromatin spreading; and transcriptional effects(reviewed in (Koerner et al., 2009; Royo and Cavaille, 2008; Wan and Bartolomei, 2008)). While the mechanisms are unclear and may be specific to each transcript, accumulating studies show that ncRNAs likely play a vital role in imprint regulation(Koerner et al., 2009; Royo and Cavaille, 2008; Wan and Bartolomei, 2008).

The development of chromosome conformation capture (3C) and its derivatives have enabled the analysis of three dimensional (3D) chromatin interactions within imprinted domains(Dekker et al., 2002; Dostie et al., 2006; Fullwood and Ruan, 2009; Lieberman-Aiden et al., 2009; Simonis et al., 2006; Zhao et al., 2006b) (discussed in Section 1.4.2).

These chromatin loop structures alter gene proximity to regulatory sites, such as enhancers, DMRs and matrix-attachment regions (MARs). To date, loop structures have been studied at three imprinted domains: *H19/Igf2*(Burke et al., 2005; Kurukuti et al., 2006; Li et al., 2008; Murrell et al., 2004; Nativio et al., 2009; Qiu et al., 2008; Vu et al. 2010), *Dlx5*/*Dlx6*(Horike et al., 2005), and *Gtl2*/*Dlk1*(Braem et al., 2008). While we are just beginning to understand the significance and regulation of these configurations, it is clear that they require the presence of specific proteins, including the CCCTC-binding factor (CTCF) insulator protein and the Cohesin complex(Han et al., 2008; Ishihara et al., 2006; Kurukuti et al., 2006; Nativio et al., 2009) (discussed in Section 1.4.3). As technologies improve we can anticipate that a complex array of interactions between chromatin and related proteins will be uncovered at many imprinted domains.

1.3.3 Imprinted Gene Networks

Recently, several independent studies have described co-regulation of a number of imprinted genes during cellular differentiation(Andrade et al.2009; Lui et al., 2008; Varrault et al., 2006). Together, these papers suggest that many imprinted genes are involved in an epigenetically regulated gene network essential for embryonic growth and development(Andrade et al. 2010; Berg et al., 2011; Lui et al., 2008; Varrault et al., 2006) (Figure 1-3). It was proposed that during development, expression of these genes is coordinated in a context-dependent manner to facilitate adaptation to genetic and environmental changes(Andrade et al. 2010; Kernohan and Bérubé, 2010; Lui et al., 2008; Varrault et al., 2006). Within the brain, these genes are highly expressed in embryogenesis and repressed in the mature brain (e.g., *H19*, *Igf2*, *Dlk1*, *Zim1* and *Grb10* (Bartolomei et al., 1991; Kernohan et al., 2010; Kim et al., 1999; Liu et al., 2009; Svensson et al., 1995; Weber et al., 2001)). For many of these genes, this expression pattern matches their functions. For example, the growth factor *Igf2* and the apoptosis inhibitor *Grb10* are highly expressed during neurogenesis when cells are rapidly dividing, but are unnecessary and silenced in post-mitotic cells of the mature brain(DeChiara et al., 1990; Hu et al. 2010). However, for several other genes the function is not yet known (e.g., *H19)* and the purpose of postnatal suppression remains elusive. In line with the suggestion that many imprinted genes are linked and co-regulated, studies surveying

genome-wide interactions of the *H19* ICR revealed that it can interact with a number of imprinted domains on multiple chromosomes, and that these connections are cell-type specific(Ling et al., 2006; Sandhu et al., 2009; Zhao et al., 2006b). Furthermore, they demonstrated that the *H19* ICR could promote transvection of epigenetic states to other imprinted genes(Sandhu et al., 2009). From these studies, it was proposed that the *H19* ICR might function as a master regulator controlling the expression of all imprinted gene network (IGN) domains(Sandhu et al., 2009) (Figure 1-3). While we are just beginning to understand this IGN, stringent control of all its components is likely essential for proper development, and perturbations of this equilibrium could have deleterious effects.

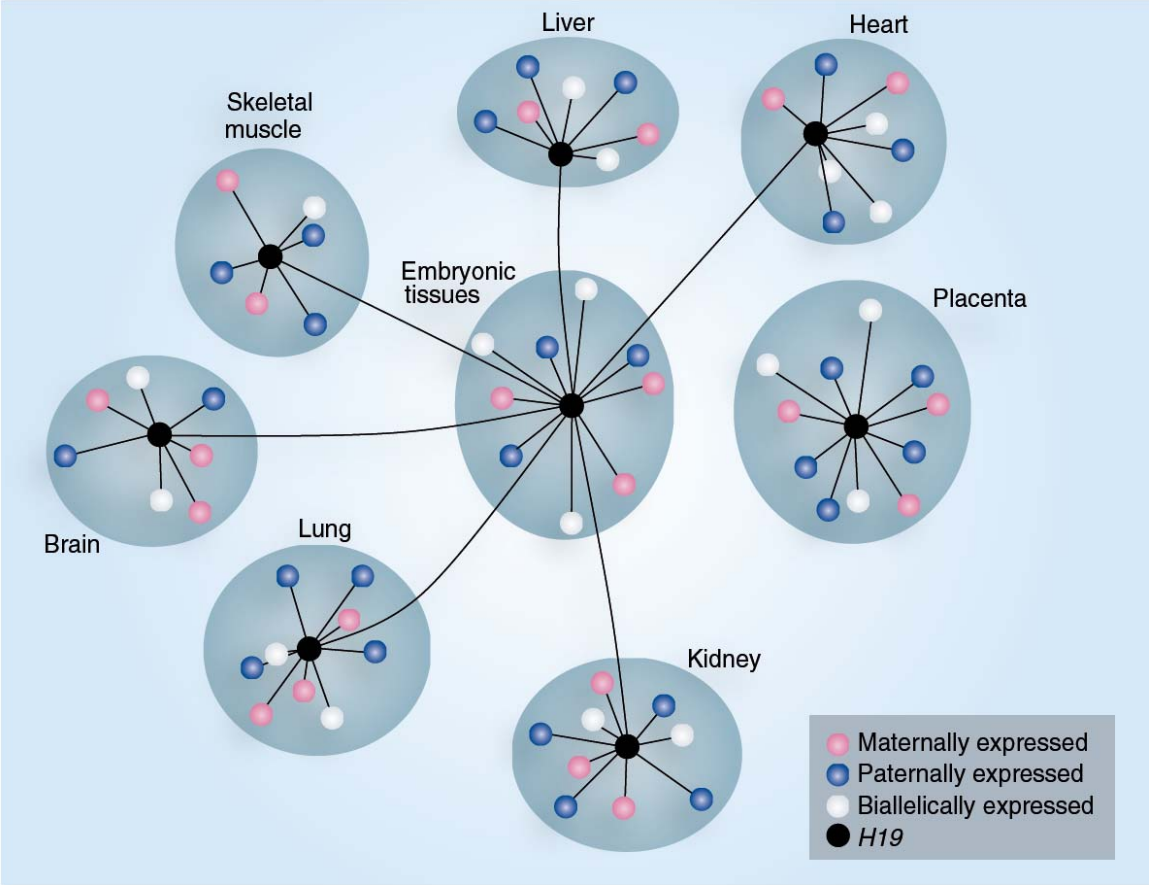

Figure 1-3. Context-dependent imprinted gene networks

A number of recent studies provide evidence for the existence of an imprinted gene network (IGN). The IGN theory posits that as cells differentiate from embryonic tissues into terminal lineages there is coordinate regulation of imprinted genes in each tissue and developmental time point(Ling et al., 2006; Sandhu et al., 2009; Zhao et al., 2006b). Furthermore, it has been proposed that the *H19* ICR may function as a master regulator of this network, as it associates with a number of imprinted domains on multiple chromosomes, and is required for trans interactions amongst imprinted genes in some cell types.

Reproduced with permission from Kernohan and Bérubé (2010) Epigenomics **2**:743-763. (Appendix A)

1.3.4 The *H19/Igf2* Imprinted Domain

The *H19*/*Igf2* imprinted domain is the most well characterized imprinted region in the mouse genome, and its regulation has been studied in depth in mouse embryonic fibroblasts, embryonic stem cells, and the liver. The *H19* gene product is a 2.3 kb maternally expressed ncRNA(Brannan et al., 1990). Loss of *H19* causes a slight overgrowth phenotype in mice(Leighton et al., 1995), while ectopic expression leads to late embryonic lethality(Brunkow and Tilghman, 1991). It was suggested this lethality might be due to exogenous expression in the brain, though the exact cause of death was never determined(Brunkow and Tilghman, 1991). The *H19* gene locus also produces a microRNA, miR-675(Cai and Cullen, 2007). miR-675 is thought to be involved in cellular proliferation and growth regulation(Keniry et al., 2012). Insulin-like growth factor 2 (*Igf2),* encodes a potent growth factor expressed from the paternal allele, except in the brain where its expression is bi-allelic(DeChiara et al., 1991). IGF2 deficiency leads to growth retardation(DeChiara et al., 1990), and its overexpression causes an overgrowth phenotype(Morison et al., 1996). Both *H19* and *Igf2* are highly expressed prenatally and downregulated postnatally in many tissues, including the brain(Svensson et al., 1995; Weber et al., 2001). The regulation of this expression pattern remains elusive.

Genomic imprinting of the 90 kb *H19/Igf2* region is primarily accomplished by four paternally methylated DMRs, including the ICR that lies 2 kb upstream of the *H19* gene(Bartolomei et al., 1993; Brandeis et al., 1993; Feil et al., 1994; Ferguson-Smith et al., 1993; Frevel et al., 1999; Moore et al., 1997; Thorvaldsen et al., 1998; Tremblay et al., 1997; Tremblay et al., 1995) (Figure 1-4A). DNA methylation from the paternal *H19* ICR has also been reported to spread to the *H19* promoter and aid in silencing of the paternal *H19* gene(Bartolomei et al., 1993; Ferguson-Smith et al., 1993; Sasaki et al., 1995; Srivastava et al., 2000; Tremblay et al., 1995). In addition to DNA methylation, the *H19* ICR is enriched for several active post-translational histone modifications on the unmethylated maternal allele, including acetylation of histone H3 and H4, while the silent paternal allele is marked with repressive histone modifications and variants, including H3K27, H3K9 and H4K20 methylation and the variant macroH2a(Choo et al., 2006;

Choo et al., 2007; Grandjean et al., 2001; Kacem and Feil, 2009). Together, the epigenetic marks within the ICR likely serve as recognition sites for numerous proteins, including CTCF and Cohesin. The *H19* ICR contains four maternal CTCF binding sites which are responsible for imprinted *H19/Igf2* expression and the maintenance of an unmethylated state on the maternal allele(Bell and Felsenfeld, 2000; Choo et al., 2006; Choo et al., 2007; Hark et al., 2000; Schoenherr et al., 2003) (Figure 1-4A). Finally, the *H19* ICR produces a number of small ncRNAs, though these transcripts do not affect imprinting and are of unknown function(Takahashi et al., 2012).

In addition to the *H19* ICR the *H19/Igf2* domain contains several other regulatory sequences, including a MAR(Greally et al., 1997), and three enhancers specific for mesoderm, endoderm, and the brain (brain enhancer known as centrally conserved domain (CCD))(Ainscough et al., 2000; Ainscough et al., 1997; Charalambous et al., 2004; Ishihara et al., 2000; Jones et al., 2001; Leighton et al., 1995; Yoo-Warren et al., 1988) (Figure 1-4A). These regulatory sequences are brought together within the nucleus to cooperate in regulating proper *H19* and *Igf2* expression(Burke et al., 2005; Guibert et al., 2012; Han et al., 2008; Kurukuti et al., 2006; Li et al., 2008). As a result of differential methylation, the *H19* ICR interacts with the other DMRs and regulatory elements to form disparate looping structures of the maternal and paternal alleles, positioning *H19* and *Igf2* into active and silent chromatin domains(Burke et al., 2005; Guibert et al., 2012; Han et al., 2008; Kurukuti et al., 2006; Li et al., 2008) (Figure 1-4B). These interactions require the presence of CTCF and Cohesin at the maternal *H19* ICR(Han et al., 2008; Kurukuti et al., 2006; Nativio et al., 2009). Studies have also reported that MeCP2 binds within the *H19* ICR and can repress *H19* transcription *in vitro*(Drewell et al., 2002), however *in vivo* or allele-specific binding of MeCP2 has not yet been demonstrated.

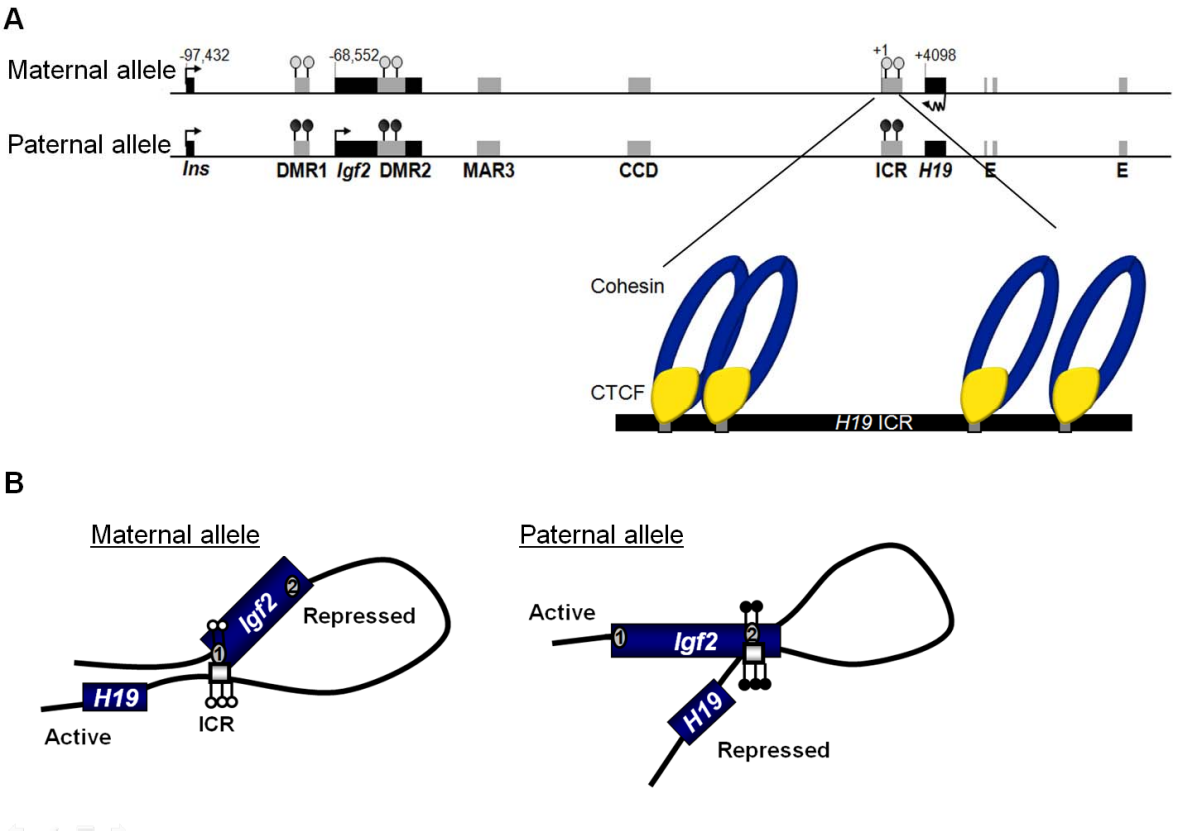

Figure 1-4. Genomic organization and regulation of the *H19/Igf2* imprinted domain

(A) Schematic of the *H19/Igf2* domain. Genes are depicted in black and regulatory elements in grey. Numbers indicate the relative position from the start of the *H19* ICR. Methylated DNA is represented as black circles, and unmethylated DNA as grey circles. The enlarged image of the *H19* ICR displays the four CTCF binding sites as grey boxes with CTCF and Cohesin enrichment demonstrated. (B) Simplified diagram representing chromatin interactions of the *H19* ICR. The unmethylated maternal ICR interacts with the unmethylated DMR1, while the methylated paternal ICR interacts with the methylated DMR2(Burke et al., 2005; Guibert et al., 2012; Han et al., 2008; Kurukuti et al., 2006; Li et al., 2008).

1.3.5 Imprinted Genes in the Brain

The majority of imprinted genes are expressed at at least one developmental stage in the brain; however, most of their functions remain to be determined(Davies et al., 2005). From the genes characterized thus far, it is evident that many are essential for neurological processes(Davies et al., 2005). Davies et al. categorized these functions, which include intracellular signaling (*Gnas* and *RasGrf1*), protein trafficking and processing (*Copg2*, *Ube3a* and *Usp29*), transcriptional regulation (*Peg3* and *Mash2*), RNA processing (*Snrpn*), and growth and cell cycle control (*Cdkn1c*, *Ndn,* and *Zac1*)(Davies et al., 2005). Additionally, some imprinted genes have an obvious biochemical function in neuronal cells. For example, *Dlx5* is required for the migration of neural progenitor cells and for the differentiation of immature precursors into GABAergic neurons(Anderson et al., 1997; Stuhmer et al., 2002). Given the multitude of roles imprinted genes play in the brain, it is not surprising that the disruption of genomic imprinting in humans has been linked to a number of neurodevelopmental syndromes, including Angelman Syndrome(Kishino et al., 1997; Matsuura et al., 1997), Prader–Willi Syndrome(Ledbetter et al., 1981; Miller et al., 2009; Muscatelli et al., 2000; Ren et al., 2003), and Turner Syndrome(Kesler et al., 2003; McCauley et al., 1987; Skuse et al., 1997)(reviewed in (Kernohan and Bérubé, 2010)). Together, the human and mouse data underscore the importance of imprinted gene expression in development and implicate the nervous system as primarily affected.

1.4 Chromatin Structure and Protein Regulators

1.4.1 Chromatin Structure and Remodeling Proteins

DNA in the nucleus is wrapped around an octamer of histone proteins in 147 base pair increments to form nucleosomes, which are then organized into a condensed fiber and folded into chromosomes. This composite of DNA and proteins is known as chromatin, and plays multiple roles within the cell, including packaging the DNA into a small volume, regulating gene expression, and facilitating mitosis. Chromatin from inactive regions of the genome is densely packaged into heterochromatin, effectively maintaining gene silencing. In transcriptionally active regions, DNA is in an open highly accessible state known as euchromatin. Post-translational histone modifications and DNA

methylation contribute to the formation and maintenance of both euchromatin and heterochromatin. Additionally, a number of chromatin remodeling proteins can alter or maintain chromatin states. In general, chromatin remodelers are a diverse group of proteins that utilize the energy of ATP to disrupt or remodel protein-DNA complexes, often to govern gene transcription. Transcriptional effects are accomplished through either facilitating or blocking polymerase binding and regulating nucleosomes. Nucleosome remodeling processes includes nucleosome removal, destabilization, repositioning or replacement with histone variants. In general, the combination of activities by chromatin remodelers is essential for proper chromatin structure and thus gene regulation. (reviewed in (Quina et al., 2006))

1.4.2 Higher-Order Chromatin Architecture

Within the interphase nucleus, chromosomes occupy a defined space, termed chromosome territories, where loops are formed in *cis* to strategically fold subregions of a chromosome(reviewed in (Cremer and Cremer, 2001; Cremer and Cremer, 2010; Cremer et al., 2006; Zhao et al., 2009)). These *cis* loops facilitate interactions between genes, and local and long-range regulatory sequences. In addition, chromosome loops can sometimes extend beyond the confines of these territories and bring genomic regions from different chromosomes into close proximity. These short- and long-range chromosomal interactions can enhance or inhibit gene expression and thus are highly relevant to genomic regulation, including the mono-allelic and coordinated expression of imprinted genes.

To date, chromatin looping has been studied at a number of regions, including alpha-globin(Bau et al., 2011; Kim et al., 2009a; Vernimmen et al., 2007), beta-globin(Junier et al., 2012; Kim and Dean, 2004; Noordermeer and de Laat, 2008; Splinter et al., 2006), T_H2(Yao et al., 2012), *IFNG*(Hadjur et al., 2009; Sekimata et al., 2009), *MHC class II*(Majumder and Boss, 2010; Ribeiro de Almeida et al., 2012), *IgH*(Ju et al., 2011), *H19/Igf2*(Burke et al., 2005; Guibert et al., 2012; Han et al., 2008; Kurukuti et al., 2006; Li et al., 2008), *Dlx5/Dlx6*(Horike et al., 2005), and *Gtl2/Dlk1*(Braem et al., 2008). With the increasing number of chromatin architecture studies, it is clear that we are only beginning to understand the complex network of chromosomal interactions that exists

throughout the genome. Furthermore, very little is known about the proteins and mechanisms that form and maintain these structures, or the consequences of disrupting these systems. Recently, the CTCF and Cohesin proteins have emerged as major players in chromatin structure and are proposed to act as global architectural regulators, though their effects have only been investigated at a limited number of sites(Gause et al., 2008; Wendt and Peters, 2009) (discussed in Section 1.4.3). Additionally, a few site-specific regulatory proteins have been identified, including BRG1 within the alpha-globin domain(Kim et al., 2009a), and GATA-1 , FOG-1, EKLF, NLI, and BRG1 at the beta-globin locus(Drissen et al., 2004; Kiefer et al., 2011; Kim et al., 2009b; Song et al., 2007; Vakoc et al., 2005), though little is known about the mechanism of many of these proteins. Overall, as we advance our understanding of this new and exciting field of 3D genome architecture, we will likely uncover many novel protein functions and mechanisms.

1.4.3 Cohesin and CTCF

The Cohesin complex consists of four subunits: structural maintenance of chromosome (SMC)1, SMC3, radiation mutant 21 (Rad21) and stromal antigen 1/2 (SA1/SA2). Cohesin was initially discovered and characterized for its role in maintaining sister chromatid cohesion during mitosis(Barbero, 2011; Michaelis et al., 1997; Moser and Swedlow, 2011; Uhlmann and Nasmyth, 1998). The Cohesin complex is proposed to function as a ring, encircling DNA strands to tether them together(Anderson et al., 2002; Gruber et al., 2003; Guacci et al., 1997; Losada et al., 1998; Michaelis et al., 1997; Toth et al., 1999). The protein interactions within the ring have been mapped: the N and C terminal domains of each SMC protein, called the head region, fold together to form an ATPase domain, while the connecting coiled-coil region forms a hinge. To form the ring, both the head and hinge regions of SMC1 and SMC3 interact, though the interaction of the head domains is mediated by Rad21 and stabilized by SA1/SA2(reviewed in (Remeseiro and Losada, 2013; Seitan and Merkenschlager, 2012)) (Figure 1-5). In addition to the Cohesin proteins, several accessory factors are involved in loading and maintaining Cohesin on DNA, including precocious dissociation of sisters 5 (PDS5), nipped- B-like protein (NIPBL) and wings apart-like (WAPL)(Gause et al., 2010; Kueng

et al., 2006; Panizza et al., 2000; Seitan et al., 2006; Vaur et al., 2012). Cohesin is loaded onto chromatin in telophase and released with sister chromatid separation in prophase/anaphase(Shamu and Murray, 1992; Shintomi and Hirano, 2010; Sumara et al., 2000; Wang et al., 2008; Watrin et al., 2006). The dynamics of loading and unloading of Cohesin onto chromatin throughout the cell cycle are complex and are being resolved, though will not be discussed here((reviewed in (Mehta et al., 2012; Remeseiro and Losada, 2013; Seitan and Merkenschlager, 2012)). Interestingly, human mutation in the genes encoding Cohesin proteins (SMC1A and SMC3) as well as the NIPBL loading factor causes Cornelia de Lange Syndrome (CdLS), which is characterized by numerous developmental and neurological defects(Ben-Asher and Lancet, 2004; Krantz et al., 2004; Revenkova et al., 2009).

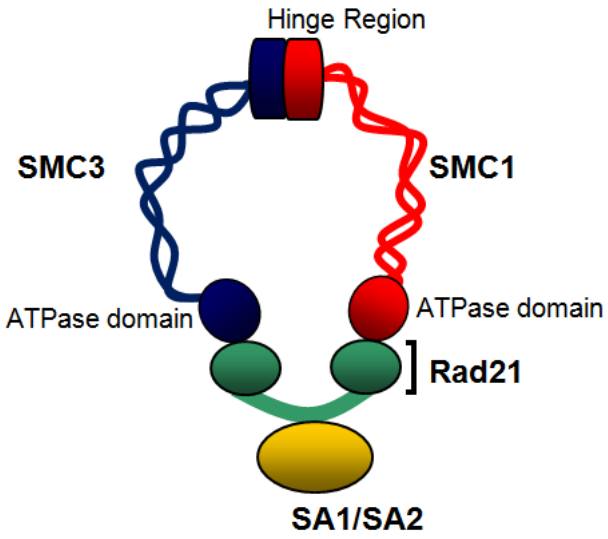

Figure 1-5. Structure of the Cohesin complex

Cohesin contains four subunits, SMC1, SMC3, Rad21 and SA1/SA2. These subunits interact to form a ring that can encircle DNA.

Emerging studies suggest that in addition to Cohesin's canonical role in sister chromatid cohesion and cell division, it may also function in DNA damage repair and transcriptional regulation(Remeseiro and Losada, 2013). It is predicted that the CdLS phenotype results from transcriptional effects as Cohesin mutants defective for chromosome cohesion are not viable(Dorsett, 2007; Nasmyth and Haering, 2009; Revenkova et al., 2009). Cohesin is proposed to govern gene transcription by tethering DNA loops together to regulate chromatin architecture(Gause et al., 2008; Rubio et al., 2008; Stedman et al., 2008). This effect has been reported at multiple genomic sites, including the T cell receptor alpha locus(Seitan et al., 2011) and the *H19/Igf2* imprinted domain(Nativio et al., 2009). Furthermore, genome-wide ChIP studies have demonstrated that Cohesin co-localizes with the insulator protein CTCF at more than 8,000 genomic locations(Rubio et al., 2008). CTCF is required for Cohesin enrichment at these sites, and it is predicted that together CTCF and Cohesin establish DNA-DNA interactions throughout the genome(Rubio et al., 2008).

CTCF is a sequence-specific DNA binding protein that functions in both transcriptional regulation and higher-order genomic organization. Genome-wide studies have shown a staggering 14,000-20,000 CTCF binding sites throughout the genome, including a disproportionately large number within gene-enriched regions(Kim et al., 2007). The sequence specificity of CTCF is thought to be conferred by its zinc finger domain, which contacts particular genomic sites using different combinations of its 11 zinc fingers(Filippova et al., 1996). Intriguingly, it has also been shown that on average 10 nucleosomes are precisely positioned surrounding each CTCF binding site, with CTCF located in a larger than normal linker region(Fu et al., 2008; Kanduri et al., 2002; Zhao et al., 2006a). As a transcriptional regulator, CTCF is recognized as a vertebrate insulator protein and can function to block enhancer promoter interactions, or as a boundary between chromatin states(Bell et al., 1999). Regarding chromatin architecture, CTCF recruits the Cohesin complex throughout the genome to facilitate 3D genomic organization, earning it the title "master weaver of the genome"(Phillips and Corces, 2009; Rubio et al., 2008). Overall, it is clear that CTCF is important throughout the genome, though we still lack a complete understanding of its regulation and specific functions.

1.5 Chromosome Conformation Capture Technology

With the development of chromosome conformation capture (3C)(Dekker et al., 2002) our understanding of the genome evolved from a linear organization to a complex three dimensional one. 3C is a cutting-edge molecular technique used to study chromatin interactions *in vivo*. It accomplishes this through first cross-linking interacting chromatin within the cell. Following DNA isolation and restriction enzyme digest, an enzyme is used to ligate the cross-linked fragments together. The cross-link is reversed, leaving a library of DNA fragments containing the once distant binding regions joined together. The analysis of 3C libraries involves choosing a region of interest contained within one restriction fragment (termed bait sequence), and surveying its interactions. Interactions are quantified by Taqman qRT-PCR using a primer and probe to the bait sequence, and a series of primers that amplify restriction fragments of interest (Figure 1-6). As 3C is a technically complex protocol, establishing reliable controls for data analysis is essential. These include evaluating digestion, PCR primer, and fixation efficiencies, and DNA concentrations(Dekker, 2006). Studies utilizing 3C have already uncovered many genomic sites involved in higher-order interactions and it is anticipated that this number will continue to grow substantially.

While very informative, 3C studies are limited to a candidate approach that is biased and not appropriate to survey larger genomic regions. To facilitate a large scale approach, a number of groups have developed 3C variants. These include circular chromosome conformation capture and chromosome conformation capture on chip (4C)(Gheldof et al., 2012; Simonis et al., 2006; Zhao et al., 2006b), chromosome conformation capture carbon copy (5C)(Dostie et al., 2006), combined chromosome conformation capture ChIP cloning (6C)(Tiwari and Baylin, 2009), Global 3C interactions (Hi-C)(van Berkum et al., 2010), and chromatin interaction analysis by paired-end tag sequencing (ChIA-PET)(Li et al., 2010)(techniques reviewed in (de Wit and de Laat, 2012)). While each technique has its advantages and disadvantages, I utilized 4C in the present study because it provides an unbiased genome-wide screen of interaction sites from a bait sequence of interest (Figure 1-6). Briefly, following 3C library production, the samples are digested with a secondary restriction enzyme and self-ligated to form circular 3C recombined

molecules. The samples are then PCR amplified with primers directed from the bait across the unknown interacting sequence. Traditionally, these 4C products were analyzed by custom tiled microarrays. Recently a few groups, including ours, have utilized next generation sequencing to establish an unbiased interactome(Gheldof et al., 2012; Papantonis et al., 2012; Splinter et al., 2012; Xu et al., 2011) (Figure 1-6). Overall, 3C and 3C derivatives shed light on how chromatin is organized within the cell.

