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Thyroxine-Dependent and -Independent Effects on Premature Aging and Myelination in ATRX Mutant Mice

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Abstract

ATRX is an ATP-dependent chromatin remodeler required to safeguard genomic integrity. Conditional deletion of *Atrx* in the mouse embryonic forebrain and anterior pituitary in $Atrx^{Foxg1Cre}$ mice phenocopies mouse models of progeria which display increased DNA damage, coupled with reduced lifespan, growth and subcutaneous fat. These mice also have severely low circulating levels of insulin like growth factor 1 (IGF-1) and (T4) which have been reported in models of premature aging. Based on evidence that *Igf1* is activated by the ligand-bound thyroid hormone receptor, I tested whether T4 supplementation could restore IGF-1 levels and ameliorate premature aging phenotypes in $Atrx^{FoxG1Cre}$ mice. However, restoration of normal serum T4 levels failed to rescue circulating IGF-1 levels, growth defects or reduced lifespan. This could be explained in part by an unexpected deletion of ATRX in hepatocytes where ATRX promotes thyroid hormone receptor mediated *Igf1* expression. In the second half of this thesis, I demonstrated that $Atrx^{FoxG1Cre}$ mice are also characterized by nervous system hypomyelination, another aging-related phenotype. Myelin is produced by oligodendrocytes and ensures rapid propagation of action potentials necessary for higher cognitive functioning. Systemic thyroxine supplementation improved myelin levels by promoting oligodendrocyte differentiation. However, myelin restoration was incomplete, as the number of oligodendrocyte precursor cells (OPCs) was still diminished, pointing to additional roles of ATRX in the generation or maintenance of these cells. Using directed inactivation of *Atrx* in postnatal OPCs or neurons, I established that ablation of ATRX in the former, but not the latter, results in hypomyelination. ATRX-null OPCs displayed a more plastic state *in vitro* and *in vivo*, allowing a shift from a strict oligodendrocyte differentiation program toward ectopic astrogliogenesis, ultimately leading to hypomyelination. Mechanistically, I provide evidence that ATRX associates with the histone deacetylase HDAC3 in OPCs to promote transcription of the *Olig2* fate specification gene. Collectively this study identified thyroxine-dependent and -independent effects of *Atrx* gene activation on premature aging-like phenotypes in mice.

Keywords

ATRX, premature aging, thyroid hormone, thyroxine, IGF-1, gene regulation, liver, myelination, glial cell fate, lineage plasticity, Olig2, HDAC3

Summary for Lay Audience

ATRX is a protein that alters the way DNA is packaged in cells. This function is necessary to prevent DNA damage and ensure the proper development of many different cell types and tissues. Mouse models can be used to study the function of ATRX in development by genetically removing the *Atrx* gene. Deletion of *Atrx* from the mouse brain and pituitary caused symptoms that appear in other mouse models of premature aging. These abnormalities included a reduction in growth, fat, glucose levels and lifespan. ATRX deficient mice also exhibited low levels of the thyroid hormone thyroxine and insulin-like growth factor 1 (IGF-1), as seen in other premature aging models and both of which are necessary for growth and development. Others have reported that thyroid hormone can increase IGF-1 levels to promote growth and development. With this in mind, we thought that supplementing ATRX deficient mice with thyroid hormone may rescue these harmful developmental symptoms by increasing IGF-1 levels. However, due to inherent problems with genetic manipulation in mice, we found that ATRX was unexpectedly absent in the liver. The liver is the primary site for production of IGF-1 and thyroid hormone was unable to increase IGF-1 levels due to the absence of ATRX in the liver. Excitingly, thyroid hormone treatment rescued hypomyelination in ATRX-deficient mice. The cell type in the brain that produces myelin is the oligodendrocyte which insulates neurons to speed up electrical current in the brain. However, the rescue in myelin was only partial, leading us to investigate whether ATRX is important in oligodendrocytes to produce myelin. Using another mouse model, ATRX was specifically deleted in oligodendrocytes. In this model, we found that ATRX is necessary for the development of oligodendrocytes and in its absence, these cells become another brain cell type called astrocytes, ultimately leading to reduced myelin in the brain. Collectively, this study has identified a role for ATRX in the prevention of premature aging, both dependent and independent of the thyroid hormone thyroxine.

Co-Authorship Statement

I designed, conducted, analyzed and interpreted all experiments presented in this thesis with the following exceptions:

In chapter two, qRT-PCR was completed in collaboration with Yan Jiang in Figure 2-3 and 2-4.

In chapter three, Dr. Mike Levy performed the RNA isolations for the microarray analysis. Matt Edwards generated the western blot in Figure 3-1 and Yan Jiang the western blot in figure 3-2. Dr. Sarfraz Shafiq performed the FANs sorting to obtain purified nuclei as described in Figure 3-4. Miguel Pena-Ortiz designed and optimized the protocol for INTACT used to isolate nuclei for qRT-PCR analysis in Figures 3-4, 3-5 and 3-6.

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Abbreviations

Abbreviation	Meaning
°C	Degrees Celsius
µg	Microgram
µL	Microliter
µm	Micrometer
Ab	Antibody
ADD	ATRX-DNTM3-DNTM3L
Ag	Antigen
ANOVA	Analysis of variance
aPAR	Ancestral pseudoautosomal region
APC	Adenomatous polyposis coli
ATAC	Assay for transposase-accessible chromatin
ATMDS	α-thalassemia myelodysplasia syndrome
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate synthase
ATRX	ATRX Chromatin Remodeler
ATR-X	Alpha-Thalassemia X-Linked Intellectual Disability Syndrome
ATRXt	Truncated form of ATRX Chromatin Remodeler
BHLH	Basic-helix-loop-helix
BRCA1	BRCA1 DNA Repair Associated
Brg1	Brahma related gene 1
CBP	CREB-binding protein
CC	Corpus callosum
cDNA	Complimentary DNA
CHD7	Chromodomain helicase DNA binding protein 7
CHD8	Chromodomain helicase DNA binding protein 8
ChIP	Chromatin immunoprecipitation
cKO	Conditional knockout
CNPase	2,3'-Cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CO_2	Carbon dioxide
cRNA	Complimentary RNA
Csf2rα	Colony stimulating factor 2 receptor, alpha
Cspg4	Chondroitin sulfate proteoglycan
Ct	Cycle threshold
CTCF	CCCTC binding factor
Ctx	Cortex

d	Days
D	Dermis
DAPI	4′,6-diamidino-2-phenylindole
DAXX	Death domain associated protein 6
Dhrsx	Dehydrogenase/reductase (SDR family) X-linked
DID	DAXX interacting domain
Dio1	Type 1 deiodinase
Dio2	Type 2 deiodinase
Dio3	Type 3 deiodinase
DIV	Days *in vitro*
dL	Decilitre
DNA	Deoxyribonucleic acid
DNMT3	DNA methyltransferase 3
DNMT3L	DNA methyltransferase 3 like
dNTP	Nucleoside triphosphate
DSB	Double strand breaks
DTT	Dithiothreitol
E#	Embryonic day #
ECL	Enhanced chemiluminescent
EGS	Ethylene glycol bis(succinimidyl succinate)
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERCC	DNA excision repair protein
EZH2	Enhancer of zeste homolog 2
FAM126A	Family with sequence similarity 126 member A
FANS	Fluorescent activated nuclear sorting
FBS	Fetal bovine serum
FoxG1	Forkhead box G1
g	Grams
GATA	GATA Binding Protein
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GH	Growth hormone
Ghr	Growth hormone receptor
GJA1	Gap junction protein alpha 1, Connexin 43
GJC2	Gap junction protein gamma 2; Connexin 47
Gpr17	G protein-coupled receptor
H&E	Hematoxylin and Eosin
H3	Histone variant H3
H3.3	Histone variant H3.3
H3.3S31	Histone H3.3 serine 31

H3K27	Histone 3 lysine 27
H3K36me3	Histone 3 lysine 36 trimethylation
H3K4me0	Histone 3 lysine 4 unmethylated
H3K9me3	Histone 3 lysine 9 trimethylation
HAT	Histone acetyltransferase
HDAC	histone deacetylase
HeLa	Henrietta Lacks (cervical cancer cells)
Hes	Hes family bHLH transcription factor
HGPS	Hutchinson-Gilford progeria syndrome
HP1α	Heterochromatin protein 1, alpha
HR	Homologous recombination
HRP	Horseradish peroxidase
HSD	Honestly significant difference
HU	Hydroxyurea
ICR	Imprinting control region
ID	Intellectual disability
Id2	Inhibitor of DNA binding 2
Id4	Inhibitor of DNA binding 4
IGEPAL-630	Octylphenoxypolyethoxyethanol
IGF-1	Insulin-like growth factor 1
IGF-1R	IGF-1 receptor
Igf2	Insulin-like growth factor 2
Igf2r	Igf2 receptor
IGFALS	IGF acid-labile subunit
IGFBP	IGF binding proteins
INTACT	Isolation of nuclei tagged in specific cell types
IP	Immunoprecipitation
IRS	Insulin receptor substrates
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
Ki67	Marker f proliferation Ki-67
KOH	Potassium hydroxide
LAT	L-type amino acid transporter
LMNA	Lamin A
Lox	Lysyl oxidase
M	Molar
MAG	Myelin associated glycoprotein
MBD2	Methyl-CpG binding domain protein 2
MBP	Myelin basic protein
MCT	Monocarboxylate transporters

MeCP2	Methyl-CpG-binding protein
mg	Milligram
$MgCl_2$	Magnesium chloride
mins	Minutes
mM	Millimolar
MOG	Myelin oligodendrocyte glycoprotein
MRE11	MRE11 homolog, double strand break repair nuclease
Mrf	Myelin regulatory factor
MRN Complex	MRE11-RAD50-NBS1
mtDNA	Mitochondrial DNA
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NaF	Sodium fluoride
NBS1	Nijmegen breakage syndrome 1
NCOR	Nuclear receptor corepressor
NER	Nucleotide excision repair
NeuN	Neuronal nuclei
Neurod6	Neuronal differentiation 6
Nex	Neuronal differentiation 6
NG2	Nanogram
NG2	Neural glial antigen 2
NHEJ	Non-homologous end joining
Nkx2.2	NK2 Homeobox 2
Nlgn 4	Neuroligin 4
NP-40	Nonyl phenoxypolyethoxylethanol 40
NPC	Neural progenitor cell
OATP	Organic anion transporter
OL	Oligodendrocyte
Olig1	Oligodendrocyte transcription factor 1
Olig2	Oligodendrocyte transcription factor 2
OMIM	Online Mendelian Inheritance in Man
OPC	Oligodendrocyte precursor cell
p	Probability value
P#	Postnatal day #
p53	Tumour suppressor protein 53
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
Pdgfrα	Platelet derived growth factor receptor, alpha
PFA	Paraformaldehyde

pH	Potential of hydrogen
Pit-1	POU domain, class 1, transcription factor 1
PLP	Proteolipid protein
PML bodies	Promyelocytic leukemia nuclear bodies
PMSF	Phenylmethylsulfonyl fluoride
POLR	RNA polymerase III
PRC2	Polycomb repressive complex 2
PriOPCs	Primitive OPCs
Prlr	Prolactin receptor
qRT-PCR	Quantitative reverse transcriptase-PCR
RAD50	RAD50 double strand break repair protein
rDNA	Ribosomal DNA
RFP	Red fluorescent protein
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
rT3	Reverse T3; 3,3,5'-triiodothyronine
RXR	Retinoid X receptor
S100β	S100 calcium-binding protein, β
SC	Subcutaneous
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SETDB1	SET Domain Bifurcated Histone Lysine Methyltransferase 1
SF	Subcutaneous fat
SIRT	Sirtuin
SmarcA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin
SNF2	Sucrose non-fermenting 2
Sox10	SRY-Box Transcription Factor 10
Sox9	SRY-Box Transcription Factor 9
Sp1	Sp1 transcription factor
SRC	Steroid receptor coactivator
SVZ	Subventricular zone
SWI/SNF	Switch/sucrose non-fermenting
T2	3,5-Diiodothyronine
T3	Triiodothyronine; 3,5,3' triiodothyronine
T4	Thyrxoine; L-3,5,5'-tetraiodothyronine
Tam	Tamoxifen
TBG	Thyroxine binding globulin

Tbl1	Transducin (beta)-like 1
TR	Thyroid hormone receptor
Thrsp	Thyroid hormone responsive protein
TRE	Thyroid hormone response element
TRIM28	Tripartite Motif Containing 28
Tris	Tris(hydroxymethyl)aminomethane
TTR	Transthyretin
VNTR	Variable number tandem repeat
WS	Werner syndrome
XPF	Xeroderma pigmentosum group F-complementing protein
Zfp24	Zinc finger protein 24
ZMPSTE	Zinc Metallopeptidase STE24
ZNF274	Zinc finger protein 274

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Chapter 1

1.1 ATRX is a chromatin remodeling protein essential for mouse and human development

1.1.1 The *ATRX* gene and protein

The *ATRX* gene is located on the long arm of the X chromosome (Xq21.1), contains 36 exons and spans approximately 300 kb of genomic DNA sequence. Mutations in *ATRX* were originally identified as the cause of a rare, X-linked syndrome, characterized by psychomotor delay, characteristic facial features, genital abnormalities, and alpha-thalassemia (ATR-X Syndrome; OMIM 301040) (Gibbons, Picketts et al. 1995). The ATRX protein is a large (280 kDa), nuclear protein that specifically localizes to heterochromatin (McDowell, Gibbons et al. 1999). In addition to the full length 280 kDa ATRX protein, characterization of *ATRX* revealed it gives rise to at least two other alternative transcripts. An alternative splicing of exon 6 results in a 265 kDa protein (Picketts, Higgs et al. 1996). The third transcript, ATRXt results from a premature stop codon, failure to remove intron 11 and C-terminal truncation producing a 180 kDa protein (Bérubé, Smeenk et al. 2000, Garrick, Sharpe et al. 2006). It has been proposed that ATRXt regulates the full-length ATRX protein as the two interact with one another (Garrick, Sharpe et al. 2006).

Chromatin remodeling primarily involves histone modifying enzymes and ATP-dependent chromatin remodeling complexes that alter chromatin accessibility. ATRX falls into the latter category, using ATP to change the association between DNA and histones by removing, replacing or changing the position of nucleosomes. ATRX is a member of the SNF2 Switch/Sucrose non-fermenting (SWI/SNF) protein superfamily that often functions within large multi-subunit complexes to remodel chromatin via ATP hydrolysis (Picketts, Higgs et al. 1996). ATRX shows sequence similarities to Rad54, a translocase protein involved in nucleotide excision repair and the initiation of transcription (Stayton, Dabovic et al. 1994), as well as other SWI/SNF proteins involved

in transcriptional regulation, recombination, replication and DNA repair (Picketts, Higgs et al. 1996).

ATRX contains two highly conserved domains important for its function (Picketts, Tastan et al. 1998). The N-terminus of ATRX contains an ADD domain (ATRX-DNMT3-DNMT3L) which has a unique combination of a GATA-like zinc finger, a plant homeodomain and an alpha-helical region that fold to form a single globular domain (Argentaro, Yang et al. 2007) (Figure 1-1A). Furthermore, the ADD region shows homology to the DNMT3 DNA methyltransferase family responsible for *de novo* methylation during development (Xie, Wang et al. 1999, Aapola, Shibuya et al. 2000). ADD is able to bind K4me0/K9me3 histone H3 tails within chromatin (Dhayalan, Tamas et al. 2011, Eustermann, Yang et al. 2011, Iwase, Xiang et al. 2011) (Figure 1-1B). The C-terminus of ATRX contains a SWI/SNF domain which provides the ATPase and translocase activity of ATRX necessary for nucleosome remodeling (Xue, Gibbons et al. 2003, Tang, Wu et al. 2004) (Figure 1-1A). ATRX has several other domains that facilitate its functions at chromatin. ATRX can associate with heterochromatin protein HP1α via a variant PxVxL motif (LYVKL) (Le Douarin, Nielsen et al. 1996, Bérubé, Smeenk et al. 2000, Lechner, Schultz et al. 2005) (Figure 1-1A). ATRX has also been shown to bind enhancer of zeste homolog 2 (EZH2) (Cardoso, Timsit et al. 1998, Sarma, Cifuentes-Rojas et al. 2014), however, recently their interaction could not be confirmed (Qadeer, Valle-Garcia et al. 2019). Near the middle of the ATRX protein, there is a death domain associated protein 6 (DAXX) interacting domain (DID) (Tang, Wu et al. 2004) (Figure 1-1A). Within the conserved ATPase domain there is a methyl-CpG-binding protein (MeCP2) binding domain (Nan, Hou et al. 2007) (Figure 1-1A). Lastly, there is a promyelocytic leukemia nuclear body (PML) targeting domain at the C-terminus of ATRX (Xue, Gibbons et al. 2003, Berube, Healy et al. 2008) (Figure 1-1A).

1.1.2 ATRX localizes to heterochromatin

Chromatin is a highly ordered structure comprised of DNA, RNA, histones and other proteins (Kouzarides 2007). Euchromatin contains transcriptionally active, hyperacetylated, hypomethylated chromatin (Kouzarides 2007). In contrast,

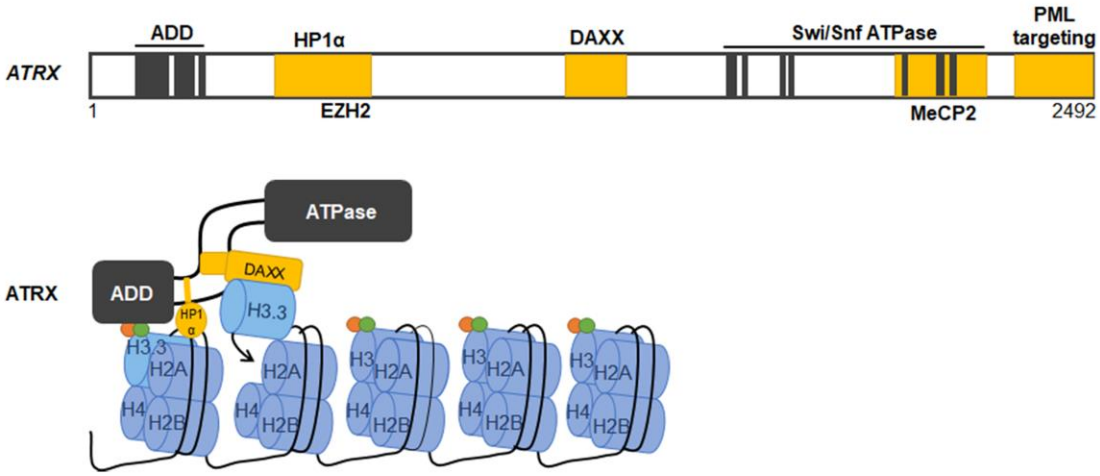

Figure 1-1 The structure and function of the ATRX protein

(a) Structure of the ATRX protein. Conserved domains are displayed in grey and protein interaction sites are indicated in yellow. (b) ATRX function at chromatin. ATRX is targeted to chromatin by binding histone H3 containing H3K9me3 and H3K4me0 (orange and green circles) chromatin marks via the N-terminal ADD domain and its interaction with heterochromatin protein 1 α (HP1α) via the LxVxL motif. ATRX interacts with the H3.3 chaperone DAXX through the DAXX interacting domain (DID) and facilitates the deposition of the histone variant H3.3 into chromatin via its C-terminal ATPase domain.

heterochromatin is transcriptionally inactive and is in large part hypoacetylated and hypermethylated (Kouzarides 2007). ATRX is an exclusively nuclear protein and localizes to the nuclear matrix during interphase (Bérubé, Smeenk et al. 2000). As the cell enters mitosis, ATRX is phosphorylated (Bérubé, Smeenk et al. 2000) and localizes to pericentromeric heterochromatin where it interacts with HP1α (McDowell, Gibbons et al. 1999, Bérubé, Smeenk et al. 2000, Lechner, Schultz et al. 2005). Heterochromatin contains an abundance of the repressive histone mark H3K9me3 and evidence supports that the ADD domain and the interaction of ATRX with HP1α contribute to recruitment to heterochromatin (Eustermann, Yang et al. 2011) (Figure 1-1B). Loss of HP1α results in delocalized ATRX from pericentric heterochromatin (Kourmouli, Sun et al. 2005). In addition, mutation of the LxVxL HP1 motif or the ADD domain disrupts ATRX localization to heterochromatin, suggesting that both interactions contribute to recruitment (Eustermann, Yang et al. 2011). ATRX can also bind another chromatin remodeler, MeCP2, through its C-terminal ATPase domain (Nan, Hou et al. 2007) (Figure 1-1A). In MeCP2-deficient neurons ATRX assumes a dispersed nuclear distribution, suggesting MeCP2 is required for heterochromatin localization of ATRX in neurons (Nan, Hou et al. 2007).

ATRX is enriched at heterochromatic repetitive sequences like telomeres, ribosomal DNA and pericentromeric major satellite repeats (McDowell, Gibbons et al. 1999, Gibbons, McDowell et al. 2000, Law, Lower et al. 2010). Furthermore, ATRX localizes to the heterochromatic inactive X chromosome (Baumann and De La Fuente 2009) as well as the pericentric heterochromatin of the Y chromosome in neonatal spermatogonia (Baumann, Schmidtmann et al. 2008). ATRX also maintains H3K9me3 levels at atypical chromatin signatures with high levels of H3K9me3 and H3K36me3 in complex with the H3K9me3-specific histone methyltransferase SETDB1 as well as TRIM28 and ZNF274 (Valle-Garcia, Qadeer et al. 2016).

ATRX is able to bind G-rich sequences that have a propensity to form bulky secondary structures called G-quadruplexes (Law, Lower et al. 2010) as well as RNA-DNA hybrids known as R-loops (Nguyen, Voon et al. 2017). These secondary structures can inhibit

accurate DNA replication and proper gene expression and tend to form at telomeres (Law, Lower et al. 2010, Nguyen, Voon et al. 2017). Evidence points to a role for ATRX in suppressing these secondary structures to allow the replication machinery to easily access DNA and preserve DNA replication (Law, Lower et al. 2010, Watson, Solomon et al. 2013, Levy, Kernohan et al. 2015, Nguyen, Voon et al. 2017).

1.1.3 ATRX forms a chromatin remodeling complex with DAXX

Early proteomics studies reported that DAXX forms a stable, DNA-independent complex with ATRX and that the majority of both proteins are usually in complex together (Xue, Gibbons et al. 2003). DAXX was originally identified as a mediator of apoptosis (Yang, Khosravi-Far et al. 1997) but other studies speculated it may have a role in transcriptional repression (Torii, Egan et al. 1999). Indeed, DAXX was shown to repress basal gene expression in HEK293 cells and regulate both gene activation and repression in B cell lines (Li, Pei et al. 2000, Emelyanov, Kovac et al. 2002).

Together ATRX and DAXX form a complex that deposits histone variant H3.3 at telomeres, pericentromeric heterochromatin and rDNA (Drane, Ouararhni et al. 2010, Goldberg, Banaszynski et al. 2010, Lewis, Elsaesser et al. 2010) (Figure 1-1B). ATRX and DAXX interact through the DID located between the ADD and ATPase domains of ATRX (Tang, Wu et al. 2004) (Figure 1-1B). DAXX is responsible for targeting ATRX to PML bodies (Tang, Wu et al. 2004) and acts as a chaperone for H3.3 (Drane, Ouararhni et al. 2010, Lewis, Elsaesser et al. 2010). ATRX provides the ATPase activity and is able to alter DNA-histone interactions and stimulate translocation of histones (Xue, Gibbons et al. 2003, Tang, Wu et al. 2004) (Figure 1-1B). Histone H3.3 is unique in that it is deposited in a replication-independent manner (Ahmad and Henikoff 2002). H3.3 can be deposited at actively transcribed genes (Ahmad and Henikoff 2002), aiding transcription through ease of nucleosome eviction potentially due to the lower energy cost of H3.3 ejection (Drane, Ouararhni et al. 2010). Additionally, the ATRX-DAXX complex acts in a specialized chromatin assembly pathway at repetitive regions and deposition of H3.3 at these sites helps to maintain their repetitive structure (Drane,

Ouararhni et al. 2010, Goldberg, Banaszynski et al. 2010, Lewis, Elsaesser et al. 2010, Levy, Kernohan et al. 2015).

1.1.4 ATRX maintains genomic integrity

ATRX is recruited to heterochromatin and telomeres during S phase (Wong, McGhie et al. 2010, Huh, Price O'Dea et al. 2012, Watson, Solomon et al. 2013). Depletion of ATRX results in an increased DNA damage response (Watson, Solomon et al. 2013, Clynes, Jelinska et al. 2014, Valle-Garcia, Qadeer et al. 2016), double strand breaks (DSBs) at telomeres (Wong, McGhie et al. 2010, Huh, Price O'Dea et al. 2012, Watson, Solomon et al. 2013) and prolonged S phase (Huh, Price O'Dea et al. 2012, Leung, Ghosal et al. 2013, Clynes, Jelinska et al. 2014). Telomeres have many kilobases of the repeat sequence $(TTAGGG)_n$ and these tandem repeats have the propensity to form secondary structures like G-quadruplexes and R-loops which form in the presence of G-rich DNA (Parkinson, Lee et al. 2002, Lipps and Rhodes 2009). ATRX is recruited to R-loops and cells deficient for ATRX accumulate R-loops, revealing a role for ATRX in their suppression (Nguyen, Voon et al. 2017). When G-quadruplexes are drug-stabilized in ATRX-null neuroprogenitor cells, there is an increase in DNA damage and reduced cell viability (Watson, Solomon et al. 2013). ATRX-null cells are also hypersensitive to hydroxyurea (HU) treatment, a drug that inhibits DNA synthesis and causes replication fork stalling (Watson, Solomon et al. 2013). Treatment of ATRX-deficient cells with HU results in an increase in stalled replication forks, slow replicating forks and a reduction in new origin firing (Leung, Ghosal et al. 2013, Clynes, Jelinska et al. 2014) indicating that ATRX is involved in the restart of stalled replication forks and required for efficient DNA replication (Clynes, Jelinska et al. 2014).

The MRN complex is essential for DSB repair and the restart of stalled replication forks (Robison, Elliott et al. 2004). ATRX and DAXX are both able to directly bind three proteins in the MRN complex: RAD50, MRE11 and NBS1 (Clynes, Jelinska et al. 2014). Furthermore, ATRX co-localizes with the MRN complex during S phase (Clynes, Jelinska et al. 2014). Homologous recombination (HR) is required for the removal of G-quadruplexes (Feng and Zhang 2012) and HR proteins RAD51 and BRCA1 are critical

for the protection of replication forks (Feng and Zhang 2012). In the absence of ATRX, RAD51 and BRCA1 colocalization is perturbed (Huh, Ivanochko et al. 2016). Subsequently it was shown that ATRX is required for the sequestration of MRE11 (Clynes, Jelinska et al. 2015) to inhibit its exonuclease activity. In the absence of ATRX, rampant MRE11 activity may deplete RAD51 pools leading to stalled replication forks, excessive DNA damage and delayed S phase (Huh, Ivanochko et al. 2016). In addition, it has been demonstrated that ATRX incorporates H3.3 during extended DNA repair synthesis, a form of HR during DNA repair (Juhász, Elbakry et al. 2018). This evidence suggests that ATRX facilitates the replication of complex secondary structures by depositing H3.3 during post replicative repair (Ray-Gallet, Woolfe et al. 2011, Clynes, Jelinska et al. 2015, Juhász, Elbakry et al. 2018).

ATRX becomes hyperphosphorylated during mitosis, indicating that it may acquire specialized functions at the onset of the cell cycle (Bérubé, Smeenk et al. 2000). ATRX is essential for mitotic (Ritchie, Seah et al. 2008, Ritchie, Watson et al. 2014) and meiotic (Baumann, Viveiros et al. 2010, De La Fuente, Baumann et al. 2011) integrity. Deletion of *Atrx* in neuroprogenitor cells results in micronuclei and dispersed chromosomes indicating abnormal chromosome segregation (Ritchie, Seah et al. 2008). In addition, deletion of *Atrx* in the forebrain causes metaphase chromosome fusions and telomere defects (Watson, Solomon et al. 2013). Following *Atrx* deletion in the mouse forebrain, there is extensive genomic instability that contributes to massive p53-mediated apoptosis (Berube, Mangelsdorf et al. 2005, Seah, Levy et al. 2008). The cells that survive undergo premature cell cycle exit during early neurogenesis and premature differentiation, resulting in irregular neocortical layering (Ritchie, Watson et al. 2014). Similarly, oocytes lacking ATRX display abnormal meiotic chromosome segregation, and chromosome instability with a high incidence of aneuploidy (Baumann, Viveiros et al. 2010, De La Fuente, Baumann et al. 2015).