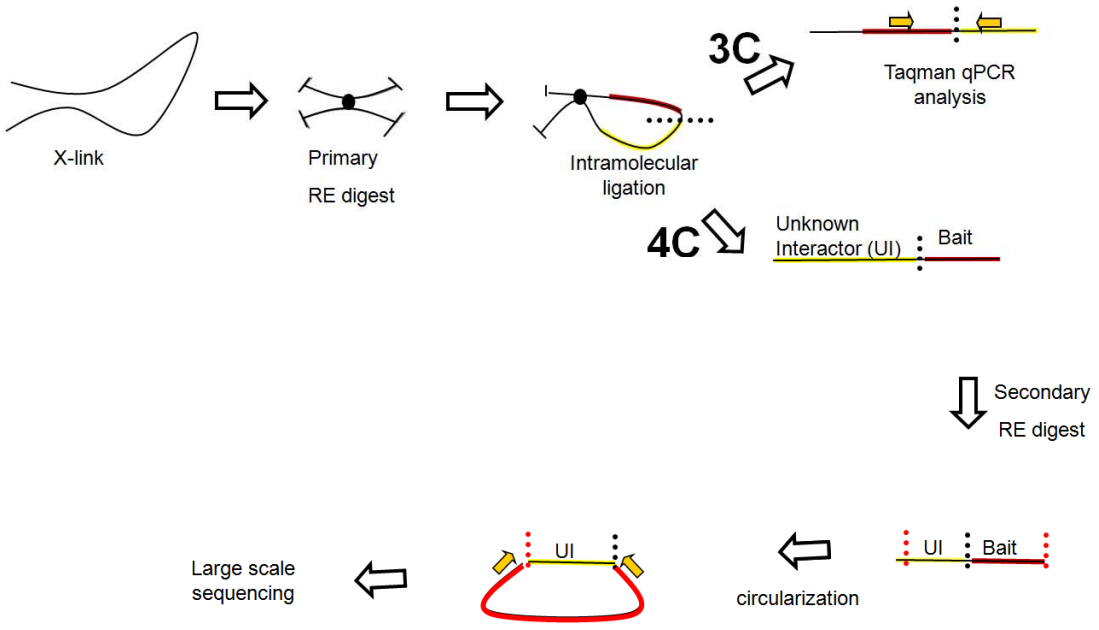

Figure 1-6. Schematic of the 3C and 4C protocols

Interacting chromatin is first cross-linked within the cell, then digested with a restriction enzyme and ligated. The cross-link is then reversed, leaving a library of 3C recombined DNA fragments. 3C studies are quantified by Taqman real-time PCR. For 4C, this library of interacting fragments is then digested with a secondary enzyme and self-ligated to form circular DNA molecules. PCR is conducted with primers extending away from the bait fragment, creating a library of unknown interacting sequences. This 4C library is then analyzed by high-throughput sequencing or microarray. UI, unknown interactor.

1.6 Thesis Overview

The overarching objective of this study was to identify genes regulated by the ATRX chromatin remodeling protein, and to define the underlying mechanisms for gene regulation by ATRX.

1.6.1 Rationale and Hypothesis

Disruption of ATRX function in humans causes ATR-X Syndrome, a severe mental retardation disorder; however, we currently do not fully understand the role of ATRX in the brain. While ATRX has been classified and characterized as a chromatin remodeling protein, no direct gene targets had been identified at the onset of the present study. Transcriptional profiling of a forebrain specific ATRX-null mutant mouse suggested that imprinted genes, a class of genes regulated by chromatin structure and epigenetic modifications, might be affected by the loss of ATRX. The misregulation of imprinted genes in humans has also been linked to neurological disorders. I therefore hypothesized that ATRX regulates the expression of imprinted genes in the brain, by binding to regulatory sites within imprinted domains and modifying the epigenetic environment and chromatin structure.

1.6.2 Chapter Two: ATRX Partners with Cohesin and MeCP2 and Contributes to Developmental Silencing of Imprinted Genes in the Brain

This initial study describes a requirement for ATRX in the postnatal silencing of a neuronal IGN. The loss of ATRX causes an increase in IGN transcripts, including *H19* and *Igf2,* in the postnatal brain. Using the *H19/Igf2* domain as a model, the mechanism of ATRX regulation at imprinted genes was examined. In the wild-type forebrain, we could show that ATRX forms a complex with MeCP2 and Cohesin and that this complex is enriched on the maternal allele of the *H19* ICR. Loss of ATRX results in diminished binding of Cohesin to this region and altered enrichment of post-translational histone modifications. Similar effects were detected at a DMR within a second imprinted domain, *Gtl2/Dlk1*. I concluded that ATRX, along with its binding partners MeCP2 and Cohesin,

regulates the neuronal IGN, and propose that this regulation is accomplished by controlling *cis* and *trans* chromatin interactions of imprinted domains.

1.6.3 Chapter Three: ATRX is Recruited by MeCP2 to Alter Nucleosome Positioning, CTCF Occupancy, and Long-Range Chromosomal Interactions

Higher-order chromatin structure is essential for the regulation of imprinted genes, though the proteins and mechanisms responsible are largely unknown. To expand our understanding of the role of ATRX at imprinted domains, I examined the effects of ATRX loss on chromatin structure. Again, I used the *H19/Igf2* domain as a model. In other cell types, *cis* and *trans* interactions involving the *H19* ICR are thought to regulate *H19/Igf2* and IGN expression, respectively(Burke et al., 2005; Guibert et al., 2012; Han et al., 2008; Kurukuti et al., 2006; Li et al., 2008; Ling et al., 2006; Sandhu et al., 2009; Zhao et al., 2006b). I utilized 3C and 4C techniques to analyze chromatin structure. The results show that the *H19* ICR forms numerous *cis* and *trans* chromosomal interactions in the mouse brain. Loss of ATRX affects *cis* interactions throughout *H19/Igf2* and these structural deficits are accompanied by an altered nucleosome distribution within the ICR and a failure to maintain CTCF binding. I demonstrate that ATRX is recruited to the *H19* ICR by MeCP2. Predictably, MeCP2 deficiency also resulted in loss of chromatin interactions. Finally, I show ATRX binding within many IGN domains and propose a model where MeCP2 targets ATRX to each imprinted gene of the IGN in a temporally regulated manner. ATRX then enacts a transcriptional switch through the control of nucleosome positioning, CTCF binding, and chromatin looping to repress imprinted genes in the postnatal brain. Overall, this thesis identifies a novel mechanism of ATRX in the control of gene expression by altering nucleosome positioning in an allele-specific manner, thus enabling CTCF occupancy and chromatin looping.

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Chapter 2

2 ATRX Partners with Cohesin and MeCP2 and Contributes to Developmental Silencing of Imprinted Genes in the Brain

Prior to the research presented in this chapter, no direct gene expression targets of the ATRX protein had been identified. A previous study from our laboratory reported a series of microarray experiments in control and ATRX-null forebrains at E13.5 and P0.5(Levy et al., 2008). Further analysis of these microarrays revealed that the expression of several imprinted genes, including *H19, Igf2, Sgce, Peg10* and *Dlx5* were upregulated at P0.5, but not at E13.5. In this chapter I sought to investigate whether ATRX was required for the direct regulation of imprinted gene expression in the mouse forebrain and began to investigating a mechanism for this regulation.

This chapter was previously published as (Kernohan et al., 2010). Permission for reproduction is found in Appendix C.

2.1 Introduction

Mounting evidence indicates that the maintenance of chromatin architecture is essential for normal human development and cognitive function. Several human disorders, such as Alpha-Thalassemia mental Retardation, X linked (ATR-X), Rett (RTT), Cornelia de Lange (CdLS), Roberts, Rubinstein-Taybi and Immunodeficiency, Chromosome instability and Facial anomalies (ICF) Syndromes, are caused by mutations in key regulators of chromatin structure and function(Amir et al., 1999; Deardorff et al., 2007; Gibbons et al., 1995; Hansen et al., 1999; Krantz et al., 2004; Musio et al., 2006; Petrij et al., 1995; Vega et al., 2005). Although clearly distinct from one another, many of these disorders share similar clinical features. Whether common symptoms are due to underlying interlinked molecular mechanisms is still poorly understood.

ATR-X Syndrome, caused by mutations in the *ATRX* gene, is one of the prototypical disorders of chromatin dysfunction(Gibbons et al., 2008). ATRX belongs to the sucrose

non-fermenting 2 (SNF2) family of chromatin remodeling proteins, a class of enzymes that utilize the energy of adenosine tri-phosphate (ATP) hydrolysis to disrupt nucleosome stability(Eisen et al., 1995; Picketts et al., 1996). ATR-X patients typically exhibit severe mental retardation, lack of speech development, seizures, microcephaly, alpha-thalassaemia and other developmental defects(Gibbons and Higgs, 2000). *Atrx* loss-of-function studies in mice have revealed its requirement for the normal development of the extra-embryonic trophoblast(Garrick et al., 2006) and of the cerebral cortex and hippocampus(Bérubé et al., 2005; Seah et al., 2008). At the molecular level, epigenetic alterations have been detected, including abnormal levels of DNA methylation at repetitive elements(Gibbons et al., 2000). Recently, it was reported that ATRX and methyl CpG binding protein 2 (MeCP2) interact *in vitro*, and that they co-localize at pericentromeric heterochromatin(Nan et al., 2007). MeCP2, like ATRX, is essential for normal brain function, and females with heterozygous mutations develop Rett syndrome, an autism-spectrum neurodevelopmental disorder(Amir et al., 1999). Importantly, MeCP2 is required for the proper localization of ATRX at pericentromeric heterochromatin in mature neurons of the mouse brain as determined by immunofluorescence studies(Nan et al., 2007).

We previously reported that loss of ATRX results in altered expression of certain genes in the mouse forebrain, including the ancestral pseudoautosomal genes(Levy et al., 2008). However, we also found that depletion of ATRX in human somatic cells resulted in chromosome misalignment and sister chromatid cohesion defects during mitosis(De La Fuente et al., 2004; Ritchie et al., 2008). Thus, it appears that ATRX plays a dual role in the regulation of cohesion during mitosis and the control of gene expression in interphase. This is reminiscent of the Cohesin complex, which has well-established functions in both mitotic chromosome cohesion and gene expression. The regulation of gene expression by Cohesin and its regulatory factors is thought to underlie developmental defects seen in patients with CdLS(Borck et al., 2007; Deardorff et al., 2007; Kawauchi et al., 2009; Krantz et al., 2004; Liu et al., 2009; Musio et al., 2006).

We now demonstrate that ATRX, MeCP2 and Cohesin interact in the mouse brain *in vivo* and co-localize at the *H19/Igf2* and *Gtl2/Dlk1* imprinted regions. At the *H19/Igf2*

domain, this interaction is maternally-biased at the *H19* ICR. We further show that ATRX is required for normal recruitment of MeCP2, Cohesin and the insulator protein CTCF and alters the expression of a connected network of imprinted genes in the postnatal brain. We speculate that ATRX, along with its binding partners Cohesin and MeCP2, regulates the expression of this imprinted gene network (IGN) by controlling higher-order chromatin structure.

2.2 Materials and Methods

2.2.1 Animal Husbandry

Conditional deletion of *Atrx* in the mouse forebrain was achieved by crossing $Atrx^{loxP}$ females with heterozygous *Foxg1Cre* knock-in males, as previously described(Bérubé et al., 2005). The $Atrx^{loxP}$ line was kindly provided by D. Higgs (Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom). For the developmental studies, midday of vaginal plug discovery was considered E0.5. For allele-specific expression studies, pups were obtained by mating 129Sv female mice with *Mus musculus castaneus* male mice (CAST; The Jackson Laboratory). To generate polymorphic ATRX-deficient animals, heterozygous $Atrx^{loxP/wt}$ $Foxg1Cre^{+/-}$ females (129Sv) were mated with CAST males. $Mecp2^{tm2Bird}$ mice were generated by crossing B6;129P2-$Mecp2^{tm2Bird}$ heterozygous females (The Jackson Laboratory) with wild-type males (C57BL6, The Jackson Laboratory). All animal studies were conducted in compliance with the regulations of The Animals for Research Act of the province of Ontario, the guidelines of the Canadian Council on Animal Care, and the policies and procedures approved by the University of Western Ontario Council on Animal Care.

2.2.2 Co-Immunoprecipitation

Nuclear lysates, obtained from forebrain tissue using the Nuclear and Cytoplasmic Extraction kit (NE-PER, Thermo Scientific) were incubated with anti-ATRX antibody (Fxnp5, gift of Richard Gibbons), anti-SMC3 antibody (Bethyl Laboratories) or anti-MeCP2 (gift of Janine M. LaSalle) for 2 hours at 4 ^{o}C. Normal sheep and rabbit IgG (Santa Cruz), and chicken IgY (Santa Cruz) were used as negative controls. Samples were then incubated with Dynabeads protein G (Invitrogen) for one hour at 4 ^{o}C. The

supernatant was then re-incubated 1 hour with Dynabeads protein G for one hour. The two aliquots were then combined. Immunoprecipitates were washed 5 times with 1ml of 0.1% Tween-20 in phosphate buffered saline (PBS), eluted, and resolved on 8% SDS-PAGE. Western blot analysis was carried out using an anti-ATRX antibody (H300, Santa Cruz), anti-MeCP2 (gift of Janine M. LaSalle), anti-SMC1, anti-SMC3, and anti-Rad21 (Bethyl Laboratories).

2.2.3 ChIP Analysis

Mouse forebrain tissue was rinsed in cold PBS, cut and homogenized. Minced tissue was diluted with DMEM (Sigma-Aldrich) and passed through a 70 μm cell strainer (BD Falcon) to ensure single cell suspension. For chromatin immunoprecipitation, an EZ-ChIP (Upstate) kit was used according to the manufacturer's instructions. Briefly, cells were cross-linked in 1% formaldehyde, lysed in SDS buffer and sonicated. Immunoprecipitation was performed using the following antibodies: anti-ATRX (Fxnp5, gift of Richard Gibbons), anti-ATRX (Bethyl laboratories), anti-SMC1 (Bethyl laboratories), anti-SMC3 (Bethyl laboratories), anti-CTCF (Upstate) and antibodies to MeCP2, H3Ac, H4Ac, H3K9me2, H3K9me3 and H4K20me3 (Upstate). Rabbit (Upstate), sheep (Santa Cruz), and goat (Santa Cruz) IgGs were used as controls. Input samples represent 1/25 of total chromatin input. Conditions for amplification were as follows: 95 °C for 5 minutes followed by 30 cycles of 95 °C for 30 seconds, 55.5 °C for 30 seconds, and 72 °C for 30 seconds. A final extension was performed at 72 °C for 5 minutes. Real-time PCR experiments were conducted with 95 °C for 5 minutes followed by 35 cycles of 95 °C for 10 seconds, 55.5 °C for 20 seconds, and 72 °C for 30 seconds. ChIP re-ChIP experiments were performed as described above, with the following exceptions: subsequent to incubation with the first antibody, samples were washed with low and high salt buffers (Upstate) and eluted in 10 mM DTT (Invitrogen) for 40 minutes. Eluted samples were diluted with re-ChIP buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100) and immunoprecipitated overnight with the second antibody. This procedure was repeated for the sequential triple ChIP experiment. Allele-specific ChIP analysis was performed as described on 129Sv/CAST F_1 forebrain tissue. PCR amplification was performed for the *H19* ICR region 5' and the

resulting product digested with SacI and MfeI (NEB) for 2 to 4 hours at 37 ^{o}C and resolved on a 7% polyacrylamide gel. The gel was stained with ethidium bromide and visualized via a UV trans-illuminator (BioRad). Primer sequences are available upon request.

2.2.4 Quantitative ChIP

ChIP products obtained from P17 *Atrx*^{Foxg1Cre} and littermate control forebrain tissue were amplified in duplicate or triplicate with iQ™ SYBR® Green master mix (BioRad) on a Chromo-4 thermocycler using the following conditions: 95 °C for 5 minutes followed by 40 cycles of 95 °C for 10 seconds, 55 °C for 20 seconds, 72 °C for 30 seconds, and a final melting curve generated from 55 to 95 °C in increments of 1°C per plate read. Fold change and % input formulas were adapted from (Mukhopadhyay et al., 2008) as follows: Fold change = $2^{(\Delta Ct_{Control}-\Delta Ct_{Control})-(\Delta Ct_{Control}-\Delta Ct_{KO})}$; $\Delta Ct= (Ct_{target} - Ct_{Gapdh\ promoter})$. % Input = $100*[2^{(\Delta Ct_{Input}-\Delta Ct_{Input})-(\Delta Ct_{Input}-\Delta Ct_{Ab})}] /25$. Error bars represent the standard error of the mean. The significance of non-uniform relative enrichment was determined via repeated-measure ANOVA across target binding sites. Primer sequences for all regions are available upon request.

2.2.5 Semi-Quantitative and Quantitative RT-PCR

Total RNA was obtained from *Atrx*^{Foxg1Cre} and littermate control forebrains using the RNeasy mini kit (QIAGEN) and reverse-transcribed into complementary DNA (cDNA) as described(Ritchie et al., 2008). Control reactions without reverse transcriptase were prepared in parallel. cDNA was amplified using gene-specific primers using the following conditions: 25-35 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute. For quantitative RT-PCR, cDNA was amplified with iQ™ SYBR® Green master mix (BioRad) using the standard curve Ct method of quantification. Experiments were performed on a Chromo-4 thermocycler (MJ Research) and analyzed with Opticon Monitor 3 and GeneX (BioRad Laboratories) software. Gene expression analysis was repeated in triplicate for each sample. Conditions for amplification were as follows: 35 cycles of 95 °C for 10 seconds, 55 °C for 20 seconds, 72 °C for 30 seconds, and a final melting curve generated in increments of 0.5 °C per plate read. Standard

curves were generated for each primer pair using three-fold serial dilutions of control cDNA. Primer efficiency was calculated as $E = [10^{(-1/slope)} - 1] * 100\%$, where a desirable slope is –3.32 and $r^2 > 0.990$. All data was corrected against *beta-actin* or *Gapdh* as an internal control. Unless otherwise indicated, the error bars represent the standard error of the mean.

2.2.6 Allele-Specific Expression Analysis

H19 and *Snrpn* allelic expression assays were performed on cDNA obtained from P0.5 129Sv/CAST F_1 forebrains using the LightCycler Real Time PCR System (Roche Molecular Biochemicals). *H19* analysis was conducted as described previously(Mann et al., 2003; Thorvaldsen et al., 2002). Briefly, 0.3 μm primers (Sigma Genosys), 0.15 μm labeled probes (TIB Molbiol), and 3 mM $MgCl_2$ were added to Ready-to-Go PCR beads (Invitrogen). Following amplification, a melting curve analysis was conducted at 95 °C 0 sec, 50 °C 1 min, and then the temperature was raised in increments of 0.2°C from 50 to 85°C with continuous fluorescence acquisition. For *Snrpn* analysis, 0.5 μm primers (Sigma Genosys), 0.3 μm labeled probes (TIB Molbiol), and 3 mM $MgCl_2$were added to Ready-to-Go PCR bead, and PCR amplification was performed(Mann et al., 2003). The melting curve analysis was performed at 95 °C for 2 minutes, 45 °C for 2 minutes, and fluorescence acquisition occurred continuously as the temperature was increased from 45 to 85 °C in 0.2 °C increments. The data was analyzed using the LightCycler Software Data Analysis function.

2.2.7 Bisulfite Mutagenesis

Genomic DNA isolated from the forebrain of two pairs of $Atrx^{Foxg1Cre}$ and littermate control mice was mutagenized with sodium bisulfite using an EpiTect Bisulfite Conversion Kit (QIAGEN) according to the manufacturer's instructions. PCR amplification was carried out with primers specific for bisulfite-treated DNA. All DMRs were amplified by the nested or semi-nested PCR approach using previously described conditions(Lopes et al., 2003; Lucifero et al., 2002; Olek et al., 1996; Takada et al., 2002). The resulting nested PCR products were ligated into the pCR2.1 vector using a TOPO-TA cloning kit (Invitrogen), according to the manufacturer's instructions.

Positive clones were sequenced (DNA Sequencing Facility at the Robarts Research Institute) using an ABI GeneAmp Thermocycler and analyzed using an Applied Biosystems 3730 Analyzer. Clones were only accepted at ≥95% cytosine conversion. Non-converted cytosine residues and mismatched base pairs were used to ensure that accepted clones originated from a unique template DNA.

2.3 Results

2.3.1 Interaction of ATRX, MeCP2 and Cohesin in the Mouse Brain *in vivo*

We previously demonstrated that depletion of ATRX resulted in reduced sister chromatid cohesion(Ritchie et al., 2008) and aberrant expression of specific genes in the mouse brain(Levy et al., 2008), suggesting similar modes of action for ATRX and Cohesin. Based on the observed effects on chromosomal cohesion, we examined whether Cohesin was associated with ATRX in SH-SY5Y neuroblastoma cell extracts. Co-immunoprecipitation experiments showed that a portion of nuclear SMC1, one of the components of the Cohesin ring complex, interacts with ATRX in these cells (Supplementary Figure 2-7A). To determine if this interaction also occurs *in vivo*, we performed similar immunoprecipitation experiments using mouse forebrain tissue. We identified interactions between ATRX and the Cohesin subunits SMC1 and SMC3 in mouse forebrain at postnatal day 17 (P17) (Figure 2-1A). At this developmental time point, most cells within the forebrain are post-mitotic, indicating that the observed interactions are probably unrelated to sister chromatid cohesion. We verified specificity of interactions using mice with conditional deletion of the long isoform (but not the short isoform, which lacks the ATPase domain) of ATRX in the mouse forebrain ($Atrx^{Foxg1Cre}$ mice, defined as "ATRX KO" here on)(Bérubé et al., 2005). Interaction between ATRX and SMC1 was greatly reduced in ATRX KO forebrain, demonstrating the specificity of the immunoprecipitation (Supplementary 2-7B). Similar results were obtained for SMC3 in HeLa cell extracts transiently depleted of ATRX protein using RNA interference (Supplementary Figure 2-7C).

The methyl-binding protein MeCP2 was recently shown to associate with ATRX(Nan et al., 2007), but interaction of the endogenous proteins had not yet been validated in mouse brain. We show that endogenous MeCP2 co-immunoprecipitates with ATRX, and also with SMC1 and SMC3 in wild-type forebrain, but not in forebrain tissue from MeCP2-null mice (*MeCP2*^{tm2Bird}), suggesting that these proteins are part of a macromolecular complex in the mouse brain (Figure 2-1A.B). The interactions between ATRX, MeCP2 and Cohesin subunits still occurred upon treatment with DNAse I (Supplementary Figure 2-7D). We also confirmed the specificity of the MeCP2 antibody by Western blot analysis of wild-type and MeCP2-null forebrain tissue (Supplementary Figure 2-7E). Thus, we identified protein interactions in the mouse brain between several regulators of chromatin structure that are associated with developmental congenital disorders.

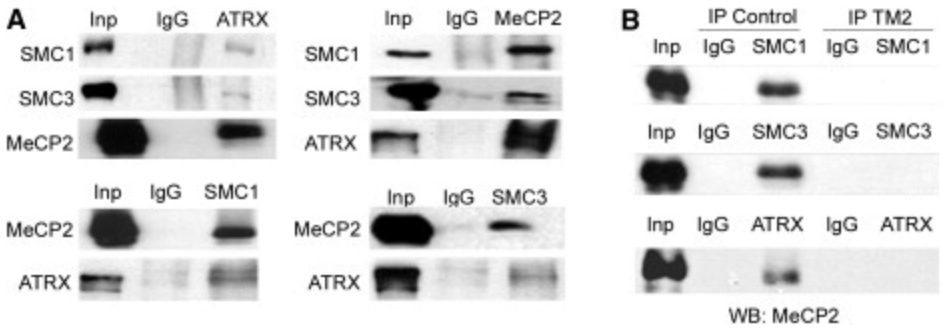

Figure 2-1. ATRX, MeCP2, and Cohesin interact *in vivo* in the mouse forebrain

(A) ATRX was immunoprecipitated from P17 mouse forebrain extracts, and Western blot analysis was performed for SMC1, SMC3, and MeCP2 (top left panel). The results show that these three proteins are immunoprecipitated with ATRX. Top right panel: in a similar manner, SMC1, SMC3, and ATRX were detected in MeCP2 immunoprecipitates. Bottom panels: the reverse immunoprecipitations were also performed, showing that SMC1 and SMC3 immunoprecipitates contain ATRX and MeCP2 protein, confirming the interactions between these proteins. Control reactions were done with IgG. (B) SMC1, SMC3, and ATRX were immunoprecipitated from control and MeCP2 KO (TM2) forebrain extracts, followed by Western blot analysis with an anti-MeCP2 antibody. In all cases, no band was observed in the MeCP2 KO (TM2) immunoprecipitates, demonstrating the specificity of these interactions.

2.3.2 Co-Occupancy of ATRX, Cohesin and MeCP2 at the Maternal *H19* ICR

Preliminary data from a microarray study of ATRX–deficient mouse brains demonstrated that the *H19* imprinted gene is among the genes upregulated in the absence of ATRX(Levy et al., 2008). The *H19/Igf2* imprinted domain located on mouse chromosome 7 contains several differentially methylated regions (DMRs), including one positioned 2 kb upstream of *H19* that acts as an imprinting control region (ICR). The *H19* ICR is methylated on the silent paternal allele in many tissues, including the brain(Bartolomei et al., 1993; Ferguson-Smith et al., 1993), and we hypothesized that this genomic site could be a target of ATRX and its interacting partners MeCP2 and Cohesin in the mouse brain. Chromatin immunoprecipitation (ChIP) was performed along a large region of the *H19* ICR as well as flanking genomic sites using an antibody specific for ATRX (depicted in Figure 2-2A). We detected substantial enrichment of ATRX at the mid-portion of the *H19* ICR, with primers *H19-5* (Figure 2-2B). The interaction of ATRX at this site was greatly decreased in the ATRX KO forebrain, demonstrating the specificity of the ATRX ChIP reaction (Figure 2-2B). To determine whether the ChIP-PCR represented a true enrichment of ATRX, we performed quantitative ChIP analysis at the *H19* ICR and outlying regions using two different ATRX antibodies, and detected significant enrichment at the *H19*-5 region of the *H19* ICR compared to flanking sequences (Figure 2-2C; ATRX-1 p=0.003, ATRX-2 p=0.005). We also showed similar enrichment of MeCP2 and of SMC1 at the same site (MeCP2 p=0.008, SMC1 p=0.03), while analysis of nonspecific IgG and the transcription factor PROX1 revealed nominal enrichment (Figure 2-2C, Supplementary 2-8A). Additionally, quantitative profiling of ATRX, MeCP2 and SMC1 across the unrelated *Gapdh* promoter region revealed no peaks of binding, demonstrating the specificity of enrichment of these proteins at the *H19* ICR (Supplementary Figure 2-8B). Although binding of ATRX, Cohesin and MeCP2 was enriched at the *H19* ICR, it was possible that binding to this site occurred independently in different cells of the forebrain. To address this question, we performed sequential ChIP for ATRX and SMC1, ATRX and MeCP2, and ATRX, SMC3 and MeCP2, and could show co-occupancy at region *H19*-5' of the *H19* ICR (a smaller

region of *H19*-5) (Figure 2-2D). Sequential ChIP was negative at the *Gapdh* promoter region, demonstrating that the interaction with the *H19* ICR is specific (Figure 2-2D).

Since *H19* is expressed in an allele-specific manner, we wanted to examine whether ATRX, MeCP2 and Cohesin displayed allele-specific binding at the *H19* ICR. To achieve this, we generated 129Sv/CAST F_1 mice that are polymorphic within this region, thereby allowing the parental alleles to be distinguished. F_1 forebrain tissue was processed for ChIP followed by allele-specific restriction digest analysis of amplified DNA. ATRX, MeCP2, SMC1 and SMC3 were all preferentially enriched on the maternal allele of the *H19* ICR (Figure 2-2E and Supplementary Figure 2-10B). We validated allele-specificity by confirming the paternal-specific deposition of the histone variant macroH2A(Choo et al., 2007), and the maternal enrichment of acetylated histone H3 (H3Ac) and H4 (H4Ac) (Figure 2-2F). In all cases, even with controls, we did not observe 100% allelic enrichment, perhaps reflecting the mosaic nature of the tissue (i.e. neurons vs. glia). Taken together, this data suggests that ATRX preferentially binds to the maternal allele of the *H19* ICR with Cohesin and MeCP2.

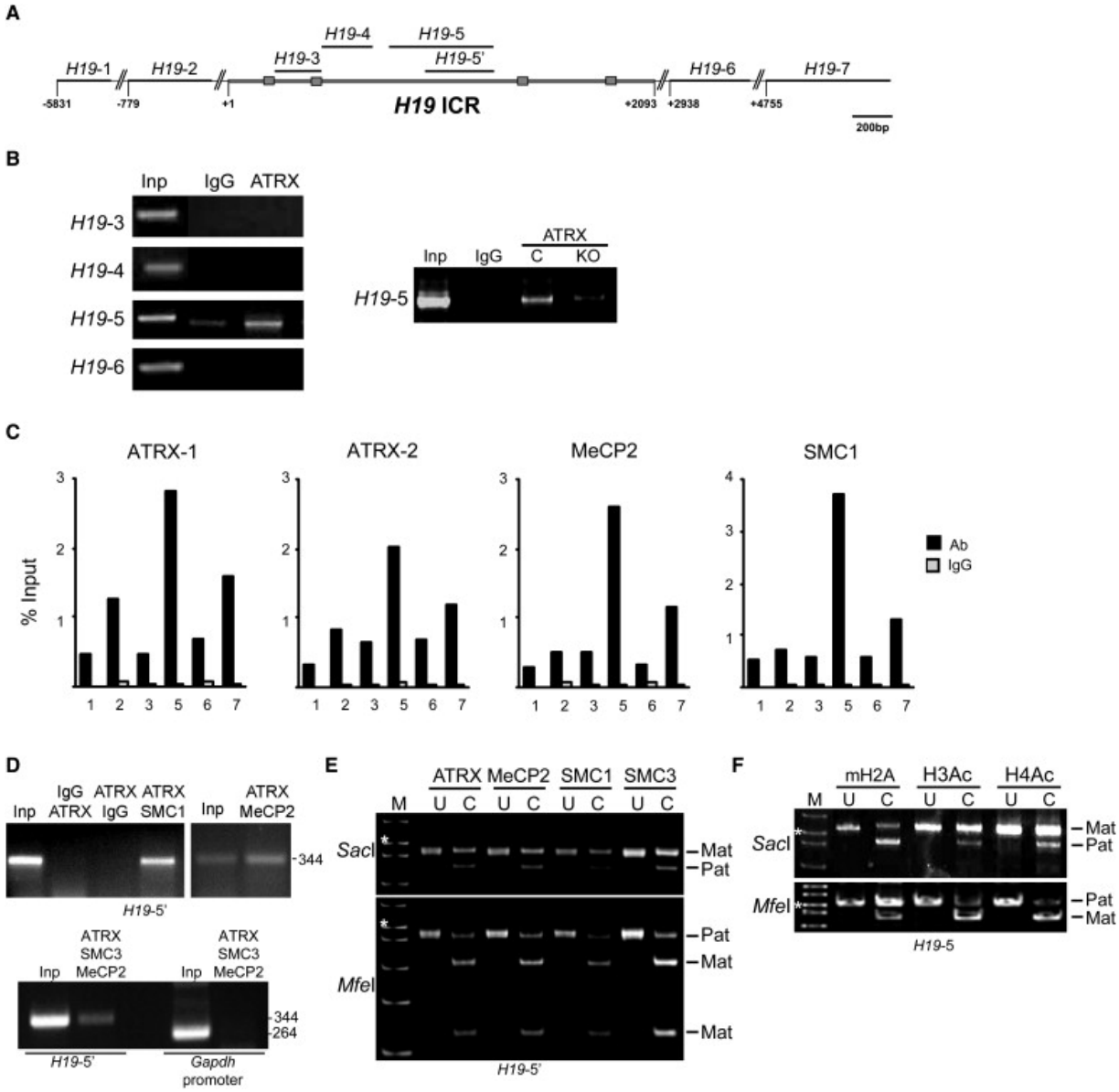

Figure 2-2. ATRX, MeCP2, and Cohesin are preferentially bound to the maternal *H19* ICR in mouse forebrain

(A) Genomic organization and alignment of primers utilized for PCR amplification of ChIP reactions. Numbers indicate the relative nucleotide position from the start of the *H19* ICR region. Grey boxes indicate CTCF-binding sites. (B) PCR of ChIP DNA shows enrichment of ATRX at the region flanked by primer pair *H19*-5 of the *H19* ICR. ATRX ChIP of region *H19*-5 was also performed on control and ATRX KO forebrain tissue (right panel). (C) A peak of enrichment of ATRX, MeCP2, and SMC1 at region *H19*-5

was confirmed by quantitative ChIP analysis. Graphs depict a representative enrichment profile. Statistical analysis revealed that deviation from uniform binding was significant, even accounting for interexperimental variability (ATRX-1, $p = 0.003$; ATRX-2, $p = 0.005$; MeCP2, $p = 0.008$; SMC1, $p = 0.030$). (D) Co-localization of ATRX, MeCP2, and Cohesin at region *H19*-5′ of the *H19* ICR was verified by double or triple sequential ChIP experiments. Input represents one-tenth of the input sample. (E and F) Allelic analysis of ATRX, MeCP2, and Cohesin interaction with region *H19*-5′ of the *H19* ICR shows that these proteins were enriched on the maternal allele in the mouse forebrain. Similar allelic analysis was performed for macroH2A (paternal) and acetylated histones H3 and H4 (maternal) at region *H19*-5′ as controls. For allelic analysis, amplicons from ChIP-isolated 129Sv/CAST F_1 forebrain DNA were digested with MfeI (129Sv maternal-specific site) or SacI (CAST paternal-specific site) enzymes. Inp, input; mH2A, macroH2A; H3Ac, acetylated histone 3; H4Ac, acetylated histone 4; U, uncut; C, cut; Mat, maternal; Pat, paternal; M, standard marker. The asterisk indicates a 500 bp marker in the standard (Invitrogen).

2.3.3 Loss of ATRX Does Not Affect DNA Methylation at the *H19* ICR but Results in an Altered Profile of Histone Tail Modifications

DNA methylation plays a central role in the regulation of genomic imprinting at the *H19/Igf2* domain(Biniszkiewicz et al., 2002; Li et al., 1993). In light of the reported aberrant patterns of DNA methylation in ATR-X patients and in ATRX-null mouse embryonic stem (ES) cells(Gibbons et al., 2000), we assessed whether ATRX contributes to the maintenance of DNA methylation at the *H19*/*Igf2* DMRs in the mouse brain. We performed the bisulfite mutagenesis and sequencing assay on control and ATRX-null forebrain tissue. At the *H19* ICR, we detected approximately 50% methylated alleles in both control and ATRX-null samples, indicating DNA methylation of this region is preserved in the absence of ATRX (Figure 2-3A). The *H19/Igf2* genomic domain is regulated by long-range chromatin interactions in the liver, a process mediated in part by the DMRs and their DNA methylation state(Kurukuti et al., 2006; Murrell et al., 2004). We therefore extended bisulfite sequencing analysis to the *Igf2* DMR1 and DMR2 regulatory regions, and determined that they are largely unmethylated in both control and ATRX KO newborn forebrain (Supplementary Figure 2-9A). To verify whether ATRX deficiency can affect DNA methylation at other genomic sites in the brain, we also investigated DNA methylation at ribosomal DNA (rDNA) gene repeats. Loss of ATRX in the forebrain resulted in hypomethylation of the 18S rDNA repeats (Supplementary Figure 2-9B) as was previously reported in mouse ES cells and ATR-X patients(Garrick et al., 2006; Gibbons et al., 2000). However, no change in DNA methylation was observed at the 28S rDNA repeats, major satellite, minor satellite (except for one sample) or IAP*gag* repeats in the ATRX KO forebrain nor in relative amplification of the latter three repeats (Supplementary Figure 2-9C,D). Thus, ATRX deficiency perturbs DNA methylation at specific repetitive elements in the brain, but not at the *H19* ICR and *Igf2* DMRs, suggesting alternative mechanistic effects of ATRX at this imprinted domain.

The potential link between ATRX and a change in epigenetic markings at the *H19* ICR was further investigated by quantitative ChIP analysis of various histone tail post-translational modifications at the *H19* ICR. We observed increased acetylation of histones H3 and H4 (H4Ac and H3Ac) in the ATRX-null forebrain, suggesting that loss

of ATRX induces a more accessible, open chromatin state (Figure 2-3B). Histone modifications including H3K9me2, H3K9me3 and H4K20me3 are generally characteristic of condensed chromatin states. We observed no change in H3K9me2 enrichment, but detected a trend towards decreased levels of H4K20me3 and H3K9me3 in the ATRX-null forebrain (Figure 2-3B). Collectively, these analyses demonstrate that the absence of ATRX at the maternal *H19* ICR does not induce changes in DNA methylation but correlates with an altered pattern of histone tail modifications reflecting reduced chromatin compaction.

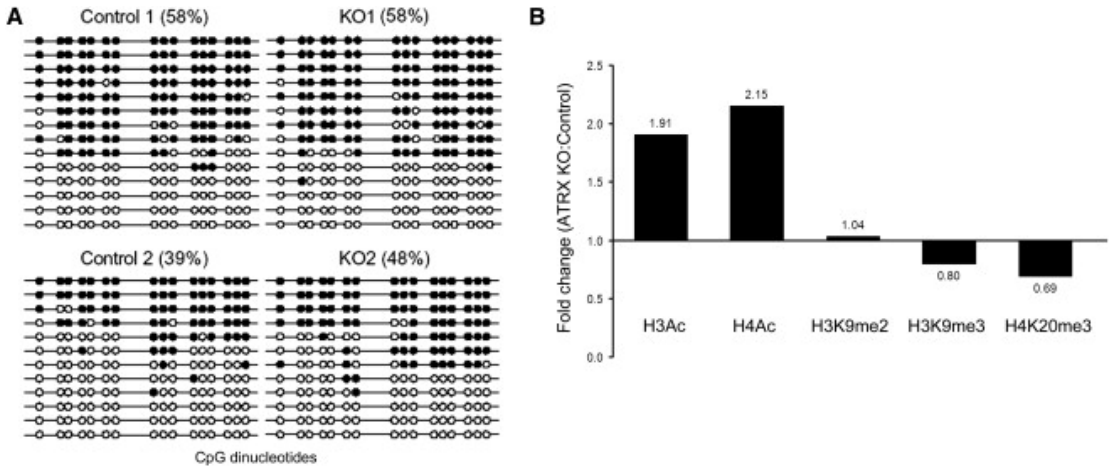

Figure 2-3. Effects of ATRX loss of function on the epigenetic state of the *H19* ICR

(A) Bisulfite mutagenesis and sequencing analysis of the *H19* ICR region revealed no change in DNA methylation upon loss of ATRX in forebrain tissue. At least 12 alleles from each sample were analyzed, and individual alleles are represented as a string of 16 CpGs. The total percent methylation for each sample is indicated in parentheses. Unmethylated CpGs are represented as empty circles, and methylated CpGs are represented as filled circles. (B) Quantitative ChIP analysis of histone H3 and H4 modifications in control and ATRX KO forebrain tissue at region *H19*-5′ of the *H19* ICR. Enrichment of activating marks H3Ac and H4Ac were increased, whereas repressive marks H3K9me3 and H4K20me3 were decreased in the absence of ATRX. Graphed data represent the mean fold change in enrichment across three control and ATRX KO littermate pairs. Data were normalized to amplification of the *Gapdh* promoter region. See also Supplementary Figure 2-9.

2.3.4 Allele-Specific Regulation of *H19* by ATRX

The presence of ATRX, Cohesin and MeCP2 at the maternal *H19* ICR, combined with the changes in histone marks suggested that these proteins could play a direct role in the regulation of *H19* expression, and perhaps of the neighboring *Igf2* gene. Semi-quantitative expression analysis of three ATRX KO and control newborn forebrain samples showed increased expression of *H19* and *Igf2* in the absence of ATRX (Figure 2-4A). Quantitative expression analysis by real-time PCR of the forebrain at various developmental time points showed that *H19* and *Igf2* gene expression is not altered at embryonic day (E) 13.5. In contrast, expression of these genes was increased 2 to 4 fold in the ATRX deficient forebrain at postnatal days (P) 0.5 and 17 (Figure 2-4B). Thus, loss of ATRX does not affect *H19* and *Igf2* gene expression during the peak of neurogenesis in the developing forebrain, but results in increased expression of both genes during the postnatal growth deceleration phase.

Atrx gene inactivation in the forebrain of the ATRX KO mice occurs at approximately E8.5, when *H19* and *Igf2* imprinted expression has already been established. Increased levels of *H19* might therefore be due to failure to maintain paternal allelic silencing, or to an imprint-independent mechanism that would alter maternal *H19* transcript levels. To determine the allelic source of additional *H19* transcripts, we mated *Atrx* heterozygous females ($Atrx^{loxP/+:Cre+/-}$) with wild-type CAST males to generate F_1 polymorphic ATRX KO and control mice. We obtained a 129Sv/CAST P0.5 *Atrx* heterozygous female that displayed decreased *Atrx* expression and increased *H19* expression compared to a control littermate (Figure 2-4C), allowing for allelic analysis of *H19* using a Lightcycler allelic melting assay(Thorvaldsen et al., 2002). As previously reported, *H19* and *Snrpn* were expressed solely from the maternal and paternal alleles, respectively, in control mouse forebrain tissue (Figure 2-4D,E)(Leff et al., 1992; Svensson et al., 1995b). Analysis of *H19* expression in the F_1 ATRX-deficient forebrain revealed that increased expression of *H19* was not due to re-activation of the paternal allele, since transcripts were still derived solely from the maternal allele (Figure 2-4F). We conclude that ATRX-deficiency results in aberrant *H19* gene transcription and that this effect is specific to the maternal allele.

Postnatal silencing of the *H19* gene in the brain was previously reported(Svensson et al., 1995a). We examined the temporal expression pattern of *H19* in the forebrain and confirmed that transcript levels decrease over the first two weeks after birth (Supplementary Figure 2-10A). We also observed an enrichment of repressive histone modifications (H3K9me2, H3K9me3 and H4K20me3) on the *H19* ICR maternal allele in the P17 brain, along with ATRX and MeCP2 (Supplementary Figure 2-10B). Taken together, these results suggest that ATRX participates in the silencing of the maternal *H19* gene in the postnatal mouse brain.

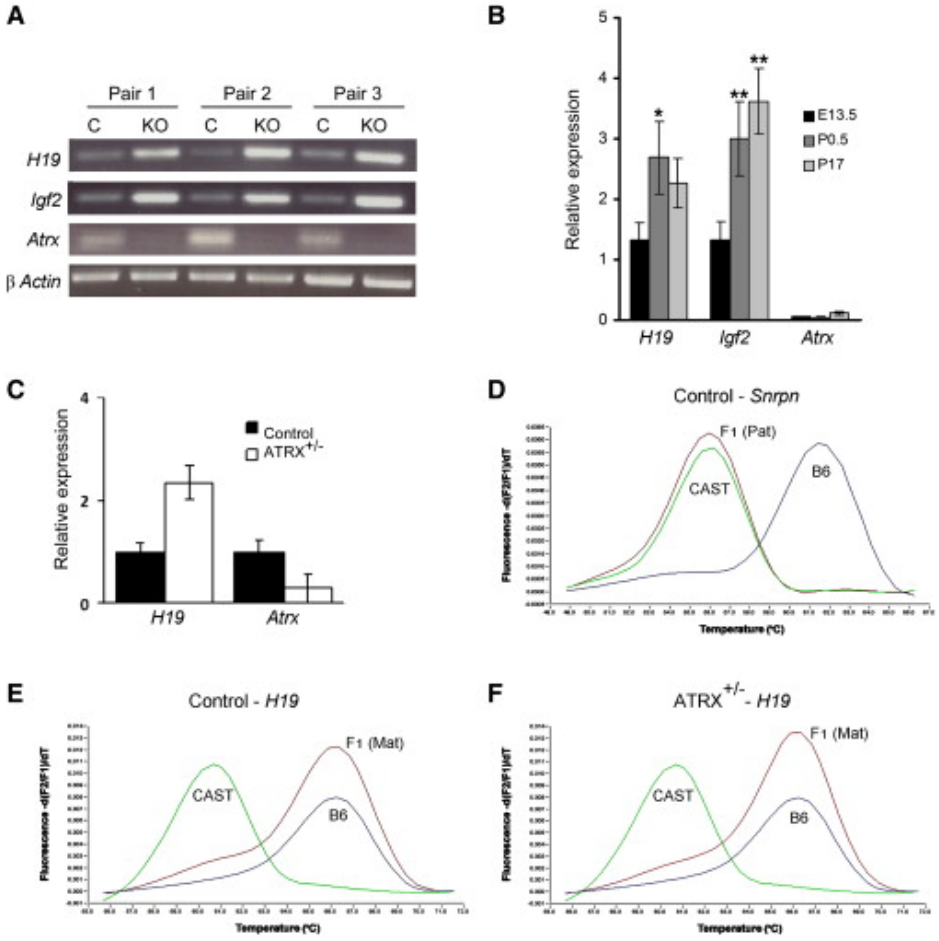

Figure 2-4. Allele-specific control of *H19* gene transcription by ATRX

(A) Semiquantitative RT-PCR analysis showing increased transcript levels of *H19* and *Igf2* in three sets of control and ATRX KO littermate-matched neonatal (P0.5) forebrains, whereas *beta-actin* transcript levels were not altered. (B) Real-time PCR analysis shows that *H19* and *Igf2* upregulation occurred in the ATRX KO postnatal forebrain (P0.5 and P17), but was not affected in the embryonic period (E13.5). Expression data for *Atrx* with primers specific for the long isoform were included as a control. Graphed data represent the mean relative expression level, and error bars depict standard error of the mean from biological replicates. The asterisk indicates $p < 0.05$, and double asterisks denote $p < 0.005$, as determined by a two-tailed t test. All results are normalized to *beta-actin* expression. (C) Quantitative RT-PCR analysis confirms upregulation of *H19* and

decreased *Atrx* expression in the $Atrx^{+/-}$ forebrain tissue (bottom right). Error bars depict standard deviation of technical replicates. Real-time RT-PCR of *H19* and *Atrx* in an $F_1Atrx^{+/-}$ forebrain. (D and E) Allelic melting curve analyses with hybridization probes (C57BL/6 and 129Sv homologous, CAST mismatched) revealed paternal-specific expression of (D) *Snrpn* and maternal-specific expression of (E) *H19* in control 129Sv/CAST F_1 forebrain. (F) Analysis of a 129Sv/CAST F_1 forebrain heterozygous for ATRX revealed that *H19* transcripts are still largely produced from the maternal allele. Homozygous C57BL/6, which has an identical sequence to 129Sv, and *Mus musculus casteneous* (CAST) samples were included as controls. Mat, maternal; Pat, paternal; B6, C57BL/6; $Atrx^{+/-}$, ATRX heterozygote

2.3.5 Co-Occupancy of ATRX, MeCP2 and Cohesin at the *Gtl2/Dlk1* Imprinted Cluster

We next wanted to determine whether ATRX, MeCP2 and Cohesin co-occupancy is specific to the *H19* ICR or also occurs at other imprinted regions in the forebrain. We chose to investigate the *Gtl2/Dlk1* imprinted cluster on mouse chromosome 12 because it shares many features with the *H19/Igf2* region, including the presence of several similarly positioned DMRs(Takada et al., 2002; Wylie et al., 2000). One DMR overlaps with the *Gtl2* promoter and extends into exon 1 and intron 1 of the gene (Figure 2-5A). This DMR was a logical candidate genomic region to investigate, as it is required for normal imprinting of *Gtl2/Dlk1*(Steshina et al., 2006), is bound by MeCP2 in mouse cerebellum(Jordan et al., 2007), and contains a putative Cohesin/CTCF-consensus site(Paulsen et al., 2001). We performed quantitative ChIP analysis to investigate the level of enrichment of ATRX, MeCP2 and SMC1 at three sites within the DMR and one site 5' of the DMR. Region GD-2 was previously reported to display asymmetric enrichment of histone H3 and H4 acetylation(Carr et al., 2007), GD-3 was shown to be bound by MeCP2(Jordan et al., 2007), and GD-4 contains a putative CTCF-binding site (see diagram in Figure 2-5A). All three proteins were associated with the DMR in P17 mouse brain (Figure 2-5B), but unlike the situation observed at the *H19* ICR, the peaks of enrichment did not overlap but were adjacent within the DMR region. We observed highest binding of ATRX at GD-2, the site of allelic histone acetylation (Figure 2-5B), while MeCP2 was most enriched at GD-3 and SMC1 at GD-4 (Figure 2-5B). ChIP using IgG or an antibody against the transcription factor PROX1 did not show enrichment across this region (Figure 2-5B, Supplementary Figure 2-8A).

Direct binding at the *Gtl2* DMR suggested that ATRX may be required to maintain normal *Dlk1* and *Gtl2* gene expression during brain development. We measured transcript levels of both genes in control and littermate-matched ATRX KO forebrain tissue. Similar to what we observed for *H19* and *Igf2*, *Dlk1* expression was not changed at E13.5, but was considerably increased in mutants in the postnatal period, at P0.5 and P17 (Figure 2-5C). In contrast, *Gtl2* expression was not affected in the ATRX KO forebrain (Figure 2-5C). A temporal survey of *Dlk1* and *Gtl2* expression revealed that

Dlk1 is gradually silenced postnatally while *Gtl2* expression levels stayed more constant (Supplementary Figure 2-10A). We conclude that ATRX binds to the *Gtl2* DMR with Cohesin and MeCP2 and silences *Dlk1*, without influencing the expression of *Gtl2*.

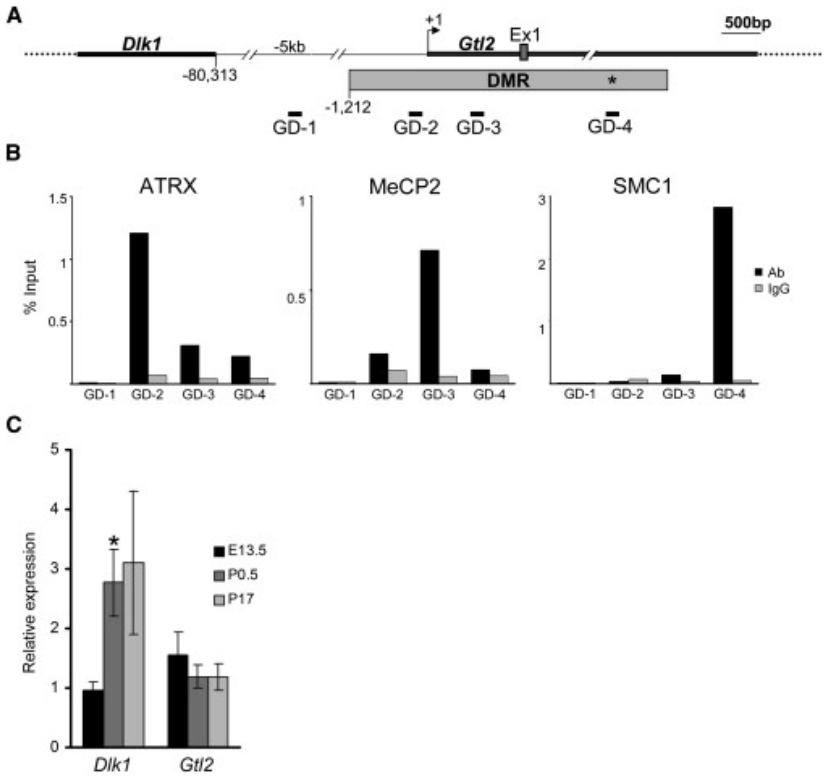

Figure 2-5. ATRX, MeCP2, and Cohesin bind within the *Gtl2/Dlk1* imprinted domain

(A) Genomic organization and alignment of regions analyzed by ChIP (GD-1, GD-2, GD-3, and GD-4). Numbers indicate the relative nucleotide position from the *Gtl2* transcription start site. The asterisk denotes the predicted CTCF-binding site. (B) Quantitative ChIP analysis of ATRX, SMC1, and MeCP2 within the *Gtl2* DMR. Regions analyzed are indicated on the x axis. ATRX (n = 4), p = 8.868e-07; MeCP2 (n = 2); SMC1 (n = 3), p = 0.05218. (C) Real-time RT-PCR analysis of *Dlk1* and *Gtl2* mRNA expression at E13.5, P0.5, and P17. Graphed data represent the mean relative expression level, and error bars depict standard error of the mean from biological replicates. Expression of *Dlk1* is increased in postnatal forebrain tissue lacking ATRX protein, whereas expression of *Gtl2* remains unaffected at all developmental time points examined. The asterisk indicates p < 0.01.GD, *Gtl2* DMR

2.3.6 ATRX is Required for Chromatin Occupancy of Cohesin, CTCF and MeCP2

The effects of ATRX deficiency on *H19* and *Dlk1* transcript levels led us to postulate that ATRX controls occupancy of its binding partners at the *H19* ICR and at the *Gtl2* DMR. Using quantitative ChIP analysis of control and ATRX KO forebrain, we established that loss of ATRX in the mouse forebrain causes a decrease in SMC1 and CTCF occupancy at the *H19* ICR (Figure 2-6A) and could show a significant decrease specifically at region *H19-5* (Figure 2-6B), indicating that the presence of ATRX at the maternal *H19* ICR is required for SMC1 and CTCF occupancy. Conversely, ATRX was not required for occupancy of MeCP2 at this site (Figure 2-6A,B) .

We also examined the outcome of ATRX deficiency at the *Gtl2* DMR. Again, loss of ATRX reduced occupancy of SMC1 and CTCF at the DMR, at region GD-4 (Figure 2-6C). In contrast to the results obtained at the *H19* ICR, MeCP2 enrichment was also decreased in the absence of ATRX within the *Gtl2* DMR at region GD-3 (Figure 2-6C). We conclude that the presence of ATRX at the maternal *H19* ICR and *Gtl2* DMR is necessary for the full recruitment of SMC1 and CTCF, but the requirement of ATRX for MeCP2 binding differs between sites.

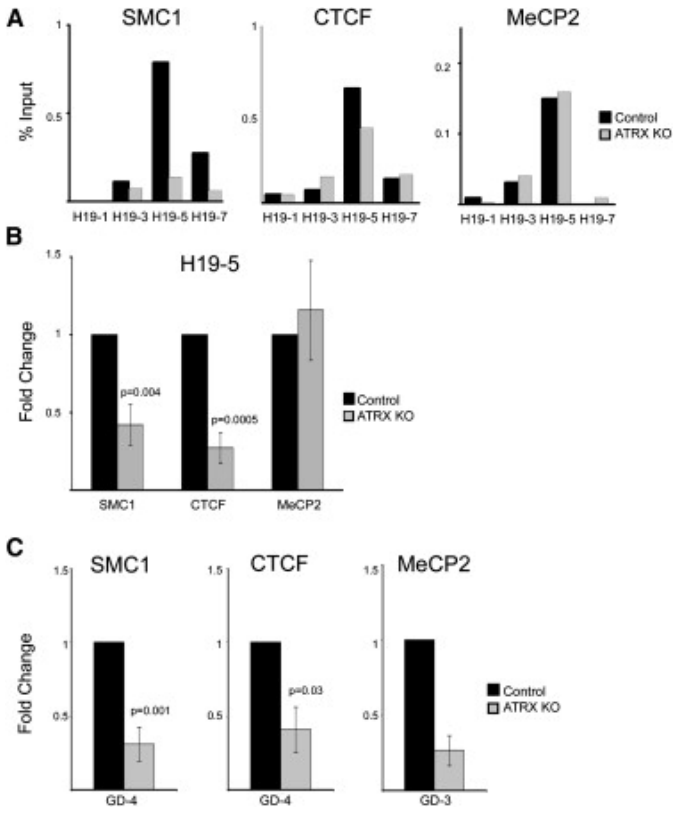

Figure 2-6. Occupancy of SMC1, MeCP2, and CTCF at the *H19* ICR and *Gtl2* DMR

(A) ChIP analysis was performed across the *H19* ICR in control and ATRX KO littermate-matched forebrains. Occupancy of CTCF and SMC1 was decreased in the ATRX KO samples at region *H19*-5, whereas MeCP2 occupancy was unchanged at this site. (B) Enrichment of SMC1, CTCF, and MeCP2 at region *H19*-5 was further quantified in additional brains (n = 3). (C) ChIP analysis was performed at the *Gtl2* DMR in control and *Atrx* null forebrain (SMC1 and CTCF, n = 3; MeCP2, n = 2). Loss of ATRX in forebrain causes decreased occupancy of SMC1, CTCF, and MeCP2 at regions GD-4, GD-4, and GD-3, respectively (depicted in Figure 2-5A). Error bars depict standard error of the mean. p values were determined by a two-tailed t test.

2.3.7 Regulation of an Imprinted Gene Network (IGN) by ATRX in the Mouse Brain

H19, *Igf2* and *Dlk1* have been linked to networks of co-regulated imprinted genes(Gabory et al., 2009; Lui et al., 2008; Varrault et al., 2006; Zhao et al., 2006). One network was identified by a meta-analysis of microarray datasets, revealing co-regulation of imprinted genes. Expression of these genes was also found to be altered in mice lacking the imprinted *Zac1* gene, a member of the identified network(Varrault et al., 2006). A second study reported a group of imprinted genes (including *H19* and *Igf2*) that were coordinately downregulated in a DNA methylation-independent manner during postnatal growth deceleration in multiple organs(Lui et al., 2008). Interestingly, in addition to regulating the *H19/Igf2* imprinted domain, the maternal *H19* ICR also mediates *inter*chromosomal interactions of a network of imprinted genes(Zhao et al., 2006). This network was different in mouse liver compared to ES cells, indicating tissue specificity of these interactions. The presence of ATRX, Cohesin and MeCP2 at the maternal *H19* ICR suggested that they might participate in *trans*-regulation of imprinted genes and therefore would affect the expression of other imprinted genes. Expression of several imprinted genes was evaluated by qRT-PCR in control and ATRX KO forebrain at E13.5, P0.5 and P17. In addition to *H19*, *Igf2* and *Dlk1*, we identified twelve other imprinted genes that displayed increased expression in the postnatal ATRX-null forebrain, including *Slc38a4*, *Dcn, Peg10, Mest, Grb10, Zac1, Sgce, Copg2, Cdkn1c, Nnat, Rian, and Ndn* (Table 1). None of the imprinted genes tested were upregulated at E13.5, however *Copg2as2*, *Nnat* and *Rian* were moderately downregulated at this embryonic time point (data not shown). The list of imprinted genes displaying increased expression in the absence of ATRX show a substantial overlap with the imprinted gene networks (IGNs) previously reported (Table 1)(Lui et al., 2008; Varrault et al., 2006).