1.1.5 ATRX regulates gene expression

ATRX has been implicated in both the activation and repression of gene expression. ATRX inhibits the gene expression of several imprinted genes in the brain (Kernohan,

Jiang et al. 2010, Kernohan, Vernimmen et al. 2014), in the liver (Chatzinikolaou, Apostolou et al. 2017) and in embryonic stem cells (Voon, Hughes et al. 2015). In the newborn mouse cortex, the methyl binding protein MeCP2 is required for recruitment of ATRX, cohesin and the insulator protein CTCF (CCCTC binding factor) to mono-allelically suppress several imprinted genes (Kernohan, Jiang et al. 2010). Following embryonic deletion of *Atrx* there is an alteration in nucleosome configuration at CTCF-binding sites of the *H19* imprinting control region, resulting in reduced CTCF occupancy and abnormal long-range chromatin interactions within the *H19-Igf2* imprinted locus (Kernohan, Vernimmen et al. 2014). Intriguingly, there is evidence suggesting that ATRX and CTCF-cohesin also silence imprinted genes in the liver (Chatzinikolaou, Apostolou et al. 2017). Deletion of *Ercc1*, part of the ERCC1-XPF complex involved in nucleotide excision repair (NER), prompts the dissociation of CTCF-cohesin and ATRX from promoters, resulting in abnormal activation of imprinted genes during liver development (Chatzinikolaou, Apostolou et al. 2017). This inability to suppress the expression of imprinted genes in the absence of ATRX may have detrimental effects on both liver and brain development.

The role of ATRX in gene activation has been studied much more extensively. One of the first clues to suggest ATRX may activate transcription was observed in ATR-X syndrome patients, who display mild α-thalassemia, indicating decreased α-globin gene expression in patients (Gibbons and Higgs 2000). Additionally, somatic mutations in *ATRX* can cause α-thalassemia myelodysplasia syndrome (ATMDS) which results in α-thalassemia in association with a myelodysplasia (Gibbons, Pellagatti et al. 2003, Steensma, Higgs et al. 2004). ATRX has been shown to negatively regulate the histone variant macroH2A and *Atrx* deletion in human erythroleukemic cells results in macroH2A accumulation at the α-globin gene cluster and a subsequent reduction in α-globin expression (Ratnakumar, Duarte et al. 2012). Furthermore, the human α-globin cluster is in very close proximity to the telomere on human chromosome 16 and ATRX is known to bind telomeres (Law, Lower et al. 2010). Chromatin immunoprecipitation (ChIP) sequencing in human erythroblasts revealed ATRX binding in the α-globin locus within a variable number tandem repeat (VNTR) comprised of GC rich DNA (Law, Lower et al. 2010). Interestingly, the severity of gene repression correlated with increased length of the

VNTR (Law, Lower et al. 2010) and G-quadruplex formation within the VNTR (Li, Syed et al. 2016).

Deletion of *Atrx* in the mouse forebrain embryonically, results in many genes with decreased expression (Levy, Fernandes et al. 2008). The most severely downregulated genes in the ATRX-null mouse forebrain are ancestral pseudoautosomal region (aPAR) genes (Levy, Fernandes et al. 2008) which are rich in repetitive sequence (Gianfrancesco, Sanges et al. 2001). Two of the most affected aPAR genes, dehydrogenase/reductase (SDR family) X-linked (*Dhrsx*) and colony stimulating factor 2 receptor, alpha (*Csf2rα*), are located near the subtelomeric regions of mouse chromosomes 4 and 19 respectively, similar to the α-globin cluster (Levy, Fernandes et al. 2008). Further investigations showed that in the mouse brain, ATRX and H3.3 are enriched at G-rich regions of several aPAR genes and deletion of *Atrx* results in decreased H3.3 incorporation in nucleosomes of the gene bodies and impairment of RNA polymerase II progression (Levy, Kernohan et al. 2015). Interestingly, ATRX recruitment to G-rich repeats is dependent on the presence of R-loops at repeats that are actively being transcribed (Nguyen, Voon et al. 2017). Similar to its function in DNA replication, it appears that ATRX deposits H3.3 to facilitate transcriptional elongation at G-rich genes prone to G-quadruplex formation that present challenges for RNA polymerase II progression.

In mouse testes, ATRX is a co-factor for the nuclear hormone receptor androgen receptor (AR) (Bagheri-Fam, Argentaro et al. 2011). When *Atrx* is deleted in Sertoli cells there is a downregulation in the AR target gene *Rhox5* (Bagheri-Fam, Argentaro et al. 2011). Indeed, ATRX can enhance *Rhox5* promoter activity in the presence of AR in the TM4 Sertoli cell line (Bagheri-Fam, Argentaro et al. 2011). Comparably, when *Atrx* is deleted from the mouse forebrain and anterior pituitary early in development, there is a decrease in thyroid hormone responsive gene expression indicating that ATRX may play a role in mediating nuclear hormone receptor gene activation (Watson, Solomon et al. 2013).

1.1.6 Hypomorphic mutations in *ATRX* cause ATR-X syndrome

The *ATRX* gene was originally discovered because mutations cause a rare X-linked disorder (Weatherall, Higgs et al. 1981, Gibbons, Wilkie et al. 1991) characterized by intellectual disability, developmental abnormalities and α-thalassemia (ATR-X syndrome; OMIM #301040) (Gibbons, Picketts et al. 1995). The most recent analysis reports over 200 cases from 182 families with 113 different mutations (Gibbons, Wada et al. 2008). The affected individuals are almost exclusively male with female carriers displaying skewed X-inactivation protecting them from symptoms (Gibbons, Suthers et al. 1992). Pregnancy occurs normally and birth weight is normal in 90% of patients (Gibbons and Higgs 2000). During the neonatal period, ATR-X syndrome babies have severe hypotonia, feeding difficulties, abnormal movement and seizures (Gibbons and Higgs 2000).

Intellectual disability is the most prevalent symptom in ATR-X syndrome. In a comprehensive analysis of 145 cases from more than 80 different families, 95% display intellectual disability, 75% have microcephaly and 35% that have seizures (Gibbons and Higgs 2000). There tends to be a wide spectrum of severity when it comes to intellectual delay, however, most patients are characterized by delayed milestones, limited speech, situational understanding and display a high degree of dependence (Gibbons and Higgs 2000). Brain imaging has allowed the diagnosis of white matter abnormalities (40%) (Wada, Ban et al. 2013, Lee, Lee et al. 2015) as well as nonspecific brain atrophy in 63% in patients analyzed (Wada, Ban et al. 2013). There is a high prevalence of facial (94%), skeletal (91%), genital (80%) and gut (75%) abnormalities (Gibbons and Higgs 2000). Short stature (66%), cardiac defects (18%) and renal/urinary anomalies (14%) are less common developmental symptoms of ATR-X syndrome (Gibbons and Higgs 2000). Lastly, the eponymous α-thalassemia is not present in all patients diagnosed with ATR-X syndrome (Ion, Telvi et al. 1996, Villard, Lacombe et al. 1996), occurring in 87% of patients (Gibbons and Higgs 2000).

ATR-X syndrome's mutation spectrum is diverse, with mutations grouped around the highly conserved ADD (50%) and ATPase (30%) domains (Gibbons, Wada et al. 2008).

The majority of the mutations in *ATRX* are missense (Gibbons, Wada et al. 2008). Two common mutations 536A>G and 736C>T stand out in 9 and 35 families, respectively (Gibbons, Wada et al. 2008). It has been speculated that complete absence of *ATRX* may be lethal as mutations that cause ATR-X syndrome are hypomorphic (Gibbons, Wada et al. 2008). Interestingly, even in the case of nonsense mutations, there is mutation skipping (Howard, Malik et al. 2004) which allows phenotypic rescue and is present for all stop codons upstream of the ATPase domain (Gibbons, Wada et al. 2008).

1.1.7 ATRX is required for proper development

Abnormal ATRX expression or activity results in severe developmental abnormalities, as demonstrated by the associated syndrome. Mouse models have allowed the elucidation of ATRX's function in specific organ systems. First, gain of function studies allowing over expression of ATRX resulted in a high lethality with the surviving mice displaying several developmental abnormalities, including the excessive generation of neuroprogenitors (Berube, Jagla et al. 2002). Second, the attempt to delete *Atrx* using transgenic approaches did not yield any clones, suggesting ATRX is required for embryonic stem cell growth and survival (Garrick, Sharpe et al. 2006). In order to investigate *Atrx* loss of function, an *Atrx* floxed allele was generated (Berube, Mangelsdorf et al. 2005). Deletion of *Atrx* using the Gata1Cre driver, which removes the *Atrx* floxed allele at the morula stage of embryonic development, is embryonic lethal before embryonic day 9.5 (E9.5) due to trophectoderm failure (Garrick, Sharpe et al. 2006). Embryos lacking *Atrx* at E7.5 were extremely small as a result of proliferation defects (Garrick, Sharpe et al. 2006). These data indicate that achieving precise levels of ATRX is very important, as defects are present in both gain and loss of function studies.

To circumvent embryonic lethality and study ATRX function in a tissue specific manner, tissue specific Cre lines have been used. Due to the interest in the role of ATRX in the brain, several conditional mouse models were established by crossing the *Atrx* floxed mice with various Cre driver lines. FoxG1Cre and NestinCre, which delete *Atrx* in all cells of the forebrain ($Atrx^{FoxG1Cre}$ mice) or central nervous system (CNS) ($Atrx^{NestinCre}$ mice) respectively, have provided a great deal of information (Berube, Mangelsdorf et al.

2005). Following deletion of *Atrx* in either case, there was a drastic increase in cortical neuroprogenitor apoptosis embryonically, resulting in microcephaly (Berube, Mangelsdorf et al. 2005, Watson, Solomon et al. 2013). Subsequent studies determined that ATRX is required to maintain the neuroprogenitor pool, influencing both the timing of cell cycle exit and neuronal differentiation (Ritchie, Watson et al. 2014). Focusing on the hippocampus, a brain structure critical in learning and memory, ATRX was found to be essential for the survival of dentate granule cells and GABAergic interneurons, similar to what is observed in cortical neural progenitors (Seah, Levy et al. 2008). In line with research in the brain, ATRX is also important for the proliferation and survival of embryonic Sertoli cells and the subsequent elongation of the testis cords (Bagheri-Fam, Argentaro et al. 2011). Deletion of *Atrx* in skeletal muscle causes reduced myoblast expansion and increased DNA damage resulting in underdeveloped muscle and kyphosis postnatally, reiterating the role for ATRX in progenitor maintenance (Huh, Price O'Dea et al. 2012). Additionally, deletion of *Atrx* in the limb mesenchyme led to shortened digit length and brachydactyly due to increased cell death, recapitulating a symptom in ATR-X syndrome (Solomon, Russell et al. 2013).

Lastly, there may be a role for ATRX in the regulation of insulin like growth factor 1 (IGF-1) that can link ATRX to several developmental pathways. Inactivation of *Atrx* in either half the cells of the CNS in heterozygote female $Atrx^{NestinCre}$ mice or in all cells of the forebrain ($Atrx^{FoxG1Cre}$ mice) results in low levels of circulating IGF-1, skeletal abnormalities and reduced body size without altering growth hormone (GH) levels (Watson, Solomon et al. 2013, Tamming, Siu et al. 2017). In target organs, IGF-1 binds its receptor (IGF-1R) to activate the protein kinase B (AKT) signaling pathway, promoting stem cell proliferation, growth and development (Guntur and Rosen 2013). Moreover, there is off-target Cre expression that deletes *Atrx* in the anterior pituitary of the $Atrx^{FoxG1Cre}$ mice and results in low levels of the thyroid hormone, thyroxine (T4) (Watson, Solomon et al. 2013). In addition to the growth and skeletal abnormalities, $Atrx^{FoxG1Cre}$ mice display several other extremely severe defects which include decreased lifespan, hypoglycemia, reduced subcutaneous fat, cataracts and small organ size (Watson, Solomon et al. 2013). Thyroid hormone has been shown to regulate IGF-1 expression during the perinatal and prepubertal period with growth hormone taking over

at later stages of postnatal development (Xing, Govoni et al. 2012). The RNA transcript levels of numerous genes involved in thyroid hormone and IGF-1 signaling are altered in the brain, pituitary and the liver of *Atrx*^{FoxG1Cre} mice, suggesting that *Atrx* deletion may affect liver function (Watson, Solomon et al. 2013).

Intellectual disability, urogenital abnormalities, skeletal deformities and neonatal hypotonia are all symptoms of ATR-X syndrome. Studying mouse models that specifically delete *Atrx* in the brain, testes, muscle and limb mesenchyme has allowed the identification of the crucial role for ATRX in the development of tissue size by maintaining progenitor cell proliferation and survival. Interestingly, deletion of *Atrx* in chondrocytes did not result in any skeletal defects (Solomon, Li et al. 2009) indicating that *Atrx* functions in a tissue and cell type-specific manner during development.

1.2 Premature aging

1.2.1 Theories of aging

Aging is defined as the functional decline in physiological integrity, resulting in an increased susceptibility to death (López-Otín, Blasco et al. 2013). Degeneration due to aging gives rise to several pathologies such as atherosclerosis and heart failure, sarcopenia, osteoporosis, macular degeneration, renal failure, loss of myelin and neurodegeneration (Campisi 2013). Aging begins to escalate at mid-life (~50 years of age in humans (Campisi 2013), ~18 months in mice (Flurkey, Currer et al. 2007)) with degeneration beginning in one or more tissues (Campisi 2013).

Of the several identified hallmarks of aging, genomic instability, telomere attrition and epigenetic alterations are deemed to be the primary culprits (López-Otín, Blasco et al. 2013). Deregulated nutrient sensing and cellular senescence, although initially intended as an adaptive response to aging, eventually exacerbate the aging process due to stem cell exhaustion, altered intercellular communication and functional deterioration (López-Otín, Blasco et al. 2013). Each of these hallmarks is sufficient on its own to cause aging,

however, two or more often present at the same time, demonstrating some level of interconnectedness.

A major cause of aging is excessive DNA damage, such as nucleotide deletions, insertions or substitutions, single- or double-stranded DNA breaks and telomere shortening (Moskalev, Shaposhnikov et al. 2013). This genetic damage can be a result of several exogenous (e.g. chemical, radiation) and endogenous (e.g. DNA replication errors, reactive oxygen species (ROS)) pressures (López-Otín, Blasco et al. 2013). Organisms have developed a complex array of DNA repair mechanisms to combat DNA damage (López-Otín, Blasco et al. 2013, Moskalev, Shaposhnikov et al. 2013). Processes are also in place to maintain the integrity of mitochondrial DNA (mtDNA) (Kazak, Reyes et al. 2012). In addition to direct DNA damage, defects in nuclear architecture can result in genomic instability (Worman 2012). When repair mechanisms can no longer cope with the level of damage, cells undergo cell cycle arrest, apoptosis or cellular senescence (Erol 2011).

Age-associated DNA damage can occur anywhere in the genome but telomeres are frequently affected (Blackburn, Greider et al. 2006). Due to the fact that polymerases are unable to completely replicate the ends of DNA, telomeres of somatic cells are eventually eliminated from the ends of chromosomes, leading to replicative senescence *in vitro* and aging *in vivo* (Blasco 2007). Telomeres are also unique in that they are bound by the shelterin complex which is responsible for precluding DNA repair proteins from telomeres to prevent chromosome fusions (Palm and de Lange 2008). Because of this, when DNA damage does occur at telomeres it is efficient at inducing senescence or apoptosis (Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012). Embryonic stem cells have telomerase, the telomere specific DNA polymerase which is able to replicate telomeric repeats to preserve telomere length and cellular immortality (Hiyama and Hiyama 2007). Remarkably, if telomerase is activated in mice, aging can be reversed (Jaskelioff, Muller et al. 2011, Bernardes de Jesus, Vera et al. 2012).

Particular histone modifications (Fraga and Esteller 2007, Han and Brunet 2012) and decreased maintenance of heterochromatin (Tsurumi and Li 2012) contribute to aging

pathologies. Therefore, manipulating the epigenome might help to extend lifespan. The family of NAD-dependent histone deacetylase, Sirtuins (SIRTs), have been implicated in aging and were first identified as a determinant of lifespan in yeast (Kaeberlein, McVey et al. 1999). The SIRT regulation of aging is also present in mammals, in particular SIRT6 which has been shown to be important in maintaining genomic instability (Mostoslavsky, Chua et al. 2006) and metabolism (Kanfi, Peshti et al. 2010). Mice deficient in SIRT6 exhibit premature aging (Mostoslavsky, Chua et al. 2006) and those overexpressing it have a longer lifespan (Kanfi, Naiman et al. 2012). Additionally, SIRT3 has been shown to improve lifespan by reducing oxidative damage (Someya, Yu et al. 2010) and improving the regenerative capacity of hematopoietic stem cells (Brown, Xie et al. 2013). Global heterochromatin loss is often associated with aging (Tsurumi and Li 2012). All of the epigenetic defects accumulated throughout life contribute to aging and may specifically affect the integrity of stem cells, thus further contributing to aging by depletion of the stem cell pool (Pollina and Brunet 2011).

Deregulated nutrient sensing and cellular senescence are two of the responses that initially alleviate deleterious outcomes of damage but can exacerbate symptoms if left unrestricted (López-Otín, Blasco et al. 2013). The hypothalamic-pituitary-somatotropic axis involves the secretion of growth hormone from the somatotropes of the pituitary gland into circulation, ultimately, stimulating the production of insulin-like growth factor 1 (IGF-1) by several tissues, primarily the liver (Renaville, Hammadi et al. 2002) (Figure 1-2). The intracellular signaling pathway of IGF-1 is the same that is elicited by insulin and is thus referred to as the insulin and IGF-1 signaling pathway. It is the most conserved pathway in aging and its attenuation extends lifespan in several model organisms (Fontana, Partridge et al. 2010, Kenyon 2010). The predominating theory is that mitigation of the insulin and IGF-1 signaling causes a shift from that of growth and metabolism to one of cellular maintenance, leading to lower levels of damage and increased survival (Fontana, Partridge et al. 2010, Kenyon 2010, López-Otín, Blasco et al. 2013). Cellular senescence is the stable arrest of the cell cycle (Hayflick 1965), which can occur naturally due to telomere shortening or in response to stress(Campisi and d'Adda di Fagagna 2007). The number of senescent cells increases with aging (Wang, Jurk et al. 2009), however, its ultimate purpose is to halt the propagation of damaged

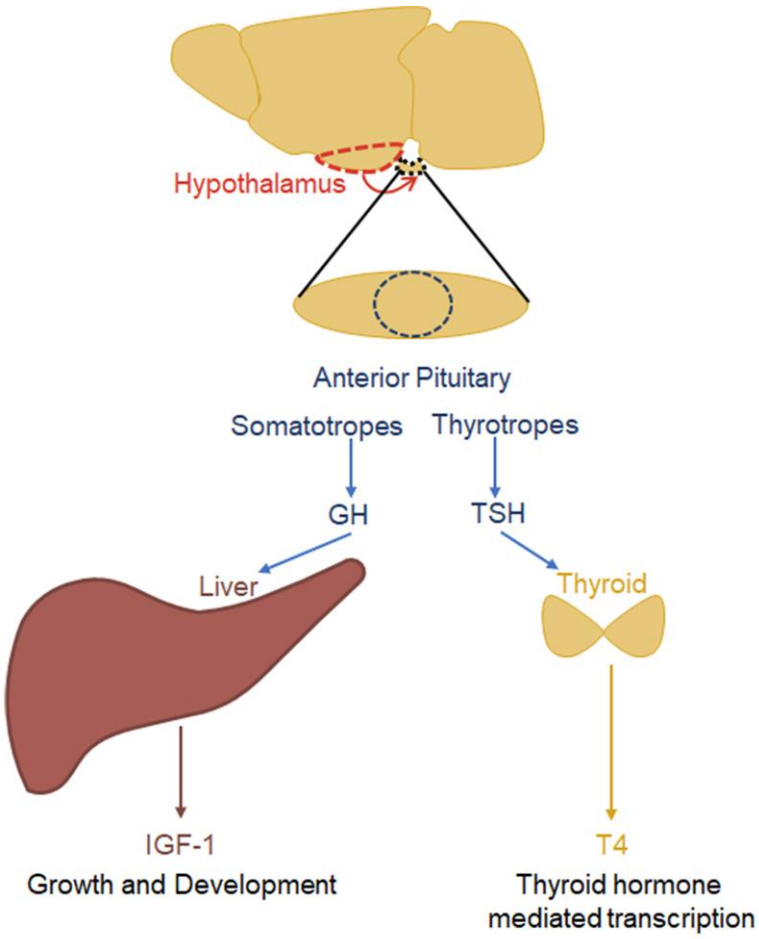

Figure 1-2 The hypothalamic-pituitary axis controls growth, development, metabolism and gene expression.

The hypothalamic-pituitary-somatotropic axis (left) results in the production of insulin-like growth factor 1 (IGF-1) from the liver. Whereas the hypothalamic-pituitary-thyroid axis (right) results in the production of the thyroid hormone thyroxine (T4) from the thyroid. The hypothalamus synthesizes and secretes growth hormone-releasing hormone and thyrotropin-releasing hormone that act on anterior pituitary to induce the production of growth hormone (GH) from somatotropes or thyroid-stimulating hormone (TSH) from thyrotropes. GH then acts on the liver to stimulate production of IGF-1, leading to growth and development, and TSH acts on the thyroid to stimulate production of T4, allowing for thyroid hormone mediated transcription, growth and metabolism.

cells (López-Otín, Blasco et al. 2013). Removal of senescent cells and proliferation of progenitors to replace their aged counterparts is critical to maintain the function of aging organ systems (López-Otín, Blasco et al. 2013). There is evidence that senescent tumour cells are actively phagocytized (Xue, Zender et al. 2007). As the immune system declines with age, clearance of senescent cells could become inefficient, leading to their accumulation (Conboy and Rando 2012, López-Otín, Blasco et al. 2013). Cellular senescence acts to compensate for accumulated DNA damage that ultimately accelerates aging when regenerative capacity declines.

Lastly, a combination of DNA damage and the responses to DNA damage lead to stem cell collapse and altered intercellular communication, which are responsible for the functional decline seen in aging (López-Otín, Blasco et al. 2013). Decline in the regenerative ability of tissues is prominent with aging (López-Otín, Blasco et al. 2013) as is diminished stem cell capacity in several different tissues (Molofsky, Slutsky et al. 2006, Conboy and Rando 2012, López-Otín, Blasco et al. 2013). As mentioned previously, an increase in senescence and a decrease in serum IGF-1, originally intended as protective for the stem cell niche, becomes deleterious when there is no longer an active pool of stem cells for regeneration of aged tissues (López-Otín, Blasco et al. 2013). The final consequence of DNA damage is altered cellular communication in which inflammation, decreased immunosurveillance and environmental changes result in a failure of the immune system to clear damaged or infected cells (López-Otín, Blasco et al. 2013).

1.2.2 Premature aging syndromes

Progeroid syndromes are rare genetic disorders that mimic the clinical and molecular aspects of aging prematurely (Navarro, Cau et al. 2006, Vidak and Foisner 2016). The majority of progeroid syndromes are considered segmental, meaning that multiple tissues are affected yet they do not fully recapitulate all symptoms of physiological aging (Navarro, Cau et al. 2006, Vidak and Foisner 2016). Studying the genetic and molecular aspects of premature aging disorders can give insight into mechanisms contributing to

natural aging. Premature aging syndromes arise when DNA damage accumulates due to a compromised nuclear membrane or with defects in DNA repair pathways, highlighting the importance of genomic integrity in aging (Eriksson, Brown et al. 2003).

Two progeroid syndromes closely echo the features of physiological aging: Hutchinson-Gilford Progeria Syndrome (HGPS) and Werner Syndrome (WS). HGPS is a premature aging disease caused by mutations in the *LAMIN A* (*LMNA*) gene (Gonzalo, Kreienkamp et al. 2017). Maintaining the structure of the nuclear membrane is critical for nuclear architecture and genome stability (Burke and Stewart 2014). The nuclear lamina is made up of type V intermediate filament lamin proteins whose expression is directly proportional to the stability of the nucleus (Swift, Ivanovska et al. 2013, Burke and Stewart 2014). A-type lamins (lamin A/C) are expressed primarily in differentiated cells (Constantinescu, Gray et al. 2006) and arise from alternative splicing of the *LMNA* gene (Burke and Stewart 2014).

The prelamin A protein is a precursor to the mature Lamin A protein. Post-translational processing of prelamin A by the endoprotease ZMPSTE24 results in a mature lamin A protein (Davies, Fong et al. 2009). The mutant form of lamin A, termed progerin, induces abnormalities in nuclear processes leading to decline in cellular functioning and disease (Davies, Fong et al. 2009). Symptoms of HGPS include, short stature, failure to thrive, skin abnormalities, alopecia, atherosclerosis, bone and joint abnormalities and subcutaneous fat loss (Merideth, Gordon et al. 2008). HGPS cells display several of the hallmarks of aging. Cells derived from patients with HGPS are characterized by loss of repressive histone marks at heterochromatin (Shumaker, Dechat et al. 2006). There are also reversals in methylation of CpG sites (Heyn, Moran et al. 2013) and hypermethylation of ribosomal RNA genes (Osorio, Varela et al. 2010). Interestingly, expression of progerin and chromatin changes observed in HGPS cells are observed in cells in normal aging (Scaffidi and Misteli 2006). Unsurprisingly, there are defects in nuclear morphology in cells that have mutations in *LMNA*. It has been reported that HGPS fibroblasts have accelerated telomere shortening (Decker, Chavez et al. 2009) and undergo premature senescence (Benson, Lee et al. 2010). Furthermore, in a mouse model

of HGPS, mice exhibit dysregulated IGF-1 signaling with a progressive decline in serum IGF-1 levels (Mariño, Ugalde et al. 2010).

WS arises due to mutations in the Werner syndrome ATP-dependent helicase (*WRN)* gene which encodes a RecQ helicase that uses ATP hydrolysis to unwind double-stranded DNA (Brosh, Waheed et al. 2002). Symptoms of WS include many of the symptoms of classic segmental progeria: lack of the pubertal growth spurt (Shamanna, Croteau et al. 2017), greying hair, cataracts, abnormal glucose and lipid metabolism and osteoporosis (Takemoto, Mori et al. 2013). Mutations in WRN cause several hallmarks of aging. WRN regulates double strand break repair pathway choice and interacts with several proteins that are involved in HR, non-homologous end joining (NHEJ) and single-strand annealing (Croteau, Popuri et al. 2014). There is an excess in DNA damage in WS as well as telomere fusions (Laud, Multani et al. 2005, Crabbe, Jauch et al. 2007) and chromosome translocations (Melcher, von Golitschek et al. 2000). WRN is also important at telomeres and is involved in the replication of secondary structures normally found at telomeres like g-quadruplexes (Johnson, Cao et al. 2010). Loss of heterochromatin has been reported in WS cells (Zhang, Li et al. 2015) and is speculated to be the cause of increased senescence and stem cell exhaustion observed in WS (Norwood, Hoehn et al. 1979).

1.2.3 The role of IGF-1 in premature aging

Paradoxically, studies have shown that reduced IGF-1 signaling occurs in both long-lived model organisms and progeria models (Garinis, van der Horst et al. 2008). It is hypothesized that in models of progeria, mutations in genome repair genes result in increased DNA damage and a switch from a state of growth to one of maintenance, reducing IGF-1 levels in order to survive the damage (Garinis, van der Horst et al. 2008). This down regulation of IGF-1 and the resulting reduction in the stem cell pool is detrimental during the developmental period, leading to premature aging syndromes. Correspondingly, IGF-1 reduction has been observed in several mouse models of progeria (de Boer, Andressoo et al. 2002, Mariño, Ugalde et al. 2010, Kanfi, Naiman et al. 2012).

Circulating IGF-1 is largely produced by liver hepatocytes (Ohlsson, Mohan et al. 2009), but small amounts can also be produced in peripheral tissues, including bone (Mohan and Baylink 1996, Canalis 1997) and brain (Bondy, Werner et al. 1992). IGF-1 is bound by six IGF binding proteins (IGFBP) that regulate the action of IGF-1 (Jones and Clemmons 1995). IGFBPs transport IGFs from the bloodstream to target tissues (IGFBP-1, 2 and 4), maintain IGF-1 levels in the serum (IGFBP-3) and can even inhibit the actions of IGF-1 (Jones and Clemmons 1995). Additionally, most of IGF-1 in the serum is in complex with IGFBP-3, IGFBP-5 and IGF acid-labile subunit (IGFALS) (Boisclair, Rhoads et al. 2001).

In target organs, IGF-1 binding to its receptor results in tyrosine kinase activation followed by phosphorylation of substrates which include insulin receptor substrates (IRS1, IRS2) (Fumihiko and Shin-Ichiro 2018). Phosphorylated substrates are recognized by molecules that contain an Src homology 2 (SH2) domain which is important for intracellular signal transduction (Fumihiko and Shin-Ichiro 2018). These interactions are followed by activation of the MAP kinase or PI 3-kinase cascades that mediate many cellular processes like cell growth, differentiation, survival and protein synthesis (Fumihiko and Shin-Ichiro 2018).

An example that highlights the detrimental effect of IGF-1 reduction during development is metallopeptidase *Zmpste24*-null mice (Mariño, Ugalde et al. 2010). Zmpste24 is a metallopeptidase involved in processing LMNA from prelamin A (Davies, Fong et al. 2009). *Zmpste24*-null mice, which phenocopy HGPS, have severely low levels of IGF-1 as well as many of the classic symptoms seen in HGPS (Mariño, Ugalde et al. 2010). Treatment of *Zmpste24*-null mice with IGF-1 ameliorates the premature aging features and extends lifespan, indicating that reduced IGF-1 is causative of premature aging phenotypes in these mice (Mariño, Ugalde et al. 2010).