Table 1. Imprinted gene expression in the ATRX KO forebrain

Gene	P0.5 Fold Change (n)	P0.5 SEM	P17 Fold Change (n)	P17 SEM	Varrault et al. (2006) IGN	Lui et al. (2008) IGN
Igf2	3.00 (10)	0.60[a]	3.62 (3)	0.54	*	*
Slc38a4	2.80 (9)	2.71[a]	3.75 (3)	0.52[a]	*	*
Dlk1	2.77 (11)	0.56[a]	3.10 (3)	1.20	*	*
H19	2.69 (10)	0.60[a]	2.26 (3)	0.41	*	*
Dcn	2.06 (7)	1.44[b]	1.81 (3)	1.01	*	
Peg10	1.83 (7)	0.92[a]	2.55 (3)	0.63[b]		
Mest	1.63 (4)	0.04[a]	n/c	-	*	*
Grb10	1.61 (4)	0.25[a]	1.74 (3)	0.29[b]	*	*
Zac1	1.61 (7)	0.61[a]	n/c	-	*	*
Sgce	1.39 (7)	0.48[b]	0.69 (3)	0.19	*	
Copg2	1.37 (8)	0.50[b]	n/c	-		
Cdkn1c	1.37 (4)	0.03[a]	n/c	-	*	
Nnat	1.37 (4)	0.10[a]	n/c	-		*
Ndn	n/c	-	1.42 (3)	0.21	*	*
Rian	n/c	-	1.51 (3)	0.52		*

n/c = not changed. n = number of C/ATRX KO biological replicates. The asterisk denotes genes involved in the gene network reported by the authors cited in the column heading.

a $p < 0.05$.

b $p < 0.08$.

2.4 Discussion

Our data link ATRX to two other important regulators of chromatin structure, to the proper occupancy of Cohesin and CTCF at two imprinted genes, and to the control of a network of imprinted genes in the developing brain. We identified direct target genes of ATRX in the mouse brain, and demonstrated that ATRX, MeCP2, and Cohesin bind to chromatin with a bias towards the maternal allele. It is unlikely that the effects on gene expression are merely due to morphological defects in the ATRX-null forebrain, since we could show direct binding of ATRX at *cis*-regulatory sites. An important aspect of our findings is their possible relevance to human developmental diseases. We provide the first glimpse of the cooperation between ATRX, Cohesin and MeCP2 in the regulation of common gene targets, perhaps explaining similarities between the associated human syndromes. Thus, the failure to properly suppress a subset of imprinted genes in the brain could potentially contribute to cognitive deficiencies characteristic of ATR-X, RTT and CdLS Syndromes.

The co-localization of ATRX and MeCP2 previously reported at pericentromeric heterochromatin in the mouse brain(Nan et al., 2007) raised the question whether they also interact at specific target genes along chromosomal arms. We now provide evidence that ATRX and MeCP2 converge to regulate common target genes. Several of the imprinted genes that we found to be affected by loss of ATRX also show altered expression in MeCP2-null tissues, including *H19*, *Dlk1* and *Zac*1(Fuks et al., 2003; Urdinguio et al., 2008). However, our results show that ATRX is required for MeCP2 binding to the *Gtl2* DMR, but not the *H19* ICR, suggesting that additional factors influence the dynamics of binding at different genomic sites.

The observation that both ATRX and MeCP2 are enriched on the maternal allele of the *H19* ICR was unexpected, as enrichment on the silenced methylated paternal allele would have seemed more likely. However, their presence on the maternal allele might enable the postnatal silencing of *H19* in the mouse brain(Pham et al., 1998). In this context, one would indeed expect to observe the recruitment of suppressive factors to the active maternal allele. This is further corroborated by the unchanged methylation status and altered chromatin composition observed in the ATRX KO forebrain. We speculate that

MeCP2 and ATRX are required to silence imprinted genes with functions that are essential during neurogenesis, but unnecessary or detrimental in the mature brain.

There is supporting evidence that mechanistically, MeCP2 and Cohesin function in chromosomal looping(Hadjur et al., 2009; Horike et al., 2005; Mishiro et al., 2009; Nativio et al., 2009). The presence of ATRX at target genes with Cohesin and MeCP2 suggests that it may also modulate chromatin loop formation by promoting specific long-range interactions. The involvement of Cohesin in this complex sheds new light on the importance of cohesion for accurate gene regulation. Much excitement was generated by recent publications describing the co-localization of Cohesin with the CTCF insulator protein at multiple genomic sites, and the requirement of Cohesin in the insulator functions of CTCF(Parelho et al., 2008; Wendt et al., 2008). More recently, Nativio et al reported the involvement of Cohesin in long-range chromatin interactions within the *IGF2-H19* locus in human cells(Nativio et al., 2009). The fact that ATRX is essential to achieve full occupancy of Cohesin and CTCF at target imprinted domains, potentially implicate ATRX in the regulation of higher order chromatin conformation, insulator functions, or mono-allelic gene regulation. It is possible that CTCF and Cohesin perform their insulator functions in different tissues in collaboration with various SNF2 chromatin remodeling proteins(Ishihara et al., 2006).

We determined that ATRX is able to suppress the expression of many imprinted genes in the postnatal period. The affected genes show a striking overlap with the previously reported ZAC1-related IGN, linked by their coordinated pattern of expression in many tissues (Table 1). Importantly, our data show that expression of *Zac1* itself is deregulated upon loss of ATRX in the brain. The maternal *H19* ICR has been shown by 4C analysis to mediate interactions with several imprinted genes(Zhao et al., 2006). These *inter*chromosomal interactions differed substantially between mouse liver and embryonic stem cells, suggesting that the interaction network involving the *H19* ICR is tissue specific, or reflects the proliferative capacity of cells. Viewed in the context of the neonatal brain, the coordination of this network of imprinted genes might be required to ensure proper brain maturation and synchronization could be enabled by the recruitment of specific genes into close proximity via chromatin interactions. The concept of *trans*-

interaction and *trans*-regulation of imprinted genes is still controversial and the possible involvement of ATRX in the regulation of an IGN in postnatal brain will require further investigation.

The role of the imprinted genes controlled by ATRX has not yet been well characterized in the brain, perhaps not surprisingly if these genes are largely suppressed during brain maturation. However, many of the genes within this network have defined functions during mouse placentation, which is intriguing considering that ATRX is an essential placental regulator(Garrick et al., 2006). Furthermore, there is evidence that ATRX is required for imprinted X chromosome inactivation in extraembryonic tissues, and somatic cells(Baumann and De La Fuente, 2009; Garrick et al., 2006). Several important questions remain to be answered, namely whether ATRX controls IGNs in other tissues, especially in the placenta, and whether ATRX's mono-allelic effects also come into play during imprinted X chromosome inactivation. Future studies will be directed at identifying additional common target genes of ATRX, Cohesin, MeCP2 and CTCF in the brain and other tissues, and to investigate the mechanism of regulation at target genes. Although it is unlikely that ATRX, Cohesin and MeCP2 only target genes that are imprinted, our findings show that these chromatin proteins can bind specific genomic sites in an allele-specific manner. Whether ATRX's function is often influenced by mono-allelic features will require further investigation. It will be important to further probe the underlying molecular regulation by these chromatin proteins, in order to gain insight into the control of mono-allelic expression, long-range chromatin gene regulation, and a better understanding of the molecular neuropathogenesis underlying the associated human disorders.

2.5 Supplementary Figures

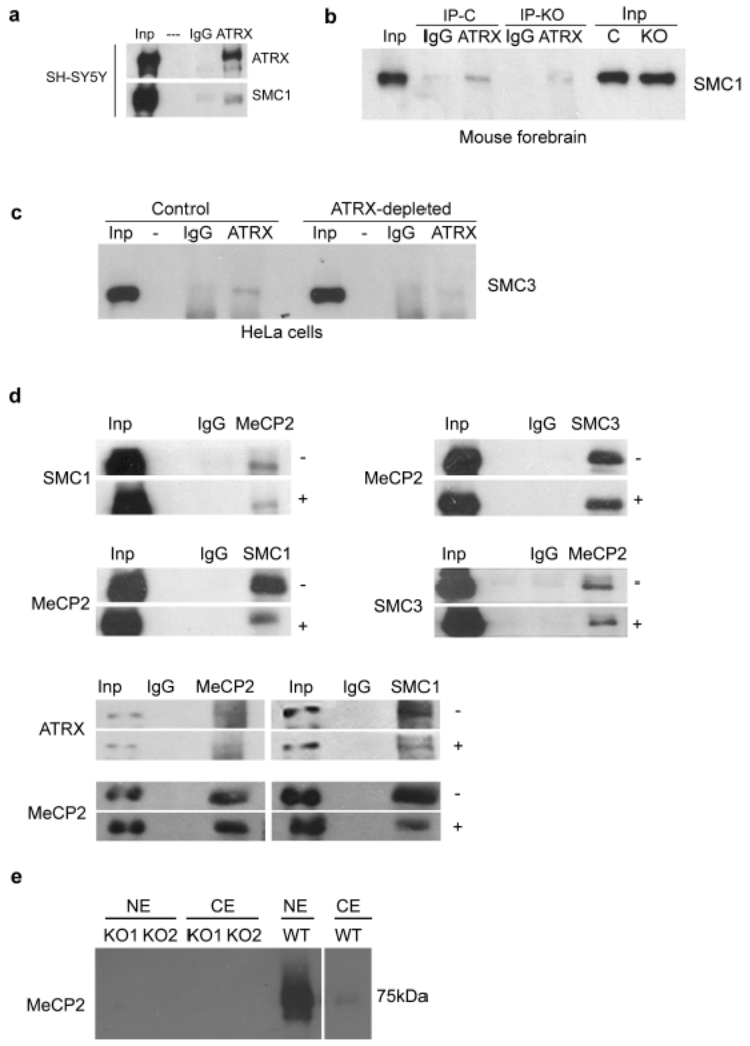

Figure 2-7. Co-immunoprecipitation of ATRX and SMC1 in SH-SY5Y cells, forebrain tissue and HeLa cells and effects of DNAse I treatment on interactions between ATRX, MeCP2 and Cohesin subunits

(A) ATRX was immunoprecipitated from human SH-SY5Y neuroblastoma cells and Western blot analysis revealed the presence of SMC1. (B) ATRX protein was immunoprecipitated from control and ATRX KO forebrain tissue and Western blot analysis showed that association with SMC1 is reduced in the KO samples.

(C) Co-immunoprecipitation experiments were repeated in HeLa cell extracts transiently depleted of ATRX protein by RNA interference. (D) Different combinations of co-immunoprecipitation experiments were performed in the absence (-) or presence (+) of DNAse I. (E) Western blot analysis of MeCP2 KO and control forebrain protein extracts demonstrates specificity of MeCP2 antibody used for co-immunoprecipitation experiments. NE: nuclear protein extracts, CE: cytoplasmic protein extracts, Inp: Input, KO: ATRX KO, C: control.

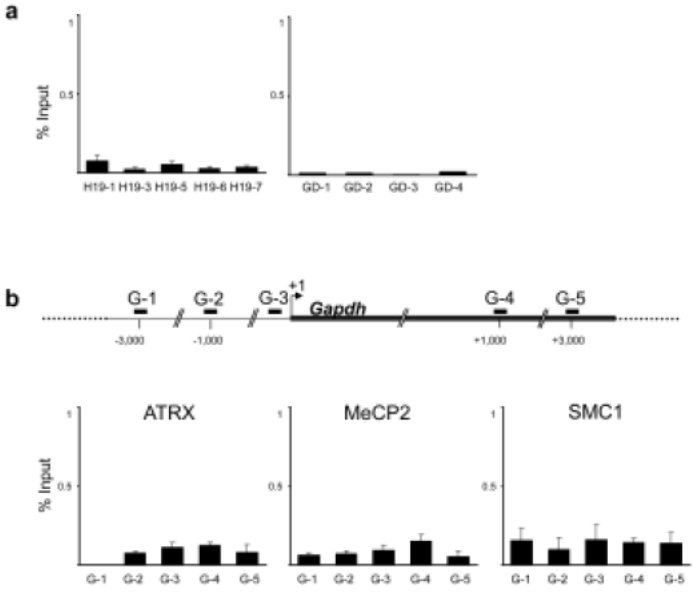

Figure 2-8. ChIP controls demonstrate specificity of protein enrichment at the *H19* ICR and *Gtl2* DMR

(A) ChIP of the transcription factor PROX1 across the *H19* ICR and *Gtl2* DMR reveals minimal enrichment, demonstrating the specificity of ATRX, MeCP2 and Cohesin enrichment at these sites. Refer to Figures 2-2 and 2-5 for primer alignment of *H19* and *Gtl2* domains, respectively. (B) Genomic organization and alignment of primers utilized for PCR amplification of ChIP reactions at the *Gapdh* locus (top). Numbers indicate the relative nucleotide position from the transcription start site. ChIP for ATRX, MeCP2 and SMC1 indicates minimal enrichment across the *Gapdh* region.

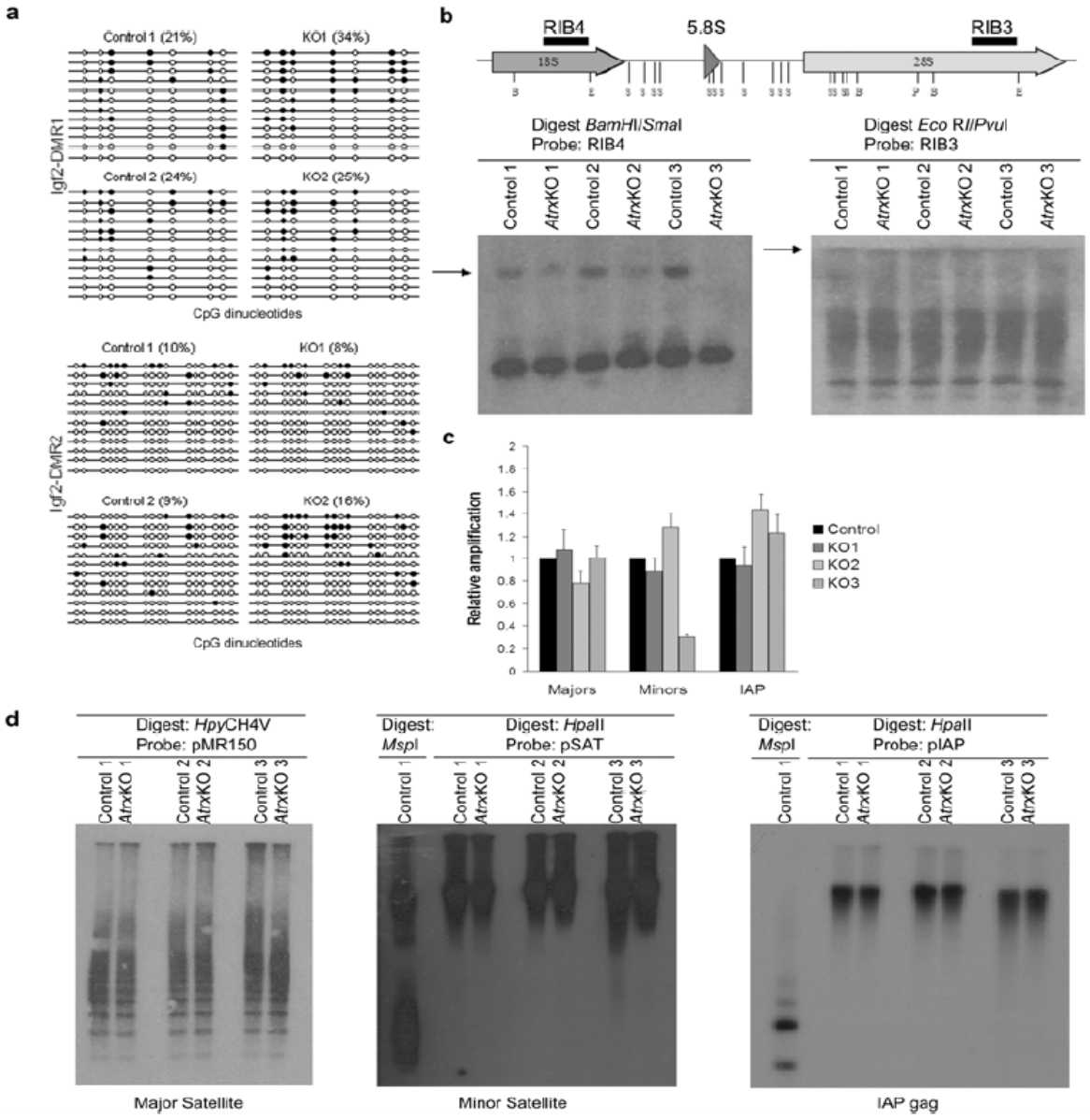

Figure 2-9. DNA methylation analysis of *Igf2* DMR1, *Igf2* DMR2, rDNA and repetitive elements in the ATRX null forebrain

(A) Bisulfite mutagenesis and sequencing analysis of the *Igf2* DMR1 and *Igf2* DMR2 in two control and ATRX KO littermate pairs. Individual alleles are represented as a line, unmethylated CpGs are represented as empty circles, and methylated CpGs as filled circles. The total percent methylation for each sample is indicated in parentheses.

(B) Southern blot analysis of DNA methylation at 18S and 28S rDNA repeats. (Top) Restriction map of the transcribed portion of the rDNA genes. Alignments of the methylation sensitive probes RIB4 and RIB3 are indicated. (Bottom) 18S rDNA methylation was analyzed by digestion with BamHI/SmaI and probed with RIB4 while 28s rDNA repeat methylation was analyzed by digestion with EcoRI/PvuI and probed with RIB3. (C) McrPCR analysis of DNA methylation at major satellite, minor satellite and IAP*gag* repeats. DNA methylation at repeat sequences was measured by the inverse ability of unmethylated DNA fragments to amplify PCR products with primer sets specific to each repeat class. Samples were normalized against equal input levels of internal control undigested DNA. (D) Southern blot analysis of DNA methylation at major satellite, minor satellite and IAP*gag* repeats. Major satellite methylation was analyzed by digestion with HpyCH41V and probed with pMR150. Minor satellite methylation was determined by digestion with HpaII and probed with pSAT. IAP*gag* methylation was assessed by digestion with HpaII and probed with pIAP.

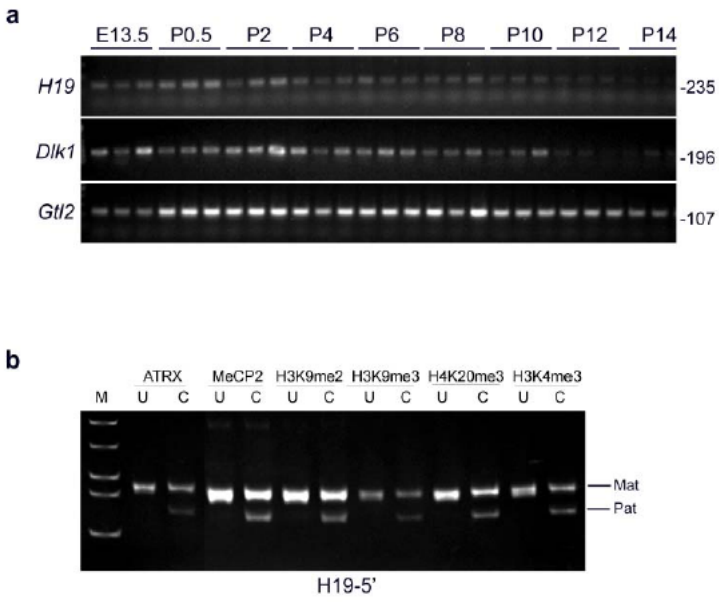

Figure 2-10. Temporal profile of *H19, Dlk1* and *Gtl2* expression and accumulation of suppressive histone marks at the maternal *H19* ICR in the postnatal brain

(A) Semi-quantitative RT-PCR expression analysis of the mouse forebrain from E13.5 to P14 illustrates gradual downregulation of the *H19* and *Dlk1* genes in the postnatal period and a more constant expression level of *Gtl2.* Numbers on the right indicate expected size of the PCR amplicons. (B) Allelic ChIP analysis of histone modifications on 129Sv/CAST F_1 forebrain at the *H19* ICR demonstrates enrichment of repressive marks H3K9me2, H3K9me3 and H4K20me3 on the maternal allele, reflecting postnatal repression of *H19* in the forebrain. Inp: input, U: uncut, C: cut, Mat: Maternal, Pat: paternal, M: standard marker.

2.6 Supplementary Methods

2.6.1 Cell Culture

SH-SY5Y and HeLa cells were cultured in DMEM high glucose medium (Sigma) supplemented with 10% FBS, in 5% CO2 atmosphere. Transient ATRX depletion in HeLa cells was achieved using siRNA interference as previously described(Ritchie et al., 2008).

2.6.2 Co-Immunoprecipitations

Extracts from SH-SY5Y cells, HeLa cells, and mouse forebrain were immunoprecipitated as described in the main text, with the following exceptions: antibodies were incubated with lysates overnight at 4 ^{o}C followed by incubation for 1 hour with protein G-Sepharose (Pharmacia). For DNAse digestion experiments, prior to immunoprecipitation, samples were incubated with 100U/mL DNAse1 (Sigma) at room temperature for 15 minutes.

2.6.3 Western Blot Analysis

Nuclear and cytoplasmic protein extracts were obtained from MeCP2 KO (B6.129P2(C)-*Mecp2*$^{tm1.1Bird}$/J, Jackson Laboratories #003890) and control forebrains using the Nuclear and Cytoplasmic Extraction kit (NE-PER, Thermo Scientific). Protein extracts were resolved on 8% SDS-PAGE, and incubated with anti-MeCP2 (gift of Janine M. LaSalle) for one hour.

2.6.4 ChIP Analysis

ChIP was performed on P17 wild-type forebrains as described in the main text. Additionally, immunoprecipitation was performed with anti-PROX1 (Covance). Samples were analyzed by real-time RT-PCR as described in the main text. Primer sequences for all regions are available upon request.

2.6.5 Methylation-Sensitive Southern Blot Analysis

P0.5 forebrain tissue was collected, digested with Proteinase K and purified using the standard phenol:chloroform extraction method. For the IAP*gag* and minor satellite

analyses, DNA from three ATRX KO and littermate control pairs was digested with the methylation-sensitive restriction enzyme HpaII and its methylation insensitive isoschizomer MspI. DNA for the major satellite blot was digested with methylation-sensitive HpyCH41V. DNA for the 18S rDNA blot was digested with BamHI and methylation-sensitive SmaI, while DNA for the 28S rDNA blot was digested with EcoRI and methylation-sensitive PvuI. pIAP, pMR150 and pSAT probes were obtained from F. Dick (London Regional Cancer Program; University of Western Ontario, London, Canada) and have been previously published(Lehnertz et al., 2003). RIB3 and RIB4 probes were generated by PCR as previously described(Gibbons et al., 2000).

2.6.6 McrPCR Methylation Analysis

Genomic DNA was digested with the restriction enzyme McrBC (New England Biolabs), which only cleaves methylcytosine of the form $(G/A)^{m}C$ in the recognition sequence 5′…$Pu^{m}C(N_{40\text{-}30000})Pu^{m}C$…3′. Quantitative real-time PCR was used to determine the relative levels of unmethylated DNA at IAP*gag*, major satellite, minor satellite and rDNA repeats, following a previously published protocol(Martens et al., 2005). Conditions for amplification were as follows: 95 °C for 5 minutes followed by 25-35 cycles of 95 °C for 10 seconds, 55 °C for 20 seconds, 72 °C for 30 seconds, and a final melting curve generated in increments of 0.5 °C per plate read. Major satellite and minor satellite samples were diluted 100-fold due to high copy number in the mouse genome (Martens et al., 2005). Standard curves were generated for each pair using three-fold serial dilutions of control littermate cDNA. Primer efficiency was calculated as $E = [10^{(-1/slope)} - 1] * 100\%$, where a desirable slope is –3.32 and $r^2 > 0.99$. All data was corrected against equal input levels of internal control undigested DNA.

2.6.7 Semi-Quantitative RT-PCR

RNA was extracted and cDNA generated as described in the main text from male wild-type forebrains at days E13.5, P0.5, P2, P4, P6, P8, P10, P12 and P14. cDNA was amplified using gene-specific primers under the following conditions: 95 °C for 5 minutes, followed by 29 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute.

2.7 References

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Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nat Genet* **38**:1341-1347.

Chapter 3

3 ATRX Regulates Nucleosome Positioning, CTCF Occupancy and Long-Range Chromosomal Interactions

My initial studies revealed that ATRX controls the expression of imprinted genes and promotes CTCF and Cohesin binding within the *H19* ICR and *Gtl2* DMR. At the onset of this study CTCF and Cohesin had recently been recognized as proteins vital for chromatin interactions throughout the genome, including within the *H19/Igf2* imprinted domain and between *H19/Igf2* and other IGN members(Han et al., 2008; Kurukuti et al., 2006; Nativio et al., 2009; Zhao et al., 2006b). This led me to investigate whether the loss of ATRX would also affect chromatin interactions at *H19/Igf2* and perhaps other imprinted domains, providing a potential mechanism for ATRX regulation of imprinted genes.

3.1 Introduction

Recent technological advancements have greatly evolved our understanding of the genome, from a linear organization to a complex three dimensional structure(de Wit and de Laat, 2012). Chromosomes within the nucleus are strategically folded to facilitate interactions of genes and regulatory sequences, both within and between chromosomes(reviewed in (Cremer and Cremer, 2001; Cremer and Cremer, 2010; Cremer et al., 2006; Zhao et al., 2009)). These short- and long-range interactions can inhibit or enhance gene expression, and are highly relevant to genomic control. Regulation of higher-order chromatin structure is not well understood, but likely involves chromatin remodeling factors. Chromatin remodeling factors are a diverse group of proteins that utilize the energy of ATP to alter the histone-DNA interface, thus affecting chromatin structure(Travers et al., 2012).

Alpha-thalassemia mental retardation, X-linked (ATRX) is a switch/sucrose non-fermenting (SWI/SNF)-like chromatin remodeling protein, implicated in neurodevelopmental syndromes and cancer in humans(Gibbons et al., 1995a; Gibbons et al., 1995b; Gibbons et al., 1992; Je et al., 2012; Jiao et al., 2012a; Liu et al., 2012;

Weisbrod et al., 2013). The ATRX protein has two main conserved domains, an ATRX-DNMT3-DNMT3L (ADD) domain and a switch/sucrose non-fermenting (SWI/SNF) domain(Picketts et al., 1998). The SWI/SNF domain confers ATPase-dependent translocase activity(Tang et al., 2004;Flaus et al., 2006), while the ADD domain recognizes and interacts with DNA and other proteins(Argentaro et al., 2007; Cardoso et al., 2000; Dhayalan et al., 2011; Wong et al., 2010). ATRX binds throughout the genome to enact diverse functions. To date, roles have been reported in DNA replication(Leung et al., 2013; Watson et al., 2013), mitosis(Ritchie et al., 2008), meiosis(De La Fuente et al., 2004), telomere stability(Bower et al., 2012; de Wilde et al., 2012; Heaphy et al., 2011; Lewis et al., 2010; Lovejoy et al., 2012; Watson et al., 2013; Wong et al., 2010) and gene expression(Law et al., 2010; Levy et al., 2008). Genome-wide studies have reported that ATRX is enriched at GC-rich and repetitive sequences, such as CpG-islands, DNA repeats, and telomeres, including many predicted to form secondary DNA structures called G-quadruplexes(Law et al., 2010). ATRX is thought to translocate along DNA to aid in the resolution of G-quadruplexes and facilitate DNA replication and transcription(Law et al., 2010). This mechanism of ATRX-dependent gene regulation was proposed at the alpha-globin domain, where ATRX binds G-rich tandem repeat sequences and modulates gene expression in *cis*(Law et al., 2010). However, ATRX deficiency causes the misexpression of numerous genes throughout the genome(Levy et al., 2008). Given the diverse roles of the ATRX protein, it is likely that ATRX also has multiple functions in transcriptional regulation.

Imprinted genes are a class of genes expressed in a parent-of-origin manner and regulated by epigenetic factors, including higher-order chromatin looping(Kernohan and Bérubé, 2010). We previously demonstrated a requirement for ATRX in the transcriptional repression of a network of imprinted genes, including *H19* and *Igf2*, in the mouse brain(Kernohan et al., 2010). At the *H19/Igf2* domain, we found that ATRX forms a complex with methyl CpG binding protein 2 (MeCP2) and Cohesin on the maternal allele of the *H19* imprinting control region (ICR)(Kernohan et al., 2010). Loss of ATRX caused a reduction in CCCTC-binding factor (CTCF) and Cohesin binding that correlated with an increase in *H19* and *Igf2* transcripts(Kernohan et al., 2010). However, the

function of ATRX at the *H19* ICR, and its role in repressing *H19/Igf2* expression is not known.

Transcription from *H19/Igf2* is regulated by chromatin looping which is mediated by the *H19* ICR and requires CTCF and Cohesin(Burke et al., 2005; Guibert et al., 2012; Han et al., 2008; Kurukuti et al., 2006; Li et al., 2008). As ATRX affects CTCF and Cohesin occupancy, we set out to investigate a possible role for ATRX in mediating looping of this domain. We utilized circular chromosome conformation capture (4C) and quantitative chromosome conformation capture (3C) to evaluate interactions of the *H19* ICR in the neonatal brain. We demonstrate that the loss of ATRX significantly reduces chromosomal interactions with specific *H19/Igf2* sites. Architectural changes across *H19/Igf2* coincide with a failure to maintain CTCF binding, likely as a result of ATRX-mediated nucleosome occupancy within the 5' *H19* ICR. Finally, we report that MeCP2 recruits ATRX to the *H19* ICR, and that the loss of MeCP2 also results in defects in *H19/Igf2* chromatin structure. We propose a model where MeCP2 recruits ATRX to the maternal *H19* ICR to repress *H19/Igf2* expression. Once recruited to the ICR, ATRX modifies nucleosome occupancy to maintain an extended linker region which accommodates CTCF binding, thus promoting long-range chromosomal interactions and gene repression.

3.2 Materials and Methods

3.2.1 Animal Husbandry

The *Atrx* gene was conditionally deleted in the mouse forebrain by mating $Atrx^{loxP}$ female mice with heterozygous *Foxg1Cre* male mice as previously described(Bérubé et al., 2005). $Atrx^{loxP}$ line was kindly provided by D. Higgs (Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom). For the developmental studies, midday of vaginal plug discovery was considered E0.5. For allele-specific expression studies, pups were obtained by mating 129Sv female mice with *Mus musculus castaneus* male mice (CAST; The Jackson Laboratory). $MeCP2^{null}$ mice were generated by crossing $MeCP2^{loxP}$ females (Jackson Laboratories Stock # 007177) with a ubiquitous *Cre* line driven by the EIIa promoter (Jackson Laboratories Stock #003724). All animal

studies were conducted in compliance with the regulations of The Animals for Research Act of the province of Ontario, the guidelines of the Canadian Council on Animal Care, and the policies and procedures approved by the University of Western Ontario Council on Animal Care.

3.2.2 Circular Chromosome Conformation Capture (4C)

4C protocol was based on (Gheldof et al., 2012). Briefly, following 3C library preparation, 100 μg of DNA was digested with MseI (*H19* ICR) (100 U; New England Biolabs) overnight at 37 ^{o}C. MseI was chosen to provide a minimum 200 base pairs from the primary to secondary restriction sites, allowing for efficient circularization. The enzyme was deactivated for 25 minutes in 1.3% SDS at 65 ^{o}C and DNA was recovered by standard phenol/chloroform extraction. Digestion efficiency was confirmed to be $\geq$96% by real-time PCR across 5 sites throughout the genome. DNA was resuspended in ligation buffer with 50 U T4 DNA ligase (Roche Diagnostics) and 1 μM ATP and incubated at 16 ^{o}C for 5 days. DNA was purified by phenol/chloroform extraction and amplified with the Expand Long Template PCR system (Roche Diagnostics) and site specific reverse primers directed from the *H19* ICR. PCR products were resolved on a 1% agarose gel, and extracted in 3 aliquots using a QIAquick gel extraction kit (Qiagen); undigested band, <230 base pairs and >230 base pairs. The >230 base pair fraction was sheared enzymatically using the Ion Shear Plus Reagents incubating at 37 ^{o}C for 5 minutes, and then combined with the <230 base pair fraction and 1/6th of the undigested self-ligation fragment before barcoding with the Ion Xpress Barcode Adapters 1-16 kit. Sequencing was performed using the Ion Torrent Personal Genome Machine (Life Technologies) with 318 chips and 200 base pair sequencing chemistry according to manufacturers protocols at the London Regional Genomics Centre.

3.2.3 4C Analysis

Ion Torrent reads were mapped by TMAP Suite 3.2.1 and supplied in BAM format. These were converted to SAM format using samtools(Li et al., 2009) and sorted by chromosome and position prior to analysis. The individual chromosome sequences of the Genome Reference Consortium Mouse Build 38 were downloaded from the UCSC

genome browser web site. A custom script, written in Perl, was used to generate a file for each chromosome containing the EcoRI restriction endonuclease coordinates. A second custom script parsed the SAM file and enumerated the total number of reads that mapped to each restriction endonuclease cleavage interval, and the number of unique reads mapping to each interval. Reads were assumed to be identical if they were in the same orientation and had the same starting positions. The total number of unique mapping reads for each dataset was between 52,162 and 303,823. Because the ratio of reads in any interval to the total was very small, standard statistical techniques were used to construct a robust estimator of the underlying proportions. Specifically, underlying proportions were estimated using a multinomial-Poisson model in a Bayesian context using a minimally-informative reference prior(Berger and Bernardo, 1992; Berger et al., 2009). Since fold-change is the usual measure of effect-size, all expectations were taken with respect to log_2-proportions. For compatibility with the UCSC genome browser, the expected log_2-proportions were mapped back to linear-space and multiplied by an arbitrary integer scaling factor.

3.2.4 Chromosome Conformation Capture (3C)

3C libraries were prepared essentially as previously described(Vernimmen et al., 2007), with the same controls. Briefly, neonatal mouse forebrain and/or liver tissue was dissected, rinsed in DMEM (Sigma-Aldrich) and cut. Minced tissue was diluted in DMEM (Sigma-Aldrich) with 10% FBS (Sigma-Aldrich) and passed through a 70 μm cell strainer (BD Falcon) to ensure single cell suspension. The cell suspension was incubated at 37 ^{o}C for 30 minutes to equilibrate. Cells were cross-linked in 1% formaldehyde (Sigma-Aldrich), rinsed 3x with cold PBS (Sigma-Aldrich) and lysed [Lysis buffer: 10mM Tris pH 8 (Sigma-Aldrich), 10mM NaCl (Sigma-Aldrich), 0.2% NP40 (Sigma-Aldrich), and 1x protease inhibitors (Roche Diagnostics)]. Nuclei were resuspended in 1.2x restriction buffer (Roche Diagnostics buffer H) and 3% SDS (Sigma-Aldrich) and incubated at 37 ^{o}C for 1 hour, followed by addition of 2% TX-100 (Sigma-Aldrich) and incubation for a second hour. EcoRI (400 U; Roche Diagnostics) was added and incubated overnight at 37 ^{o}C. The enzyme was deactivated for 25 minutes in 1.3% SDS at 65 ^{o}C. Digested DNA was incubated in lysis buffer with 1% TX-100 (Sigma-

Aldrich) for one hour, followed by the addition of T4 DNA ligase (100 U; Roche Diagnostics). Ligation was performed for 4 hours at 16 ^{o}C followed by 30 minutes at room temperature. The cross-link was reversed and protein degraded by addition of 300 µg Proteinase K(PK) (BioShop Canada Inc.) and incubation overnight at 65 ^{o}C. RNA was degraded by addition of 300 µg RNAse A (Roche Diagnostics) and incubated for 30 minutes at 37 ^{o}C. DNA was purified by phenol/chloroform extraction. Samples were prepared in parallel that lacked either the EcoRI or T4 DNA ligase enzymes. Digestion efficiency was confirmed to be ≥96% by real-time PCR across 5 sites spanning the *H19/Igf2* domain.

3.2.5 Quantitative 3C Analysis

Library amplification and quantification was conducted as described previously(Vernimmen et al., 2007), with the same controls. Briefly, PCR reactions, primers and probes were optimized on a library of randomly ligated BAC DNA containing the *H19/Igf2* domain (RP23-50N22) and XPB (RP23-148C24) (clones were obtained from TCAG Genome Resource Facility, The Hospital for Sick Children Toronto). All *H19/Igf2* primer combinations were tested to amplify in linear correlation with the amount of BAC DNA and within 2 Cts. All 3C data was corrected to primer efficiency and calculated relative to XPB/ERCC3 amplification, a genomic region demonstrated to have equivalent looping structures in many tissues, including liver and brain(Tiwari et al., 2008). This also controls for differences in chromatin concentrations and cross-linking efficiency between samples. 3C template obtained from P0.5 control and $Atrx^{null}$ and littermate control forebrains were amplified in duplicate with Taqman Universal PCR Master Mix (Applied Biosystems) on a Chromo-4 thermocycler (BioRad) using the following conditions: 50 °C for 2 minutes, then 95 °C for 10 minutes, followed by 50 cycles of 95 °C for 10 seconds, 60 °C for 1 minute. All primer combinations were visualized on polyacrylamide gels to confirm amplification of the correct sequences. For all reactions, a negative bait site located approximately 100 kb downstream of *H19* was also used; the lack of interactions from this site ensured specificity of ICR interactions.

3.2.6 DNA FISH and Immunofluorescence

DNA FISH protocol was adapted from (Dorin et al., 1992). Neonatal brains were fixed overnight in 4% paraformaldehyde (Sigma-Aldrich), equilibrated in 30% sucrose-PBS, frozen in O.C.T. (Tissue Tek) and sectioned at 8 μm. Antigen retrieval was performed using 0.3% sodium citrate (Sigma-Aldrich) for an hour. Slides were dehydrated in an ethanol series of 70% for 2 minutes, 90% for 2 minutes, and 100% for 5 minutes, followed by denaturation in 70% formamide/2xSSC for 5 minutes at 65 ^{o}C. Slides were again dehydrated as described above and then incubated with 0.05 μg of DIG and/or biotin-labeled probe/hybridization buffer [83% formamide (Sigma-Aldrich), 3.3x SSC (Sigma-Aldrich), 0.02 μM dextran sulfate, and 30 μg salmon sperm DNA (Sigma-Aldrich)] overnight at 37 ^{o}C in a humidified chamber. Probes were prepared by nick translation of BAC DNA (*H19/Igf2*:RP23-50N22, *Gapdh*:RP23-319C23, *Peg10/Sgce*: RP23-327D3, *Dcn*:RP23228L10, *Slc38a4*: RP23-304B5, *Grb10*: RP23-298L21, *Dlk1:*RP23-385B6, *Zac1*:RP23-259L24, *Mest*:RP23-269K7 (all clones obtained from TCAG Genome Resource Facility, The Hospital for Sick Children Toronto) using the Biotin and DIG- Nick Translation Kits (Roche Diagnostics) and purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) as per manufacturer's instructions. Slides were washed in 50% formamide/2xSSC for 2 x 5 minutes, and 2xSSC for 2 x 5minutes. Sections were incubated with the primary antibody for 1 hour at room temperature, washed for 15 minutes in PBS, and incubated with the secondary antibody for 1 hour. Sections were counterstained with DAPI (Sigma-Aldrich; D9542) and mounted in Slowfade Gold Antifade Reagent (Invitrogen). Primary antibodies used were as follows: anti-ATRX H-300 (1:250; Santa Cruz Biotechnology) anti-DIG (1:100; Roche Diagnostics), and anti-Biotin (1:500; Abcam). Secondary antibodies used were as follows: goat-anti rabbit Alexa 594 (1:800; Invitrogen) and goat anti-mouse Alexa 488 (1:800; Invitrogen). Images were taken at 0.3 μm intervals across the 8 μm section using the Olympus FV1000 confocal microscope and FV10-ASW 2.1 image acquisition software (Olympus). Volocity software (PerkinElmer) was used to compile 3D images and make distance measurements in 3D. For co-localization analysis, FISH signals with a centre-to-centre distance of less than 1 μm were considered to be interacting(Sandhu et al., 2009).

3.2.7 ChIP-Sequencing Analysis

Raw sequencing data for ATRX embryonic stem cell ChIP-sequencing was downloaded from the NCBI Sequence Read Archive (Accession number GSE22162), and aligned to the mouse genome using Bowtie version 0.12.8 in the -n alignment mode. During alignment duplicate sequences were removed, up to 3 mismatches were allowed, and reads that aligned to more than one location were discarded. Genome-wide data tracks were generated using custom Perl scripts to extend reads to their fragment lengths and normalized to 20 million reads. Data was visualized in the UCSC Genome Browser(Kent et al., 2002).

3.2.8 Chromatin Immunoprecipitation

ChIP was conducted as previously described(Kernohan et al., 2010) with the following exceptions: cells were dissected and fixed immediately at 37 °C, then washed in PBS containing 1x protease inhibitor cocktail (Roche Diagnostics), immunoprecipitation was conducted with anti-ATRX (H300; Santa Cruz) and anti-CTCF (Cell Signaling), DNA-antigen complexes were retrieved by incubation with protein A agarose beads (Cell Signaling), and LiCl wash was omitted. ChIP products were amplified in duplicate with iQ™ SYBR® Green master mix (BioRad) on a Chromo-4 thermocycler using the following conditions: 95 °C for 5 minutes followed by 40 cycles of 95 °C for 10 seconds, 57.5 °C for 20 seconds, 72 °C for 30 seconds, and a final melting curve generated from 55 to 95 °C in increments of 1.0 °C per plate read. Ct values were obtained and % input and fold-change calculated as previously described(Kernohan et al., 2010).

3.2.9 Nucleosome Density Analysis

Neonatal mouse forebrain was dissected, rinsed in 37 ^{o}C DMEM (Sigma-Aldrich) and passed through a 70 μm cell strainer (BD Falcon) to ensure single cell suspension. The cell suspension was incubated at 37 ^{o}C for 30 minutes to equilibrate. Cells were fixed in 1% formaldehyde (Sigma-Aldrich) for 5 minutes and rinsed 3x with cold PBS containing protease inhibitors (Roche Diagnostics). Cells were resuspended in lysis buffer [0.34 M sucrose, 60 mM KCl, 15 mM Tris-HCl, 15 mM NaCl, 0.5% NP-40 and 1x protease inhibitors (Sigma Aldrich)] and flash-frozen and thawed 3x, nuclei were spun down, and

resuspended in micrococcal nuclease digestion buffer (NEB). Micrococcal Nuclease (2 U) was added and incubated at 37 ^{o}C for 5 minutes then quenched with EDTA. Cells were lysed with 1% SDS and cross-links reversed by incubation at 65 ^{o}C for 5 hours, followed by RNAse and PK digestion and phenol/chloroform extraction. DNA was amplified in duplicate with iQ^{TM} SYBR$^{®}$ Green master mix (BioRad) on a Chromo-4 thermocycler (MJ Research) using the following conditions: 35 cycles of 95 °C for 30 seconds, 57.5 °C for 30 seconds, and 72 °C for 1 minute. Quantification was conducted using the standard curve Ct method of quantification and normalized to amplification from *Gapdh* and *beta-actin*.

3.3 Results

3.3.1 ATRX Mediates Higher-Order Chromatin Structure of the *H19/Igf2* Imprinted Domain

We previously reported that ATRX localizes to the *H19* ICR where it mediates binding of CTCF and Cohesin(Kernohan et al., 2010), proteins known to be required for *H19/Igf2* higher-order chromatin structure(Guibert et al., 2012; Han et al., 2008; Kurukuti et al., 2006; Li et al., 2008; Nativio et al., 2009; Rubio et al., 2008). This led us to postulate that ATRX is also required for proper *H19/Igf2* chromatin looping, providing an explanation for elevated *H19* and *Igf2* transcript levels in the *Atrx^{null}* forebrain. To evaluate interactions of the *H19* ICR we utilized 4C, a 3C based molecular technique used to screen for genome-wide interactions *in vivo*(reviewed in (Sajan and Hawkins, 2012)). 3C libraries were generated from P0.5 forebrains utilizing EcoRI, then redigested with MseI and self-ligated to form circular 3C recombined molecules. The samples were then PCR amplified with primers directed from the *H19* ICR 'bait sequence' across the interacting fragments and sequenced to provide a genome-wide unbiased analysis. Sequencing results were aligned to an EcoRI digested genome. While chromatin looping of *H19/Igf2* has been reported elsewhere(Burke et al., 2005; Guibert et al., 2012; Han et al., 2008; Kurukuti et al., 2006; Li et al., 2008), none of these studies utilized whole-genome sequencing to generate an unbiased screen and none have been conducted on forebrain tissue. We began by establishing the pattern of interactions in the control forebrain and found a series of contacts, including the insulin (*Ins)* and insulin like

growth factor 2 (*Igf2)* genes, *Igf2* differentially methylated region 1 (DMR1), *Igf2* DMR2, matrix-attachment region 3 (MAR3), centrally conserved domain (CCD), and the *H19* promoter and downstream enhancers (Figure 3-1A). To determine if the loss of ATRX affects interactions of the *H19* ICR, we compared 4C libraries from control and $Atrx^{null}$ forebrains. We found reduced interaction frequencies across numerous sites including the CCD, MAR3 and DMR1 (Figure 3-1A), demonstrating that ATRX is required for interactions of the *H19* ICR across the *H19/Igf2* domain.

To confirm ATRX-dependent effects on chromatin structure within *H19/Igf2,* we utilized quantitative 3C. We designed this analysis with the same EcoRI primary digestion, thereby dividing the 140 kb region into 45 fragments stretching from the *Ins* gene to the *H19* enhancers (Figure 3-1B). We designed a forward primer and Taqman probe to the *H19* ICR (EcoRI restriction fragment used as bait), and numerous reverse primers in other EcoRI fragments covering intergenic regions as well as key elements identified in the 4C screen. To provide further confirmation of interaction frequencies at a subset of sites, a second primer was designed to the other end of the restriction fragment. For validation of the 3C approach, we began with an analysis of neonatal liver and forebrain. A similar interaction profile of the *H19* ICR was observed in the liver and forebrain. Thus, the interactions previously reported(Qiu et al., 2008) and confirmed here in neonatal liver are also present in the neonatal brain (Supplementary Figure 3-7B). These profiles also mirror the interactions observed in our 4C experiments, demonstrating the reliability and robustness of our *in vivo* 3C and 4C analyses. We next quantified interactions in control and $Atrx^{null}$ forebrains (Figure 3-1C,D). Similar to our 4C experiments, we found that the loss of ATRX diminished interactions across the *H19*/*Igf2* domain, with significant reductions at the *Igf2* DMR1 (region E/F; p=0.001 and 0.0003), MAR3 (region I; p=0.0052 and 0.0038), CCD (region L; p=0.0001 and 0.0001) and the endodermal enhancer (region Q; p=0.0004 and 0.0105) (Figure 3-1C,D). Conversely, interactions with *Ins* (region B), the intergenic site between MAR3 and the CCD (region K) and the region downstream of the *H19* enhancers (region T) were unaltered. For a subset of samples, expression analysis was performed in tandem with the 3C analysis to confirm that gene expression and chromatin looping were changed in the same brain

samples (Supplementary Figure 3-7C). We conclude that ATRX is required for *intra*chromosomal interactions of the *H19* ICR in the neonatal forebrain.

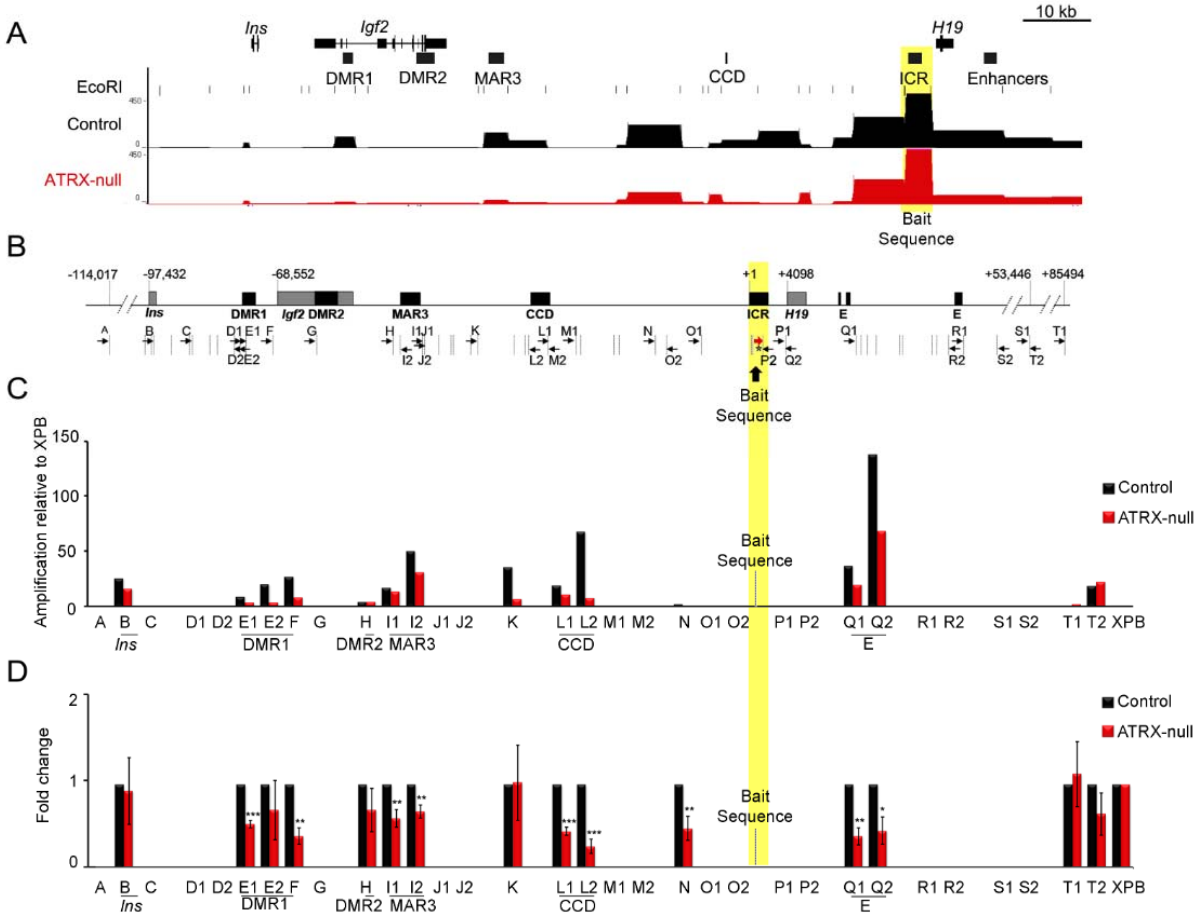

Figure 3-1 ATRX is required for *intra*chromosomal interactions across the *H19/Igf2* imprinted domain

(A) 4C interactions profile of the *H19* ICR in neonatal control and $Atrx^{null}$ forebrain tissue reveals that ATRX is necessary for chromosomal contacts across *H19/Igf2*. The *H19* ICR bait sequence is highlighted in yellow. (B) Schematic representation of the *H19/Igf2* imprinted domain and alignment of primers utilized for PCR amplification of 3C reactions. Numbers indicate the relative nucleotide position from the start of the *H19* ICR. Grey boxes represent the position of genes and black boxes demarcate regulatory elements. Vertical black lines indicate the position of EcoRI restriction site and black arrows represent primers used for analysis. The bait sequence primers and probe are

marked as a red arrow and black asterisk, respectively. (C) 3C analysis was performed across the *H19/Igf2* domain in control and *Atrx*^{null} littermate matched forebrains. Interaction frequencies were significantly reduced at the DMR1, MAR3, CCD, and the endodermal enhancer. Image depicts a representative interaction profile. (D) Interaction frequencies across the region were quantified in additional brains (n=5). p values were determined by a two tailed t-test, *$p<0.05$, **$p<0.01$, ***$p<0.0001$.

3.3.2 ATRX Acts in *Cis* to Regulate the Expression of Imprinted Genes

In addition to *intra*chromosomal interactions, the *H19* ICR can interact in *trans* with other imprinted domains on multiple chromosomes(Ling et al., 2006; Sandhu et al., 2009; Zhao et al., 2006). We investigated if these interactions occur in the brain and if they are mediated by ATRX, perhaps providing a mechanism for ATRX regulation of the imprinted gene network (IGN). To determine if the *H19/Igf2* genomic region co-localizes with other imprinted genes in the brain, we conducted DNA fluorescent in situ hybridization (FISH) analysis on newborn brain sections, followed by confocal microscopy and three dimensional (3D) image analysis. To facilitate comparison with published embryonic stem cell data, we defined an interaction as FISH signals with a centre-to-centre distance of less than 1 μm in 3D(Sandhu et al., 2009). We quantified the localization of *H19/Igf2* with the top eight imprinted genes affected by the loss of ATRX (*Slc38a4*, *Grb10*, *Dlk1*, *Dcn*, *Zac1*, *Mest* and *Peg10/Sgce*)(Kernohan et al., 2010). We also included *Gapdh* as a control that should display random localization. All gene regions, with the exception of *Peg10/Sgce*, co-localize with *H19/Igf2* with interaction frequencies characteristic of transient events that are consistent with previously reported embryonic stem cell data (Figure 3-2A,B). *Peg10/Sgce* did not co-localize with *H19*/*Igf2*, but rather exhibited a random localization profile similar to that of *Gapdh*. Therefore, the regulation of this imprinted domain is likely independent of *H19* and/or other IGN members. We next assessed whether ATRX is required for interactions between *H19*/*Igf2* and other IGN domains. 3D DNA FISH analysis of cortical sections from three control/$Atrx^{null}$ littermate matched pairs revealed no significant difference in interaction frequencies upon loss of ATRX (Figure 3-2A,B). This result suggests that while IGN domains co-localize transiently in the forebrain, their interaction with the *H19/Igf2* genomic region is not regulated by ATRX.

We previously reported that ATRX also localizes within a second imprinted domain on mouse chromosome 12, *Gtl2*/*Dlk1*(Kernohan et al., 2010) suggesting that ATRX may be recruited to several imprinted domain to regulated gene expression in *cis*. Analysis of previously published ATRX ChIP-sequencing data from embryonic stem cells(Law et al.,

2010) reveals that ATRX directly binds in proximity to many imprinted genes, with the majority of binding sites overlapping known DMRs and ICRs (Figure 3-3). We were able to confirm ATRX localization to these sites in P0.5 forebrains by ChIP, and propose that ATRX is likely required to regulate chromosomal interactions in *cis* at individual imprinted genes, but not by promoting their co-localization in the nucleus.

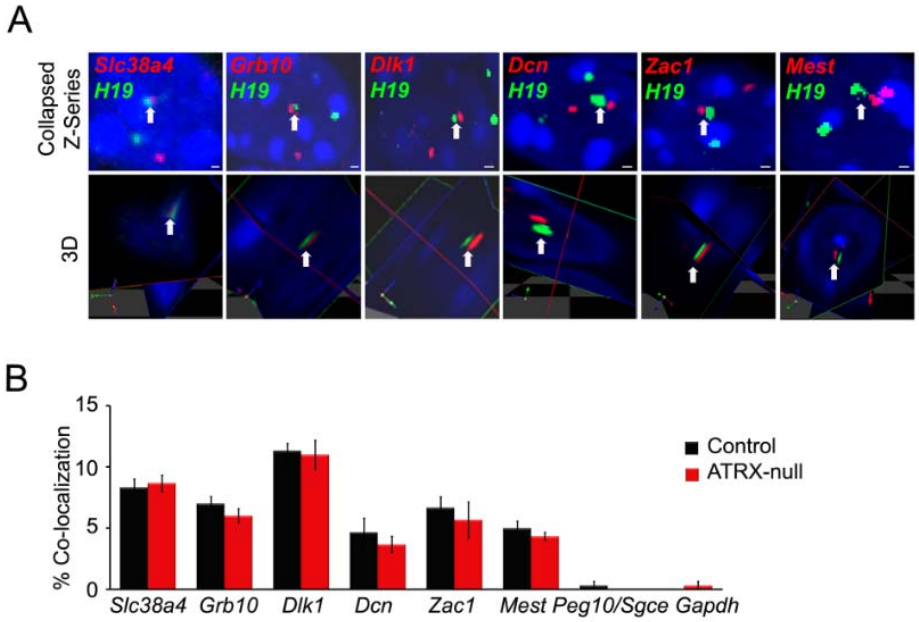

Figure 3-2 The *H19/Igf2* imprinted domain forms ATRX-independent *inter*chromosomal interactions with specific IGN members

(A) Collapsed confocal series (top), and 3D reconstructed serial confocal images (bottom) of neonatal cortical nuclei showing superimposed hybridization signals for *H19/Igf2* (green) and other IGN members (red). Cells are counterstained with DAPI (blue). Scale bar: 0.5 μm. (B) Frequency of hybridization signals from IGN member either overlapping or in close physical proximity to *H19/Igf2* DNA FISH signal. Graphed data represent the mean interaction frequency from three control and ATRX-null littermate pairs. One hundred cells were counted for each animal and signals with a 3D centre-to-centre distance of less than 1 μm were considered to represent an interaction. *Slc38a4*, *Grb10*, *Dlk1*, *Dcn*, *Zac1* and *Mest* display co-localization with *H19/Igf2*, while *Peg10/Sgce* and *Gapdh* do not. Interaction frequencies were not affected by the loss of ATRX in the forebrain.