1.2.4 The role of thyroid hormone in premature aging

The hypothalamic-pituitary axis is essential for the maintenance of homeostasis, metabolism, reproduction, growth, and lactation (Scully and Rosenfeld 2002, Mendoza

and Hollenberg 2017). The synthesis and secretion of trophic hormones is controlled by the hypothalamus which subsequently signals to the pituitary gland (Scully and Rosenfeld 2002, Mendoza and Hollenberg 2017) (Figure 1-2). The anterior pituitary is derived from ectoderm in Rathke's pouch where organogenesis begins at E8.5 and is followed by proliferation and patterning of several different cell types (Scully and Rosenfeld 2002). Thyrotropes in the anterior pituitary produce thyroid-stimulating hormone (TSH) which stimulates the thyroid to produce the prohormone T4 (Scully and Rosenfeld 2002, Chiamolera and Wondisford 2009) (Figure 1-2). T4 is then converted to its active form triiodothyronine (T3) in the target tissue (Scully and Rosenfeld 2002). Thyroid hormone participates in a positive and negative feedback loop from peripheral organ systems to both the hypothalamus and pituitary to regulate hormone synthesis in the hypothalamic-pituitary-thyroid axis (Mendoza and Hollenberg 2017) (Figure 1-2). Thyroid hormone is essential for proper development, growth, cellular differentiation and metabolic regulation (Gereben, Zavacki et al. 2008).

Thyroid hormone exerts its effects by binding to the thyroid hormone receptors (TRs) to control the expression of several genes. TRs act as transcription factors to either enhance or repress transcription depending on whether T3 is bound or not (Xu, Glass et al. 1999, Grøntved, Waterfall et al. 2015). The thyroid receptors TRα (Sap, Munoz et al. 1986) and TRβ (Koenig, Warne et al. 1988) both bind thyroid hormone and recognize the same thyroid hormone response elements (TREs) (Lazar 1993). There appears to be some degree of compensation amongst the TR isoforms (Gauthier, Chassande et al. 1999); TRα is ubiquitously expressed whereas TRβ is restricted to particular tissues, including the liver, later in development (Bradley, Towle et al. 1992). TRα has been shown to be important for bone growth (Gauthier, Chassande et al. 1999) with mice null for both isoforms of TRα exhibiting low levels of thyroid hormone, growth defects and early postnatal death (Fraichard, Chassande et al. 1997). Deletion of TRβ on its own did not result in any defects in growth (Forrest, Hanebuth et al. 1996).

The actions of TRs are mediated by both systemic T4 and local T3 availability (Gereben, Zavacki et al. 2008), transport of thyroid hormone into the cell (Visser, Friesema et al. 2011), the distribution of TR isoforms (Mendoza and Hollenberg 2017) and nuclear

corepressors and coactivators (Astapova, Lee et al. 2008, Mendoza and Hollenberg 2017). Both T3 and T4 are normally bound to transport molecules in the bloodstream like albumin, thyroxine binding globulin (TBG) and transthyretin (TTR) (Mendoza and Hollenberg 2017). Neither of the thyroid hormones are able to passively cross cell membranes (Mendoza and Hollenberg 2017). T4 is transported from the circulation into the cytoplasm by monocarboxylate transporters (MCT8 and MCT10), the organic anion transporter (OATP1) and L-type amino acid transporter (LAT) (Mendoza and Hollenberg 2017). Once T4 enters the cell, it is converted to T3 by type 1 deiodinase (Dio1) or type 2 deiodinase (Dio2) (Mendoza and Hollenberg 2017). The availability of the thyroid hormones can also be regulated by deiodinase 3 (Dio3), producing reverse T3 (rT3) and T2 which are the deactivated forms of T4 and T3 respectively (Mendoza and Hollenberg 2017). The action of T3 is dependent on the availability of its receptor, which is mostly found as a heterodimer with retinoid X receptor (RXR) (Mendoza and Hollenberg 2017). Following ligand binding, the heterodimer undergoes a conformational change allowing for differential recruitment of coregulators (Ramadoss, Abraham et al. 2014, Grøntved, Waterfall et al. 2015, Mendoza and Hollenberg 2017). In the absence of T3 ligand, TRs bind nuclear receptor corepressor (NCOR1), which forms a corepressor complex with transducin (beta)-like 1 (Tbl1) and histone deacetylase (HDAC3) (Sun, Feng et al. 2013, You, Lim et al. 2013, Ramadoss, Abraham et al. 2014, Grøntved, Waterfall et al. 2015, Mendoza and Hollenberg 2017). Following T3 ligand binding to TRs, the corepressor complex is replaced by coactivators which include the steroid receptor coactivator (SRC) family and p300/CBP (CREB-binding protein) leading to histone acetylation and gene activation (Astapova 2016, Mendoza and Hollenberg 2017). Additionally, liganded TRs can repress genes (Ramadoss, Abraham et al. 2014) and TRs have a myriad of target genes (Yen, Feng et al. 2003) emphasizing the complexity of TR-mediated gene regulation. It is important to note that low thyroid hormone could contribute to hypomyelination observed in the premature aging syndromes like, Cockayne Syndrome (Koob, Laugel et al. 2010, Revet, Feeney et al. 2012), as ligand-bound TR is an important activator of myelin gene expression (Farsetti, Mitsuhashi et al. 1991, Ibarrola and Rodriguez-Pena 1997, Dong, Yauk et al. 2009).

The overall role of thyroid hormone in aging appears to be similar to that of IGF-1; subclinical levels of thyroid hormone may lead to extended longevity (Gussekloo, van Exel et al. 2004, Over, Mannan et al. 2010) but can be detrimental during the developmental period. Low thyroid hormone and excessive Dio3 expression, the enzyme responsible for deactivating thyroid hormone, have been reported in premature aging mouse models and aged mice (Visser, Bombardieri et al. 2016). The mechanism by which thyroid hormone exerts a protective effect in aging has yet to be elucidated. IGF-1 levels are controlled by GH, but there is evidence to suggest *Igf1* expression may be controlled by thyroid hormone, particularly in the prepubertal period (O'Shea, Bassett et al. 2005, Wang, Shao et al. 2010, Xing, Govoni et al. 2012). TR is able to bind a thyroid hormone response element in intron one of *Igf1* to stimulate its expression (Xing, Govoni et al. 2012). Mice deficient in thyroid hormone show greater than 50% decrease in *Igf1* expression in liver and bone, and T4 treatment can reverse this effect (Xing, Govoni et al. 2012). These mice also exhibit phenotypes of premature aging, including skeletal abnormalities and low IGF-1, both of which were rescued following treatment with thyroid hormone (Xing, Govoni et al. 2012).

1.3 Myelination

1.3.1 Oligodendrocyte development

The balance of the four major cell types in the brain (neurons, microglia, astrocytes and oligodendrocytes (OLs)) is essential for the development of a normal functioning nervous system. Neural stem cells generate neurons, astrocytes and OLs in the CNS. After the initial production of neurons, neural stem cells shift to a gliogenesis program to generate astrocytes and OLs (Sauvageot and Stiles 2002). Astrocytes are involved in synapse formation, regulation of blood flow, ion homeostasis, metabolism and blood brain barrier function (Sofroniew and Vinters 2010). OLs are responsible for producing the myelin sheath which is essential for proper signal propagation in neurons. (Stadelmann, Timmler et al. 2019). Oligodendrocyte precursor cell (OPC) birth occurs early in development followed by the migration and proliferation of OPCs. OPCs then go through several stages of maturation ultimately differentiating into mature myelinating OLs. Lastly, the

production of myelin occurs as OLs make contact with and ensheathe target axons with their plasma membrane.

In the embryonic mouse brain, OPCs exhibit compensatory redundancy, by being produced in three distinct waves. Beginning at E12.5, OPCs originate in the subventricular zone of the medial ganglionic eminence and the anterior entopeduncular area, migrating in a ventral to dorsal wave (Kessaris, Fogarty et al. 2006). OPC generation continues to move dorsally towards the neocortex, in a medial wave, with production stemming from the lateral and caudal ganglionic eminences at E15.5 (Kessaris, Fogarty et al. 2006). A final wave of OPC production occurs at birth from the dorsal subventricular zone into the cortex (Kessaris, Fogarty et al. 2006). OPC tracing has demonstrated that the P10 cortex is populated by OPCs from the medial and dorsal waves of OPCs (Kessaris, Fogarty et al. 2006) (Figure 1-3), while the first ventral wave of OPCs is completely lost in the postnatal brain. Interestingly, ventrally produced OPCs do survive if the second or third waves are perturbed (Kessaris, Fogarty et al. 2006). The overabundance of OPC production suggests a competitive and flexible system in which the amount of myelin produced can be adjusted to suit unfavourable conditions, neuronal activity or even diminished when there is a reduced need (Hill, Patel et al. 2014).

OPCs are generated from the commitment of proliferative neural stem cells through the expression of the oligodendrocyte basic-helix-loop-helix (bHLH) transcription factors Olig2 and Olig1 to generate OPCS (Figure 1-3). *Olig2* is necessary for OPC generation whereas *Olig1* is involved in OL differentiation (Lu, Cai et al. 2001, Zhou, Choi et al. 2001, Othman, Frim et al. 2011). Knockout of *Olig1/2* individually or together, results in defects in myelin production (Lu, Sun et al. 2002, Zhou and Anderson 2002, Zhu, Zuo et al. 2012). Olig2 is also transiently expressed in developing astrocytes (Cai, Chen et al. 2007). OPC migration is controlled by several signaling molecules, growth factors and extracellular matrix components (de Castro and Bribián 2005, Elbaz and Popko 2019). Of particular note is platelet derived growth factor (PDGF) (Fruttiger, Karlsson et al. 1999) which is critical in general OPC development and is normally secreted by neurons and astrocytes. Following OPC migration to the correct brain locations, OPCs rapidly proliferate, mediated by PDGF signalling, and continue to divide until homeostasis is

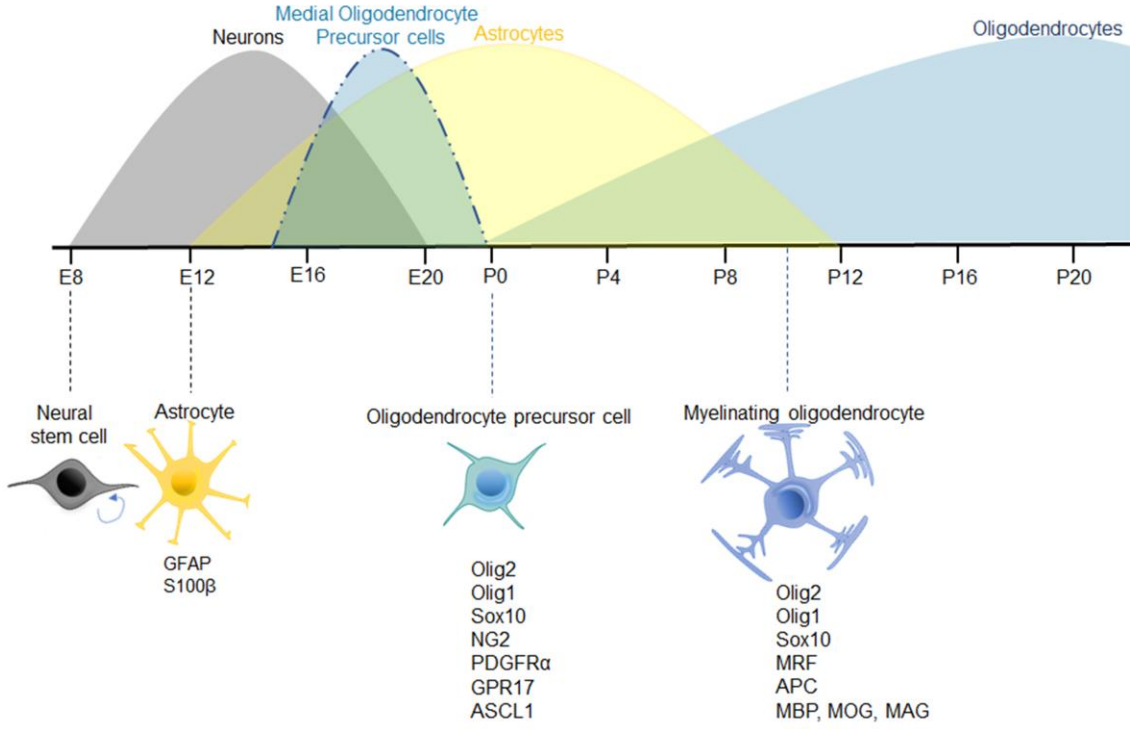

Figure 1-3 Developmental timeline of neuron, astrocyte and oligodendrocyte production in the mouse brain.

Neurons, astrocytes and oligodendrocytes are all produced form the commitment of neural stem cells that have the ability to self-renew or become committed to one of these lineages. Neuron development occurs earliest in development starting at embryonic day (E)8 continuing until just before birth at E20. Astrocyte development begins at E12 and continues postnatally until P12. During this time, oligodendrocyte development also occurs. At E15.5, the medial oligodendrocyte precursor cells are born, however, only a portion go on to produce myelinating oligodendrocytes, represented by the dashed line in the developmental timeline. The majority of oligodendrocytes come from oligodendrocyte precursor cells that appear at birth (P0) which continue into myelinating oligodendrocytes starting at P10 and throughout postnatal development. Additionally, there is a resident population of oligodendrocyte precursors that exist in adulthood. Astrocytes, oligodendrocyte precursors and myelinating oligodendrocytes express several cell-type specific proteins which are listed below each cell type.

reached (van Heyningen, Calver et al. 2001). Id2 (inhibitor of DNA binding 2) (Samanta and Kessler 2004), Id4 (inhibitor of DNA binding 4) (Samanta and Kessler 2004), Hes1 (Hes family bHLH transcription factor 1) (Kondo and Raff 2000), Hes5 (Hes family bHLH transcription factor 5) (Kondo and Raff 2000) and G protein-coupled receptor (Gpr17) (Chen, Wu et al. 2009) are responsible for the prevention of premature differentiation of OPCs. The rate of OPC cell division is dependent on location and declines with age (Young, Psachoulia et al. 2013). OPCs make up ~5% of cells in the adult brain and constitute the major dividing population in the adult CNS (Dawson, Polito et al. 2003). OPCs in the adult CNS are important for normal myelin maintenance as well as in remyelination in cases of injury (Rivers, Young et al. 2008, Kang, Fukaya et al. 2010, Young, Psachoulia et al. 2013). In the case of injury, demyelinated axons make synaptic contacts with OPCs and the resulting neuronal activity via glutamate signalling has been shown to cause OPC differentiation (Gallo, Zhou et al. 1996, Mangin, Li et al. 2012, Gibson, Purger et al. 2014, Gautier, Evans et al. 2015).

In addition to Olig2, several additional cofactors contribute to OL specification. Olig2 activates Sox10 (SRY-Box Transcription Factor 10), which participates in a positive feed back loop with Olig2 to establish the OPC lineage (Finzsch, Stolt et al. 2008, Kuspert, Hammer et al. 2011, Yu, Chen et al. 2013). Sox10 expression in primitive OPCs (priOPCs) induces OPC development through the activation of chondroitin sulfate proteoglycan (*Cspg4*; protein: neural glial antigen 2 (NG2)) and *Pdgfrα* (Pringle, Mudhar et al. 1992, Nishiyama, Lin et al. 1996). NG2 is a surface type I transmembrane core protein involved in cell migration and proliferation (Makagiansar, Williams et al. 2007). PDGFRα promotes OPC proliferation, migration and survival (Noble, Murray et al. 1988, Pringle, Collarini et al. 1989). NG2 and PDGFRα have been shown to interact and co-localize (Nishiyama, Lin et al. 1996, Nishiyama, Lin et al. 1996) providing further evidence that they work together to maintain the OPC population. NG2 (Kucharova and Stallcup 2010) and PDGFRα-null mice have a reduction in the OPC pool and subsequent hypomyelination due to precocious OPC differentiation (Fruttiger, Karlsson et al. 1999).

Further commitment to the OL lineage starts with the generation of pre-OLs which, after cell cycle exit, express myelin regulatory factor (*Mrf*) (Emery, Agalliu et al. 2009).

Sox10 is responsible for the activation of *Mrf* and both proteins are responsible for the activation of many other myelin critical genes (Hornig, Frob et al. 2013). As the OLs mature and contact axons, they become highly branched and upregulate several myelin sheath proteins while encompassing the axon. Several nuclear receptors are also important for OL development (Elbaz and Popko 2019). There is an abundance of evidence suggesting that TRs are important for the differentiation of OPCs. Myelination is delayed in hypothyroid rats and accelerated in hyperthyroid rats (Walters and Morell 1981). Both TRα and TRβ are expressed in OLs *in vitro* (Baas, Fressinaud et al. 1994) and *in vivo* (Carlson, Strait et al. 1994) and contribute to their differentiation. TRα1 is involved in initial cell cycle exit (Billon, Jolicoeur et al. 2002) and TRβ1 in promoting differentiation and myelination (Farsetti, Mitsuhashi et al. 1991, Billon, Tokumoto et al. 2001). In line with this, studies have shown that expression of essential myelin proteins, myelin basic protein (MBP), myelin associated glycoprotein (MAG), proteolipid protein (PLP) and 2,3'-Cyclic nucleotide 3'-phosphodiesterase (CNPase), are reduced in models of hypothyroidism (Rodriguez-Peña, Ibarrola et al. 1993, Ibarrola and Rodríguez-Peña 1997, Barradas, Vieira et al. 2001). Furthermore, both TRs can directly bind and activate the *Mbp* and *Mag* genes (Farsetti, Mitsuhashi et al. 1991, Ibarrola and Rodríguez-Peña 1997, Dong, Yauk et al. 2009).

Epigenetic regulation is critical for OPC formation, differentiation and subsequent myelination. The OL lineage can be manipulated by changing histone acetylation state; histone acetyltransferases (HATs) make chromatin more accessible, while the opposite is true for histone deacetylases (HDACs) (Kouzarides 2007). HDAC1 and HDAC2 promote OPC differentiation in the mouse CNS (Ye, Chen et al. 2009). Of note, only concurrent deletion of HDAC1 and HDAC2 results in a defect in myelination, suggesting a functional redundancy for these two proteins (Ye, Chen et al. 2009). At the onset of OPC differentiation, HDAC1 is recruited to repress inhibitors of differentiation like *Id2*, *Id4* and *Hes5* (He, Dupree et al. 2007, Shen, Sandoval et al. 2008). HDAC3 supresses neuronal specification (He, Dupree et al. 2007, Ye, Chen et al. 2009) and paradoxically, has also been shown to promote *Olig2* expression with the p300 HAT (Zhang, He et al. 2016). Additionally, the acetyltransferase CREB-binding protein (CBP) acetylates *Olig1* and promotes OL differentiation (Dai, Bercury et al. 2015).

Methylation states have not been fully characterized in OL development. Histone demethylases have not been associated with OL development, however, during OL differentiation the level of H3K9me3 increases (Sher, Boddeke et al. 2012, Liu, Magri et al. 2015, He, Wang et al. 2017). Furthermore, EZH2, which catalyzes the trimethylation of H3K27, has been shown to support OPC lineage commitment in neural stem cells while suppressing astrocyte development (Sher, Rossler et al. 2008). In fact, the PRC2 complex, which contains EZH2, has been shown to control the balance of OPC maintenance and OL differentiation, favoring the latter (He, Wang et al. 2017).

Several members of the ATP-dependent SWI/SNF chromatin remodeling family have been implicated in OL development in a cascade of chromatin remodeling events. The Snf2 chromatin remodeler CHD8 promotes the expression of the *Smarca4* (Brg1) which encodes the ATPase domain of the multi-subunit SWI/SNF chromatin remodeling complex (Zhao, Dong et al. 2018). Brg1 is recruited to the *Olig2* promoter (Matsumoto, Banine et al. 2016) as well as other OL-differentiation genes (Yu, Chen et al. 2013). Brg1 and Olig2 then activate the chromatin remodeler CHD7 during OL differentiation (He, Marie et al. 2016) and CHD7 triggers expression of *Mrf* and *Olig1* to stimulate OL maturation (He, Marie et al. 2016). This cascade of chromatin remodeling events ensures the proper timing and region-specific progression of the OL lineage.

1.3.2 The fate potential of oligodendrocyte precursor cells

OPCs have been shown to become astrocytes (Raff, Miller et al. 1983, Rao and Mayer-Proschel 1997) and neurons (Kondo and Raff 2000, Belachew, Chittajallu et al. 2003, Liu, Han et al. 2007, Rivers, Young et al. 2008) under particular *in vitro* conditions. In spite of this, there is limited evidence as to whether OPCs have an expanded fate potential. There is *in vivo* evidence to suggest OPCs may be bipotential glial progenitors. When cells of the OPC cell line CG4 are transplanted into glial deficient areas of the mouse spinal cord, they develop into both astrocyte and OL-like cells (Franklin, Bayley et al. 1995, Windrem, Nunes et al. 2004). Additionally, reports have suggested that 40% of protoplasmic astrocytes specifically in the grey matter of the ventral posterior forebrain, are derived from NG2-expressing cells, however, this was not observed at all

in white matter tracts (Zhu, Bergles et al. 2008). Genetic manipulation has allowed the mechanisms governing OPC potential to be further clarified. It was shown that deletion of *Olig2* or *Hdac3* in OPCs results in ectopic astrocyte development in the dorsal cortex at the expense of the OL lineage. (Cai, Qi et al. 2005, Zhu, Zuo et al. 2012, Zhang, He et al. 2016, Zuo, Wood et al. 2018). These data suggest that OPCs have the ability to differentiate into astrocytes *in vitro* but are restricted to the OL lineage in normal *in vivo* conditions.

1.3.3 Myelin function and structure

White matter tracts in the CNS are made up of many myelinated axons with very few cell bodies. Myelination begins at P10 and continues until 3 months of age in the mouse brain (Hammelrath, Skokic et al. 2016). Myelination of axons occurs by the stacking of the OL membrane, twisting around the axon and subsequent membrane expansion (Stadelmann, Timmler et al. 2019). The membrane is then primarily compacted by MBP to prevent current leakage along the axon and to allow for saltatory conduction of action potentials (Salzer and Zalc 2016).

Myelin is also required for axon integrity and survival (Funfschilling et al., 2012; Saab et al., 2016). OLs have been shown to produce energy for axons in times of high neuronal activity (Lee, Morrison et al. 2012, Saab, Tzvetavona et al. 2016). As the compaction of the myelin sheath prevents the axon from contacting nutrients, cytoplasmic channels within the myelin sheath allow metabolic coupling between the axon and the OL (Stadelmann, Timmler et al. 2019). OLs express MCTs (Lee, Morrison et al. 2012) which transport lactate, pyruvate, ketone bodies and hydrogen ions across membranes (Pierre and Pellerin 2005). Additionally, OLs can react to neuronal activity, by sensing axonal glutamate release and responding with increased production of glucose transporters to meet the energy needs of the neuron (Saab, Tzvetavona et al. 2016).

Myelin is unique in that it is composed of an unusually high amount of lipids (Norton and Poduslo 1973) which make up about 70% of the myelin sheath (Salzer and Zalc 2016). Electron microscopy has allowed myelin structure to be studied in depth. Myelin was first

characterized as a multilayered stack of membranes which later become the cytoplasmic (major dense line) and extracellular (interperiod line) membrane bilayers which occur in repeating patterns of 12 nm (Salzer and Zalc 2016). The major dense line is a unique structure in which there is a 3 nm wide compartment between two lipid bilayers (Salzer and Zalc 2016). The major dense line is primarily stabilized by MBP which is essential for the compaction of the two extracellular membrane bilayers (Salzer and Zalc 2016, Raasakka, Ruskamo et al. 2017). MBP is a small, unstructured polypeptide chain that assumes both α-helical and β-sheet structures upon interaction with the membrane (Harauz, Ishiyama et al. 2004). This interaction is mediated by electrostatic forces between the basic residues of MBP and negatively charged lipids of the membrane (Musse, Gao et al. 2008, Nawaz, Kippert et al. 2009, Raasakka, Ruskamo et al. 2017).

Upon MBP binding, the membrane is compacted, resulting in protrusion of cytosol and the exclusion of most proteins from the myelin sheath (Aggarwal, Yurlova et al. 2011). To maintain cytoplasmic channels necessary for molecule transport, CNPase interacts with actin to oppose MBP-mediated membrane compaction (Brunner, Lassmann et al. 1989, Snaidero, Velte et al. 2017). Myelin is also compacted by PLP, a transmembrane protein within the myelin sheath (Boison, Bussow et al. 1995). Glycoproteins are myelin minor proteins (Poduslo 1983) that mediate glial-glial and glial-neuron interactions. The myelin sheath is attached to an axon via the paranodal axonal-glial junction (Salzer and Zalc 2016). MAG coats the circumference of the adaxonal myelin membrane (Quarles 2007) and its dimerization restricts the cytosolic regions to a diameter of 10 nm (Pronker, Lemstra et al. 2016). MAG also interacts with neuronal gangliosides (Schnaar and Lopez 2009) and activates extracellular signal-regulated kinase, which is involved in axonal cytoskeleton maturation (Dashiell, Tanner et al. 2002). Similarly, MOG is expressed on the surface of the myelin sheath (Brunner, Lassmann et al. 1989) and although its structure (Martini and Schachner 1986) is known, its exact role within white matter remains elusive. However, MOG is a member of the immunoglobulin superfamily (Pham-Dinh, Mattei et al. 1993) and is a target for immune cells, resulting in demyelination in experimental models of MS (Sun, Link et al. 1991) and in MS patients (Weber, Derfuss et al. 2018).

Both neuron and astrocyte communication are necessary to achieve a fully myelinated brain. Electrical activity from neurons has been shown to induce OPC proliferation (Gibson, Purger et al. 2014) and there is some evidence to show that electrical activity induces myelination of certain axons (Gautier, Evans et al. 2015). Furthermore, astrocytes secrete many signaling molecules during OL development and myelination, including PDGF (Gard, Burrell et al. 1995) and ciliary neurotrophic factor (Stankoff, Aigrot et al. 2002).

1.3.4 Myelin pathology in development

Myelin pathologies represent a heterogenous group of disorders with several etiologies ranging from genetics to OL attack during inflammation. Disorders of the myelin sheath can be classified in three categories; hypomyelination, where there is a permanent deficiency in myelin production (Pelizaeus–Merzbacher Disease (Koeppen, Ronca et al. 1987)), dysmyelination, where there is abnormal deposition of myelin (Metachromatic Leukodystrophy (Nandhagopal and Krishnamoorthy 2006)) or demyelination, where myelin is formed but subsequently destroyed (Multiple Sclerosis (MS) (Karussis 2014)). Hypomyelination and dysmyelination occur during development and demyelination occurs later in life.

Hypomyelinating disorders are the largest category of leukoencephalopathies of unknown origin and are often undiagnosed (van der Knaap, Breiter et al. 1999, Schiffmann and van der Knaap 2009, Steenweg, Vanderver et al. 2010). It has been shown that hypomyelinating disorders are easily identified by magnetic resonance imaging (Schiffmann and van der Knaap 2009, Steenweg, Vanderver et al. 2010). They often stem from mutations in genes involved in myelination like *PLP1*, (Pelizaeus–Merzbacher disease) (Koeppen, Ronca et al. 1987) *SOX10*, (Waardenburg-Shah Syndrome) (Elmaleh-Berges, Baumann et al. 2013), and *FAM126A* (Hypomyelinating Leukodystrophy 5) (Zara, Biancheri et al. 2006). Additionally, mutations in gap junctions (*GJC2*, Pelizaeus–Merzbacher like Disease (Gotoh, Inoue et al. 2014); *GJA1,* Oculodentodigital Dysplasia (Bugiani, Al Shahwan et al. 2006)), DNA excision repair (*ERCC,* Tay Syndrome (Weeda, Eveno et al. 1997, Viprakasit, Gibbons et al. 2001), Cockayne Syndrome

(Karikkineth, Scheibye-Knudsen et al. 2017)), RNA polymerase III (POLR3A, Hypomyelinating Leukodystrophy 7 (Wolf, Vanderver et al. 2014); POLR3B, Hypomyelinating Leukodystrophy 8 (Saitsu, Osaka et al. 2011)), as well as partial deletion of the long arm of chromosome 18 (18q, Chromosome 18q Deletion Syndrome (Chen, Lin et al. 2006)) have all been implicated in hypomyelinating disorders. In addition to hypomyelination, clinical manifestations of these pathologies range from dystonia, developmental anomalies, seizures, cataracts, hypotonia, motor delay and cognitive impairment (Barkovich and Deon 2016). ATR-X syndrome patients display hypomyelination (Wada, Ban et al. 2013, Lee, Lee et al. 2015) with a prevalence of 44% in patients examined, with no correlation with mutation site (Wada, Ban et al. 2013). White matter abnormalities were widespread and scattered (Wada, Ban et al. 2013, Lee, Lee et al. 2015) or concentrated around the trigones of the lateral ventricle (Wada, Ban et al. 2013).

1.4 Thesis Overview

The overall aim of this thesis was to determine if restoration of serum T4 levels in mice with a forebrain and anterior pituitary specific deletion of *Atrx* (*Atrx*^{FoxG1Cre} mice) would rescue the premature aging phenotypes observed in this model. This hypothesis was supported by the reported reduction in serum IGF-1 and T4 in other mouse models of premature aging (Mariño, Ugalde et al. 2010, Xing, Govoni et al. 2012, Visser, Bombardieri et al. 2016). Additionally, in one study, restoration of serum T4 levels rescued premature aging phenotypes and IGF-1 levels (Xing, Govoni et al. 2012). Despite complete restoration of serum T4 levels to that of controls, *Atrx*^{FoxG1Cre} mice still exhibited growth abnormalities, short lifespan, loss of subcutaneous fat and low glucose levels. Accordingly, several thyroid hormone responsive genes, including *Igf1*, were not re-established in the liver of *Atrx*^{FoxG1Cre} mice following T4 treatment, nor were serum IGF-1 levels. The lack of rescue of IGF-1 levels points to unexpected Cre-mediated *Atrx* deletion in the liver of *Atrx*^{FoxG1Cre} mice, the primary site of IGF-1 production. Indeed, *Atrx* was absent in a subset of hepatocytes and in its absence, thyroid hormone mediated gene expression could not occur for a subset of genes, indicating *Atrx* may be required for their expression.