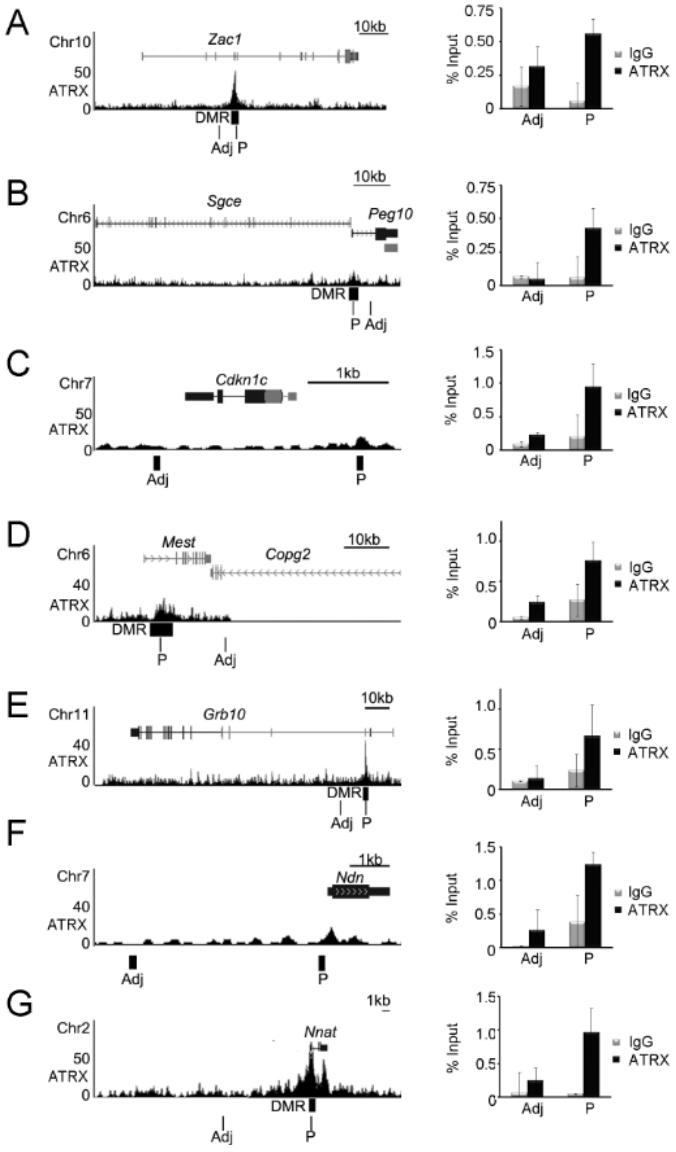

Figure 3-3 ATRX binds DMRs and ICRs throughout the IGN

Previously published ChIP-sequencing from embryonic stem cells was analyzed(Law et al., 2010) and demonstrates binding of ATRX at many imprinted domains. ATRX enrichment at these sites was confirmed in the forebrain, demonstrating ATRX recruitment is a common IGN regulatory mechanism. Graphs depict average % input, n=3, error bars represent SEM

3.3.3 ATRX is Recruited to the *H19* ICR to Regulate Nucleosome Positioning and Maintain CTCF Occupancy

ATRX-mediated silencing of imprinted genes is limited to the postnatal brain(Kernohan et al., 2010). This led us to enquire whether ATRX is present at the *H19* ICR in the embryonic brain and then functions upon neuronal maturation, or whether it is absent embryonically before being recruited in the late gestational/neonatal period to affect chromatin looping. To address this question, we conducted chromatin immunoprecipitation (ChIP) for ATRX on embryonic day 13.5 (E13.5) and P0.5 forebrains. We found ATRX enrichment within the *H19* ICR at P0.5 but not E13.5 (Figure 3-4B), demonstrating that ATRX must be recruited to the *H19* ICR in the late gestational/neonatal period. ATRX-deficiency in the forebrain at P17 results in decreased CTCF binding at the *H19* ICR(Kernohan et al., 2010), leading us to question if ATRX functions to recruit or maintain CTCF at this site. ChIP for CTCF on E13.5 and P0.5 control forebrains revealed similar patterns of CTCF binding with high enrichment at sites *H19*-2 and *H19*-4 (Figure 4C), indicating CTCF is present at the *H19* ICR prior to ATRX occupancy. Furthermore, we find that CTCF enrichment in ATRX-deficient forebrains is normal at E13.5 but reduced at P0.5 (Figure 3-4D). We conclude that ATRX recruitment to the *H19* ICR in the neonatal brain functions to maintain CTCF binding.

Recent reports have suggested ATRX targets DNA enriched for G-quadruplex structures, where it can aid in insertion of histone H3.3(Goldberg et al., 2010; Law et al., 2010; Wong et al., 2010) and we hypothesized that this mechanism may account for the differences in CTCF binding at the *H19* ICR. However, we found no sequences predicted to form G-quadruplexes in the *H19* ICR(Scaria et al., 2006). ChIP for H3.3 in control and ATRX-null neonatal forebrains showed that the loss of ATRX caused a small increase in H3.3 occupancy across the 5' region of the *H19* ICR, which is inconsistent with decreased H3.3 deposition observed at ATRX-deficient telomeres(Goldberg et al., 2010) (Supplementary Figure 3-8B). To determine if this increase was specific to H3.3, we conducted ChIP for histone H2A and found a similar increase in enrichment

(Supplementary Figure 3-8B). Based on these findings, we propose that ATRX does not target G-quadruplex DNA or function to insert H3.3 at the *H19* ICR.

Several studies have noted that CTCF bound regions are often devoid of nucleosomes(Davey et al., 2003; Fu et al., 2008; Kanduri et al., 2002; Kelly et al., 2012; Teif et al., 2012). Given that ATRX has DNA translocase activity(Xue et al., 2003; Mitson et al., 2011), and that we observed a slight increase in histone proteins in the absence of ATRX at the *H19* ICR, we speculated that ATRX could be shuttling nucleosomes within the ICR, resulting in nucleosome devoid regions. To test if ATRX affects nucleosome positioning, we digested control and $Atrx^{null}$ neonatal forebrains with micrococcal nuclease, which digests all DNA not protected by proteins, including histones. This analysis is more precise and sensitive than the ChIP for histones as it generates smaller fragments. Samples were evaluated by qRT-PCR with primers tiling the *H19* ICR in 100 bp fragments (Figure 3-4A). Amplification revealed a trend towards an increase at site B overlapping the CTCF bound region (Supplementary Figure 3-9B). ATRX binds only the maternal allele(Kernohan et al., 2010), and the presence of the paternal allele might attenuate the detection of maternal allele-specific effects. To circumvent this problem, we further digested micrococcal nuclease processed samples with McrBC, an enzyme that degrades GC-rich methylated DNA, including the paternal allele of the *H19* ICR (Figure 3-4E). Paternal-specific McrBC digestion was confirmed by allelic analysis in 129Sv/Castaneous polymorphic mice (Supplementary Figure 3-9C,D). McrBC digested $Atrx^{null}$ brains displayed significantly increased amplification over controls in regions B and C (Region B, $p=0.016$; Region C, $p=0.05$), indicating an increase in nucleosome protection within this area in mutant compared to control brains (Figure 3-4F). Regions B and C of the *H19* ICR overlap the two previously reported ATRX-dependent CTCF binding sites(Kernohan et al., 2010), and are in close proximity to the ATRX-enriched region. Conversely, we find no significant changes in protection elsewhere in the *H19* ICR, illustrating a site-specific effect on nucleosome occupancy (Figure 3-4F). This substantiates our hypothesis that ATRX regulates nucleosome occupancy within the 5' region of the *H19* ICR and provides a mechanism for the decrease in *H19* ICR CTCF binding and chromatin interactions in the $Atrx^{null}$ brain.

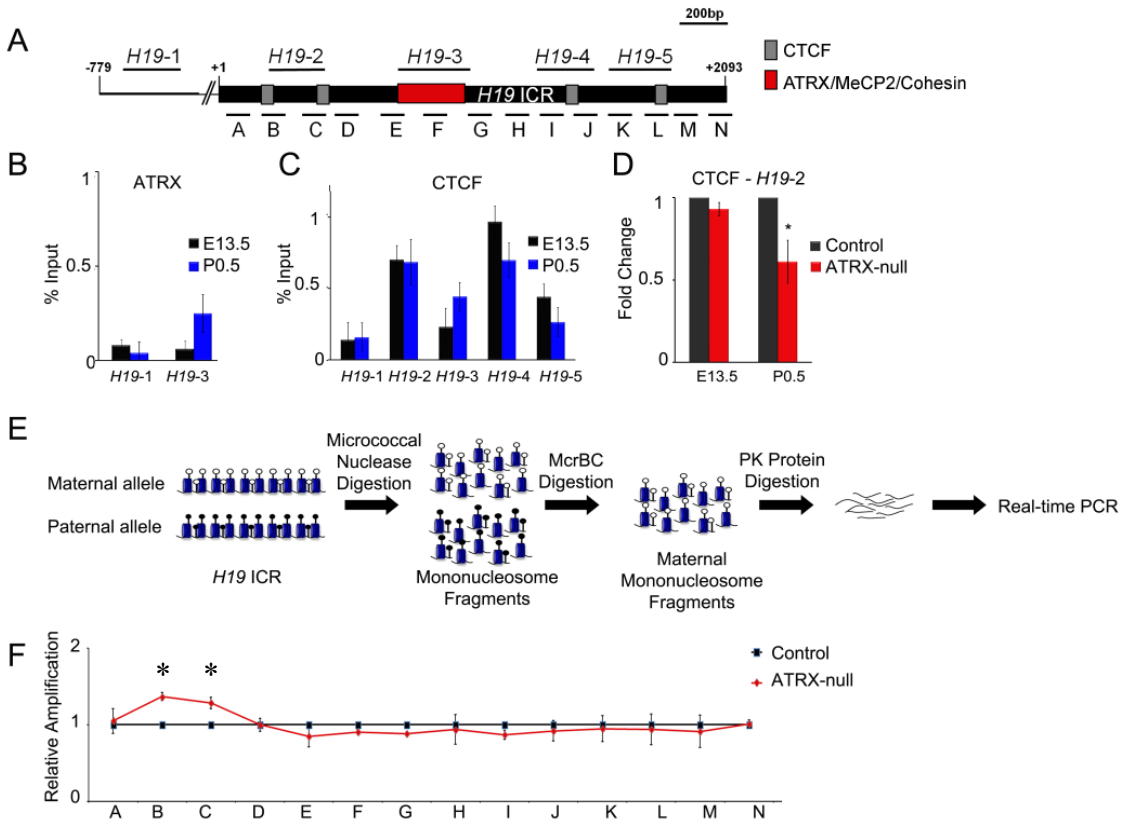

Figure 3-4 ATRX is recruited to the *H19* ICR to govern nucleosome occupancy and CTCF binding in the neonatal brain

(A) Schematic representation of the *H19* ICR and alignment of primers used for qPCR of ChIP (top) and nucleosome occupancy (bottom) analysis. Grey boxes indicate the position of CTCF binding sites, and the ATRX/MeCP2 site is marked in red. Numbers indicate the relative position from the start of the *H19* ICR. ChIP for ATRX (B) and CTCF (C) in E13.5 and P0.5 forebrains shows that while CTCF is enriched at both E13.5 and P0.5, ATRX is limited to the neonatal brain and is required for neonatal CTCF enrichment (D) (n=3 for each, error bars depict SEM). (E) Schematic of allele-specific micrococcal nuclease digestion protocol. (F) qPCR of micrococcal nuclease and McrBC digested DNA depicts maternal DNA protected by histones. A significant increase in nucleosome occupancy was observed within regions B and C of the *H19* ICR. Graphs depict average fold change and statistical analysis was performed by a two-tailed t-test (n=3, errors bars depict SEM). *=$p<0.05$

3.3.4 MeCP2 Recruits ATRX to the *H19* ICR in the Neonatal Brain to Govern *intra*chromosomal Interactions

We previously reported co-localization of ATRX with MeCP2 at the *H19* ICR, and that loss of ATRX did not affect MeCP2 enrichment at this site(Kernohan et al., 2010). MeCP2 has been shown to recruit ATRX to PCH(Baker et al., 2013; Nan et al., 2007), leading us to question if MeCP2 also recruits ATRX to genic regions. To address this question, we performed ChIP for ATRX in control and *MeCP2*^{null} neonatal brains and found that in the absence of MeCP2, ATRX failed to occupy the *H19* ICR (Figure 3-5A,B). Therefore, MeCP2 binding at the *H19* ICR is required for the recruitment of ATRX.

As MeCP2 is required for ATRX binding at the *H19* ICR, and the loss of ATRX causes defects in *intra*chromosomal interactions of this region, we predicted that the loss of MeCP2 would also affect *H19/Igf2* architecture. We conducted 4C and 3C in control and *MeCP2*^{null} neonatal forebrains and found by both methods that MeCP2-deficiency results in chromatin structure defects more dramatic than those observed following the loss of ATRX. 4C analysis displayed a decrease in interactions across *H19/Igf2* including the CCD, MAR3 and DMR1 regions (Figure 3-5C). Quantitative 3C analysis revealed that, like ATRX, *MeCP2*^{null} brains had significantly decreased interaction frequencies with the *Igf2* DMR1 (region E/F; p=0.008 and 0.001), MAR3 (region I; p= 0.001), CCD (region L; p= 0.0007) and the endodermal enhancer (region Q; p=0.0008). Additionally, decreased interactions were observed with the *Ins* gene (Region B; p=0.05), and the intergenic regions K and N (region K; p=0.009; region N; p=0.001) (Figure 3-5D,E). Together these data demonstrate that MeCP2 recruits ATRX, and likely additional unidentified factors, to the *H19* ICR in the neonatal brain to govern long-range chromosomal interactions at the *H19/Igf2* genomic region.

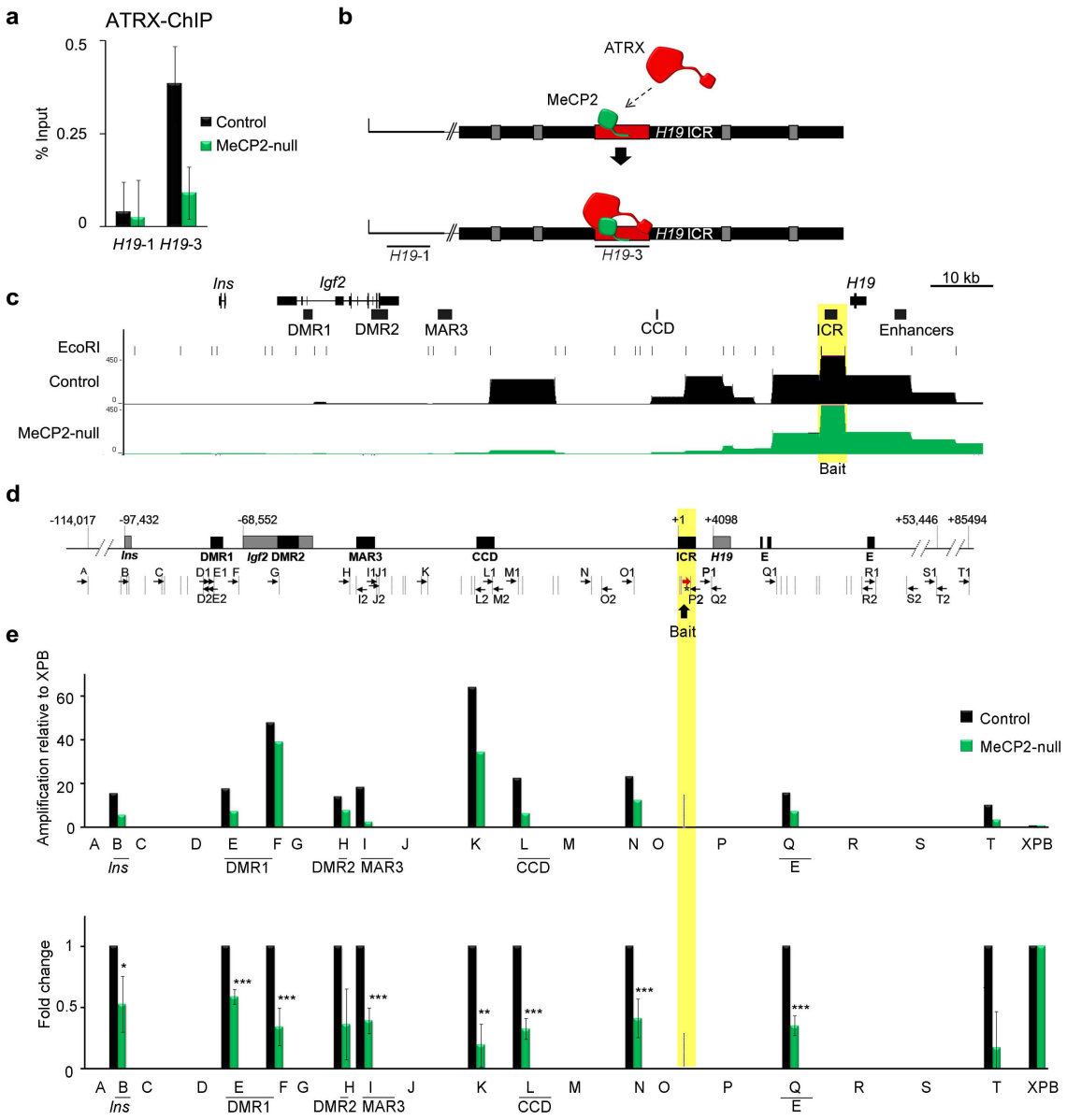

Figure 3-5 MeCP2 is required for ATRX binding and *intra*chromosomal interactions of the *H19/Igf2* domain

(A) Schematic of *H19* ICR as depicted in Figure 3-4, with hypothesized recruitment of ATRX by MeCP2. (B) Analysis of ChIP for ATRX in control and MeCP2^{null} neonatal brains demonstrated a requirement of MeCP2 for ATRX enrichment within the *H19* ICR. (C) 4C interaction profile of the *H19* ICR in neonatal control and *MeCP2*^{null} forebrains demonstrates a requirement for MeCP2 in *H19* ICR chromatin interactions. (D) Genomic

organization and primer alignment for 3C analysis as depicted in Figure 3-1. (E) 3C analysis was performed across the *H19/Igf2* domain in control and MeCP2^{null} littermate matched forebrains. Interaction frequencies were decreased at all sites across the region. Image depicts a representative interaction profile. (F) Interaction frequencies across the region were further quantified in additional brains (n=3). p values were determined by a two tailed t-test, *$p<0.05$, **$p<0.01$, ***$p<0.0001$.

3.4 Discussion

Our findings demonstrate a novel mechanism for the ATRX chromatin remodeling protein in the control of gene expression through the regulation of higher-order chromatin interactions. We define an *in vivo* three-step mechanism where MeCP2 recruits ATRX to the *H19* ICR to regulate nucleosome occupancy, thus maintaining CTCF binding and facilitating *intra*chromosomal contacts. In the absence of ATRX, these mechanisms are disrupted, leading to improper chromatin organization and a failure to properly silence imprinted genes (Figure 3-6).

In view of recent work showing that Cohesin and CTCF enable higher-order chromatin looping within imprinted domains and that ATRX alters CTCF and Cohesin dynamics within these regions(Guibert et al., 2012; Han et al., 2008; Kernohan et al., 2010; Kurukuti et al., 2006; Li et al., 2008; Nativio et al., 2009; Rubio et al., 2008), a logical prediction was that the loss of ATRX would disrupt chromatin looping. We detected changes in *H19* ICR interactions with the DMR1, MAR3, CCD, and enhancer sequences. The disruptions in chromatin folding we observe (formation of an ICR-DMR1-MAR3 complex) parallel the maternal-specific effects following loss of CTCF(Kurukuti et al., 2006). A maternal allele-specific effect is corroborated by our previous study showing that ATRX localizes to the maternal *H19* ICR and affects maternal *H19* expression(Kernohan et al., 2010). These findings link the ATRX chromatin remodeling protein to the control of chromatin looping. As the loss of ATRX affects the expression of numerous genes in the brain(Levy et al., 2008), it is possible that ATRX may regulate chromatin looping at multiple sites throughout the genome to govern gene expression.

ATRX recognizes G-quadruplex DNA structures and is proposed to resolve these formations to facilitate DNA replication and transcription(Goldberg et al., 2010; Law et al., 2010; Wong et al., 2010). This mechanism has been proposed at telomeres(Goldberg et al., 2010; Law et al., 2010; Wong et al., 2010) and some specific genes(Law et al., 2010; Levy,M., unpublished data). We now define a novel mechanism of ATRX targeting and function which is independent of G-quadruplexes. We find that MeCP2 recruits ATRX to *H19/Igf2*, where ATRX then functions to modulate nucleosome

occupancy within the 5' *H19* ICR. Genome-wide studies have demonstrated that CTCF binds in an extended linker region. At the *H19* ICR, improper placement of a nucleosome within a CTCF binding site abrogates CTCF binding and compromises insulator activity *in vitro*(Kanduri et al., 2002). Thus, the positioning of nucleosomes within the ICR is essential. ATRX may redistribute nucleosomes by sliding along the chromatin fiber in a process termed translocation. Accordingly, ATRX has been demonstrated to have DNA translocase activity(Mitson et al., 2011; Xue et al., 2003). In the absence of ATRX, improper nucleosome distribution leads to CTCF eviction from the *H19* ICR. This mechanism of CTCF regulation is similar to that proposed to regulate the chicken lysozyme locus(Lefevre et al., 2008), and may occur throughout the genome by site-specific chromatin remodelers. ATRX recruitment to maintain CTCF binding suggests that a 'developmental switch' must occur at the *H19* ICR to elicit gene silencing. While we still lack a complete picture of the events within the *H19* ICR at this time, gene silencing likely requires protein recruitment (including ATRX), epigenetic modifications and changes in long-range chromatin interactions. Overall, these observations provide a novel mechanism for ATRX regulation of CTCF binding and chromatin looping.

Several years ago it was suggested that MeCP2 binding surrounding imprinted genes may indicate a role in chromatin looping(Yasui et al., 2007), and that loss of MeCP2 alters chromatin interactions within a small area surrounding the *Dlx5* imprinted gene(Horike et al., 2005). We now provide definitive evidence that MeCP2 recruits ATRX to regulate chromatin looping and that the loss of MeCP2 abrogates chromatin interactions across the 90 kb *H19/Igf2* imprinted domain. Looping defects in the absence of MeCP2 are more severe and affect more sites than those observed in the absence of ATRX, implying that MeCP2 recruits additional factors that promote chromatin interactions or has other unidentified functions in promoting chromatin structure. Genome-wide ChIP studies have shown that MeCP2 binds throughout the genome and affects the expression of multiple genes(Yasui et al., 2007). In light of our results, it is possible that MeCP2 functions at many locations to recruit site-specific chromatin remodeling proteins and control chromatin architecture. Importantly, our analysis was conducted in the neonatal brain, a time when MeCP2 is thought to have limited or no

function due to low expression levels. Our data clearly demonstrates that MeCP2 regulates chromatin structure during early development, despite low protein levels in the nucleus. This is an important difference in the ATRX-MeCP2 partnership at PCH versus the *H19* ICR; at PCH it was reported that MeCP2 did not affect ATRX enrichment until seven weeks of age(Baker et al., 2013), while MeCP2 is required for ATRX localization to the *H19* ICR at birth. The expansion of studies on MeCP2 and gene regulation to earlier developmental stages will help to elucidate the full role of MeCP2 in early neurodevelopment.

An emerging theory proposes that some imprinted genes are jointly regulated in a cell-type specific network(Andrade et al. 2010; Kernohan et al., 2010; Lui et al., 2008; Sandhu et al., 2009; Varrault et al., 2006; Zhao et al., 2006). In the nervous system, this sort of coordinated control of gene expression might be necessary during cellular differentiation and/or neuronal maturation, and could be facilitated by close subnuclear proximity or even direct allelic interactions. ATRX is required for the postnatal silencing of this connected network of imprinted genes in the brain(Kernohan et al., 2010). We now extend these studies and show that neuronal IGN members indeed co-localize in the brain, but were not able to find any evidence of ATRX regulation of these interactions. Instead, we show that ATRX independently localizes to imprinted domains throughout the IGN, and propose that ATRX governs *intra*chromosomal interactions across each domain in parallel.

The repercussions of failing to suppress the expression of *H19* and other imprinted genes in the brain are unknown, as the role of these genes has not yet been fully characterized. Given that misexpression of imprinted genes causes neurodevelopmental syndromes(reviewed in (Kernohan and Bérubé, 2010), the failure to suppress IGN components in the brain could potentially contribute to cognitive deficiencies characteristic of ATR-X and Rett Syndromes. Additionally, *ATRX*-null mutations and over-expression of imprinted genes have been linked to cancer, including central nervous system cancers(Bower et al., 2012; de Wilde et al., 2012; Jelinic and Shaw, 2007; Jiao et al., 2012b; Jiao et al., 2011; Kannan et al., 2012; Liu et al., 2012; Lovejoy et al., 2012; Schwartzentruber et al., 2012; Weisbrod et al., 2013), suggesting that ATRX regulation

of imprinted genes could also have important implications for tumorigenesis. *H19* is upregulated in many types of cancer, including bladder(Ariel et al., 1995; Ariel et al., 2000; Byun et al., 2007; Elkin et al., 1995; Luo et al., 2013a; b; Verhaegh et al., 2008), ovarian(Kim et al., 1998; Tanos et al., 1999), breast(Berteaux et al., 2008; Berteaux et al., 2005; Yballe et al., 1996), leukemia/lymphoma(Takeuchi et al., 2007), and lung cancers(Kondo et al., 1995). While the role of this non-coding RNA in tumorigenesis remains elusive, *H19* overexpression has been shown to increase tumor progression in mice(Lottin et al., 2002). Treatments are currently in development utilizing BC-819, a vector carrying the diptheria toxin A (DTA) gene, encoding a strong inhibitor of protein synthesis, under the control of the *H19* promoter(Mizrahi et al., 2009). As normal cells in mature tissues do not express *H19*, utilizing the *H19* promoter selectively targets and destroys cancerous cells(Mizrahi et al., 2009). If imprinted genes, including *H19*, are identified as upregulated in ATR-X null cancers, this treatment could also be utilized to treat the array of ATRX-null tumors.

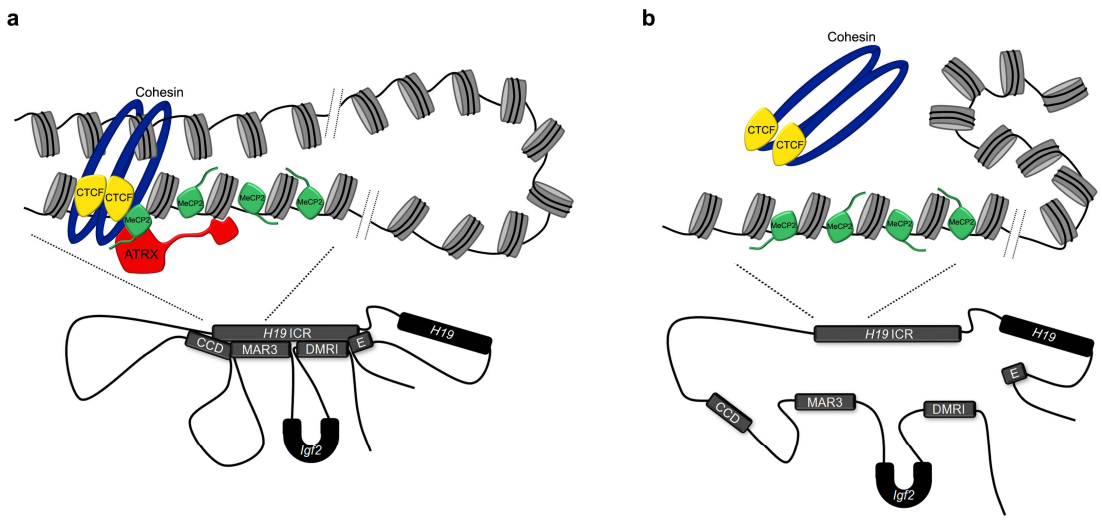

Figure 3-6 Proposed model

(a) In the wildtype brain, MeCP2 recruits ATRX to the maternal *H19*-ICR in the late embryonic/neonatal period. ATRX translocates along the chromatin fiber and alters nucleosome positioning to generate an extended linker region and promote CTCF occupancy. CTCF then dictates *intra*chromosomal interactions. (b) In the absence of ATRX, increased nucleosome occupancy disrupts CTCF binding, leading to a loss of *intra*chromosomal interactions.