I also observed drastic decreases of several myelin proteins in *Atrx*^{FoxG1Cre} mice, a phenotype also known to occur in models of premature aging. Myelin gene expression is known to be activated by ligand-bound TR and in this case, T4 administration to *Atrx*^{FoxG1Cre} mice increased the number of mature OLs and myelin protein expression was partially rescued. However, the number of OPCs did not increase in *Atrx*^{FoxG1Cre} mice treated with T4, hinting at an intrinsic role for ATRX in their development. Targeted inactivation of *Atrx* in OPCs (*Atrx*^{Sox10CreER} mice) but not in neurons also caused hypomyelination. Deletion of *Atrx* in OPCs resulted in a decrease in the fate-specifying transcription factor Olig2. Mechanistically, ATRX occupies the *Olig2* gene locus and binds HDAC3, a known co-activator of *Olig2* expression. Following *Olig2* downregulation in ATRX-null OPCs, these cells lose several OPC markers and gain the ability to differentiation intro astrocytes, causing the hypomyelination phenotype observed in both *Atrx*^{FoxG1Cre} mice and *Atrx*^{Sox10CreER} mice.

It has previously been shown in a mouse model of premature aging that T4 administration can directly activate *Igf1* expression and subsequently rescue premature aging phenotypes. *Atrx*^{FoxG1Cre} mice phenocopy models of premature aging and also display low levels of both T4 and IGF-1. Based on this, I hypothesized that T4 administration to *Atrx*^{FoxG1Cre} mice would induce *Igf1* transcription and salvage phenotypes of premature aging. My results show that ATRX is required to facilitate transcription of several thyroid hormone responsive genes, including *Igf1*, and in its absence, rescue of premature aging phenotypes by T4 is perturbed. Despite the fact that T4 administration did not rescue several premature aging phenotypes, hypomyelination was partially rescued in a T4-dependent manner. This led to the discovery that ATRX is required in OPCs independently of T4 to promote the expression of the key OL transcription factor *Olig2*, maintain the OL lineage and drive myelination in the corpus callosum. Overall, this thesis highlights the importance of ATRX in the prevention of premature aging, both dependent and independent of thyroxine.

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Chapter 2

2 Inactivation of hepatic ATRX in *Atrx*^{FoxG1Cre} mice prevents reversal of aging-like phenotypes by thyroxine

Based on emerging evidence that T4 stimulates expression of IGF-1 in pre-pubertal mice, we tested whether T4 supplementation in *Atrx*^{FoxG1cre} mice could restore IGF-1 levels and ameliorate premature aging-like phenotypes. Despite restoration of normal serum T4 levels, we did not observe improvements in circulating IGF-1. In the liver, thyroid hormone target genes were differentially affected upon T4 treatment, with *Igf1* and several other thyroid hormone responsive genes failing to recover normal expression levels. These findings hinted at Cre-mediated *Atrx* inactivation in the liver of *Atrx* Foxg1cre mice, which we confirmed. Premature aging phenotypes observed in the *Atrx* Foxg1cre mice can be explained in part by a role of ATRX in the liver to promote T4-mediated *Igf1* expression, thus explaining the inefficacy of T4 therapy observed in this study.

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2.1 Introduction

The α-Thalassemia/mental retardation X-linked (ATRX) chromatin remodeler is a component of constitutive heterochromatin (McDowell, Gibbons et al. 1999, Baumann, Schmidtmann et al. 2008, Goldberg, Banaszynski et al. 2010, Sadic, Schmidt et al. 2015, Voon, Hughes et al. 2015) and is exclusively nuclear (McDowell, Gibbons et al. 1999). The C-terminus of ATRX contains a Switch/Sucrose non-fermenting (Swi/Snf) domain (Picketts, Higgs et al. 1996) which confers the ATPase and translocase activity of ATRX necessary for nucleosome remodeling (Xue, Gibbons et al. 2003). The N-terminus of ATRX contains an ADD domain (ATRX-DNTM3-DNTM3L) with homology to the

DNA methyltransferase family and is capable of binding methylated histone tails within chromatin (Aapola, Shibuya et al. 2000).

ATRX is essential for heterochromatin (Baumann, Schmidtmann et al. 2008, Law, Lower et al. 2010) and telomere integrity (Law, Lower et al. 2010, Watson, Solomon et al. 2013), as well as normal gene expression (Levy, Fernandes et al. 2008, Bagheri-Fam, Argentaro et al. 2011, Ratnakumar, Duarte et al. 2012, Levy, Kernohan et al. 2015, Li, Syed et al. 2016). Deletion of ATRX in neuroprogenitor cells causes DNA damage at telomeres and pericentromeric heterochromatin (Watson, Solomon et al. 2013). We have previously shown that telomestatin-mediated stabilization of G-quadruplexes exacerbated DNA damage and decreased viability of neuroprogenitors in the absence of ATRX (Watson, Solomon et al. 2013). ATRX may be facilitating the progression of DNA replication and transcriptional machinery through these non-canonical DNA structures (Watson, Solomon et al. 2013, Levy, Kernohan et al. 2015).

We previously reported that postnatal health and longevity are severely affected in mice with forebrain and anterior pituitary-specific deficiency for ATRX ($Atrx^{FoxG1cre}$) (Watson, Solomon et al. 2013). Many of the abnormalities detected in these mice resemble those previously reported in mouse models of progeria, including low circulating levels of IGF-1 (Niedernhofer, Garinis et al. 2006, van der Pluijm, Garinis et al. 2006, Varga, Eriksson et al. 2006, Mariño, Ugalde et al. 2010, Kanfi, Naiman et al. 2012). Progeroid syndromes are a group of disorders characterized by accelerated physiological aging. These heritable disorders produce signs of premature aging in many, but not all tissues and therefore are termed segmental progerias. It has been postulated that in progeroid syndromes, mutations in genome repair genes result in increased genomic and heterochromatin instability (Shumaker, Dechat et al. 2006, Liu, Barkho et al. 2011). This is paired with the downregulation of genes involved in growth, such as *Igf1*, presumably in order to survive the damage (Garinis, Uittenboogaard et al. 2009). However, if this occurs during development, it is detrimental to the organism, resulting in metabolic defects and premature aging. Several progeroid syndromes have been described, such as Cockayne syndrome (Karikkineth, Scheibye-Knudsen et al. 2017), Werner syndrome (Oshima, Sidorova et al. 2017) and Hutchinson-Gilford progeria (Gonzalo, Kreienkamp et al.

2017). Symptoms of these disorders typically include reduced growth, decreased lifespan, bone and organ abnormalities and loss of subcutaneous fat.

The role of insulin signaling in aging and longevity is highly conserved (Giannakou and Partridge 2007). IGF-1 is predominantly produced by the liver and important for stem cell proliferation in many organs and some evidence points to depleted stem cell pools as a main cause of aging (Burtner and Kennedy 2010, Guntur and Rosen 2013). A case in point is Hutchinson-Gilford progeria, which is caused by mutations in *Zmpste24*, a metallopeptidase involved in the processing of lamin A (Mariño, Ugalde et al. 2010). When lamin A is mutated, the nuclear membrane is unable to form properly, resulting in DNA damage and genome instability (Mariño, Ugalde et al. 2010). *Zmpste24*-null mice exhibit accelerated aging phenotypes and low levels of circulating IGF-1 (Mariño, Ugalde et al. 2010). Remarkably, treatment of these mice with IGF-1 results in amelioration of aging features and extends lifespan, demonstrating that reduction of IGF-1 levels is a cause rather than an effect of premature aging (Mariño, Ugalde et al. 2010).

Xing et al. (2012) showed that mice deficient in thyroid hormone also exhibit premature aging phenotypes associated with greater than 50% decrease in *Igf1* expression in liver and bone (Xing, Govoni et al. 2012). In this case, treatment with both the prohormone thyroxine (T4) and the active hormone triiodothyronine (T3) rescued IGF-1 levels as well as the phenotypic defects (Xing, Govoni et al. 2012). Thyroid hormone mediated gene expression is regulated through the binding of T3 to the thyroid hormone receptors (TRs). TRs act as transcription factors to either enhance or repress transcription depending on whether T3 is bound or not (Xu, Glass et al. 1999, Grøntved, Waterfall et al. 2015). Moreover, Visser et al. (2016) reported suppressed thyroid hormone signaling and increased expression of the thyroid hormone deactivating enzyme deiodinase 3 (Dio3) in mouse models of Xeroderma pigmentosa, Cockayne syndrome and in wild type aged mice (Visser, Bombardieri et al. 2016). Taken together, this evidence suggests that thyroid hormone signaling is implicated in progeria through the regulation of IGF-1 in early postnatal development.

Given that the *Atrx*^{FoxG1cre} mice exhibit signs of accelerated aging as well as reduced levels of T4 and IGF-1, we hypothesized that T4 treatment could rescue abnormalities in these mice and perhaps extend longevity. Our findings demonstrate that, contrary to our expectation, T4 administration does not rescue IGF-1 serum levels nor the associated adverse phenotypes. Upon further investigation, we found that *Atrx* is partially deleted in the liver of *Atrx*^{FoxG1cre} mice and is required for the expression of a subset of thyroid hormone responsive genes, including *Igf1*, providing a potential explanation for the lack of rescue in this model system.

2.2 Materials and methods

2.2.1 Mouse husbandry and genotyping

Mice were exposed to 12-hour light/12-hour dark cycles and fed tap water and regular chow ad libitum. The *Atrx*^{loxP} mice have been described previously (Berube, Mangelsdorf et al. 2005, Garrick, Sharpe et al. 2006). *Atrx*^{loxP} mice, when mated to mice expressing Cre recombinase under the control of the *Foxg1* promoter (*Foxg1KiCre*, RRID:MGI:3767191) (Hebert and McConnell 2000), produce male progeny with *Atrx* deficiency in the forebrain and anterior pituitary (*Atrx*^{loxP}*;Foxg1KiCre* or *Atrx Foxg1cre* for simplicity, RRID:MGI:3530074). Ear or tail genomic DNA was used for genotyping. *Atrx*, *Cre*, and *Sry* genotyping was performed by PCR as previously described (Berube, Mangelsdorf et al. 2005).

2.2.2 Thyroxine injections

Control and *Atrx*^{FoxG1cre} mice were injected subcutaneously from birth (P0) until P14 with 0.1, 0.5 or 1.0 mg/kg L-thyroxine (T4) (Sigma, T2376-100MG).

2.2.3 Measurements of T4, T3, IGF-1 and glucose

Plasma samples were collected from P14 mice. Blood was collected from the inferior vena cava. EDTA pH 7.0 was added to the blood sample and centrifuged at 10000 RPM

for 10 minutes at 4°C. Plasma supernatant was collected and kept frozen at –80°C. Plasma T4 was assayed using a mouse T4 ELISA kit (Calbiotech, T4044T-100) according to the manufacturers' instructions. Plasma T3 was assayed using a mouse T3 ELISA kit (T3043T-100) according to the manufacturers' instructions. Plasma IGF-1 content was measured using a mouse IGF-1 ELISA kit (R&D Systems, MG100) according to the manufacturers' instructions. Blood glucose levels were measured prior to sacrifice using the ReliOn Prime Blood Glucose Meter according to the manufacturer's instructions.

2.2.4 Hematoxylin and eosin staining of skin sections

P20 mice were perfused with 4% paraformaldehyde before PBS washes and dehydration. Skin tissue was flash-frozen in liquid nitrogen using Cryomatrix (ThermoFisher Scientific) cryoprotectant (Berube, Jagla et al. 2002) and sectioned at 8µm (Leica CM 3050S). H&E staining was performed as follows: CAT hematoxylin (Biocare Medical) for 2 minutes, wash for 30 seconds, Tasha's bluing solution (Biocare Medical) for 30 seconds, wash for 10 minutes, eosin Y (Biocare Medical) for 2 minutes, 70% ethanol for 1 minute, 90% ethanol for 1 min, 100% ethanol for 4 min, Xylene for 15 minutes. Slides were mounted with permount (Fisher Scientific).

2.2.5 Immunofluorescence and antibodies

P14 or P20 mice were perfused with 4% paraformaldehyde before PBS washes and dehydration. Liver tissue was flash-frozen in liquid nitrogen using Cryomatrix (ThermoFisher Scientific) cryoprotectant (Berube, Jagla et al. 2002) and sectioned and sectioned at 8µm (Leica CM 3050S). For immunofluorescence staining of cryosections, antigen retrieval was performed on slides by warming 10 mM sodium citrate pH 6 solution to approximately 95°C and microwaving slides in solution for 10 minutes on low. After cooling, slides were washed, blocked with 10% normal goat serum (Sigma-Aldrich) for 1 hour and incubated with primary antibody overnight at 4°C. Slides were washed in PBS and incubated with secondary antibody for 1 hour. Sections were counterstained with DAPI (Sigma-Aldrich) and mounted with permafluor (ThermoFisher

Scientific). The following primary antibodies were used: anti-ATRX, rabbit polyclonal (1:75, Santa-Cruz Biotechnology, sc-15408, RRID:AB_2061023), anti-GFP, chicken polyclonal (1:150, ThermoFisher Scientific, PA1-9533, RRID:AB_1074893), anti-RFP, rabbit polyclonal (1:150, Rockland, 600-401-379, RRID:AB_2209751), anti-Albumin, goat polyclonal (1:600, Bethyl, A90-134, RRID:AB_67120), anti-F4/80, rat monoclonal (Abcam, ab6640, RRID:AB_1140040), anti-GFAP, mouse monoclonal (Sigma-Aldrich Cat# G3893, RRID:AB_477010). The secondary antibodies used were goat anti-rabbit-Alexa Fluor 594 (1:800 dilution; ThermoFisher Scientific, A-11012, RRID:AB_2534079), goat anti-chicken-Alexa Fluor 488 (1:800 dilution; ThermoFisher Scientific, A-11039, RRID:AB_2534096), goat anti-mouse-Alexa Fluor 488 (1:800 dilution; ThermoFisher Scientific, A-21121, RRID:AB_2535764) and goat anti-rat-Alexa Fluor 488 (1:800 dilution; Thermo Fisher Scientific, A-11006, RRID:AB_2534074).

2.2.6 Microscopy, imaging and cell counts

Hematoxalin and eosin images were captured using a scanscope (Aperio CS model, Leica). Scanscope console imaging software was used for automated image capture and processing was performed using Volocity software (PerkinElmer). Immunofluorescence images were captured using an inverted microscope (DMI 6000b, Leica). Digital microscopy images were captured with a digital camera (ORCA-ER, Hamamatsu). Openlab imaging software was used for manual image capture, and processing was performed using Volocity software (PerkinElmer). Cell counts in the liver were performed in a blinded and randomized manner for immunofluorescence staining of ATRX in control or experimental cryosections using Volocity (PerkinElmer Demo Version 6.0.1, RRID:SCR_002668). The counting area was recorded, and cells were counted in 2-6 sections per biological replicate with 3 biological replicates for each genotype.

2.2.7 Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was isolated from liver with the RNeasy Mini Kit (QIAGEN) and reverse transcribed into cDNA as described previously (Jiao, Killela et al. 2012). Control reactions without reverse transcriptase were prepared in parallel. cDNA was amplified with gene-specific primers under the following conditions: 25–35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. For qRT-PCR, cDNA was amplified with iQ SYBR Green Master Mix (Bio-Rad) by 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 (Bio-Rad) software. Gene expression analysis was repeated in duplicate 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. Primer sequences are listed in Table 1.

Gene Name	Forward Primer	Reverse Primer
Igf1	ACC TCA GAC AGG CAT TGT GG	GTT TGT CGA TAG GGA CGG GG
Igf1r	TGT GGT CAA GGA TGA ACC CG	CCT TGG GAT ACC ACA CCC AG
Igf2r	GCA TCT TTC CAC CAG TTC CG	GCT CGT CCT CAT TGT CAT CC
Igfals	CAC ACA ACG CCA TCA CTA GC	CGT TGA AGA GGC CAA AGA AG
Igfbp1	AGC CCA GAG ATG ACA GAG GA	GTT GGG CTG CAG CTA ATC TC
Igfbp2	GCG GGT ACC TGT GAA AAG AG	AAC ACA GCC AGC TCC TTC AT
Igfbp3	GTG ACC GAT TCC AAG TTC CA	TGT CCT CCA TTT CTC TGC GG
Serpina7	CCT TCC AAA AGA GGG ACA CA	CCA AGG TCA TAT GTG GCA GA
Dio1	GGA CAC AAT GCA GAA CCA GA	GCA AAG CTT TTC CAG GAC AG
Dio3	GTT TTT GGC TTG CTC TCA GG	CAA GTC CGA GCT GTG AA
Trβ	CAG AAC CCA CGG ATG AGG AG	GGC ATT CAC AAT GGG TGC TT
Prlr	GCA TCT TTC CAG CAG TTC CG	GCT CGT CCT CAT TGT CAT CC
Ghr	ATT CAC CAA GTG TCG TTC C	TCC ATT CCT GGG TCC ATT CA
Thrsp	ACG GAG CCC CTG ATC TCT AT	GGC TTC TAG GTC CAG CTC CT

Table 1 Primer sequences used for quantitative reverse transcriptase PCR

2.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism6 software (6.05; GraphPad Software Inc.), and all results are expressed as the mean ± SEM unless indicated otherwise. Two independent data sets were compared with the Student's t test (unpaired, 2-tailed). Multiple independent data sets were compared with a one-way ANOVA with post-hoc Tukey's test. Statistical analyses of Kaplan-Meier survival curves were performed using the log-rank test and the Gehan-Breslow-Wilcoxon test. A repeated-measures polynomial modeling analysis (SAS v.9.4, SAS Institute Inc., Cary, NC, USA) was used to compare differences between groups in weight over time. P values of 0.05 or less were considered to indicate significance.

2.2.9 Study approval

All procedures involving animals were conducted in accordance with the regulations of the Animals for Research Act of the Province of Ontario and approved by the University of Western Ontario Animal Care and Use Committee.

2.3 Results

2.3.1 T4 administration does not improve body growth of *Atrx*^{FoxG1cre} mice

We previously reported that *Atrx*^{FoxG1cre} mice have reduced weight, length and a very short lifespan associated with low T4 and IGF-1 (Watson, Solomon et al. 2013). To test whether T4 injections in the postnatal period might ameliorate deleterious outcomes in these mice, we established a protocol consisting of daily injections of three different doses of L-thyroxine (T4) or PBS as a control, based on Xing et al. 2012 (Xing, Govoni et al. 2012). Cohorts of *Atrx*^{FoxG1cre} (indicated as cKO in figures) and control mice (indicated as Ctrl in figures) were injected subcutaneously with 0.1, 0.5 or 1.0 mg/kg T4 daily from birth to P14. *Atrx* Foxg1cre mice injected with T4 did not show lifespan extension at any dose of T4 (Figure 2-1A). Furthermore, injection protocols with 0.5 and

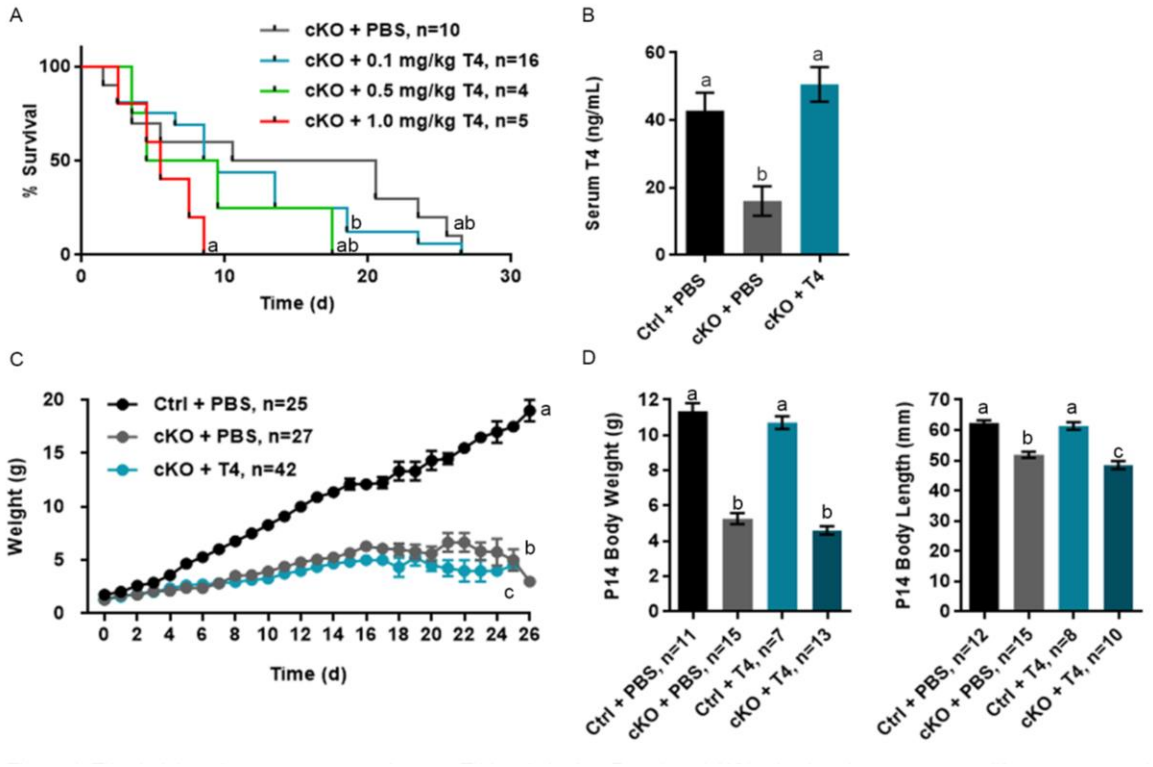

Figure 2-1 T4 administration restores normal serum T4 levels in *Atrx* Foxg1cre (cKO) mice but does not rescue life span or growth abnormalities.

(a) Kaplan-Meier curve depicting survival of *Atrx* Foxg1cre mice injected with various amount of T4. (b) Serum T4 was recovered at P14 in *Atrx* Foxg1cre mice upon T4 treatment (0.1 mg/kg T4) (c) Body weight is not restored to control levels following T4 treatment, (d) Body weight and length were not recovered to control levels at P14 in *Atrx* Foxg1cre mice following T4 treatment. Groups with the same letter have means that are not significantly different. Groups with different letters have means that are significantly different ($p<0.05$). Error bars represent SEM.

1.0 mg/kg resulted in decreased average lifespan compared to *Atrx* Foxg1cre mice + PBS (Figure 2-1A and 2-8). We thus used the lowest dose of T4 (0.01 mg/kg) for subsequent analyses. We confirmed that at this lower dose, T4 levels were restored to control levels and were significantly increased compared to sham-treated *Atrx*^{FoxG1cre} mice (Figure 2-1B). If T4 levels in *Atrx*^{FoxG1cre} mice differed from the control mean by more than 2 standard deviations, they were considered biological outliers and removed from the study (Figure 2-9A). In order to determine whether serum T3 (the active form of thyroid hormone) was increased after T4 treatment in *Atrx*^{FoxG1cre} mice, a T3 ELISA was performed (Figure 2-9B). However, due to the low levels of T3 in the serum (1 ng/mL) we were unable to detect a change in any of the groups at this low level. As previously reported, we observed that the weight of PBS-injected *Atrx*^{FoxG1cre} mice was significantly lower than PBS-injected control mice over time and this effect was not rescued following T4 treatment (Figure 2-1C). Weight and length measurements at P14 show that both PBS- or T4-injected *Atrx*^{FoxG1cre} mice weigh less than half of controls and are shorter in length (Figure 2-1D). These results indicate that, contrary to our expectation, restoration of normal circulating T4 levels in *Atrx*^{FoxG1cre} mice failed to improve their reduced size and short lifespan.

2.3.2 Low subcutaneous fat and blood glucose are not improved by T4 administration

The *Atrx*^{FoxG1cre} mice were previously reported to have very low levels of subcutaneous fat and serum glucose (Watson, Solomon et al. 2013). To measure the effects of T4 injections on these parameters, we examined skin sections and blood taken from P20 *Atrx*^{FoxG1cre} mice treated with PBS or T4. These analyses confirmed our previous findings that subcutaneous fat thickness was significantly reduced in *Atrx*^{FoxG1cre} mice compared to controls; however, it was not improved in T4-treated *Atrx*^{FoxG1cre} mice and appears to be further reduced (Figure 2-2A). In contrast, thickness of the dermal layer was not affected by the deletion of *Atrx* or T4 treatment. A similar outcome was observed with serum glucose level, which was significantly decreased in both PBS- and T4-treated *Atrx* Foxg1cre mice compared to control mice (Figure 2-2B). Additionally, the liver and

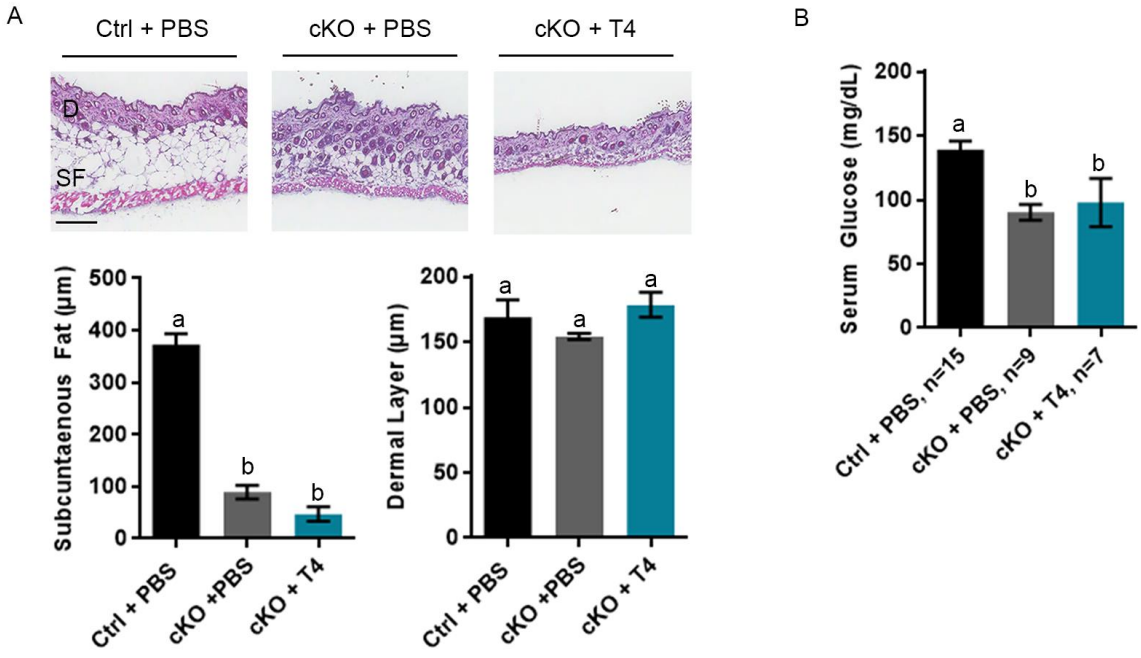

Figure 2-2 T4 administration in *Atrx*^{FoxG1cre} (cKO) mice does not ameliorate reduction in subcutaneous fat or serum glucose.

(a) H&E staining of P20 skin cryosections shows that subcutaneous fat (SF) is not restored to control levels in *Atrx*^{FoxG1cre} mice following T4 treatment (n=3). Dermal layer (D) was unchanged between control and either PBS or T4 treated *Atrx*^{FoxG1cre} mice as previously reported (Scale bar = 200 µm). (b) Serum glucose at P14 was not restored to control levels in *Atrx*^{FoxG1cre} mice following T4 treatment. Groups with the same letter have means that are not significantly different. Groups with different letters have means that are significantly different (p<0.05). Error bars represent SEM.

spleen of PBS-treated *Atrx*^{FoxG1cre} mice were decreased in size when compared to control. Upon T4 treatment, we observed a slight increase in liver and spleen weights (relative to body weight) in *Atrx*^{FoxG1cre}, but it was not significantly different from either PBS treated or control mice (Figure 2-10). At P20, heart weight was unchanged in *Atrx*^{FoxG1cre} mice treated with PBS compared to control, however following treatment with T4, heart weight increased significantly (Figure 2-10). In summary, subcutaneous fat and serum glucose levels were not rescued by T4 treatment in *Atrx*^{FoxG1cre} mice.

2.3.3 T4 administration fails to recover circulating and hepatic IGF-1 levels

We confirmed that *Atrx*^{FoxG1cre} serum IGF-1 level at P14 is 17.7% that of control mice (Figure 2-3A). However, recovery of normal T4 circulating levels did not result in the expected increase of serum IGF-1. Given that the liver is the major source of circulating IGF-1, we next examined liver *Igf1* expression by quantitative reverse transcriptase PCR (RT-qPCR) and found that *Igf1* expression is significantly decreased in the liver of *Atrx*^{FoxG1cre} mice, even after T4 supplementation (Figure 2-3A).

The decrease in serum IGF-1 in *Atrx*^{FoxG1cre} mice could be due to alterations in *Igf1* receptor (*Igf1r)* or *Igf2* receptor (*Igf2r*) expression. IGF-1 binds Igf1r, which elicits an intracellular response, whereas Igf2r acts as a scavenger receptor and no response occurs. While *Igf1r* expression is not altered in the liver, *Igf2r* expression is decreased in the *Atrx*^{FoxG1cre} compared to control as previously reported (Watson, Solomon et al. 2013) and not recovered by T4 treatment (Figure 2-3B). These data provide evidence that IGF-1 is able to bind Igf1r and is not subject to deactivation by high levels of Igf2r.

Serum IGF-1 could also be decreased due to gene expression alterations in IGF binding proteins (IGFBPs) and acid labile subunit (IGFALS). Both IGFALS and IGFBPs bind IGF-1, enhancing or diminishing IGF-1 signaling depending on the need of the cell. *Igfals* and *Igfbps* are aberrantly expressed in livers of *Atrx*^{FoxG1cre} mice compared to controls. *Igfals* expression is significantly decreased in *Atrx*^{FoxG1cre} mice + PBS compared

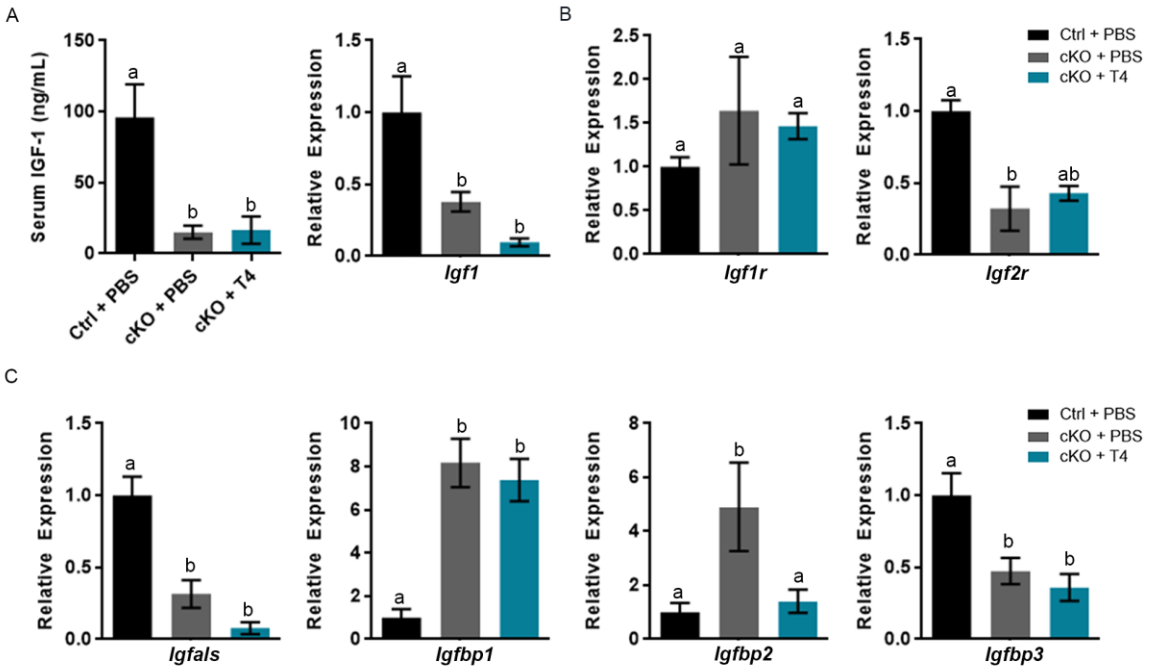

Figure 2-3 T4 administration does not rescue decreased serum IGF-1 or dysregulated gene expression in the liver of *Atrx*^{FoxG1cre} (cKO) mice at P14.

(a) Serum IGF-1 and expression of liver *Igf1* are not restored to control levels following T4 treatment in *Atrx*^{FoxG1cre} mice. (b) *Igfr* expression was not changed between control and either PBS or T4 treated Foxg1cre mice. *Igf2r* expression was not restored to control levels following T4 treatment in *Atrx* Foxg1cre mice. (c) Gene expression of IGF-1 complex proteins, *Igfbp1* and *Igfbp3* were not restored to control levels in *Atrx*^{FoxG1cre} mice, except in the case of *Igfbp2* which was restored to control levels. qRT-PCR data were normalized to β-actin expression, n=3-6. Groups with the same letter have means that are not significantly different. Groups with different letters have means that are significantly different (p<0.05). Error bars represent SEM.

to control and is not rescued by T4 treatment. *Igfbp1* and *Igfbp2* expression are significantly increased in *Atrx*^{FoxG1cre} compared to control mice. *Igfbp2*, but not *Igfbp1* expression is restored to control levels in *Atrx*^{FoxG1cre} + T4. Low *Igfbp2* expression has been reported in hypothyroidism and its increase in expression is likely due to a feedback mechanism following increased serum T4. *Igfbp3* expression is decreased in the *Atrx*^{FoxG1cre} + PBS and not restored to control levels following T4 treatment (Figure 2-3C). These results indicate that despite restoring serum T4 levels, most genes involved in IGF-1 signaling are still aberrantly expressed.

2.3.4 T4 administration restores thyroxine binding globulin gene expression and a subset of thyroid hormone responsive genes in the liver of *Atrx*^{FoxG1Cre} mice

Alterations in the gene *Serpina7* (thyroxine binding globulin (TBG)), one of the thyroid hormone transport molecules, could also affect transport of T4. *Serpina7* is significantly increased in the *Atrx*^{FoxG1cre} + PBS and is restored to control levels following treatment with T4 (Figure 2-4A). We next examined the effect of T4 injections on the expression of several thyroid hormone target genes in the liver of *Atrx*^{FoxG1cre} mice. *Dio1*, which converts T4 to its active form T3, is expressed at slightly decreased levels in *Atrx*^{FoxG1cre} liver, however not significantly. Dio3, the enzyme which converts T3 to the inactive forms rT3 and T2, is normally expressed embryonically when precise control over thyroid hormone signaling is crucial. *Dio3* was reported to be reactivated in the liver of two mouse models of premature aging and in normal mouse aging (Visser, Bombardieri et al. 2016). *Dio3* is slightly increased in *Atrx*^{FoxG1cre} mice compared to control, however not significantly (Figure 2-4B). Following T4 treatment *Dio3* expression is highly repressed and is significantly decreased in *Atrx*^{Foxg1Cre} mice + T4 compared to *Atrx*^{FoxG1cre} mice + PBS, suggesting that T4 is converted to the T3 active form. *Thyroid hormone receptor β* (*Trβ*), the active thyroid hormone receptor in the liver, is decreased in the *Atrx*^{FoxG1cre} + PBS compared to control. When treated with T4, *Trβ* expression is restored to control levels and is significantly increased compared to *Atrx*^{FoxG1cre} + PBS

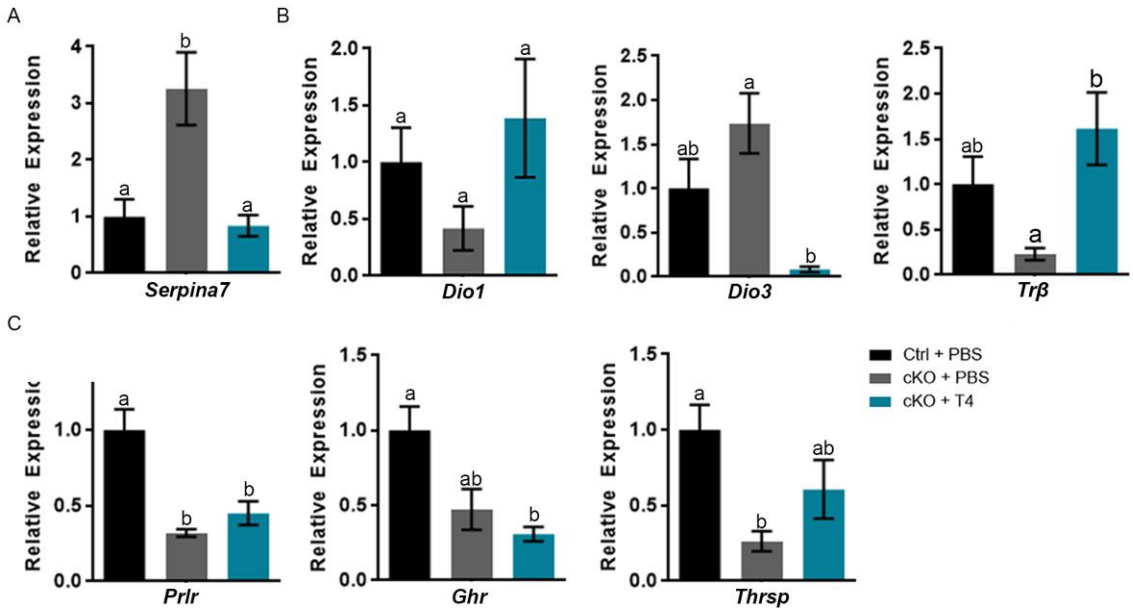

Figure 2-4 T4 administration restores thyroxine binding globulin gene expression and a subset of thyroid hormone responsive genes in the liver of *Atrx*^{FoxG1cre} (cKO) mice at P14.

(a) *Serpina7* (thyroxine binding globulin) gene expression is restored following T4 treatment. (b) Administration of T4 restores thyroid hormone responsive gene expression of *Dio1* and *Trβ* in *Atrx*^{FoxG1cre} mice to that of control mice. In addition, there is a dramatic repression of *Dio3* expression in the liver of *Atrx*^{FoxG1cre} mice at P14. (c) Despite normal expression of *Trβ*, some thyroid hormone responsive genes are not rescued following T4 treatment in *Atrx* Foxg1cre mice. qRT-PCR data were normalized to β-actin expression, n=3-6. Groups with the same letter have means that are not significantly different. Groups with different letters have means that are significantly different (p<0.05). Error bars represent SEM.

(Figure 2-4B). Despite proper regulation and activation of thyroid hormone in the liver, expression of thyroid hormone responsive genes *Prlr, Ghr* and *Thrsp* were not restored to control levels in *Atrx*^{FoxG1cre} mice treated with T4 (Figure 2-4C). While the liver of *Atrx*^{FoxG1cre} mice should essentially be normal, all these results suggested that this is not the case and that it fails to respond to normal thyroid hormone signaling. This prompted us to investigate the possibility that Foxg1Cre expression might be spuriously activated in the liver of these mice, causing *Atrx* gene inactivation.

Cre recombinase expression and *Atrx* deletion in the liver of *Atrx*^{FoxG1Cre} mice

We had previously verified by western blot analysis that ATRX protein levels were normal in the liver in P20 *Atrx*^{FoxG1cre} mice (Watson, Solomon et al. 2013). However, given the results above, we re-examined the status of ATRX in the liver. To achieve this, we introduced the *ROSAmT/mG* reporter allele into the *Atrx*^{FoxG1cre} mice to allow immunofluorescent detection of Cre-recombinase activity through the detection of green fluorescent protein (GFP), while cells that have never been exposed to Cre-recombinase activity display red fluorescent protein (RFP). Immunofluorescence detection of RFP and GFP in liver cryosections at either P14 (Figure 2-5A) or P20 (Figure 2-5C) in *Atrx*$^{wt/Y}$*; Foxg1cre; Rosa mTmG* (controls) and *Atrx*$^{f/Y}$*;Foxg1cre;Rosa mTmG* (cKO) mice revealed the presence of many GFP^{+} cells in the liver. To verify that this resulted in loss of ATRX protein, we performed immunofluorescence staining of ATRX in liver cryosections at either P14 (Figure 2-5B) or P20 (Figure 2-5D). Quantification revealed a significant reduction in the proportion of cells that express ATRX in the liver *Atrx*^{FoxG1cre} mice compared to controls at both P14 (Figure 2-5E) and P20 (Figure 2-5F). These results indicate that Cre-mediated *Atrx* inactivation occurred in the liver of *Atrx*^{FoxG1cre} mice. However, at P20, we noted an increase in the total number of cells in the *Atrx*^{FoxG1cre} liver, and that many oblong-shaped cells expressed liver, and that many oblong-shaped cells expressed high levels of ATRX when compared to control (Figure 2-5F). We speculate that these cells represent a regenerative event mediated by proliferating ATRX^{+} Kupffer or stellate cells, likely explaining the lack of difference in

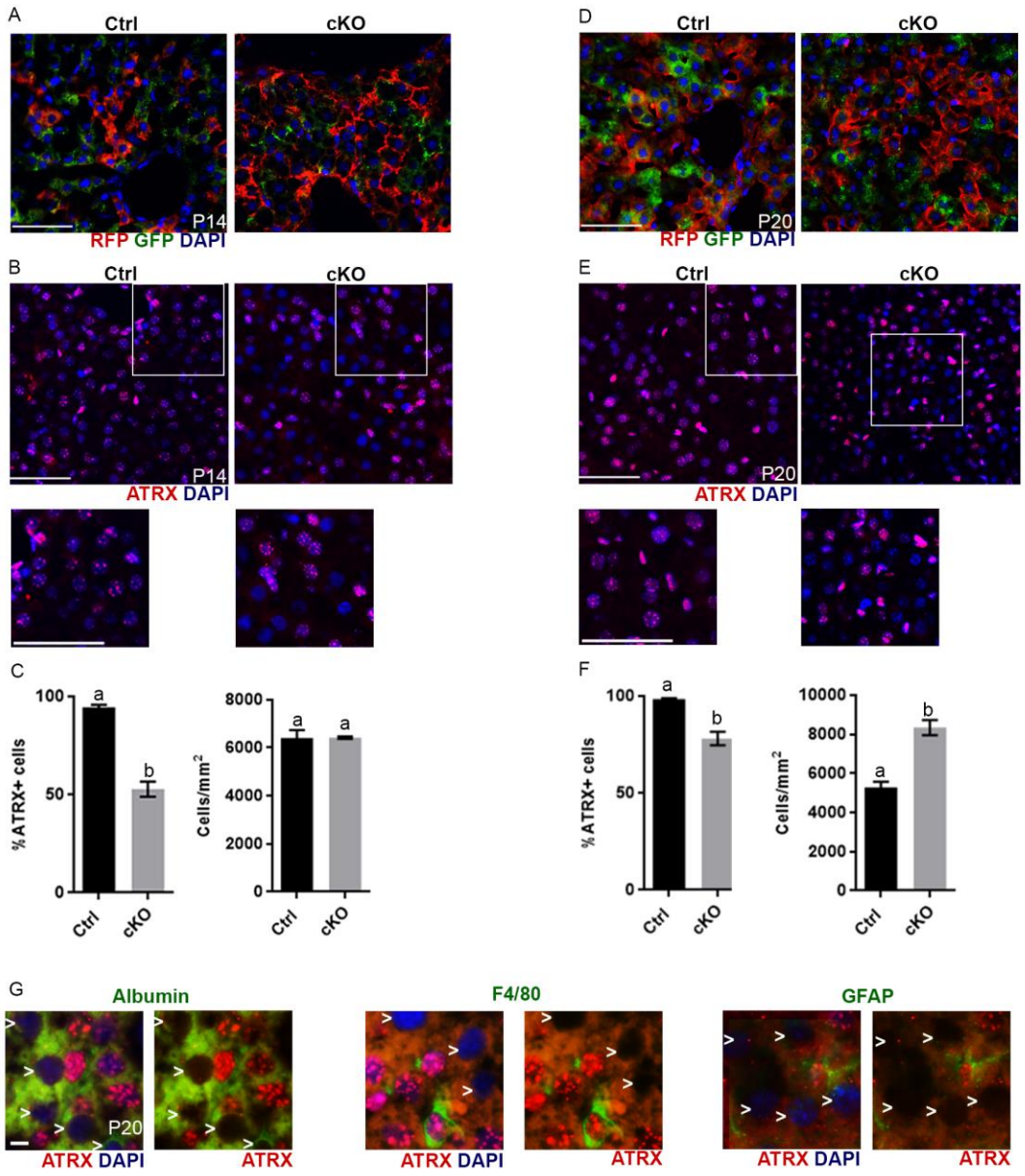

Figure 2-5 Cre recombinase activation in the liver results in fewer hepatocytes that express ATRX at P14 and P20 in *Atrx*^{FoxG1cre} (cKO) mice.

(a) Co-immunofluorescence detection of RFP and GFP in liver cryosections at P14 in ROSAmT/mG mice shows presence of GFP staining in *Atrx*^{FoxG1cre} mice indicating Cre recombinase activation. (b) Immunofluorescence staining of ATRX in liver cryosections at P14. White box indicates area of zoom (n=3). Cell counts of ATRX staining revealed a

significant reduction in % ATRX^{+} cells in *Atrx*^{FoxG1cre} compared to control at P14 despite equal number of cells. (c) Co-immunofluorescence detection of RFP and GFP in liver cryosections at P20 in ROSAmT/mG mice shows presence of GFP staining in *Atrx*^{FoxG1cre} mice indicating Cre recombinase activation. (d) Immunofluorescence staining of ATRX in liver cryosections at P20. White box indicates area of zoom (n=3). (e) Cell counts of ATRX staining revealed a significant reduction in % ATRX^{+} cells in *Atrx*^{FoxG1cre} mice compared to control at P14. (f) At P20, there is a reduction in the % ATRX+ cells in *Atrx*^{FoxG1cre} mice compared to control and there is also an increase in the number of cells in *Atrx*^{FoxG1cre} mice when compared to control. *Atrx*^{FoxG1cre} mice when compared to control. Scale bar = 50 µm. (g) At P20, immunofluorescence staining in *Atrx*^{FoxG1cre} mice shows cells that do not express ATRX protein also costain with albumin, a marker of hepatocytes and do not costain with F4/80 (Kupffer cell marker) or GFAP (stellate cell marker). Scale bar = 5 µm. White arrows indicate cells that do not express ATRX. Original magnification, 40x. Groups with the same letter have means that are not significantly different. Groups with different letters have means that are significantly different (p<0.05). Error bars represent SEM.

the levels of ATRX protein previously observed in the P20 liver of *Atrx*^{FoxG1cre} mice by Western blot analysis (Watson, Solomon et al. 2013). Immunofluorescence detection of ATRX and either albumin (hepatocyte marker), F4/80 (Kupffer cell marker) or GFAP (stellate cell marker) in *Atrx*^{FoxG1cre} mice revealed that cells lacking ATRX co-express albumin but not F4/80 or GFAP, indicating that Cre-mediated *Atrx* deletion occurs in hepatocytes (Figure 2-5G).

Increased expression of a subset of thyroid hormone responsive genes upon T4 administration require ATRX expression

Several thyroid hormone responsive genes (*Igf1*, *Prlr*, *Ghr* and *Thrsp*) failed to respond to T4 treatment in *Atrx* Foxg1cre mice, suggesting that their induction by T4 might require ATRX. To test this, we compared the effect of T4 treatment of control and *Atrx*^{FoxG1cre} mice on the expression of *Igf1*, *Prlr*, *Ghr* and *Thrsp* in the liver. The results show that these thyroid hormone responsive genes are induced by T4 in control mice, but not in *Atrx* mutants (Figure 2-6), confirming a dependence on ATRX (Figure 2-7).

2.4 Discussion

Low serum IGF-1 has been implicated as a driver of premature aging syndromes (Mariño, Ugalde et al. 2010, Xing, Govoni et al. 2012). More recently, thyroid hormone signaling suppression has been linked to both premature aging and normal aging in mice (Xing, Govoni et al. 2012, Visser, Bombardieri et al. 2016). Given the previous observation that the *Atrx*^{FoxG1cre} mice have both low T4 and IGF1 (Watson, Solomon et al. 2013), we tested the effects of T4 injections. However, despite recovery of normal T4 levels, the signaling that promotes *Igf1* expression in the liver was found to be defective, likely due to previously unrecognized Cre-mediated inactivation of the *Atrx* gene in the liver. Thus, while several thyroid hormone target genes were responsive to T4 treatment, several others, including *Igf1*, were not, suggesting that ATRX is required for the induction of these genes in the liver. As a result, T4 administration does not rescue decreased lifespan, reduced growth or other defects in *Atrx*^{FoxG1cre} mice. Higher doses of

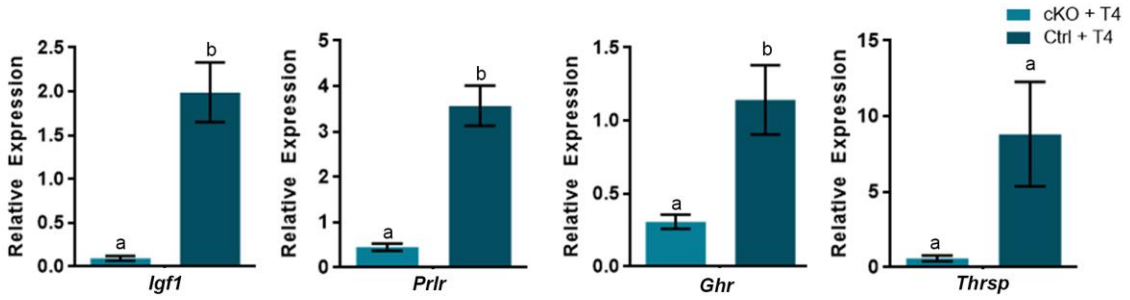

Figure 2-6 A subset of thyroid hormone responsive genes are induced in the P14 liver in the presence of excess T4, in an ATRX-dependent manner.

Administration of T4 induces gene expression at or above the level of controls treated with PBS (control set to 1) only when ATRX is present in Ctrl + T4 mice and not in *Atrx*^{FoxG1cre} + T4 mice at P14. qRT-PCR data were normalized to β-actin expression, n=3-6. Groups with the same letter have means that are not significantly different. Groups with different letters have means that are significantly different (p<0.05). Error bars represent SEM.

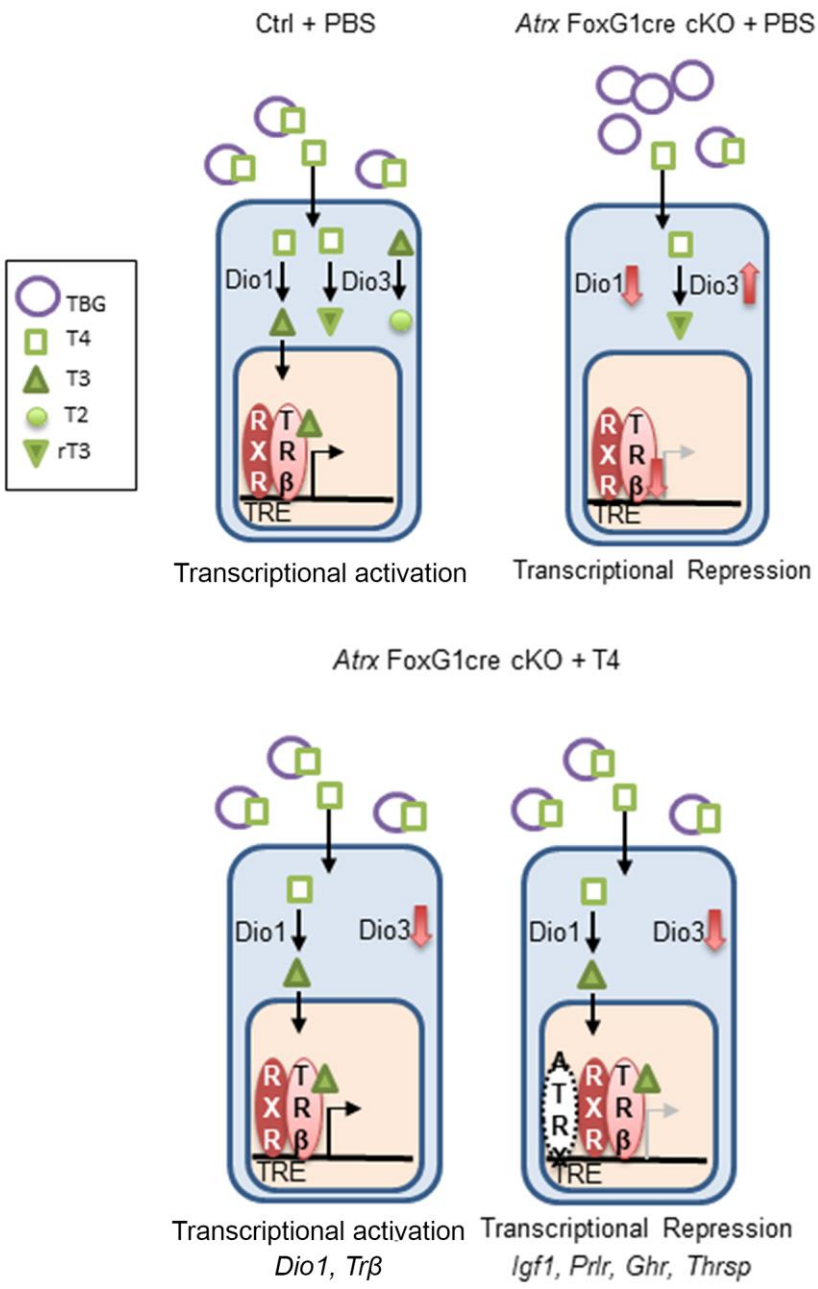

Figure 2-7 ATRX is required for the transcription of several thyroid hormone responsive genes in the liver.

In the control, T4 is bound to thyroxine binding globulin (TBG) in the blood. It is converted to T3 in the liver by *Dio1*. Both T4 and T3 can be inactivated by *Dio3* to produce the inactive molecules T2 and rT3. T3 binds its receptor *Trβ* (found as a heterodimer with retinoid x receptor), which enters the nucleus, binds a thyroid hormone

responsive element and initiates transcription. In *Atrx*^{FoxG1cre} mice there is an increase in TBG and a decrease in T4 in the serum. In the liver, there is a decrease in *Dio1* and an increase in *Dio3*. Any T4 present is likely converted to rT3. Low levels of *Trβ* acts as a transcriptional repressor. Following treatment with T4 in *Atrx* Foxg1cre mice, there are control levels of TBG and T4 in the blood. In the liver, *Dio1* is at control levels and *Dio3* is repressed. T4 is converted to T3 where it binds its receptor and transcription occurs normally. This occurs for a subset of genes (*Dio1* and *Trβ*). However, some genes (*Igf1*, *Prlr*, *Ghr* and *Thrsp*) are still transcriptionally repressed following T4 treatment. This suggests that ATRX is required for the transcription of some thyroid hormone responsive genes.

T4 that we initially tested (0.5 and 1.0 mg/kg daily) were likely inducing hyperthyroidism in these small, young mice. The lowest dose, 0.1 mg/kg T4 daily, appeared to have variable effects as some *Atrx*^{FoxG1cre} mice injected received well above control levels or levels similar to uninjected *Atrx*^{FoxG1cre} mice. Whether this was experimental error or *Atrx*^{FoxG1cre} mice have variable absorption is unknown. Nevertheless, we used only *Atrx*^{FoxG1cre} mice that expressed control levels of serum T4 after treatment for subsequent analyses. Our results suggest that restoring normal levels of serum T4 in *Atrx*^{FoxG1cre} mice does not ameliorate premature aging phenotypes. It is known that hyperthyroidism causes increased metabolic rate, reduced bone density and osteoporosis (Williams and Bassett 2017). Due to the small size of the *Atrx*^{FoxG1cre} mice (46.3% of control weight), it is possible that *Atrx*^{FoxG1cre} mice received levels of T4 that are too high for their body weight and that a lower dose is necessary to eliminate induction of hyperthyroidism in this model. Since *Igf1r* appears to be unaffected by deletion of ATRX, a future experiment would be to directly inject IGF-1 in *Atrx*^{FoxG1cre} mice. It has been shown in a Hutchinson-Gilford Progeria mouse model that IGF-1 treatment extends lifespan and delays onset of premature aging (Mariño, Ugalde et al. 2010).

The inefficacy of T4 treatment in our study could be explained by deletion of *Atrx* in the liver of *Atrx*^{FoxG1cre} mice, which was not previously reported. *Dio1*, *Serpina7* and *Trβ* transcript levels were normalized by T4 treatment, indicating that hepatic T3 is produced and that thyroid signaling is at least partially intact. Conversely, several thyroid hormone responsive genes, including *Igf1*, *Prlr*, *Ghr* and *Thrsp* were not rescued following T4 treatment. Furthermore, when controls were treated with T4, these genes all increased compared to control levels. These data suggest that ATRX must be required for T3-mediated transcription of a subset of genes in the liver.

It is still unknown why deletion of *Atrx* in the liver disrupts proper signaling and production of IGF-1. ATRX contains several LXXLL-type motifs required to bind hormone receptors and in the testes, it was shown that ATRX is able to bind the androgen receptor, facilitating transcription of androgen receptor target genes (Bagheri-Fam, Argentaro et al. 2011). Therefore, it is possible that ATRX binds and cooperates with the

thyroid hormone receptor directly to modulate gene expression in the liver. Moreover, a recent report strongly implicates ATRX in the regulation of gene expression in the liver (Chatzinikolaou, Apostolou et al. 2017). It was shown that the nucleotide excision repair (NER) structure-specific endonuclease ERCC1–XPF, a complex that is mutated in premature aging, recruits the CTCF–cohesin complex, MBD2 and ATRX to promoters and imprinting control regions (ICRs) to silence a subset of imprinted genes during hepatic development (Chatzinikolaou, Apostolou et al. 2017).