3.5 Supplementary Figures

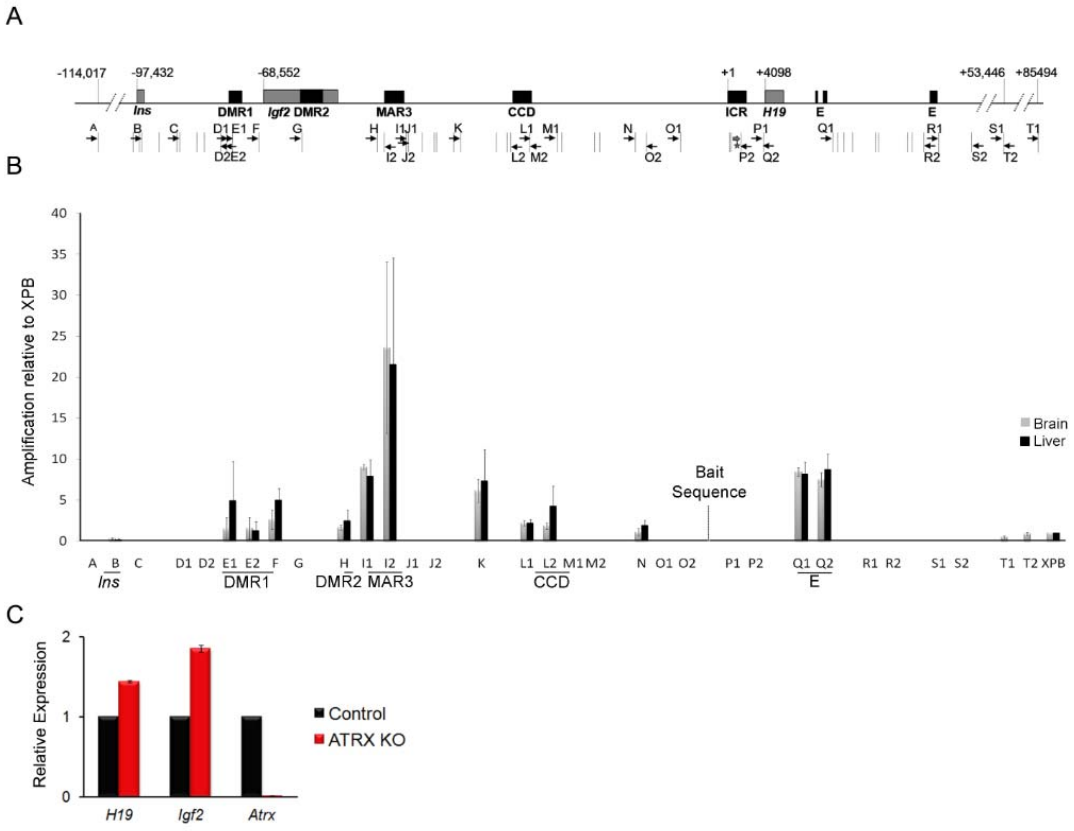

Figure 3-7 Liver and forebrain 3C analysis

(A) Schematic representation of the *H19/Igf2* imprinted domain and 3C assay as depicted in Figure 3-1. (B) Chromatin looping analysis confirms previous interactions reported in the neonatal liver(Qiu et al., 2008), and identifies a similar interaction profile in the forebrain. (C) Gene expression from the control/*Atrx*^{null} forebrain depicted in Figure 3-1C demonstrates an upregulation of *H19* and *Igf2* and misregulation of chromatin looping in the same brain. Error bars represent standard deviation of technical error.

A

B

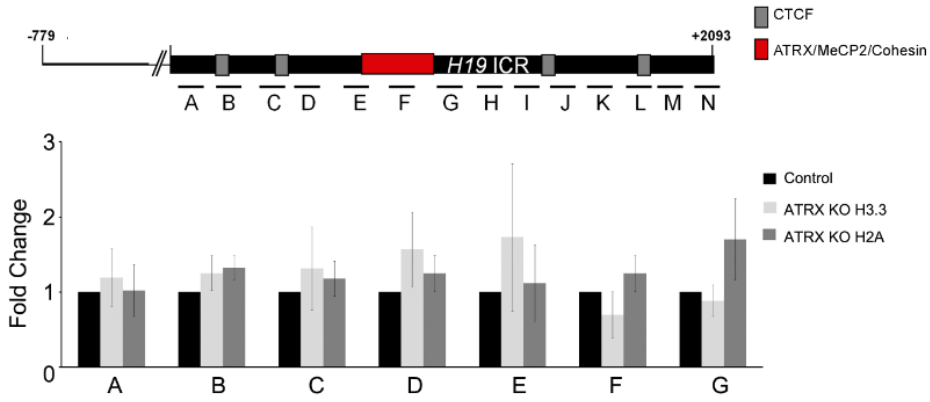

Figure 3-8 ATRX does not target G-quadruplexes or insert histone H3.3 at the *H19* ICR.

(A) Schematic of the *H19* ICR and alignment of primers. (B) ChIP in control and *Atrx*^{null} brains demonstrates a small increase in enrichment of H3.3 and H2A within the 5' region of the *H19* ICR.

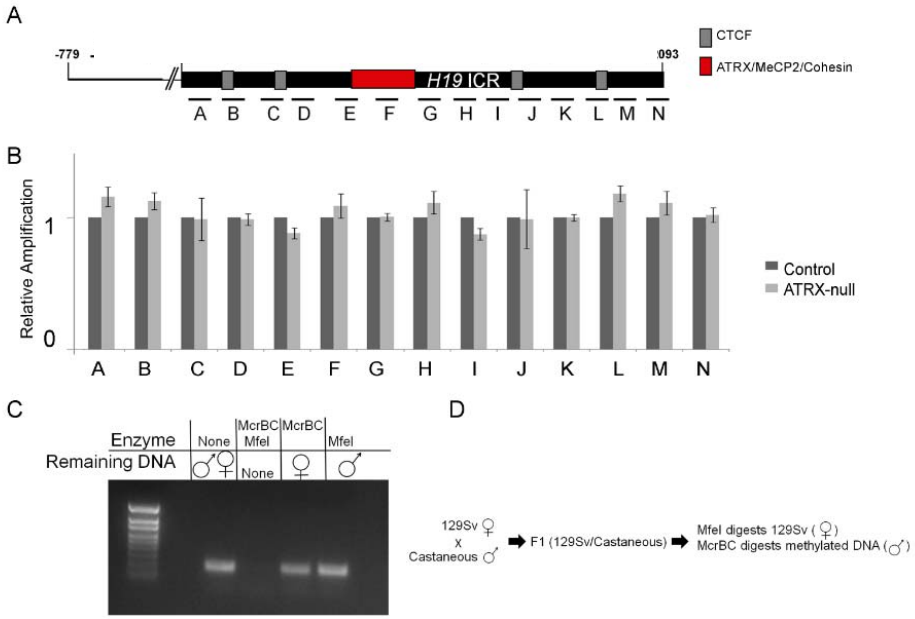

Figure 3-9 Analysis of nucleosome density in the ATRX-null forebrain

(A) Schematic of the *H19* ICR and alignment of primers as depicted in Figure 3-3. (B) qPCR of micrococcal nuclease digested DNA demonstrates quantification of DNA protected by histones. A small increase in nucleosome occupancy was observed within some regions of the *H19* ICR. Graphs depict average fold change. (C) Confirmation of paternal *H19* ICR specific digest by McrBC using F1 polymorphic 129Sv (maternal)/Castaneous (paternal) mice. MfeI digests 129Sv maternal DNA and McrBC digests methylated paternal DNA. DNA was digested and then amplified using primers spanning the MfeI restriction site. Schematic of protocol is detailed in (D).

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Chapter 4

4 Discussion and Future Directions

ATR-X Syndrome patients exhibit severe mental retardation, developmental abnormalities and alpha-thalassemia(Gibbons et al., 1995a; Gibbons et al., 1995b; Gibbons et al., 1992). While the link between *ATRX* mutations and alpha-thalassemia is indicative of ATRX's capacity to regulate alpha-globin expression, no direct gene targets have been identified and little is known about ATRX function. Overall, this body of work identified a group of genes regulated by ATRX and defined the first mechanistic role of ATRX in gene regulation.

In Chapter two, I documented a requirement for ATRX in the postnatal silencing of a network of imprinted genes in the mouse brain, including *H19, Igf2* and *Dlk1*. I began to explore the mechanism of this regulation and found that in the brain ATRX forms a complex with MeCP2 and Cohesin at the *H19* ICR and *Gtl2* DMR. Loss of ATRX results in an altered profile of post-translational histone modifications and reduced CTCF and Cohesin binding. As CTCF and Cohesin are known architectural proteins, this data suggested that ATRX might regulate imprinted gene expression through the control of higher-order chromatin structure. (Kernohan et al., 2010)

Chapter three further explores the mechanism of ATRX's regulation at imprinted genes in the brain. Using *H19/Igf2* as a model, I found that MeCP2 is required for ATRX recruitment to the *H19* ICR, where it governs nucleosome occupancy to maintain CTCF binding. I utilized 3C and 4C techniques to evaluate *in vivo* chromatin interactions and demonstrated that the loss of ATRX or MeCP2 caused a significant decrease in *cis* interactions of the *H19* ICR. Finally, I established that ATRX binds within many imprinted domains and I propose a model in which ATRX localizes to each IGN region to control local chromatin structure and modulate gene expression in the neonatal brain.

4.1 The ATRX-MeCP2-Cohesin Complex

My data demonstrates a functional connection between four important epigenetic regulators: ATRX, MeCP2, CTCF and the Cohesin complex. The co-localization of ATRX and MeCP2 has previously been demonstrated, where MeCP2 deficiency or mutation abrogates ATRX enrichment at PCH(Nan et al., 2007). A recent study demonstrated that MeCP2 binds DNA via an AT-hook domain, and that this domain is required for stable localization of MeCP2 to PCH and consequently ATRX recruitment(Baker et al., 2013). These studies raised the possibility that ATRX and MeCP2 could cooperate at other sites in the genome. I demonstrated that ATRX and MeCP2 co-localize at multiple imprinted genes, and that MeCP2 recruits ATRX to the *H19* ICR. It is plausible that MeCP2 utilizes the same AT-hook domain to bind imprinted regions, forming a stable interaction with DNA to recruit ATRX. Loss of ATRX causes an up-regulation of imprinted genes in the postnatal brain. Similarly, loss of MeCP2 has also been reported to cause an increase in several of these transcripts, namely *H19*, *Dlk1*, and *Zac1*(Fuks et al., 2003; Urdinguio et al., 2008). These data suggest that ATRX and MeCP2 can cooperate to influence gene expression, at least at imprinted genes.

Previous studies in our laboratory demonstrated that ATRX is required for proper sister chromatid cohesion(Ritchie et al., 2008), the canonical function of the Cohesin complex(Barbero, 2011; Michaelis et al., 1997; Moser and Swedlow, 2011; Uhlmann and Nasmyth, 1998). This led to our investigation and identification of an interaction between ATRX and Cohesin proteins. In addition to its mitotic role, the Cohesin complex also functions as a transcriptional regulator(Lara-Pezzi et al., 2004; Remeseiro and Losada, 2013). Cohesin is often recruited by CTCF to genic regions, where it governs chromatin architecture and subsequent gene expression(Rubio et al., 2008). This effect has been demonstrated at numerous sites, including the *H19* ICR(Rubio et al., 2008). I found that ATRX and MeCP2 localize with Cohesin to the *H19* ICR, and that the loss of ATRX caused a reduction of Cohesin and CTCF at this site. Notably, we cannot detect an interaction between CTCF and ATRX or MeCP2 (Jiang, Y., unpublished data). This could be due to a lack of interaction between these proteins or that the antigen

recognition site(s) are masked by these interactions. The interaction between MeCP2 and Cohesin is also novel and perhaps surprising as the loss of MeCP2 has not been reported to cause mitotic cohesion abnormalities. This suggests that the MeCP2-Cohesin interaction may be restricted to cells which are not undergoing mitosis, for example post-mitotic neurons, or that MeCP2 and Cohesin only function together to regulate gene expression. Alternatively, if the MeCP2-Cohesin complex functions in mitosis, the loss of MeCP2 may trigger a compensatory response, for example the recruitment of additional proteins to aid in proper mitotic progression. In the future, it will be important to determine whether the ATRX-MeCP2-Cohesin complex binds elsewhere in the genome to regulate gene expression and/or chromatin structure. One possibility is PCH, where ATRX and MeCP2 interact(Baker et al., 2013; Nan et al., 2007), and Cohesin regulates pericentric chromatin loops(Stephens et al., 2013).

In a clinical context, ATRX, MeCP2 and Cohesin are implicated in ATR-X, RTT and CdLS Syndromes, respectively(Amir et al., 1999; Ben-Asher and Lancet, 2004; Gibbons et al., 1992; Krantz et al., 2004; Revenkova et al., 2009). While each of these disorders manifests with numerous cognitive and physical symptoms, some commonalities include developmental delay, microcephaly and growth deficiencies. Though these interactions have yet to be confirmed in human cells, it is possible that the cooperation of these proteins in the brain, and potentially other tissues, may lead to similarities between their associated syndromes. With regards to ATR-X and RTT Syndromes, MeCP2 targeting of ATRX to genes in the neonatal brain could contribute to the postnatal onset of RTT neuronal deficits in patients and mice. Loss of ATRX causes misregulation of numerous genes in the mouse forebrain(Levy et al., 2008), an effect that probably contributes to neurodevelopmental defects in ATRX-null mice and potentially plays a role in ATR-X Syndrome. If a subset of these genes require the recruitment of ATRX by MeCP2, patients with either ATR-X or RTT will have misregulation of these genes and the associated consequences. A recent study reported that ATRX is not localized at PCH in neurons of mice with mutations in an AT-hook domain of MeCP2(Baker et al., 2013). This defect occurred in the mature brain and was theorized to result in compromised chromatin structure(Baker et al., 2013). I have now extended Baker et al.'s findings and

shown that MeCP2 does target ATRX to regulate chromatin structure and that this occurs in the neonatal brain. The timing of the this regulation is just prior to the onset of RTT symptoms(Armstrong, 2002; Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002; Shahbazian and Zoghbi, 2001), and therefore has potential to play a causative role in RTT etiology. While ATR-X Syndrome manifests much earlier(Gibbons et al., 1995a; Gibbons et al., 1995b; Gibbons et al., 1992), ATRX-MeCP2 targets may add to the neuronal deficits observed in patients. Overall, the identification of additional binding sites for the ATRX-MeCP2 and ATRX-MeCP2-Cohesin complex, including analysis of the mature brain, and other tissues, might aid in the identification of novel therapeutic targets to treat the array of conditions affected by ATRX, MeCP2 and Cohesin.

4.2 Allele-Specific Binding of ATRX at Intergenic Regulatory Regions

At the outset of this study, no direct gene targets for ATRX had been reported. I identified imprinted genes as affected by the loss of ATRX. ATRX affects these genes directly by binding to intergenic regulatory sequences. A subsequent study by Law et al. reported ChIP sequencing for ATRX in mouse embryonic stem cells, providing information on the enrichment profile of ATRX throughout the mouse genome(Law et al., 2010). They found 1305 ATRX binding sites, including 456 in gene bodies, 78 at promoters and 771 in intergenic regions(Law et al., 2010). Among these intergenic sites are several ICRs and DMRs in imprinted domains. However, imprinted genes are rare in the genome (approximately 150 to date (MRC Harwell, 2013); the abundance of intergenic ATRX sites suggests that ATRX targets many types of regulatory sequences, not just those near imprinted genes. The role ATRX plays at these sites remains unknown. I found that ATRX is required for chromatin looping within imprinted domains. It is possible that ATRX-bound intergenic regions are sites of chromatin-chromatin contact, and that ATRX helps to form or maintain chromatin interactions at these locations. This may also be true of some ATRX-bound genic and promoter regions. Alternatively, ATRX may have a yet undiscovered function at these sites. Notably, the ATRX ChIP sequencing experiment by Law et al. was conducted in embryonic stem

cells. It is probable that a portion of ATRX binding sites are cell-type specific, and that the binding profile in the brain may differ, at least at some sites.

I discovered that ATRX binds chromatin and regulates expression in an allele-specific manner. Within the *H19/Igf2* domain, ATRX binds the maternal allele of the *H19* ICR and silences the maternal *H19* gene. Loss of ATRX does not affect the silent paternal allele. Given that members of the ATRX-dependent IGN all exhibit postnatal repression, and imprinted genes that remain highly expressed are not affected (e.g. *Gtl2*), I predict that ATRX binds the active allele and represses active transcription of each IGN domain. Within imprinted domains, the presence of allele-specific proteins, for example CTCF(Szabo et al., 2000), is essential for the establishment and maintenance of imprinted expression. ATRX now joins the small cohort of allelic binding proteins. However, the role of ATRX is to modulate expression from the active allele, not to establish or maintain imprinted expression. Further studies are needed to determine if any of the additional ATRX binding sites are allele-specific, what attracts ATRX to these sites and what limits binding to one allele. Analysis of ATRX ChIP-sequencing by Law et al. revealed that ATRX binds preferentially at CpG-islands and repetitive sequences, many of which form G-quadruplexes(Law et al., 2010). They demonstrated that ATRX can bind G-quadruplex DNA *in vitro*, and suggested that ATRX targets genomic sites by recognizing these secondary structures in the DNA(Law et al., 2010). While the *H19* ICR is a CpG-island, it does not contain any repeat sequences or predicted G-quadruplexes. Therefore, my results have also uncovered a novel method for ATRX targeting to DNA. ATRX may recognize the ICR, and one parental allele, through an epigenetic signature, including lack of DNA methylation and a specific combination of post-translational histone modifications. Indeed, recent literature has shown that ATRX can recognize the presence of H3K9me3 and absence of H3K4me2 and H3K4me3(Dhayalan et al., 2011; Eustermann et al., 2011; Lewis et al., 2010; Wong et al., 2010). Furthermore, ATRX binding at the ICR is probably mediated by other proteins, including MeCP2. Further research is required to establish the pattern of modifications that attracts ATRX to one allele of imprinted domains, and to determine if any proteins other than MeCP2 are required.

4.3 ATRX Regulates Nucleosome Positioning and CTCF Binding

It has been accepted for many years that transcription can be controlled by the accessibility of regulatory proteins to the DNA, which largely depends on DNA packaging into nucleosomes(Muchardt and Yaniv, 1999; Travers et al., 2012). Nucleosome occupancy can be influenced by nucleosome positioning sequences in the DNA and ATP-dependent chromatin remodeling proteins(Becker and Horz, 2002; Travers et al., 2012). Together, these factors dictate whether certain DNA sequences are present in the accessible linker region between nucleosomes or are concealed by the histone octamer(Becker and Horz, 2002; Travers et al., 2012). *In vitro* studies evaluating nucleosome occupancy across the *H19* ICR have uncovered that nucleosome distribution in this 2 kb region is not random, but rather includes nucleosomes positioned at specific sequences(Davey et al., 2003; Fu et al., 2008; Kanduri et al., 2002). These sequences surround, but do not overlap, the CTCF binding sites(Davey et al., 2003; Fu et al., 2008; Kanduri et al., 2002). To test the relationship between CTCF and nucleosome occupancy, Kanduri et al. generated an *in vitro* assay that situated a CTCF consensus sequence within a positioned nucleosome(Kanduri et al., 2002). They found that nucleosome occupancy within the CTCF consensus site compromised CTCF binding and insulator function(Kanduri et al., 2002), indicating the importance of proper nucleosome distribution at the ICR. However, studies have found that DNA sequences which strongly position nucleosomes *in vitro o*ften fail to precisely localize nucleosomes *in vivo*(Li et al., 1997). Nucleosome positioning *in vivo* requires the addition of chromatin remodeling proteins, which can either translocate or evict nucleosomes(Li et al., 1997; Rippe et al., 2007). I found that ATRX affects the distribution of nucleosomes at the 5' region of the *H19* ICR, which contains two CTCF binding sites. I propose that this increase in nucleosome occupancy might be responsible for decreased CTCF enrichment at this site in the ATRX-null brain. As CTCF is already bound to the *H19* ICR prior to ATRX occupancy, I propose that ATRX controls nucleosome positioning to maintain, rather than to establish CTCF binding. To achieve this regulation, ATRX may redistribute nucleosomes by sliding along the chromatin fiber in a process termed translocation. Accordingly, *in vitro* biochemical studies have demonstrated that ATRX

can function as a DNA translocase(Mitson et al., 2011; Xue et al., 2003). Similar functions have been reported for other chromatin remodeling proteins. For example, the Chromatin Structure Remodeling (RSC) complex interacts with specific DNA sequences to translocate nucleosomes along the chromatin fiber(van Vugt et al., 2009). Alternatively, ATRX could affect nucleosome positioning indirectly through non-coding transcripts since transcription of non-coding RNAs has been shown to redistribute nucleosomes and affect CTCF binding(Lefevre et al., 2008). The *H19* ICR produces a number of non-coding RNAs(Takahashi et al., 2012) and ATRX could regulate nucleosome distribution by controlling transcription of these sequences. In the ATRX-null brain, an increase in ICR transcription could redistribute nucleosomes over the CTCF binding sites, thus evicting CTCF. This model has been previously shown at the chicken lysozyme locus(Lefevre et al., 2008), but has yet to be evaluated in a mammalian system. It is also possible that these models are not mutually exclusive, and that ATRX controls nucleosome occupancy through both nucleosome translocation and the regulation of non-coding RNAs. Further studies are required to completely elucidate the role of ATRX in governing nucleosome distribution. First it must be determined if the ICR produces non-coding transcripts in the brain, and if their expression is affected by the loss of ATRX. This can be done by qRT-PCR on cDNA from control and ATRX-null brains with primers tiling the ICR. To evaluate the contribution of ATRX translocase function to nucleosome positioning at the ICR, a mouse model could be generated with a mutation inhibiting ATRX's translocase activity. A suitable candidate is a missense mutation in the SWI/SNF domain, termed L1746S, which was recently discovered in a patient(Mitson et al., 2011). Characterization of the ATRX-L1746S protein revealed that it is present at relatively normal levels, can bind DNA, and has appropriate DNA-stimulated ATPase activity(Mitson et al., 2011). However, this mutant is unable to translocate along DNA(Mitson et al., 2011). Comparison of nucleosome positioning across the ICR in mice harbouring the L1746S mutation with controls would determine the contribution of ATRX translocation to nucleosome distribution.

4.4 A Novel Role for ATRX in the Regulation of Higher-Order Chromatin Structure

At the *H19/Igf2* imprinted domain, *cis* interactions of the *H19* ICR regulate *H19* and *Igf2* expression(Burke et al., 2005; Guibert et al., 2012; Han et al., 2008; Kurukuti et al., 2006; Li et al., 2008). These interactions require the presence of CTCF and Cohesin(Han et al., 2008; Kurukuti et al., 2006; Nativio et al., 2009; Zhao et al., 2006). As ATRX affects *H19* and *Igf2* expression, and the localization of CTCF and Cohesin to the *H19* ICR, I predicted that the loss of ATRX would also affect *H19*/*Igf2* chromatin structure. I found a significant decrease in interactions of the *H19* ICR across *H19/Igf2* in the ATRX-null brain. To further confirm a role for ATRX in *intra*chromosomal looping of imprinted domains, I have also generated preliminary 4C data analyzing interactions of the *Gtl2* DMR (Appendix D). The *Gtl2* DMR, located in the *Gtl2/Dlk1* imprinted domain on mouse chromosome 12, is the second site where I have demonstrated binding of ATRX, MeCP2, CTCF and Cohesin. I found that the loss of ATRX altered chromatin contacts across the *Gtl2/Dlk1* domain. Together, these data demonstrate for the first time that ATRX can affect higher-order chromatin structure. I propose that ATRX elicits these effects through the control of nucleosome positioning and consequently CTCF and Cohesin binding. As CTCF physically links Cohesin to chromatin(Rubio et al., 2008), the loss of CTCF binding (due to altered nucleosome occupancy), would decrease Cohesin enrichment. This is supported by an approximately equal reduction in CTCF and Cohesin at the *H19* ICR in the ATRX-null brain. Cohesin is theorized to physically encircle DNA loops to tether regions together. Thus, the loss of Cohesin in the ATRX-null brain would result in a lack of DNA tethering, and the decrease in chromatin contacts observed. Interestingly, chromatin looping at the alpha-globin(Kim et al., 2009) and Interleukin 2R-alpha domains(Yasui et al., 2002) has been shown to depend on nucleosome occupancy and chromatin remodeling proteins. It remains to be seen if nucleosome occupancy is linked with Cohesin binding at these sites, and/or if ATRX also plays a role at these domains.

The loss of ATRX affects the expression of numerous genes(Levy et al., 2008). As CTCF and Cohesin binding sites are abundant throughout the genome(Rubio et al., 2008), it is plausible that ATRX-dependent chromatin looping it not unique to imprinted genes, but rather is a common function of ATRX. One potential candidate is the alpha-globin locus. The majority of ATR-X patients present with some degree of alpha-thalassaemia, caused by a downregulation of the alpha-globin genes(Gibbons, 2006). In erythroid cells, chromatin looping positions the alpha-globin genes into an active conformation, which is not present in non-erythroid cells where alpha-globin is repressed(Vernimmen et al., 2007; Vernimmen et al., 2009; Zhou et al., 2006). Furthermore, studies evaluating alpha-globin looping in chicken and human cells have found that CTCF has a different binding profile in erythroid versus non-erythroid cells(Furlan-Magaril et al., 2011; Mahajan et al., 2009), suggesting an active role for CTCF in loop formation and gene regulation. Numerous proteins have been documented as contributors to the alpha-globin locus configuration, including GATA-1(Escamilla-Del-Arenal and Recillas-Targa, 2008), and BRG1(Kim et al., 2009); it is plausible that ATRX is also involved. Like ATRX, BRG1 is an ATP-dependent SWI/SNF chromatin remodeling protein that regulates nucleosome occupancy(Rippe et al., 2007), and was recently shown to function within an imprinted domain on human chromosome 20 (L3MBTL1/SGK)(Aziz et al., 2013). ATRX may function in concert with other chromatin remodeling proteins, including BRG1, to control looping in *cis* at *H19/Igf2*, alpha-globin, and multiple other regions throughout the genome. Like CTCF and Cohesin, ATRX may emerge as an important factor in chromatin looping, at least at some sites.

While the position of nucleosomes influences CTCF and Cohesin binding and is probably a strong contributing factor in the regulation of *H19/Igf2* architecture by ATRX, it may not be the only factor. In interphase, ATRX is tightly associated with the nuclear matrix(Bérubé et al., 2000). The nuclear matrix is a dynamic structural network within the nucleus thought to play a role in the formation and/or maintenance of nuclear architecture by tethering to specific DNA sequences known as matrix-attachment regions (MARs)(Pederson, 2000). For example, matrix mediated looping has been demonstrated at the beta-globin domain where inter-MAR association at the base of chromatin loops is

necessary for proper beta-globin expression(Wang et al., 2009). The *H19/Igf2* domain contains four MAR regions, including MAR3 located between *H19* and *Igf2*(Greally et al., 1997). While it is unknown if ATRX binds these MARs in the brain, my 3C and 4C data sets clearly demonstrate that that the *H19* ICR interacts with MAR3, and that this interaction is dependent on the presence of ATRX. It is possible that ATRX is involved in tethering *H19/Igf2* MAR sites to the matrix, aiding in the formation or maintenance of chromatin loops. Overall, it is clear that ATRX can compartmentalize DNA into higher-order chromatin structure to govern gene expression.

4.5 Chromatin Looping and Gene Expression

It has long been recognized that enhancers can exist and function at large distances from their target genes(Stadhouders et al., 2012). Technologies evaluating higher-order chromatin structure have provided evidence that looping mechanisms are responsible for this long-distance gene regulation. In the classical model, a distant enhancer loops into close proximity of a gene promoter to regulate its expression. This model is largely based on studies at the beta-globin domain, where a distal enhancer, termed the locus control region, loops from over 40 kb away to interact with the beta-globin promoter and activate gene expression(Palstra et al., 2008a). However, as studies continue to discover a complex array of chromatin interactions throughout the nucleus, it is clear that the relationship between chromatin looping and gene regulation is rarely this simple. The study of genes expressed in specific cell types has provided insight into the relationship between chromatin looping and gene expression. For example, in undifferentiated erthyroblasts the mouse alpha-globin locus is linear and alpha-globin is not expressed(Vernimmen et al., 2007). Upon differentiation, chromatin contacts are formed between alpha-globin genes and regulatory elements, inducing gene expression(Vernimmen et al., 2007). However, it is equally possible that these chromatin contacts are merely formed as a consequence of transcription, perhaps through the association of adjacent sites with the same transcription factory. The hypothesis of chromatin looping as a transcriptional by-product was disputed by a study which demonstrated that chromatin interactions of the *H19/Igf2* domains persist throughout

mitosis when genes are silent(Burke et al., 2005). These results corroborate that chromatin looping facilitates gene transcription.

I propose that ATRX regulates the expression of *H19* and *Igf2* in the postnatal brain by controlling *H19/Igf2 intra*chromosomal looping. In the ATRX-null brain, the loss of chromatin contacts could cause an increase in *H19* and *Igf2* expression through various means. These include *H19* and *Igf2* being placed into closer proximity of enhancers, or alternatively at a greater distance from yet undiscovered repressive elements. A full understanding of all genomic elements located in the *H19/Igf2* domain, and their role in silencing imprinted genes in the postnatal brain, is required to understand the relationship between ATRX, *H19/Igf2* chromatin structure and gene regulation. Importantly, my data provide the first mechanistic link between ATRX and the control of gene expression. It remains possible that altered chromatin structure in the absence of ATRX is a secondary consequence to increased transcription. To distinguish between a direct and indirect effect on chromatin looping, control and ATRX-null cells could be treated with pharmacological inhibitors to prevent transcription. For example, the chemical alpha-amanitin interacts with RNA polymerase II to inhibit transcriptional initiation and elongation(Seifart and Sekeris, 1969). Another possibility is 5,6-dichloro-1-β-D-ribofuranosylbenzamidazole which inhibits elongation by RNA polymerase II(Yamaguchi et al., 1999). Analysis of treated and untreated control cells would reveal if transcription is required for *H19/Igf2* loop formation or maintenance. A similar analysis in ATRX-null cells would determine if looping differs in ATRX-null cells in the presence or absence of extra *H19* and *Igf2* transcripts. Based on my model of nucleosome positioning governing CTCF and Cohesin occupancy, I predict that additional *H19* and *Igf2* transcription is not responsible for altered looping. Furthermore a similar study utilizing transcriptional inhibition and analysis of chromatin structures at the beta-globin locus revealed that altering transcription had no effect on beta-globin looping(Palstra et al., 2008b).