ATRX deletion has been shown to result in DNA replication stress and telomere abnormalities (Watson, Solomon et al. 2013). It is also possible that in addition to low levels of IGF-1, DNA damage is accumulating in hepatocytes lacking ATRX and these cells are dying or undergoing senescence, further exacerbating low levels of stem cells in the liver. In the future, it will be necessary to investigate the full extent of ATRX function in the liver and to further explore the link between ATRX and thyroid hormone mediated transcription.

2.5 Supplementary Figures

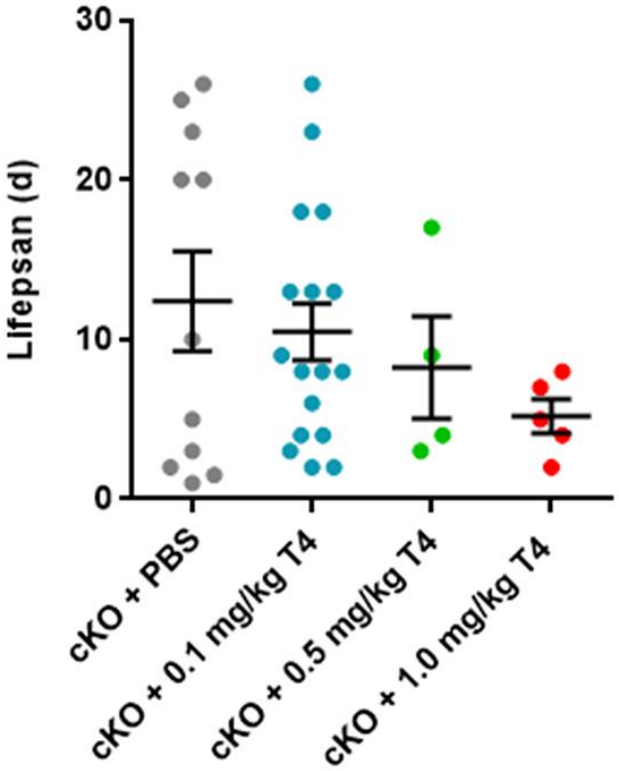

Figure 2-8 T4 supplementation does not improve lifespan in *Atrx*^{FoxG1cre} (cKO) mice.

Average lifespan was not significantly changed between PBS and T4 treated *Atrx*^{FoxG1cre} mice. Error bars represent SEM.

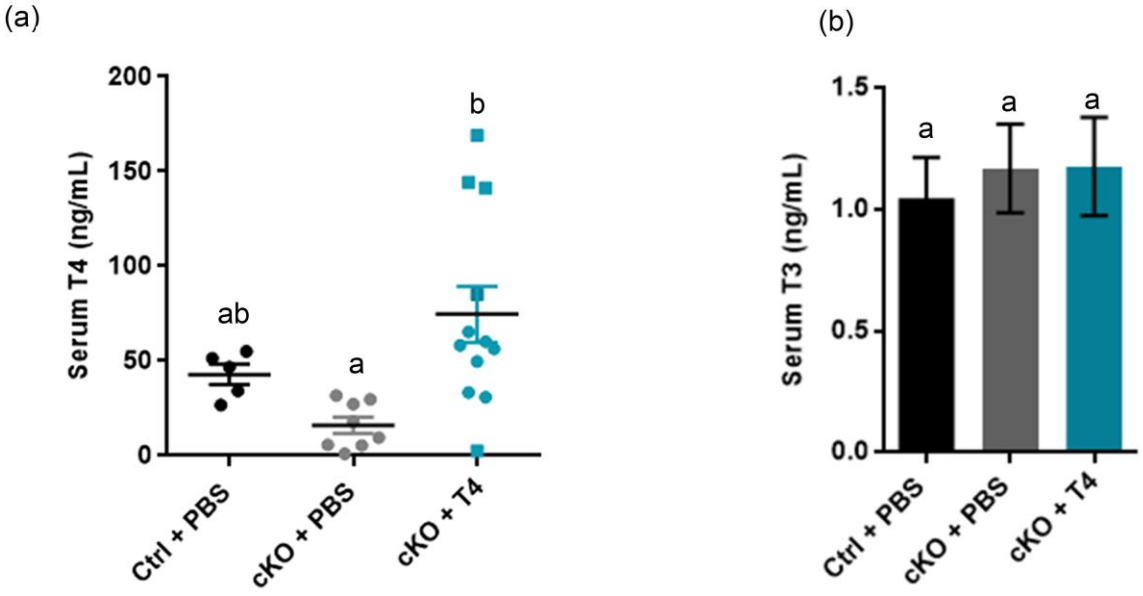

Figure 2-9 T4 supplementation results in variable serum T4 levels in *Atrx*^{FoxG1cre} (cKO) mice.

Variable serum T4 levels and unchanged serum T3 levels in *Atrx*^{FoxG1cre} (cKO) mice upon T4 supplementation. (a) Graph of Serum T4 levels in *Atrx*^{FoxG1cre} mice following T4 treatment. Biological outliers are represented as squares and these mice were excluded from further analyses. (b) Graph of serum T3 levels in *Atrx*^{FoxG1cre} mice following T4 treatment. Groups with the same letter have means that are not significantly different. Groups with different letters have means that are significantly different ($p<0.05$). Error bars represent SEM.

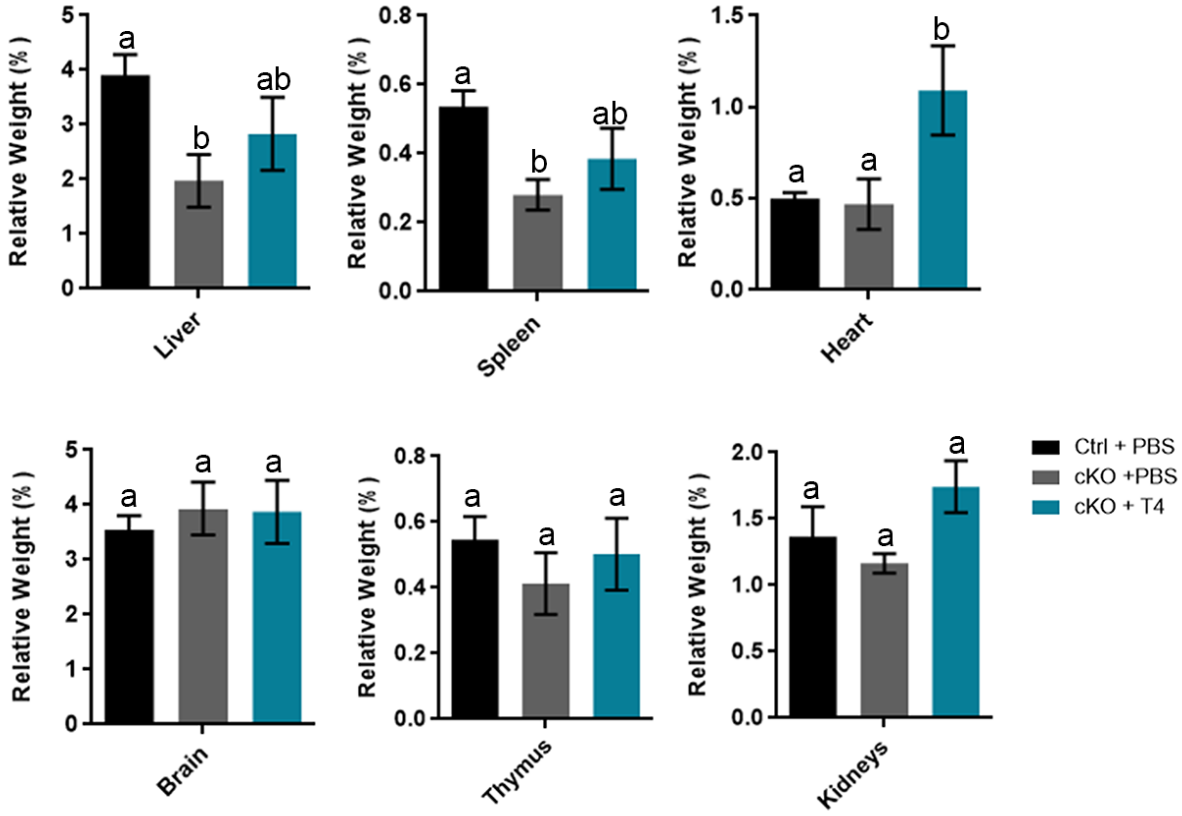

Figure 2-10 Liver and spleen abnormalities in *Atrx*^{FoxG1cre} (cKO) mice are partially rescued following T4 treatment.

Following T4 treatment in *Atrx*^{FoxG1cre} mice, abnormal relative organ weights in the liver and spleen are statistically similar to PBS treated control. There is an increase in relative heart weight in T4 treated *Atrx*^{FoxG1cre} mice compared to PBS treated control mice. There was no significant difference in brain, thymus or kidneys relative weight. Groups with the same letter have means that are not significantly different. Groups with different letters have means that are significantly different (p<0.05). Data were normalized to body weight, n=3-6. Error bars represent SEM.

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Chapter 3

3 Defective gliogenesis underlies aberrant myelination elicited by ATRX deficiency

Hypomyelination has been reported in both mouse models and patients with premature aging (Koob, Laugel et al. 2010, Revet, Feeney et al. 2012). Additionally, there is deterioration of the myelin sheath consistent with axon degeneration present in normal aging. The molecular mechanisms that control myelination during development are incompletely resolved, however, it is well documented that ligand-bound TR directly activates myelin gene expression and promotes oligodendrocyte differentiation (Farsetti, Mitsuhashi et al. 1991, Billon, Tokumoto et al. 2001, Billon, Jolicoeur et al. 2002, Dong, Yauk et al. 2009). As shown in chapter 2, postnatal systemic thyroxine administration can improve, but not completely rescue myelin levels, pointing to additional roles of ATRX in this process. Here, we show that inactivation of *Atrx* in postnatal OPCs increases plasticity of these cells *in vitro* and *in vivo*, allowing a shift from a strict oligodendrocyte differentiation program toward ectopic astrogliogenesis. Mechanistically, we provide evidence that ATRX associates with HDAC3 to promote the expression of Olig2, a key oligodendrocyte fate specification factor. These findings identify a role for ATRX in the oligodendrocyte-astrocyte fate choice in the postnatal cortex.

3.1 Introduction

Myelin is an extension of the oligodendrocyte (OL) plasma membrane that wraps itself around axons to increase conduction velocity, enabling efficient propagation of action potentials. A series of events are necessary for the production of myelin, including proliferation and migration of oligodendrocyte precursor cells (OPCs), recognition of

recipient axons, membrane expansion, wrapping of axons and the appropriate production of membrane components and myelin proteins for compaction (Nave and Werner 2014).

In the developing cortex, the generation of neurons by neural progenitor cells eventually shifts towards production of astrocytes and then OLs (Sauvageot and Stiles 2002). The majority of primitive OPCs are generated postnatally from neural stem cells through the expression of the oligodendrocyte basic-helix-loop-helix transcription factors Olig1 and Olig2 (Zhou and Anderson 2002). Olig2 is required for OPC generation and also suppresses the astrocytic lineage (Zhou, Choi et al. 2001, Cai, Chen et al. 2007, Zhu, Zuo et al. 2012, Zuo, Wood et al. 2018). Downstream of Olig2, the SRY-box transcription factor 10 (Sox10) participates in a positive feedback loop to establish the OPC lineage (Finzsch, Stolt et al. 2008, Kuspert, Hammer et al. 2011, Yu, Chen et al. 2013). Notably, Sox10 activates several OPC-critical genes, including *Chondroitin sulfate proteoglycan* (*Cspg4*; protein: neural glial antigen 2 (NG2)) and *Platelet-derived growth factor receptor α* (*Pdgfrα*) (Pringle, Mudhar et al. 1992, Nishiyama, Lin et al. 1996, Nishiyama, Lin et al. 1996). NG2 is a surface type I transmembrane core protein involved in cell migration and proliferation (Makagiansar, Williams et al. 2007), whereas PDGFRα promotes OPC proliferation, migration and survival (Noble, Murray et al. 1988, Pringle, Collarini et al. 1989). Further commitment to the OL lineage starts with the generation of pre-OLs which, after cell cycle exit, express myelin regulatory factor (MRF) (Emery, Agalliu et al. 2009). As OLs mature and contact axons, they become highly branched and upregulate several myelin sheath proteins in order to encompass the axon (Mitew, Hay et al. 2014, Nave and Werner 2014). The expression of myelin proteins is complex and regulated by several different transcription factors including the aforementioned Olig1 (Li, Lu et al. 2007), Sox10 (Stolt, Rehberg et al. 2002, Li, Lu et al. 2007) and MRF (Emery, Agalliu et al. 2009) but also Sp1 (Wei, Miskimins et al. 2004), Nkx2.2 (Qi, Cai et al. 2001) and the ligand-bound thyroid hormone receptor (Farsetti, Mitsuhashi et al. 1991, Ibarrola and Rodríguez-Peña 1997, Dong, Yauk et al. 2009).

Myelination has been studied extensively in the context of demyelinating diseases like multiple sclerosis and leukodystrophies (Franklin and ffrench-Constant 2008). However, myelin deficits are also frequently observed in neurodevelopmental cognitive

disorders like autism and schizophrenia and can contribute to intellectual disability (Courchesne, Press et al. 1993, Wolkin, Rusinek et al. 1998, Iwase, Bérubé et al. 2017). This is exemplified by the ATR-X syndrome, an intellectual disability disorder caused by abnormalities in the ATRX chromatin remodeling protein (Gibbons, Picketts et al. 1995). Affected boys often exhibit cognitive dysfunction, seizures and autistic features, among other developmental defects (Gibbons, Picketts et al. 1995, Gibbons and Higgs 2000, Wada, Ban et al. 2013, Lee, Lee et al. 2015, Giacomini, Vari et al. 2019). ATRX syndrome patients also display abnormal myelination with a prevalence of 44% (Wada, Ban et al. 2013, Lee, Lee et al. 2015) making white matter abnormalities a supporting feature of the diagnostic criteria.

ATRX belongs to the Switch/Sucrose non-fermenting (Swi/Snf) family of chromatin remodelers (Picketts, Higgs et al. 1996), and can regulate gene expression through several mechanisms. In the newborn cortex, ATRX represses expression of imprinted genes by fostering long-range chromatin interactions mediated by CCCTC-binding factor (CTCF) and cohesin (Kernohan, Jiang et al. 2010, Kernohan, Vernimmen et al. 2014). In embryonic stem cells, it promotes maintenance of the repressive H3K27me3 histone mark at polycomb target genes (Sarma, Cifuentes-Rojas et al. 2014). On the other hand, ATRX can promote gene transcription through the incorporation of histone H3.3 and facilitating RNA PolII elongation through G-rich sequences (Levy, Fernandes et al. 2008, Levy, Kernohan et al. 2015). In testes, ATRX associates with the androgen receptor and facilitates transcription of its target genes (Bagheri-Fam, Argentaro et al. 2011). Additionally, ATRX was also reported to promote gene expression at the α-globin cluster through the negative regulation of histone variant macroH2A (Ratnakumar, Duarte et al. 2012).

The molecular or cellular basis for white matter abnormalities associated with ATRX dysfunction has not yet been described. Here, we report that conditional deletion of *Atrx* in the mouse forebrain and anterior pituitary causes hypomyelination. While myelin levels and OL numbers were partially recovered upon administration of the thyroid hormone thyroxine (T4) , this treatment failed to replenish OPCs, indicating that ATRX might be required to maintain these progenitors independently of circulating T4

levels. Indeed, targeted inactivation of *Atrx* in Sox10-expressing OPCs, but not in neurons, resulted in CNS hypomyelination, revealing cell autonomous functions of ATRX in OPCs. We demonstrate that ATRX-null OPCs tend to revert to a more malleable state, and in so doing gain the ability to differentiate into astrocytes. Mechanistically, we find that ATRX occupies the *Olig2* gene locus and binds histone deacetylase 3 (HDAC3), another regulator of OL versus astrocyte fate choice.

3.2 Methods

3.2.1 Animal husbandry and genotyping

Mice were exposed to 12-hour light/12-hour dark cycles and fed water and regular chow ad libitum. The *Atrx*^{loxP} mice have been described previously (Berube, Mangelsdorf et al. 2005) (MGI:3528480). Briefly, *loxP* sites flanking exon 18 allow for deletion of this exon, destabilization of the mRNA and absence of the full-length ATRX protein (Berube, Mangelsdorf et al. 2005). Mating *Atrx*^{loxP} female mice to male mice expressing Cre recombinase under the control of the *Foxg1* promoter (129(Cg)-*Foxg1*$^{tm1(cre)Skm}$/J, RRID:IMSR_JAX:004337) (Hebert and McConnell 2000) yielded male progeny with ATRX deficiency in the forebrain and anterior pituitary (*Atrx*^{loxP};FoxG1Cre RRID:MGI:3530074) called *Atrx*^{FoxG1Cre} for simplicity. Mating *Atrx*^{loxP} female mice to males expressing Cre recombinase under the control of the *Nex* gene promoter (Schwab, Bartholomae et al. 2000, Goebbels, Bormuth et al. 2006) (Neurod6$^{tm1(cre)Kan}$, MGI:2668659) produced male progeny with ATRX deficiency in forebrain glutamatergic neurons (*Atrx*^{loxP};NexCre or *Atrx*^{NexCre} for simplicity). Mating *Atrx*^{loxP} female mice to males expressing Cre recombinase under the control of the Sox10 promoter (McKenzie, Ohayon et al. 2014) (CBA;B6-Tg(Sox10-icre/ERT2)388Wdr, MGI:5634390, RRID:IMSR JAX:027651), produced male progeny with *Atrx* deficiency in OPCs (*Atrx*^{loxP};Sox10Cre or *Atrx*^{Sox10Cre}). Two Cre-sensitive reporter lines were bred into the *Atrx*^{loxP} mice to track Cre recombinase-expressing cells. The Sun1GFP allele (B6;129-*Gt(ROSA)26Sor*$^{tm5(CAG-Sun1/sfGFP)Nat}$/J, MGI:5614796, RRID: IMSR_JAX:021039) encodes the Sun1GFP fusion protein (located in the nuclear membrane) upon Cre-mediated recombination (Mo, Mukamel et al. 2015) and the Tomato-Ai14 (Ai14) allele (B6.Cg-

Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J, MGI:3809524, RRID:IMSR_JAX:007914) (Madisen, Zwingman et al. 2010) expresses the tdTomato protein with red fluorescence in the cytoplasm and nucleus upon Cre recombination. Ear punch genomic DNA was used for PCR genotyping of the *Atrx* floxed or wildtype alleles using the primers 17F, 18R and neoR as described previously (Berube, Mangelsdorf et al. 2005). The *FoxG1Cre, NexCre, Sox10Cre, Tomato-Ai14* and *Sun1GFP* genotyping primers are listed in Table 1.

3.2.2 Tamoxifen and thyroxine injections

Tamoxifen (10mg; Cat# T5648, Sigma) was dissolved in 100 µL 95% ethanol at 65°C for 10 mins, followed by dilution in 900 µL corn oil (Cat# C8267, Sigma). Lactating mothers were injected intraperitoneally daily with 2 mg for two (mixed glial culture) or three consecutive days (Harris, Buac et al. 2013). Subcutaneous injection of L-thyroxine (0.1 mg/kg; Sigma Cat# T2376-100MG) was performed daily on control and *Atrx*^{FoxG1Cre} mice from birth (P0) until P14 as done previously (Rowland, Jiang et al. 2018).

3.2.3 Microarray analysis

Total forebrain RNA (10 µg) was isolated from three P17 pairs of littermate matched *Atrx*^{FoxG1Cre} and control mice using the RNeasy Mini kit (Qiagen Cat# 74104). cRNA was generated and hybridized to an Affymetrix Mouse Genome 430 2.0 Array at the London Regional Genomics Center (London, Canada). Probe signal intensities were generated using GCOS1.4 (Affymetrix Inc., Santa Clara, CA) using default values for the Statistical Expression algorithm parameters and a Target Signal of 150 for all probe sets and a Normalization Value of 1. Gene level data was generated using the RNA preprocessor in GeneSpring GX 7.3.1 (Agilent Technologies Inc., Palo Alto, CA). Data were then transformed (measurements less than 0.01 set to 0.01), normalized per chip to the 50th percentile, and per gene to control samples. Probe sets representing *Atrx* transcripts were removed (10 sets). The remaining probe sets were filtered by fold change ≥1.5 between control and *Atrx*^{FoxG1Cre} samples, and by confidence level of $P<0.05$. Significantly overrepresented GO categories were determined using GeneSpring: probe sets were

filtered by 1.5-fold change, P<0.05 and categorized as either up or downregulated. Where there were multiple probe sets for a gene, duplicates were removed. P<0.001 was used as the significance cut-off (Levy, Fernandes et al. 2008).

3.2.4 Western blot analysis

Protein was extracted from P14 or P20 mouse forebrain and homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 50 mM Tris pH 8.0, 0.5% deoxycholic acid, 0.1% SDS, 0.2 mM PMSF, 0.5 mM NaF, 0.1 mM Na_3VO_4, 1x protease inhibitor cocktail (Millipore Sigma Cat# 11873580001)). Homogenized tissue was incubated on ice for 20 minutes and centrifuged for 20 minutes at 10000 RPM. Supernatant protein was quantified using the Bradford assay (BioRad Cat# 500-0006). Protein (30 μg) was resolved on a 12% SDS-PAGE and transferred to a 0.45 μm nitrocellulose membrane (BioRad Cat# 1620115). The membranes were probed with anti-MAG, mouse monoclonal (1:3000, Abcam Cat# ab89780, RRID:AB_2042411), anti-MOG, rabbit polyclonal (1:3000, Abcam Cat# ab32760, RRID:AB_2145529), or anti-MBP, rat monoclonal (1:3000, Abcam Cat# ab7349, RRID:AB_305869) antibodies at 4°C overnight. This was followed by incubation with the appropriate horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature: mouse-HRP (1:3,000, Santa Cruz Cat# sc-516102, RRID:AB_2687626), rabbit-HRP (1:5000, Jackson ImmunoResearch Cat# 111-036-003, RRID:AB_2337942) or rat-HRP (1:3,000, Santa Cruz Cat# sc-2006, RRID:AB_1125219). The membrane was incubated in enhanced chemiluminescent (ECL) (Thermo Fisher Cat# 34095) before exposure using film (Progene Cat# 39-20810) or Universal Hood III (BioRad Cat# 731BR00882) and analyzed with Image Lab (BioRad, Version 4.1, 2012).

3.2.5 Immunofluorescence

P14 or P20 mice were transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were dissected and fixed in 4% paraformaldehyde overnight. The next day, brains were washed 3x 5 min in PBS and dehydrated in 30% sucrose until tissue had sunk. Fixed brain tissue was flash-frozen on dry ice using Cryomatrix

cryoprotectant (ThermoFisher Cat# 6769006) and cryosectioned coronally at 8 µm thickness (Leica CM 3050S) on superfrost slides (Thermo Fisher Cat# 22-037-246) and stored at -80°C with a desiccant (VWR, 61161-319). For immunofluorescence staining of cryosections, slides were rehydrated in 1x PBS for 5 minutes, washed with PBS + 0.1% TritonX100 (Millipore Sigma Cat# T8787), blocked with 10% normal goat serum diluted in washing solution (Millipore Sigma Cat# G9023) for 1 hour and incubated with primary antibody overnight at 4°C. Slides were washed 3 times for 5 min with PBS and incubated with secondary antibody for 1 hour in the dark followed by 2x 5 min PBS washes. Sections were counterstained with 1 µg/mL DAPI (Millipore Sigma Cat# D9542) for 5 min, washed for 5 min with PBS and mounted with Permafluor (Thermo Fisher Cat# TA-006-FM) followed by imaging. Primary mixed glial cultures were fixed for immunofluorescence staining at 3DIV or 9DIV with 3% PFA at room temperature for 15 minutes. Cells were permeabilized with PBS + 0.1% TritonX100 (Millipore Sigma Cat# T8787), blocked with PBS + 10% normal goat serum (Millipore Sigma Cat# G9023) for 1 hour and incubated with primary antibody overnight at 4°C. The next day, flasks were washed 3x 5 minutes with PBS and incubated with secondary antibody diluted in blocking solution for 1 hour at room temperature. Cells were then counter-stained with 1 µg/mL DAPI (Millipore Sigma Cat# D9542) for 5 min, washed for 5 min with PBS and mounted with Permafluor (Thermo Fisher Cat# TA-006-FM) followed by imaging. The following primary antibodies were used: anti-MOG, rabbit polyclonal (1:200, Abcam Cat# ab32760, RRID:AB_2145529), or anti-MBP, rat monoclonal (1:50, Abcam Cat# ab7349, RRID:AB_305869), anti-ATRX, rabbit polyclonal (1:75, Santa Cruz Biotechnology Cat# sc-15408, RRID:AB_2061023), anti-Olig2, rabbit polyclonal (1:200, Millipore Cat# AB9610, RRID:AB_570666), anti-S100β, rabbit polyclonal (1:200, Agilent Cat# Z0311, RRID:AB_10013383), anti-GFAP, rabbit polyclonal (1:200, Agilent Cat# Z0334, RRID:AB_10013382), Anti-NG2, rabbit polyclonal (1:200, Millipore Cat# AB5320, RRID:AB_11213678), Anti-Pdgfrα, rabbit polyclonal (Abcam Cat# ab65258, RRID:AB_1141669) and Anti-APC, mouse monoclonal (Abcam Cat# ab16794, RRID:AB_443473). The secondary antibodies used were goat anti-rabbit-Alexa Fluor 594 (1:800, Thermo Fisher Scientific, A-11012, RRID:AB_2534079), goat anti-rabbit-Alexa Fluor 488 (1:800, Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165),

goat anti-mouse-Alexa Fluor 594 (1:800 Thermo Fisher Scientific Cat# A-21125, RRID:AB_2535767), goat anti-mouse-Alexa Fluor 488 (1:800, Thermo Fisher Scientific Cat# A-11001, RRID:AB_2534069) and goat anti-rat-Alexa Fluor 488 (1:800, Thermo Fisher Scientific, A-11006, RRID:AB_2534074).

3.2.6 Microscopy, imaging and cell counts

Immunofluorescence images were captured using an inverted microscope (DMI 6000b, Leica) outfitted with a digital camera (ORCA-ER, Hamamatsu). Openlab software (PerkinElmer Version 5.0, RRID:SCR_012158) was used for image capture. Image processing was performed using Volocity (PerkinElmer Demo Version 6.0.1, RRID:SCR_002668) and Adobe Photoshop. Cell counts of the mouse corpus callosum were performed in a blinded and randomized manner for immunofluorescence staining of Olig2, PDGFRα, APC, Sun1GFP, S100β and GFAP in control or experimental cryosections using Volocity (PerkinElmer Demo Version 6.0.1, RRID:SCR_002668). The corpus callosum was outlined and the area recorded. Cells were counted in 2-10 sections per biological replicate and 3-4 biological replicates were counted for each genotype.

3.2.7 Isolation of nuclei tagged in specific cell types (INTACT) method

To circumvent the problems associated with mechanical purification techniques, the INTACT method (Mo, Mukamel et al. 2015) followed by fluorescence-activated nuclei sorting (FANS) was used to enrich for Sun1-GFP^{+} OPC nuclei. Control or *Atrx*^{Sox10Cre} forebrain tissue was homogenized in 500 µL homogenization buffer (20 mM Tricine KOH, 25 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, 0.1% IGEPAL-630, 1x protease inhibitor cocktail (Millipore Sigma Cat# 11873580001), 1 uL/mL RNase inhibitor (Thermo Fisher Scientific Cat# 10777019). Samples were diluted to 7.5 mL with homogenization buffer and filtered through a 40 µm strainer. Filtered samples were layered on top of 7.5 mL cushion buffer consisting of 0.5 mM $MgCl_2$, 0.88 M sucrose, 0.5 mM DTT, 1x protease inhibitor cocktail (Millipore

Sigma Cat# 11873580001), 1 uL/mL RNase inhibitor (Thermo Fisher Scientific Cat# 10777019) and centrifuged at 2800 g for 20 mins at 4°C. Nuclei were collected as a pellet, incubated for 10 min in 500 µL 4% FBS, 0.15 mM spermine, 0.5 mM spermidine, 1x protease inhibitor cocktail (Millipore Sigma Cat# 11873580001) and 1 uL/mL RNase inhibitor (Thermo Fisher Scientific Cat# 10777019) in PBS and resuspended by gentle pipetting. Nuclei were sorted using a Sony SH800 Cell Sorter and Sun1GFP$^+$ nuclei were collected. Total RNA was immediately isolated from GFP+ nuclei with a single cell RNA purification kit (NorgenBiotek Cat# 51800).

3.2.8 Quantitative reverse transcriptase-PCR (qRT-PCR)

cDNA was prepared using 100 ng RNA from FANS-purified GFP$^+$ nuclei or from the optic tract (RNeasy Mini kit; Qiagen Cat# 74104) and 1.5 µL 100 ng/uL of random hexamer (Integrated DNA Technologies Cat# 51-01-18-26) were diluted to 12 µL with RNAse free water. Samples were heated to 65°C followed by addition of 4 µL first strand buffer (Thermo Fisher Scientific Cat# 18064014), 2 µL 100 mM DTT (Thermo Fisher Scientific Cat# 18064014), 0.8 µL 25 mM dNTPs, 0.5 µL RNaseOut (Thermo Fisher Scientific Cat# 10777019), 0.5 uL SuperScript II Reverse Transcriptase (Thermo Fisher Scientific Cat# 18064014) and 0.5 µL RNAse free water per sample. Samples were then incubated at 30°C for 10 minutes then 42°C for 45 minutes and stored at -20°C. cDNA was amplified with gene-specific primers under the following conditions: cDNA was amplified with iQ SYBR Green Master Mix (BioRad Cat# 1708884) using the standard curve Ct method of quantification. Experiments were performed on a Chromo-4 thermocycler (MJ Research/BioRad) and analyzed with Opticon Monitor 3 and GeneX (BioRad) software. Technical duplicates were completed for each sample. Conditions for amplification were as follows: 40 cycles of 95°C for 10 seconds, 55-60°C for 20 seconds, 72°C for 30 seconds, and a final melting curve generated in increments of 0.5°C per plate read. Primer sequences are listed in Table 2**.**

Gene Name	Forward Primer	Reverse Primer	Application
AtrxWT	AGA AAT TGA GGA TGC TTC ACC	TGA ACC TGG GGA CTT CTT TG	Genotyping
Atrxfloxed	AGA AAT TGA GGA TGC TTC ACC	CCA CCA TGA TAT TCG GCA AG	Genotyping
FoxG1Cre	TGA CCA GAG TCA TCC TTA GCG	AAT GCT TCT GTC CGT TTG CC	Genotyping
NexCre	TGA CCA GAG TCA TCC TTA GCG	AAT GCT TCT GTC CGT TTG CC	Genotyping
Sox10Cre	CAC CTA GGG TCT GGC ATG T	CAG GTT TTG GTG CAC AGT CA	Genotyping
Sun1GFP	AAG GGA GCT GCA GTG GAG TA	CGG GCC ATT TAC CGT AAG TTA T	Genotyping
Ai14Tomato	GGC ATT AAA GCA GCG TAT CC	CTG TTC CTG TAC GGC ATG G	Genotyping
Gapdh	CAA CGA CCC CTT CAT TGA CCT	ATC CAC GAC CGA CAC ATT GG	qRT-PCR
Atrx	AGA AAT TGA GGA TGC TTC ACC	TGA ACC TGG GGA CTT CTT TG	qRT-PCR
Olig2	GCA GCG AGC ACC TCA AAT CT	AGA TCA TCG GGT TCT GGG GA	qRT-PCR
Sox10	CTA CAGG GAG TGC CCA CCT GG	GCT CTG TCT TTG GGG TGG TT	qRT-PCR
Pdgfrα	GTC AGG CCA CT AAA GAG GTC A	CGT GCA AGT TGA CAG CTT CC	qRT-PCR
Olig1	CTC GCC CAG GTG TTT TGT TG	CGA CGT GCC TTG CTA CCT AT	qRT-PCR
Gpr17	TCACAGCTTACCTGCTTCCC	TTGTCCGCATTGCTCAGAGT	qRT-PCR
Cspg4	CTCAAGATGGGAGCCTCAGC	ACAAAGGCGTCTGTCTGTGT	qRT-PCR
Mag	CAG AGA GCC ACT GCC TTC AA	TCA AAG GCC ACA GAG GTT C	qRT-PCR
Mog	AGA GGC AGC AAT GGA GTT GA	TGC GAT GAG AGT CAG CAC AC	qRT-PCR
Mbp	CAT TGG GTC GCC ATG GGA AA	AGC CTC TCC TCG GTG AAT CT	qRT-PCR
Plp	GGC CAC TGG ATT GTG TTT CT	GAA AGC ATT CCA TGG GAG AA	qRT-PCR
Gfap	AAA CCA GCC TGG ACA CCA AA	ACA CCT CAC ATC ACC ACG TC	qRT-PCR
S100β	TGA AGC CAG AGA GGA CTC CA	CCA GGA AGT GAG AGA GCT CG	qRT-PCR
P1 Promoter	ATT CCC CGT CTC ACT CCG TA	TAG CGG GGC TGC TAA AGA AG	ChIP qRT-PCR
P2 Proximal	GCC TGA CGC TAC AGT GAC AA	TAG CGG GGC TGC TAA AGA AG	ChIP qRT-PCR
P3 Exon1	CTG CCT CCA CCC AGC TA TAA	GGT GTT GGC TCG GTC TGT AA	ChIP qRT-PCR
P4 Exon2	ATC TTC CTC CAG CAC CTC CT	GTT CGC GGC TGT TGA TCT TC	ChIP qRT-PCR
P5 3'UTR	TAG GAG ACT CCC AGG AAC CG	GAC CAC AGG ACC CTA AGT GC	ChIP qRT-PCR
P6 3'UTR2	CCT CCC ACA CAC ACA CCT TT	AGG TGG AAG GTT AGG AGG CA	ChIP qRT-PCR

Table 2 List of primer sequences

3.2.9 Mixed glial cells primary culture

Cre recombination was induced by IP injection of 2 mg tamoxifen to lactating mothers for two consecutive days (P0-P1) (Harris, Buac et al. 2013). At P3, mouse cortices were dissected into MEM and tissue dissociated by pipetting with a P1000. Samples were then incubated at 37°C with OPC papain solution (Worthington Cat# LS003124) for 20

minutes with constant inversion to prevent tissue aggregation. Papain was deactivated for 10 min at room temperature followed by trituration with a flame polished Pasteur pipette. Samples were centrifuged at 300 g for 5 minutes and pellets resuspended in 1 mL mixed glial culture media consisting of DMEM supplemented with 10% FBS (Gibco Cat # 12483020), 1% Penicillin-Streptomycin (Gibco Cat # 15070063), 1% Antibiotic-Antimycotic (Gibco Cat # 15240062) and 1% GlutaMax (Gibco Cat # 35050061). The cell suspension from each tube was added to a pre-equilibrated poly-L-lysine (1 mg/mL) coated 6 well plate in 1 mL of media. Flasks were incubated at 8.5% CO_2 in a tissue culture incubator for 3 hours to allow cells to attach to the poly-L-lysine substrate followed by a full media change. A 2/3 volume media change was performed every three days (Chen, Balasubramaniyan et al. 2007, O'Meara, Ryan et al. 2011).

3.2.10 Co-immunoprecipitation

P20 control mouse forebrain protein (1 mg), 50 µL protein G Dynabeads (Invitrogen Cat# 10009D) and 5 µg either anti-HDAC3, mouse monoclonal (Millipore Sigma Cat# 05-813, RRID: AB_310021) or anti-IgG, rabbit polyclonal (Thermo Fisher Scientific Cat# 02-6102, RRID:AB_2532938) diluted in PBS + 0.1% Tween-20 were incubated with rotation at 4°C overnight. The Dynabeads-Ab-Ag complex was washed three times with PBS and the antigen was eluted from the beads with 30 µl SDS sample buffer (100 mM Tris (pH 6.8), 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol, 200 mM β-mercaptoethanol) heated for 10 min at 70°C with agitation. Input protein (100 µg) was treated the same. Samples were resolved on 6% SDS-PAGE and transferred onto a 0.45 µm nitrocellulose membrane (BioRad Cat# 1620115). The membrane was probed with anti-ATRX, rabbit polyclonal (1:1000, Abcam Cat# ab97508, RRID:AB_10680289) at 4°C overnight. This was followed by rabbit-anti-HRP (1:5000, Jackson ImmunoResearch Cat# 111-036-003, RRID:AB_2337942) conjugated secondary antibody for 1 hour at room temperature. The membrane was treated with enhanced chemiluminescent (ECL) (Thermo Fisher Cat# 34095) before exposure with a Hood III (BioRad Cat# 731BR00882) and analyzed with Image Lab (BioRad, Version 4.1, 2012).

3.2.11 Chromatin immunoprecipitation in cultured OPCs

Cortices from 15 control P0.5 pups were dissected and OPCs were grown for 9 DIV in a mixed glial co-culture as described in the mixed glial co-culture section. OPCs were then physically separated from resident astrocytes by shaking overnight in a tissue culture incubator at 5% CO_2 (Chen, Balasubramaniyan et al. 2007, O'Meara, Ryan et al. 2011). OPCs were pooled, homogenized and passed through a 70 µm strainer. To crosslink chromatin, the sample was diluted to 20 mL with PBS with 0.02 M EGS (ethylene glycol bis(succinimidyl succinate)) (Thermo Fisher Scientific Cat # 21565) and incubated at room temperature for 45 min with constant mixing (Truch, Telenius et al. 2018). This was followed by the addition of 1% formaldehyde and incubation at room temperature for 20 minutes with constant mixing. To quench crosslinking, 0.125 M glycine was added for 2 minutes at room temperature and samples were centrifuged at 700 g for 5 minutes. Pellets were washed twice with 10 mL cold PBS and stored at -80°.

Chromatin was sonicated following lysis with a Bioruptor Pico Sonication Device (Diagenode Cat# B01060010) for 6 cycles. For ChIP, 50 µL protein G Dynabeads (Invitrogen Cat# 10009D) were prepared with either 10 µg anti-ATRX rabbit polyclonal antibody (Abcam Cat# ab97508, RRID:AB_10680289) or anti-IgG rabbit polyclonal antibody (Thermo Fisher Scientific Cat# 02-6102, RRID:AB_2532938) for 4 hours with rotation at 4°C. Chromatin lysate (50 µg per IP) was precleared with 100 uL protein G Dynabeads (Invitrogen Cat# 10009D) for 1 hour at 4°C. Precleared lysate and antibody-bound beads were combined and incubated at 4°C overnight with rotation. The next day, beads were washed five times and eluted for 15 minutes at room temperature followed by a second elution for 20 minutes at 65°C (Truch, Telenius et al. 2018). Crosslinking was reversed by adding 5M NaCl at 65°C for 4 hours. Samples were treated with 1 µL 10 mg/mL RNase A at 37°C for 30 min then 1 µL 20 µg/mL proteinase K for 1 hour at 45°C. $1/10^{th}$ of precleared chromatin lysate was treated the same and used as input. DNA was purified (QIAquick PCR Purification Kit, Qiagen Cat# 28104) and amplified by qRT-PCR with iQ SYBR Green Master Mix (BioRad Cat# 1708884) by using the standard curve Ct method of quantification. Experiments were performed on a Chromo-4 thermocycler (MJ Research/BioRad) and analyzed with Opticon Monitor 3 and GeneX

(BioRad) software. qRT-PCR was repeated in duplicate for each sample as a technical replicate. Conditions for amplification were as follows: 40 cycles of 95°C for 10 seconds, 55-60°C for 20 seconds, 72°C for 30 seconds, and a final melting curve generated in increments of 0.5°C per plate read. Amplified DNA was resolved on a 1.5% agarose gel to confirm correct product size. Primer sequences are listed in Supplementary Table 1.

3.2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism6 software (6.05; GraphPad Software Inc.) and all results are expressed as the mean ± SEM. Two independent data sets were compared with the Student's t test (unpaired, 2-tailed). Multiple independent data sets were compared with a one-way ANOVA with post-hoc Tukey's test. P values of 0.05 or less were considered to indicate significance.

3.2.13 Study approval

All procedures involving animals were conducted in accordance with the regulations of the Animals for Research Act of the Province of Ontario and approved by the University of Western Ontario Animal Care and Use Committee.

3.3 Results

3.3.1 Decreased myelination in the *Atrx*^{FoxG1Cre} forebrain

Our previous studies have shown that postnatal health and longevity are adversely affected in mice with forebrain and anterior pituitary-specific deficiency for ATRX (*Atrx*^{FoxG1cre} mice) (Watson, Solomon et al. 2013, Rowland, Jiang et al. 2018). *Atrx*^{FoxG1cre} mice have a shortened lifespan, growth and hormone abnormalities, including low circulating levels of IGF-1 and T4 and exhibit aberrant gene expression in the liver (Watson, Solomon et al. 2013, Rowland, Jiang et al. 2018). To identify potential forebrain related differences in transcription between control and *Atrx*^{FoxG1Cre} we performed a microarray analysis on forebrain tissue of 3 littermate-matched pairs of P17

control and *Atrx*^{FoxG1Cre} mice. The microarray analysis identified a potential effect of ATRX loss of function on myelination, as several central nervous system related phenotypes including abnormal myelination, demyelination and abnormal OL morphology/physiology were enriched in pathway analysis (Figure 3-1A). The genes significantly downregulated in the abnormal myelination and demyelination categories included 25 genes associated with abnormal myelination (Figure 3-1B). Based on several eports of aberrant myelination in ATR-X patients (Wada, Ban et al. 2013, Lee, Lee et al. 2015), we explored a possible loss of myelination in these mice, examining levels of well-established myelin markers, including myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP) in control and *Atrx*^{FoxG1Cre} forebrain protein extracts. Western blot analysis demonstrates that, similar to the lower mRNA levels, the level of these myelin proteins is significantly decreased in *Atrx*^{FoxG1Cre} forebrain compared to littermate matched controls (n=4 each genotype; Student's T-test, MAG $p<0.05$) (Figure 3-1C). Immunofluorescence staining of brain cryosections reinforced these findings, showing qualitative decreased level of MOG and MBP in the corpus callosum and adjacent cortex of *Atrx*^{FoxG1Cre} compared to control mice at P20 (Figure 3-1D). These data demonstrate that the *Atrx*^{FoxG1Cre} mice display severe hypomyelination.

3.3.2 Postnatal thyroxine treatment partially restores OLs, but not OPCs in *Atrx*^{FoxG1Cre} *mice*

We previously determined that circulating levels of the thyroid hormone T4 in *Atrx*^{FoxG1Cre} mice are less than half that of controls, most likely stemming from the deletion of *Atrx* in the anterior pituitary in these mice (Watson, Solomon et al. 2013, Rowland, Jiang et al. 2018). The thyroid hormone receptor is a transcription factor that requires its ligand, triiodothyronine (T3), to promote the expression of several myelin genes (Farsetti, Mitsuhashi et al. 1991, Ibarrola and Rodríguez-Peña 1997, Dong, Yauk et al. 2009). Based on this, we tested whether postnatal injections of the prohormone T4 could rescue hypomyelination in *Atrx*^{FoxG1Cre} mice. We performed daily injections of 0.1 mg/kg L-thyroxine (T4) or PBS from P0 to P14, as outlined in Figure 3-2A, a dose

A

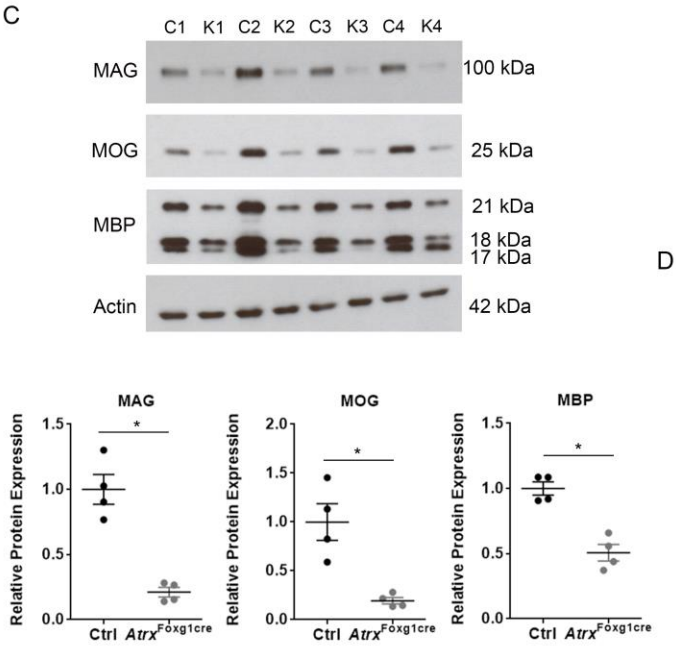
Mouse phenotypes

ID	Name	pValue	Bonferroni
MP:0000920	abnormal myelination	5.88E-09	2.73E-05
MP:0000921	demyelination	9.05E-09	4.20E-05
MP:0003633	abnormal nervous system physiology	4.33E-08	2.01E-04
MP:0000953	abnormal oligodendrocyte morphology	5.27E-07	2.45E-03
MP:0008917	abnormal oligodendrocyte physiology	1.74E-06	8.09E-03
MP:0002882	abnormal neuron morphology	3.86E-06	1.79E-02
MP:0005405	axon degeneration	5.05E-06	2.35E-02
MP:0005403	abnormal nerve conduction	6.81E-06	3.16E-02

B

Downregulated genes related to myelination

Mag	myelin associated glycoprotein
Mal	mal, T cell differentiation protein
Mbp	myelin basic protein
Ndrg1	N-myc downstream regulated 1
Ugt8a	UDP glycosyltransferase 8
Plp1	proteolipid protein 1
Mobp	myelin-associated oligodendrocyte basic protei
Cnp	2',3'-cyclic nucleotide 3' phosphodiesterase
Mog	myelin oligodendrocyte glycoprotein
Gal3st1	galactose-3-O-sulfotransferase 1
Litaf	lipopolysaccharide induced TNF factor
Fa2h	fatty acid 2-hydroxylase
Igf1	insulin like growth factor 1
Csf1r	colony stimulating factor 1 receptor
Aspa	aspartoacylase
Sox10	SRY-box 10
Itga6	integrin subunit alpha 6
Itgb4	integrin subunit beta 4
Gjb1	gap junction protein beta 1
Ptprz1	protein tyrosine phosphatase, receptor type Z1
Npc1	NPC intracellular cholesterol transporter 1
Lamc1	laminin subunit gamma 1
Gjc2	gap junction protein gamma 2
Stmn1	stathmin 1
Tspan2	tetraspanin 2



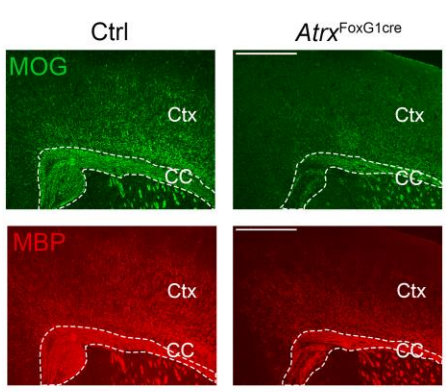


Figure 3-1 The mouse forebrain lacking ATRX expression is hypomyelinated.

(a) Microarray analysis of P17 control and *Atrx*^{FoxG1Cre} forebrain tissue reveals a significant downregulation ($p<0.05$) of genes related to myelination, axon degeneration and abnormal nerve conduction. (b) List of downregulated genes involved in myelination. (c) Western blot analysis of P20 forebrain extracts shows significantly decreased levels of MAG, MOG and MBP proteins in *Atrx*^{FoxG1Cre} compared to control mice (n=4 for each genotype) after normalization to Actin protein levels. Error bars represent $^{+}$/- SEM and asterisks indicate $p<0.05$ (Student's T-test). (d) Immunofluorescence microscopy of P20 brain cryosections stained with anti-MOG (green) and anti-MBP (red) antibodies confirm decreased levels of these myelin proteins in the cortex (Ctx) and corpus callosum (CC)

(outlined in white dashed line) areas of the forebrain in *Atrx*^{FoxG1Cre} mice compared to controls (Ctrl). Scale bar, 500 µm.

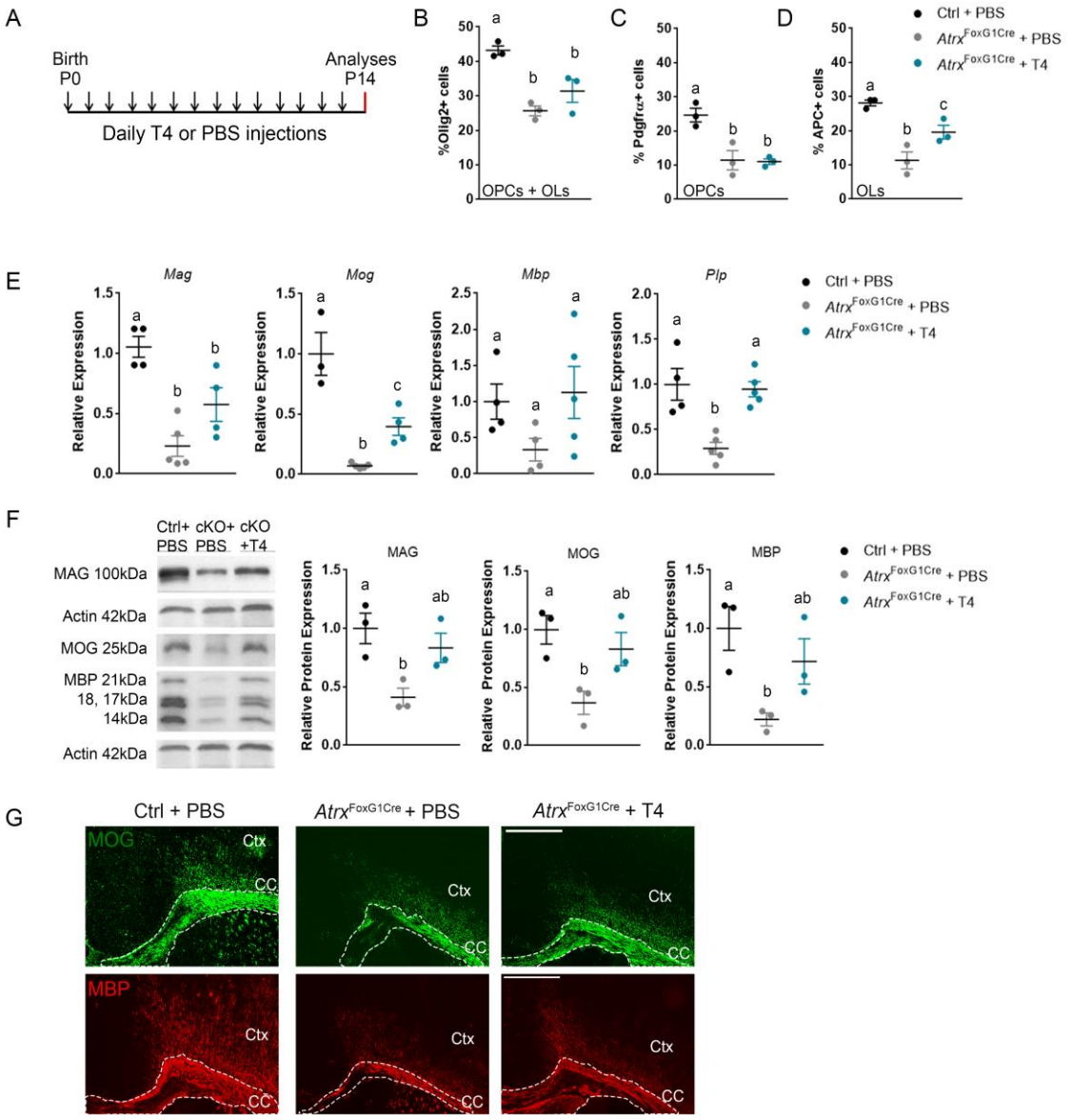


Figure 3-2 Postnatal thyroxine treatment partially rescues the number of APC$^+$ OLs and the expression of myelin proteins in the ATRX-null forebrain but does not restore the number of Pdgfrα^+ OPCs.

(a) Outline of experimental design. Each arrow represents a subcutaneous (SC) injection of 0.1 mg/kg T4 or PBS to *Atrx*^{FoxG1Cre} and control pups. Analyses were then performed

at P14. (b-c) Quantification of immunostained P14 cryosections demonstrate that the reduced number of Olig2^{+} and Pdgfrα^{+} cells observed in the *Atrx*^{FoxG1Cre} CC is not rescued by T4 treatment (n=3 each group). (d) The number of APC^{+} cells are partially rescued upon T4 treatment (n=3 each group). (e) qRT-PCR shows that transcript levels of several myelin genes are decreased in the P14 *Atrx*^{FoxG1Cre} forebrain and partially restored to control levels following T4 treatment (n=3-5 for each group). (f) Western blot analysis of MAG, MOG and MBP shows decreased protein levels in P14 *Atrx*^{FoxG1Cre} compared to control forebrain extracts and partial rescue upon T4 treatment. Graphs show quantification of 3 trios, normalized to Actin protein levels. (g) Immunofluorescence staining of P14 brain cryosections highlight the decreased expression of MOG (green) and MBP (red) protein levels in the *Atrx*^{FoxG1Cre} cortex (Ctx) and corpus callosum (CC) (outlined in white dashed line) that are ameliorated following T4 treatment. Scale bar, 500 μm. In all graphs, error bars represent $^{+}$/-SEM, and groups with different letters significantly differ from one another (one-way ANOVA with post-hoc Tukey HSD; $p<0.05$).

previously shown to successfully elevate circulating T4 to control levels in *Atrx*^{FoxG1Cre} mice (Rowland, Jiang et al. 2018).

We first quantified the number of OLs after T4 treatment by immunofluorescence staining of P14 brain cryosections, using an antibody specific for the pan-OL marker Olig2. We find that the proportion of Olig2^{+} cells (representing all OPCs and OLs) is significantly decreased in the *Atrx*^{FoxG1Cre} corpus callosum (25.7% of total DAPI^{+} cells) when compared to controls (43.2% of total DAPI^{+} cells) and this is partially recovered in *Atrx*^{FoxG1Cre} mice treated with T4 (31.4%) (n=3 for each genotype; $p<0.05$, one-way ANOVA) (Figure 3-2B). We also counted the proportion of OPCs or OLs in the corpus callosum of control and *Atrx*^{FoxG1Cre} brain cryosections at P14, identified by PDGFRα and adenomatous polyposis coli (APC) staining, respectively. The proportion of differentiated APC^{+} OLs is decreased in the *Atrx*^{FoxG1Cre} corpus callosum (11.3%) compared to controls (28.2%) and was partially recovered (19.6%) following T4 treatment (n=3 for each genotype; $p<0.05$ one-way ANOVA) (Figure 3-2D). While the proportion of PDGFRα^{+} OPCs is lower in *Atrx*^{FoxG1Cre} mice (11.5%) compared to controls (24.6%) it is not improved upon T4 treatment (11%) (n=3 each genotype; $p<0.05$, one-way ANOVA) (Figure 3-2C).

To determine if T4 administration increases the expression of myelin genes, we performed qRT-PCR using primer pairs specific for several established myelin genes: *Mag*, *Mog*, *Mbp* and *Plp1* (proteolipid protein 1). The level of RNA transcripts is generally elevated in T4- vs PBS-treated *Atrx*^{FoxG1Cre} mice, but rescue is only partial and does not reach control levels (n=3-4 each genotype; *Mag*, *Mog*, *Plp* $p<0.05$ by one-way ANOVA) (Figure 3-2 E) Given that RNA transcript levels are not always representative of protein levels, we also examined how T4 injections affect myelin protein expression. Western blot analysis of MAG, MOG and MBP reveals a significant albeit partial rescue of the level of these proteins upon T4 treatment in *Atrx*^{FoxG1Cre} forebrain extracts (n=3 each genotype $p<0.05$ by one-way ANOVA) (Figure 3-2 F). The results were similar by immunofluorescence staining of MOG and MBP in *Atrx*^{FoxG1Cre} forebrain cryosections

with or without T4 supplementation (Figure 3-2 G). Taken together, these experiments suggest that hypomyelination in *Atrx*^{FoxG1Cre} mice is due to a reduced number of OPCs and OLs. T4 treatment stimulates OL differentiation by promoting myelin gene expression but does not ameliorate OPC numbers, thus explaining the incomplete effect of T4 treatment on the extent myelination.

3.3.3 Targeted deletion of *Atrx* in neurons does not affect myelination

The results of the T4 treatment suggested that ATRX is likely required in the central nervous system to support normal myelination either cell autonomously or in other cell types to provide trophic support to OPCs. To resolve whether ATRX regulates myelination intrinsically in OPCs or indirectly via neuronal signaling (or both), we used different Cre driver lines to inactivate ATRX specifically in each cell type. We generated mice that lack ATRX in forebrain excitatory neurons using the *NexCre* driver mice for *Atrx*^{loxP} recombination (Goebbels, Bormuth et al. 2006). We confirmed the absence of ATRX nuclear protein in cortical neurons by co-immunofluorescent staining of ATRX and the neuronal marker NeuN (Figure 3-3A). We next examined myelin protein expression in the forebrain of these mice by immunofluorescence staining of P20 brain cryosections and observed no obvious difference in MBP and MOG protein between control and *Atrx*^{NexCre} mice (Figure 3-3B). Furthermore, MAG, MOG and MBP protein levels are also similar to controls when assessed by immunoblot of P20 *Atrx*^{NexCre} forebrain extracts (n=3 each genotype) (Figure 3-3C). These findings show that ATRX is not required in neurons of the developing brain for proper signaling to OPCs and for normal initiation of the myelination program.