4.6 MeCP2 Regulation of Imprinted Genes and Chromatin Architecture

Mutations in the *MeCP2* gene were identified over a decade ago as the causative factor for Rett Syndrome(Amir et al., 1999). Since then, MeCP2 has been a subject of intense investigation; however, we still lack a clear picture of its function(s). MeCP2 was originally identified and characterized as a methylated-DNA binding protein, which bound to target genes with the co-repressor Sin3A and recruited histone deacetylases(HDACs), effectively repressing local genes(Ashraf and Ip, 1998; Ballestar and Wolffe, 2001). Emerging studies continue to challenge this original view of a simple transcriptional repressor; MeCP2 is now known to largely coat the genome, binding unmethylated DNA as well as methylated DNA, and it is frequently associated with actively transcribed genes(Hansen et al., 2010; Yasui et al., 2007). While future research may reconcile these seemingly conflicting roles, it seems more likely that MeCP2 has diverse functions throughout the genome. These functions could depend on the genomic context, post translational modifications and/or protein interaction partners. Allele-specific binding of MeCP2 had previously been demonstrated within the *U2af1-rs1* and *Dlx5/Dlx6* imprinted domains(Gregory et al., 2001; Horike et al., 2005). At these sites MeCP2 binds the silent methylated allele, and is associated with either imprint establishment or maintenance(Gregory et al., 2001; Horike et al., 2005). I identified an interaction between MeCP2 and ATRX/Cohesin on the unmethylated maternal allele of the *H19* ICR. Localization to the unmethylated allele was surprising, and suggested that the role of MeCP2 at imprinted genes is complex and may differ between domains, or may be specific to developmental stages and cell types. At *H19/Igf2*, binding to the unmethylated allele could be mediated by other proteins that somehow override MeCP2's preference for methylated DNA. I predict that MeCP2 also binds the unmethylated allele of other neuronal-IGN targets and is involved in *H19*, *Igf2*, and IGN silencing, though this has not yet been evaluated. In support of this hypothesis, *in vitro* studies have demonstrated that MeCP2 binding at the *H19* ICR is repressive(Drewell et al., 2002). Understanding the role of MeCP2 at other IGN sites is complicated by many conflicting studies on MeCP2 and gene regulation. While several groups have linked MeCP2 to the

regulation of imprinted genes, including *Ube3a*(Makedonski et al., 2005; Samaco et al., 2005), *Zac1*(Urdinguio et al., 2008), *Dlk1(Urdinguio et al., 2008)*, and *Dlx5*(Horike et al., 2005; Miyano et al., 2008), there are an equal number of reports refuting these claims(Jordan and Francke, 2006; Schule et al., 2007). The irreproducibility of these studies is probably due to the small transcriptional effects produced by MeCP2, as well as differences in the cell types and developmental stages examined. My results outline the need for careful and detailed allele-specific ChIP and expression analysis in the MeCP2-null brain to determine the effect of MeCP2 on IGN transcription.

In 2005 a great deal of excitement was generated surrounding a study identifying MeCP2 as a mediator of chromatin looping and imprinting of the *Dlx5* gene(Horike et al., 2005). However, this chromatin looping analysis was not quantitative, and the regulation of *Dlx5* by MeCP2 was later contested(Horike et al., 2005; Miyano et al., 2008; Nakashima et al.2010; Schule et al., 2007). A role for MeCP2 in chromatin looping was further suggested by reports of MeCP2 binding in numerous intergenic regions throughout the genome and an *in vitro* study which utilized electron microscopy to visualize DNA loops connected by a single MeCP2 molecule(Ghosh et al., 2010; Yasui et al., 2007). However, none of the above studies provide conclusive evidence for MeCP2-mediated higher-order chromatin architecture. I have now demonstrated that MeCP2-deficiency causes a clear and dramatic loss of chromatin interactions across the 90 kb *H19/Igf2* imprinted domain. I have also generated preliminary data showing a similar result at the *Gtl2/Dlk1* imprinted domain (Appendix D). Comparison of my ATRX-null and MeCP2-null 3C and 4C data sets revealed that MeCP2 affects chromatin interactions at more sites and to a greater extent than ATRX. This suggests that, in addition to ATRX, MeCP2 probably recruits other proteins essential for chromatin looping. It will be important to determine if MeCP2 regulation of chromatin structure is unique to imprinted genes, or if this form of regulation occurs at other sites. Given the widespread abundance of MeCP2 throughout the genome(Skene et al., 2010), it is plausible that MeCP2 recruits different chromatin remodeling factors and proteins to various sites to control chromatin architecture.

In the mouse brain, MeCP2 is expressed at low levels before birth and increases dramatically during the first three weeks of life(Kishi and Macklis, 2004; Skene et al., 2010). Due to this expression pattern, and the postnatal onset of Rett Syndrome in humans and mice(Armstrong, 2002; Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002; Shahbazian and Zoghbi, 2001), the vast majority of studies concentrate on MeCP2's effects in the mature brain. The longstanding belief was that MeCP2 was not required during neurogenesis(Guy et al., 2007; Guy et al., 2001; Kishi and Macklis, 2004). My results counter these assumptions by demonstrating that MeCP2 is required for ATRX binding to the *H19* ICR and MeCP2-deficiency nearly abolishes chromatin looping in the neonatal brain. This neonatal function of MeCP2 occurs before MeCP2 levels have reached their maximum and before disease onset in mutant mice. A role for MeCP2 earlier in development is supported by several studies reporting consequences of MeCP2 deficiency beginning in the neonatal period(De Filippis et al., 2010; Forbes-Lorman et al., 2012; Gantz et al., 2011; Kurian et al., 2008; Picker et al., 2006). The identification of MeCP2 targets in the neonatal brain would be very informative, as gene expression changes before the onset of overt phenotypes may contribute to disease etiology. A few years ago, several groups demonstrated that re-expression of MeCP2 in the postnatal brain caused a partial reversal of Rett Syndrome-like symptoms in MeCP2-null mice(Giacometti et al., 2007; Guy et al., 2007; Luikenhuis et al., 2004; Tropea et al., 2009). While there may be numerous technical and biological explanations for the partial phenotypic rescue, it remains possible that the loss of MeCP2 in early development causes irreparable damage to the nervous system. Overall, my research highlights a need for studies evaluating MeCP2 function and targets much earlier in development.

4.7 *Trans*-Chromosomal Interactions of the Imprinted Gene Network

Recent technological advancements have greatly enhanced our ability to query genome-wide 3D chromatin interactions and revealed that interactions between chromosomes may be important for gene regulation(Apostolou and Thanos, 2008; Brickner and Brickner, 2012; Spilianakis et al., 2005; Williams et al., 2010). For example, in erythroid cells the active alpha-globin and beta-globin genes associate with numerous actively transcribed

genes on other chromosomes(Schoenfelder et al., 2010). It was proposed that this interaction reflects co-localization with preferred transcription partners at transcription factories(Schoenfelder et al., 2010). Studies evaluating the *H19* ICR have found that in addition to *cis* interactions, the *H19* ICR also interacts in *trans* with other imprinted domains(Sandhu et al., 2009; Zhao et al., 2006). Many of these connections are cell type specific and depend on the presence of CTCF(Sandhu et al., 2009; Zhao et al., 2006). Together, the identification of the IGN and *trans* interactions of the *H19* ICR sparked a theory of IGN regulation through one central mechanism(Andrade et al. 2008; Kernohan and Bérubé, 2010; Lui et al., 2008; Sandhu et al., 2009; Varrault et al., 2006). As ATRX regulates CTCF occupancy at the *H19* ICR and IGN expression, we predicted that ATRX might regulate interactions between imprinted domains. Using 3D-FISH of cortical sections, I demonstrated that imprinted domains indeed co-localize in a small percentage of cells in the mouse forebrain (5-10%). Nevertheless, I did not detect a change in interaction frequencies in the ATRX-null brain. This relatively low co-localization frequency might reflect transient interactions, for example as a result of co-localization at a transcription factory. FISH experiments are limited in resolution by the probe size and microscopy visualization. It is still formally possible that despite co-localization on a macro scale, loss of ATRX causes changes in interactions between the *H19* ICR and specific IGN sites on a smaller scale. My results do not rule out the presence of a central mechanism which contributes to IGN regulation. However, I have established that if such a mechanism exists, it is independent of ATRX. Furthermore, as MeCP2 recruits ATRX to imprinted domains, I predict that this mechanism is also independent of MeCP2.

4.8 Implications of Aberrant Imprinted Gene Expression to Brain Development

Accumulating evidence suggests that imprinted genes are regulated as a developmental network which is highly expressed in embryogenesis and silenced in postnatal tissues(Andrade et al. 2010; Berg et al., 2011; Lui et al., 2008; Varrault et al., 2006). In the brain, I have shown that ATRX is required for this coordinated transcriptional program which represses expression of imprinted genes following neurogenesis. These

results suggest that ATRX may function to co-regulate groups of genes. In the future, it will be important to determine if this coordinated control of gene sets is a common mechanism of ATRX, or whether this is unique to imprinted genes.

Imprinted genes are overexpressed in the ATRX-deficient mouse brain, but we can only speculate at this point what the physiological ramifications might be. This question is further complicated for targets, like *H19*, which do not yet have a clear function. The *H19* gene locus produces a 2.3 kb non-coding RNA(Brannan et al., 1990), and a microRNA, miR-675(Cai and Cullen, 2007). miRNAs are a class of small non-coding RNAs (~22 nucleotides in length) which can function in transcriptional and post-transcriptional regulation(Lee and Vasudevan, 2013). Overexpression of miR-675 results in reduced cellular proliferation(Keniry et al., 2012) perhaps due to an interaction between miR-675 and the growth-promoting insulin-like growth factor 1 receptor transcript (*Igf1r*)(Keniry et al., 2012). In the context of neuronal development, an increase in miR-675 could lead to reduced neuronal proliferation and contribute to microcephaly. Later in development, this could result in reduced proliferation of neuronal stem cells in the dentate gyrus, which are important for learning and memory. It is unclear what effect elevated levels of *H19* would have. For other genes, the potential consequences of overexpression in the central nervous system are more obvious. For example, *Dlx5* encodes a protein required for neuronal migration and differentiation(Anderson et al., 1997; Stuhmer et al., 2002). As such, misexpression of *Dlx5* could signal aberrant neuronal organization, or improper differentiation into mature neurons. Overall, imprinted genes have diverse functions and their overexpression could potentially lead to defects in a number of essential neuronal processes, including cellular replication, fate and death(Davies et al., 2005).

Genetic or epigenetic abnormalities within a number of imprinted domains cause a group of developmental syndromes known as Imprinting Disorders. These disorders include Angelman Syndrome(Kishino et al., 1997; Matsuura et al., 1997), Prader–Willi Syndrome(Ledbetter et al., 1981; Miller et al., 2009; Muscatelli et al., 2000; Ren et al., 2003), Beckweith-Wiedemann Syndrome(DeBaun et al., 2002), and Turner

Syndrome(Kesler et al., 2003; McCauley et al., 1987; Skuse et al., 1997). Together these syndromes emphasize the importance of maintaining normal expression of imprinted genes during development(reviewed in (Wilkins and Ubeda, 2011)). Of these conditions, Prader-Willi Syndrome(Gunay-Aygun et al., 2001; Holm et al., 1993), Angelman Syndrome(Williams et al., 2006), and Turner Syndrome(Elsheikh et al., 2002; McCauley et al., 1987) result in abnormal neurodevelopment, and Beckweith-Wiedemann Syndrome is frequently associated with autism(Kent et al., 2008). The link between Imprinting Disorders and neurodevelopment ascertains that the aberrant expression of imprinted genes can disrupt brain development and function. This suggests that the misexpression of imprinted genes in the ATRX-null mouse brain may contribute to the neuronal deficiencies observed in our ATRX-null mouse model, including increased cell death and microcephaly(Bérubé et al., 2005; Seah et al., 2008). As ATRX-null mice die very early (P0.5-P30), it is not possible to determine if these mice have impaired neurological function, and any potential contributions of imprinted gene misexpression. To bypass early neurodevelopmental defects, an inducible ATRX-null mouse could be generated utilizing the Cre-ERT system in conjunction with the *Foxg1* promoter. Exposure of pups to tamoxifen just before birth (via mother), would delete ATRX in the mouse forebrain, prior to imprinted gene silencing. This would hopefully circumvent early lethality and facilitate analysis of ATRX deficiency in the mature brain.

In addition to congenital disorders, misregulation of imprinted genes has also been implicated in cancer. The loss of imprinting has been documented in many cancers, including chronic myeloid leukaemia (100%)(Randhawa et al., 1998), ovarian tumors (80%)(Kamikihara et al., 2005), Wilms' tumors (70%)(Mummert et al., 2005), colorectal cancer (66%)(Nakagawa et al., 2001; Ohlsson et al., 1999), renal-cell carcinomas (50%)(Oda et al., 1998), oesophageal cancer (50%)(Hibi et al., 1996) and lung adenocarcinoma (47-85%)(Kohda et al., 2001)(reviewed in (Jelinic and Shaw, 2007)). Additionally, patients with Beckwith-Wiedemann and Prader-Willi Syndromes are at higher risk for developing childhood cancers than the general population(Davies et al., 2003; Weksberg et al., 2010). This is not surprising as a large proportion of imprinted genes play roles in embryonic or placental growth(Morison et al., 2005). Imprinted genes

linked to cancer thus far include: *H19*, *IGF2, MEST, DCN, GTL2, KCNQ1*, and *CDKN1C* (reviewed in (Jelinic and Shaw, 2007) and (Uribe-Lewis et al., 2011)). Recently, *ATRX* mutations or aberrant expression has been identified in various cancer types(Bower et al., 2012; de Wilde et al., 2012; Jiao et al., 2012; Jiao et al., 2011; Kannan et al., 2012; Liu et al., 2012; Lovejoy et al., 2012; Schwartzentruber et al., 2012; Weisbrod et al., 2013). Amongst these, Liu et al identified a subtype of gliomas that harboured *ATRX* mutations, along with *IDH1/2* and *p53*(Liu et al., 2012). They evaluated the expression profile of these tumors and reported a list of up-regulated genes, which includes many imprinted genes(Liu et al., 2012). This observation, coupled with the aforementioned statistics on imprinted gene regulation and cancer, suggests that the misregulation of imprinted genes may contribute to tumorigenesis in ATRX-deficient cancers. The identification of imprinted genes as affected in ATRX-deficient tumors is important as therapies are in development targeting these genes, and would be available to treat ATRX-null cancers.

Taken together, the clinical data clearly indicate that imprinted genes play an essential role in neurodevelopment and tumorigenesis. Reports investigating imprinted gene expression in both the developmental disorders and cancers most often observe a loss of imprinting, effectively causing a two-fold increase in transcript levels. While the loss of ATRX does not reactivate expression of the silent parental allele, we observe a two fold increase in transcript levels of many imprinted genes, effectively recapitulating transcript levels produced by reactivation of a silent allele. Furthermore, the majority of imprinting disorders and cancer phenotypes result from aberrant expression of one imprinted domain. Given that ATRX loss of function affects the expression of various imprinted genes, it is difficult to predict their additive effects. To determine if imprinted genes factor into the etiology of ATR-X Syndrome, it would be imperative to test expression in mouse models recapitulating patient mutations and neuronal samples from ATR-X individuals. ATR-X Syndrome results from hypomorphic mutations and not a complete loss of ATRX protein (as in our mouse model)(Gibbons et al., 2008). Therefore, it is possible that imprinted genes would not be affected by patient ATRX mutations, which still retain some function. It is also possible that only a subset of mutations would affect the expression of imprinted genes; for example, mutations in the SWI/SNF chromatin

remodeling domain. In my proposed model for IGN regulation, the loss of SWI/SNF activity would lead to a failure to redistribute nucleosomes, thus resulting in improper *intra*chromosomal structure and gene expression. Furthermore, future investigations should also evaluate the status of imprinted transcripts in ATRX-deficient cancers. While it is often difficult to determine cause and effect in a tumor genome, there are numerous studies linking overexpression of imprinted genes to cancer formation(Jelinic and Shaw, 2007). Though the misexpression of imprinted genes is probably not the initiating even in ATRX-null cancers (alternative lengthening of telomeres (ALT) has been identified as a main contributor(Bower et al., 2012; de Wilde et al., 2012; Heaphy et al., 2011; Lovejoy et al., 2012; Nguyen et al., 2013)), it is possible that the expression of these transcripts facilitate growth of cancerous cells. If imprinted genes are affected in ATR-X patients and ATRX-null cancers, the identification of these genes as targets may facilitate the development of novel therapies.

4.9 Proposed Model and Remaining Questions

The body of work described in this thesis demonstrates that ATRX is required for proper postnatal silencing of the neuronal IGN. My data supports a model in which ATRX is recruited by MeCP2 to DMRs, where it governs nucleosome occupancy to maintain CTCF and Cohesin binding, ultimately leading to proper formation of local chromatin architecture and gene silencing. In the absence of ATRX, chromatin is in a more relaxed and open configuration, resulting in increased transcription of *H19* and *Igf2* (Figure 3-6). This model suggests that a developmental switch must be activated at DMR/ICR sequences to initiate transcriptional repression in the neonatal brain. This switch likely involves compacting the chromatin from a euchromatic to a heterochromatic state to initiate or maintain silencing. The loss of ATRX significantly compromises silencing of imprinted genes; however, these genes still undergo some level of repression in the ATRX-null brain(Kernohan, K., data not shown). It is therefore likely that ATRX is not the only protein involved in this developmental program, and that other yet unidentified factors are also required. ATRX could recruit other proteins, or has other yet unidentified functions leading to this effect. Why is it that CTCF and Cohesin need to be actively maintained in the postnatal brain, or, if maintenance is constantly required, what serves

this function embryonically? Perhaps different chromatin remodeling factors function at the *H19* ICR when the adjacent genes are highly expressed, or the more open chromatin state negates a need for active CTCF maintenance. We do not yet know what directs MeCP2 to this site, and at what time this occurs. Given the expression pattern of MeCP2, perhaps MeCP2 becomes enriched in the late embryonic/neonatal brain when protein levels have reached a particular threshold, but this idea requires experimental confirmation. It remains to be seen what changes drive postnatal repression. This could be one or more alteration in chromatin interactions, epigenetic effects, repressive protein recruitment or activator protein exclusion. IGN silencing occurs in many tissues, including the lung, kidneys and heart(Lui et al., 2008). We do not yet understand what signals IGN repression in other tissues. Is ATRX involved in IGN regulation in many cell types, or are there tissue specific proteins and regulatory systems? My data demonstrating maternal-specific ATRX binding at the *H19* ICR and the sites of decreased chromatin interactions (ICR-DMR1-MAR3 complex) suggests a maternal specific effect. However, it remains possible that the loss of ATRX affects both parental alleles. This could be due to yet unknown ATRX binding sites outside of the ICR on the paternal allele, or signalling between the alleles. Finally, it will be important to determine if the ATRX-MeCP2-Cohesin complex or ATRX or MeCP2 independently affect looping at other non-imprinted genes. In turn, this knowledge will be essential for the understanding of the function of these proteins

Overall, the work presented in this thesis has identified the first direct gene targets of the ATRX chromatin remodeling protein. The data shows that ATRX binds intergenic regulatory regions (DMRs and ICRs) with MeCP2 to regulate gene expression in an allele-specific manner. Using the *H19/Igf2* domain as a model, I demonstrated that ATRX is recruited by MeCP2 in the neonatal brain to regulate nucleosome positioning, and consequently CTCF/Cohesin binding and higher-order chromatin interactions. These ATRX-dependent chromatin interactions are required for the coordinated transcriptional repression of imprinted genes following neurogenesis in the mouse brain. The identification of additional ATRX gene targets and the mechanism of ATRX regulation throughout the

genome, including a potential role in governing chromatin architecture, will be imperative for a full understanding of the function of ATRX.

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Kernohan KD, Jiang Y, Tremblay DC, Bonvissuto AC, Eubanks JH, Mann MR and Bérubé NG (2010) ATRX partners with Cohesin and MeCP2 and contributes to developmental silencing of imprinted genes in the brain. *Developmental cell* **18**:191-202

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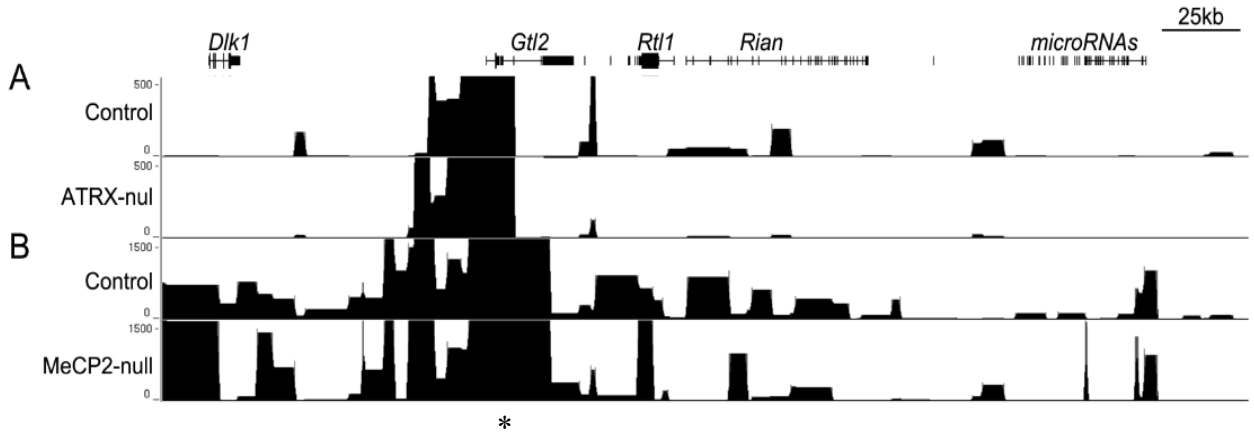
Appendix D: Preliminary 4C data supporting a role for ATRX and MeCP2 in the control chromatin looping at the *Gtl2/Dlk1* imprinted domain

4C interaction profile of the *Gtl2* DMR in neonatal control, $Atrx^{null}$ and $MeCP2^{null}$ forebrains demonstrates that the *Gtl2* DMR makes many contacts stretching from upstream of *Gtl2* to downstream of the microRNA cluster. These interactions are dependent on the presence of ATRX (A) and MeCP2(B).



Appendix E: Statement of permission for the use of animals for experimental research

All animal experimentation was conducted in compliance with the animal use protocol 2008-041-02 held by Dr. Nathalie Bérubé, principal investigator at the Schulich School of Medicine and Dentistry and the department of Paediatrics at the University of Western Ontario in London, Ontario, Canada.

Kristin D. Kernohan

Education

PhD Candidate, Western University (Formerly University of Western Ontario) September 2007-Present
- Thesis title: Regulation of imprinted genes in the brain by the ATRX chromatin remodeling protein
- Supervisor: Dr. Nathalie G. Bérubé

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- Honours thesis title: Analysis of the endoplasmic reticulum stress response in *Mist1* null mutant during pancreatitis
- Supervisor: Dr. Christopher L. Pin

Academic Awards and Funding

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Drs Madge and Charles Macklin Fellowship for Publication	2010 - 2011
Ontario Graduate Scholarship, PhD (declined)	2010 - 2011
International Rett Syndrome Symposium Graduate Student Award	2010
Western Graduate Thesis Award	2010
Ontario Graduate Scholarship, PhD	2009 - 2010
Curtis Cadman Graduate Studentship	2009 - 2010
NSERC CGS Michael Smith Foreign Study Supplements Program	2009
Western Graduate Thesis Award	2009
NSERC Canada Graduate Scholarship (CGS), MSc	2008 - 2009
Western Graduate Thesis Award	2008 - 2009
Paediatrics Graduate Scholarship, Children's Health Research Institute (CHRI)	2007 - 2008
Western Graduate Research Scholarship	2007 - 2012
Schulich Medicine and Dentistry Research Scholarship	2007 - 2012
CHRI Paediatrics Summer Scholarship	2007
Harvey F. Sullivan Scholarship - London Health Sciences Centre	2004 - 2005

Publications

Kernohan, KD and Bérubé, NG, Genetic and epigenetic dysregulation of imprinted genes in the brain. *Published Invited Review*, *Epigenomics* (2010), Volume 2(6), 743–763.

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*Highlighted in:

- Muers, M, Chromatin on the brain. *Nature Reviews Genetics* (2010).
- Muers, M, Chromatin on the brain. *Nature Reviews Genetics Neuroscience* (2010).
- Cunningham, MD. et al., Chromatin modifiers, cognitive disorders and imprinted genes. *Developmental Cell*. (2010), Volume 18, Issue 2, p 169-170.

Published Abstracts

Kernohan KD, Jiang, Y, and Bérubé, NG, ATRX regulates *H19/Igf2* expression through the control of higher order chromatin architecture in the neonatal brain. *Abstract*, Keystone Symposia on Epigenomics and Chromatin Dynamics, Keystone, Colorado, January 17-22, 2012.

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Close TE, Rose K L, **Kernohan KD**, Bérubé NG, Summers KL, Rieder MJ, Fraser D, Diabetic ketoacidosis in mice elicits a unique cytokine response between serum and brain. *Abstract*, 9th International Congress of Neuroimmunology, Fort Worth, Texas, October 29, 2008.

Invited International Presentations

Childhood disorders of chromatin dysfunction: Evidence for linked molecular pathways in imprinting control. *Invited Oral Presentation*, Texas A&M University, Developmental Biology Series, March 26, 2010. (Host: Dr. Michael Golding)

Presentations (*denotes presenter)

Kernohan KD*, Jiang, Y, and Bérubé, NG, ATRX regulates *H19/Igf2* expression through the control of higher order chromatin architecture in the neonatal brain:

- Oral Presentation: Developmental Biology Research Day, London, Ontario, May 24, 2012.
- Poster presentation: London Health Research Day, London, Ontario, March 20, 2012. ***Awarded best graduate student poster***
- Poster presentation: CHRI Paediatrics Research Day, London, Ontario, May 16, 2012.
- Poster presentation: Keystone Symposia on Epigenomics and Chromatin Dynamics, Keystone, Colorado, January 17-22, 2012.

Kernohan KD*, Jiang, Y, and Bérubé, NG, Chromatin architecture at the *H19* imprinted domain:

- Poster presentation: 1st Canadian Conference on Epigenetics, London, Ontario, May 4-7, 2011.
- Oral presentation: CHRI Paediatrics Research Day, London, Ontario, May 26, 2011. ***Awarded best senior graduate student presentation.***

Kernohan, KD*, Jiang, Y, Tremblay, DC, Bonvissuto, A, Eubanks, JH, Mann, MRW, Bérubé, NG, ATRX partners with cohesin and MeCP2 and contributes to developmental silencing of imprinted genes in the brain:

- Oral Presentation: Developmental Biology Research Day, London, Ontario, May 27, 2010.
- Poster Presentation: 11th Annual International Rett Syndrome Foundation Symposium, Leesburg, Virginia, June 27-29, 2010. ***Awarded best graduate student research***
- Poster presentation: CHRI Paediatrics Research Day, London, Ontario, May 27, 2010. ***Awarded best graduate***

student poster

- Poster presentation: Lawson Research Day, London, Ontario, March 26, 2010. ***Awarded best graduate student poster***

Kernohan, KD*, Tremblay, DC, Jiang, Y, and Bérubé, NG, Molecular analysis of imprinted genes in the ATRX-deficient forebrain. Oral Presentation: CHRI Paediatrics Research Day, London, Ontario, June 18, 2009.

Kernohan, KD*, Tremblay, DC, Jiang, Y, Mann, MRW, Bérubé, NG, The ATRX chromatin regulator interacts with cohesin and confers methylation-independent control of *H19* gene expression in the mouse forebrain. Poster presentation: Keystone Symposia: Epigenetics, Development and Human Disease, Breckenridge, Colorado, January 4-10, 2009.

Kernohan, KD*, Tremblay, DC, Jiang, Y, Mann, MRW, Bérubé, NG, Molecular analysis of imprinted genes in the ATRX-deficient forebrain:

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- Poster presentation: Lawson Research Day, London, Ontario, May 26, 2008.
- Poster presentation: CHRI Paediatrics Research Day, London, Ontario, May 21, 2008.
- Poster presentation: Margaret Moffatt Research Day, London, Ontario, May 20, 2008. ***Awarded best graduate student poster***

Professional Activities and Supervisory Experience

Student Representative, Developmental Biology Program Steering Committee 2010 - present

- Involved in student admissions, planning program initiatives and directions and organizing social and academic events.

Supervisor, Undergraduate Honours Projects (3 students) 2008 - present

- Assisted with project planning and conducting experiments. Edited research reports and presentations.

Student Representative, Developmental Biology Research Day Organizing Committee 2010

- Involved in event organization and speaker selection.