3.3.4 Inducible postnatal inactivation of ATRX in OPCs

We next sought to establish whether deletion of ATRX specifically in OPCs causes depletion of the OPC/OL pool leading to hypomyelination. ATRX deletion in OPCs was achieved using the tamoxifen inducible *Sox10CreER*^{T2} allele, which upon mating with *Atrx*^{LoxP} mice produced *Atrx*$^{Sox10\text{-}iCreERT2}$ (*Atrx*^{Sox10Cre}) mice

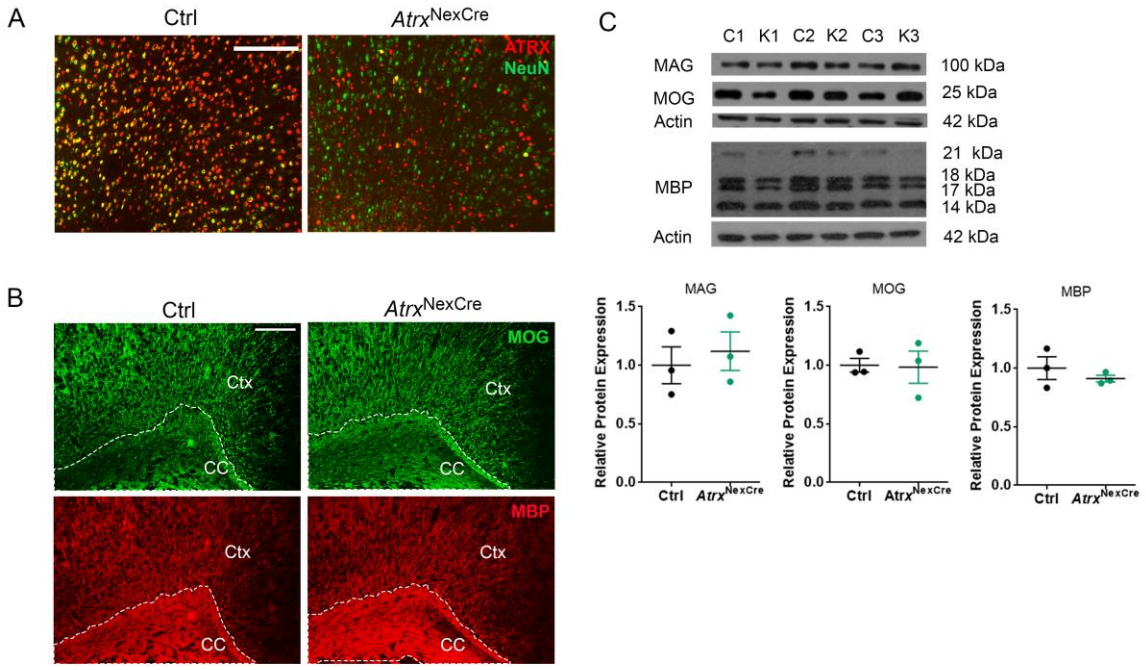


Figure 3-3 Myelination appears normal in mice with *Atrx* deletion in forebrain excitatory neurons.

(a) Immunofluorescence staining of P20 brain cryosections shows absence of ATRX protein (red) in NeuN expressing neurons (green) in the cortex of *Atrx*^{NexCre} mice. Scale bar, 100 µm. (b) MBP and MOG immunofluorescence staining of P20 *Atrx*^{NexCre} and control cortex (Ctx) and corpus callosum (CC) (outlined in white dashed line). Scale bar, 100 µm. (c) Western blot analysis of MAG, MOG and MBP and quantification below shows similar levels of these proteins in the P20 *Atrx*^{NexCre} mouse forebrain compared to controls when normalized to Actin protein levels ($p>0.05$ Student's T-test). Error bars represent $^{+}$/-SEM, n=3 each genotype.

(McKenzie, Ohayon et al. 2014). The Cre-sensitive nuclear membrane *Sun1GFP* reporter was also introduced in the *Atrx*^{Sox10Cre} mice to facilitate fate tracking of Cre-expressing cells (Figure 3-4 A) (Mo, Mukamel et al. 2015). Cre recombination was induced by daily intraperitoneal injections of nursing mothers for three successive days (pups at P1-P3) (Harris, Buac et al. 2013) and the mice were analyzed at P20 or P35 (Figure 3-4B). The absence of ATRX protein was confirmed in *Atrx*^{Sox10Cre} OPCs by immunofluorescence staining of ATRX and imaging in parallel with the endogenous Sun1GFP reporter (Figure 3-4C). To confirm depletion of *Atrx*, we obtained OPC nuclei from P20 forebrain of control and *Atrx*^{Sox10Cre} mice and enriched for Sun1GFP^{+} nuclei using FANS (Figure 3-4D). qRT-PCR analysis confirmed a 74.2% decrease in *Atrx* transcript levels *Atrx*^{Sox10Cre} Sun1GFP^{+} nuclei compared to control Sun1GFP^{+} nuclei (n=4 each genotype; Student's T-test, $p<0.05$) (Figure 3-4E). These results confirm that *Atrx* gene deletion in OPCs leads to a reduction of ATRX protein and transcript levels in *Atrx*^{Sox10Cre} mice.

3.3.5 Loss of the Olig2 fate specification factor in ATRX-null OPCs.

At all stages of development, OPCs and OLs express Olig2, an essential regulator of OL lineage specification (Lu, Sun et al. 2002, Takebayashi, Nabeshima et al. 2002, Zhou and Anderson 2002). Olig2 suppresses astrocytic fate (Cai, Chen et al. 2007, Zhu, Zuo et al. 2012, Zuo, Wood et al. 2018) and promotes OPC differentiation via regulation of the chromatin remodeling complex Smarca4/Brg1 (Yu, Chen et al. 2013). Upon staining with Olig2, we were surprised to see that in the *Atrx*^{Sox10Cre} corpus callosum, a large proportion of Sun1GFP^{+} ATRX-null OPCs do not express the Olig2 protein (47.9%) compared to Sun1GFP^{+} control OPCs (12.1%) (n=3-4 each genotype; Student's T-test, $p<0.05$) (Figure 3-5A, B). Similarly, qRT-PCR performed on sorted *Atrx*^{Sox10Cre} Sun1GFP^{+} nuclei confirms a 58% reduction in *Olig2* RNA transcripts compared to Sun1GFP^{+} control nuclei (n=3 each genotype; Student's T-test, $p<0.05$) (Figure 3-5C).

Loss of *Olig2* expression in ATRX-null OPCs is also observed in primary glial cell cultures. Cre-mediated recombination was induced by tamoxifen injections to nursing mothers for two days (age of pups P0-P1). P3 cortices of *Atrx*^{Sox10Cre} or control mice that

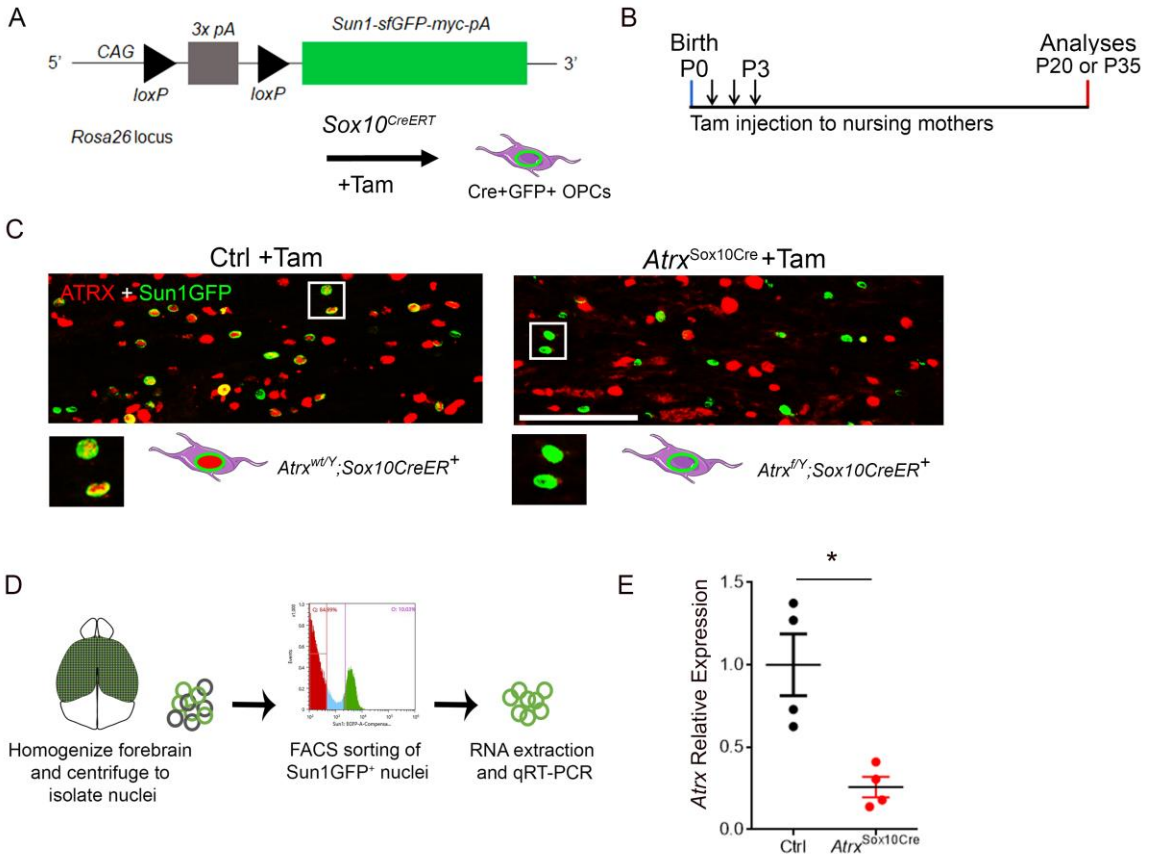


Figure 3-4 ATRX is absent in Sox10-expressing cells following tamoxifen injection to nursing mothers.

(a) Diagram of the Cre-sensitive Sun1GFP nuclear membrane reporter. (b) Nursing mothers were injected daily with tamoxifen (Tam) for three days (P1-P3 of pups, as indicated by black arrows) and pups were sacrificed for analysis at P20. (c) Immunofluorescence microscopy of P20 *Atrx*$^{wt/y}$;Sox10Cre^{+} corpus callosum shows expression of the Sun1GFP fusion protein in the nuclear membrane of OPCs upon Tam induced Sox10Cre-mediated excision of the upstream polyA transcriptional stop sequence (left). P20 Atrx$^{fl/y}$;Sox10Cre^{+} treated with Tamoxifen also express Sun1GFP but lack expression of nuclear ATRX (right). Scale bar, 100 µm. (d) Isolation of nuclei expressing Sun1GFP via FACS sorting. (e) qRT-PCR analysis shows that *Atrx* transcript levels are significantly decreased in P20 *Atrx*^{Sox10Cre} isolated nuclei (n= 4 for each genotype; Student's T-test $p<0.05$).

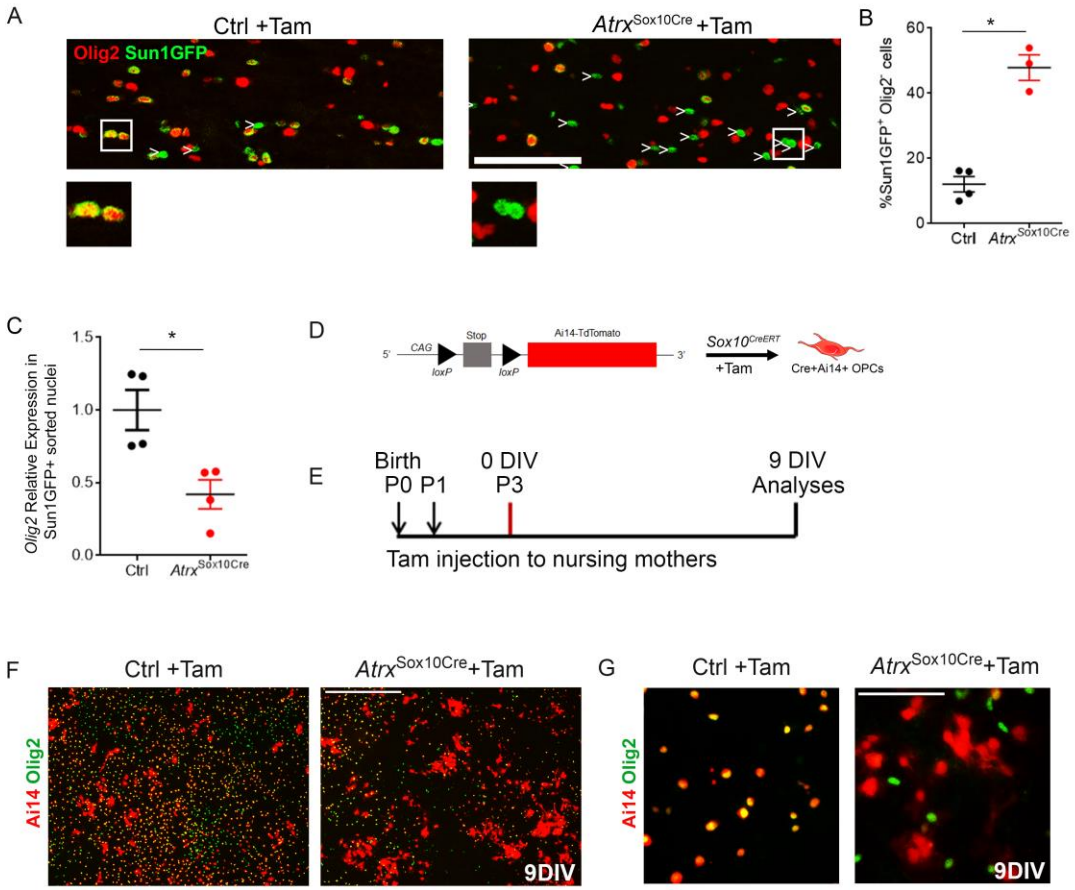


Figure 3-5 ATRX-null OPCs fail to express Olig2 and exhibit altered morphology.

Fate mapping of ATRX-null Sun1GFP$^+$ OPCs reveals frequent loss of the Olig2 transcription factor (white arrowheads). Scale bar, 100µm. (b) Quantification of Sun1GFP$^+$ OPCs that lack Olig2 staining (right) (Ctrl n=4; *Atrx*^{Sox10Cre} n=3; Student's t-test; $p<0.05$). (c) qRT-PCR analysis demonstrates significantly decreased Olig2 transcript levels in Sun1GFP$^+$ *Atrx*^{Sox10Cre} sorted nuclei compared to Sun1GFP$^+$ control nuclei (n= 4 for each genotype; Student's T-test; $p<0.05$). (d) Diagram of the Cre sensitive Ai14 allele encoding the tdTomato fluorescent reporter. (e) Experimental timeline of Tamoxifen (Tam) injections to nursing mothers at P0 and P1 (black arrows). Primary mixed glial cultures of cortices were established at P3 and maintained for 9 days in vitro (DIV). (f, g) Control and *Atrx*^{Sox10Cre} Ai14$^+$ (red) primary cultures at 9DIV immunostained for Olig2 (green) reveal limited overlap of ATRX-null Ai14$^+$ OPCs with Olig2 and flattened cell morphology. Scale bar, (f) 500 µm (g) 100 µm. In all graphs, error bars represent $^+$/-SEM and asterisks indicate $p<0.05$ (Student's T-test).

also express the Ai14-TdTomato reporter (Figure 3-5D) (Madisen, Zwingman et al. 2010) were dissociated and cells grown in mixed glial co-culture in which OPCs are expanded on top of an astrocyte monolayer (Chen, Balasubramaniyan et al. 2007, O'Meara, Ryan et al. 2011) (Figure 3-5E). After 9 days *in vitro* (DIV), we observed that many ATRX-null Ai14^{+} cells do not express Olig2and display an elongated and flattened morphology unlike the typically rounded shape of OPCs (Figure 3-5F, G). These results establish that ATRX-null OPCs fail to express Olig2 *in vivo* and *in vitro* and start to display an atypical flattened morphology.

3.3.6 Inactivation of *Atrx* downregulates the expression of OPC-specific genes

To further characterize ATRX-null OPCs and investigate the downstream consequences of Olig2 loss of expression, we evaluated the transcript level of known OPC-specific markers. We extracted RNA from ATRX-null or control Sun1GFP^{+} OPC nuclei sorted from cortical samples. RT-qPCR analysis reveals a dramatic reduction in the expression of OPC markers *Pdgfrα*, *Cspg4* (NG2) and *Gpr17* in ATRX-null OPCs (Figure 3-6A) (n=3-4 each genotype; Student's T-test, $p<0.05$). The OPC-expressed genes *Olig1* and *Sox10* were similarly reduced but did not reach the significance threshold of $P<0.05$ (Figure 3-6A) (n=3 each genotype; $p>0.05$ Student's T-test). Moreover, Sun1GFP+ ATRX-null OPCs in the corpus callosum stain less frequently for PDGFRα (24.9%) compared to controls (41.1%) (Figure 3-6B) (n=3 each genotype; $p<0.05$, Student's T-test). Immunofluorescence staining was also performed on mixed glial cultures at either early (3DIV) or late (9DIV) timepoints to examine OPC development over time as depicted in Figure 3-6C. At 3DIV, many ATRX-null Ai14^{+} cells still express PDGFRα despite the onset of an abnormal flattened and elongated morphology (Figure 3-6D). Conversely, NG2 is not expressed in ATRX-null Ai14^{+} cells at 3DIV, even in cells displaying the typical round OPC morphology (Figure 3-6E). These data suggest that downregulation of NG2 precedes that of PDGFRα in ATRX-null OPC. At 9DIV, however, there is a striking increase in ATRX-null Ai14^{+} cells displaying atypical morphology coupled with NG2 and PDGFRα loss (Figure 3-6F). We also noted that at

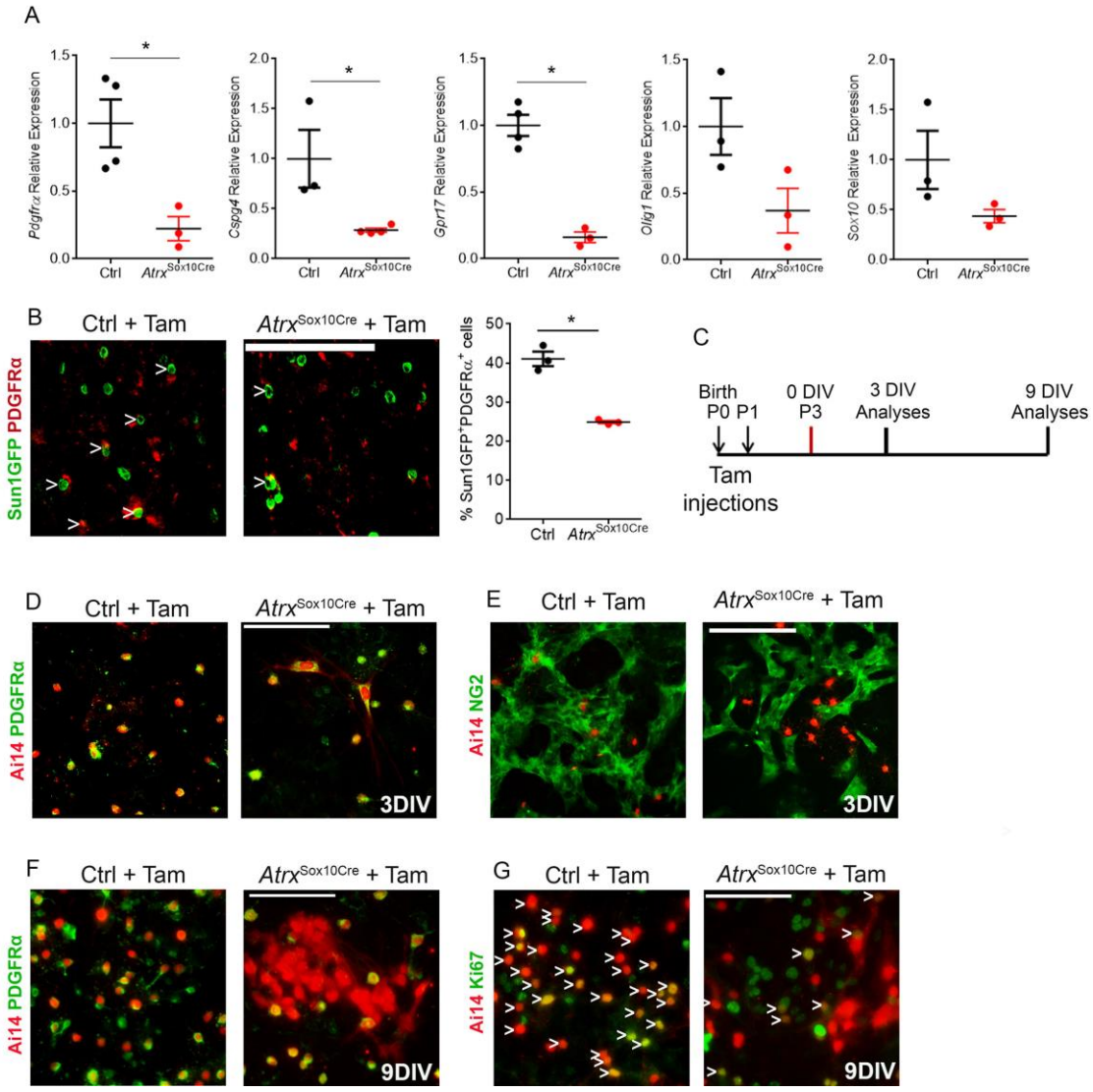


Figure 3-6 Loss of OPC identity after induction of *Atrx* deletion in Sox10$^+$ cells.

(a) Transcriptional analysis of *Atrx*^{Sox10Cre} sorted Sun1GFP$^+$ nuclei shows downregulation in OPC markers compared to control (n=3-4 each genotype; asterisks indicate $p<0.05$, Student's t-test). (b) PDGFRα immunostaining (red) shows decrease proportion of Sun1GFP$^+$ PDGFRα$^+$ cells in the corpus callosum of *Atrx*^{Sox10Cre} compared to control mice. Scale bar, 100 μm (n=3 each genotype; Student's t-test; $p<0.05$). (c) Timeline of tamoxifen injection and primary culture performed for panels (d) to (g). (d) Immunofluorescence staining for PDGFRα (green) shows all Ai14$^+$ (red) cells are PDGFRα$^+$ at 3DIV, even in cells starting to undergo morphological changes (e)

Immunofluorescence staining for NG2 shows Ai14^{+} (red) cells do not express NG2 (green) following 3DIV. (f) Loss of PDGFRα^{+} (green) staining in ATRX-null OPCs (red) at 9DIV. (g) Ki67 staining (green) reveals diminished proliferative capacity in ATRX-null OPCs (red). Scale bar, 100 μm. In all graphs, error bars represent $^{+}$/-SEM.

9DIV, there are fewer ATRX-null Ai14^{+} cells that stain positively for the proliferation marker Ki67, which could stem from the reduction of the growth promoting factor PDGFRα (Figure 3-6G). We conclude that ATRX ablation in postnatal Sox10-expressing OPCs leads to a reduction in the expression of OPC markers and a loss of progenitor identity.

3.3.7 Postnatal *Atrx* inactivation in OPCs results in fewer myelinating OLs and hypomyelination.

Considering the reduction in the normal OPC pool in the *Atrx*^{Sox10Cre} mice, we predicted a consequent decrease in the number of differentiated and myelinating OLs. To establish whether this is the case, we performed Immunofluorescence staining for APC, a marker of differentiated OLs in P20 control and *Atrx*^{Sox10Cre} cortical sections. There is a significant reduction in the number of Sun1GFP^{+} ATRX-null OPCs that co-express APC (41%) compared to Sun1GFP^{+} control OPCs (61.4%) (Figure 3-7A) (n=3 each genotype; $p<0.05$ Student's T-test), indicative of limited capacity of ATRX-null OPCs to differentiate into OLs. RNA transcript levels of the OL-specific genes *Mag, Mog, Mbp* are also decreased in P35 *Atrx*^{Sox10Cre} optic tract (n=3-4 each genotype; *Mog*, *Mbp* $p<0.05$ Student's T-test) (Figure 3-7B). Furthermore, immunofluorescence staining for MOG and MBP in brain cryosections at P20 show decreased level of these myelin proteins in the corpus callosum and cortex of *Atrx*^{Sox10Cre} mice compared to controls (Figure 3-7C). Reduced myelin protein expression in *Atrx*^{Sox10Cre} mice was confirmed by western blot analysis of MAG, MOG and MBP (n=4 each genotype; MOG, MBP $p<0.05$ Student's T-test) (Figure 3-7D). These results demonstrate that ATRX-null OPCs either do not survive or have lost the ability to differentiate into mature OLs, leading to reduced myelination.

3.3.8 ATRX-null OPCs undergo ectopic astrogliogenesis

Ablation of the transcription factor Olig2 in OPCs in the postnatal cortex has been reported to induce abnormal OPC differentiation to that of astrocytes

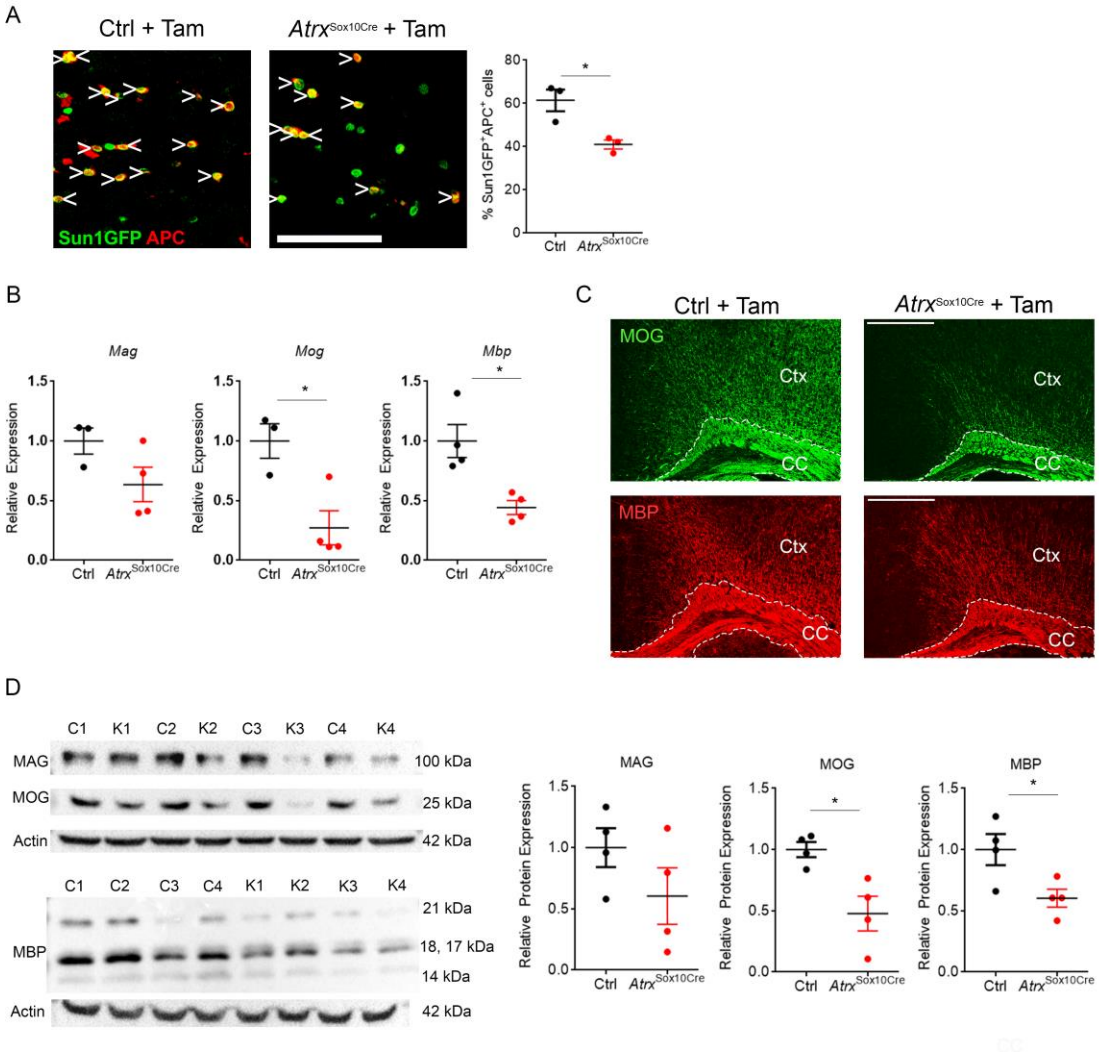


Figure 3-7 Reduced capacity of ATRX-null OPCs to differentiate into OLs leads to hypomyelination.

(a) Cell counts show significantly fewer Sun1GFP^{+} cells that co-stain for the mature OL marker APC (red) in the P20 *Atrx*^{Sox10Cre} corpus callosum compared to controls. Scale bar, 100 µm (n=3 each genotype; $p<0.05$ Student's T-test). (b) qRT-PCR analysis of *Mag*, *Mog* and *Mbp* shows reduced RNA transcript levels in *Atrx*^{Sox10Cre} P35 optic tract compared to controls (n=3-4 each genotype; $p<0.05$ Student's T-test). (c) Immunofluorescence staining of myelin proteins MOG (green) and MBP (red) shows decreased protein levels in P20 *Atrx*^{Sox10Cre} cortex (Ctx) and corpus callosum (CC) (outlined in white dashed line) compared to controls. Scale bar, 500 µm. (d) Western blot

analysis of MAG, MOG and MBP (left) and quantification (right) reveals decreased expression of these proteins in P20 *Atrx*^{Sox10Cre} mouse forebrain extracts compared to controls after normalization to actin (n=4 each genotype; $p<0.05$ Student's T-test). In all graphs, error bars represent $^{+}$/-SEM and asterisks indicate significance of $p<0.05$.

(Cai, Chen et al. 2007, Zhu, Zuo et al. 2012, Zuo, Wood et al. 2018). This, combined with the observation that many ATRX-null OPCs take on an atypical morphology reminiscent of astrocytes *in vivo* and *in vitro*, prompted us to investigate whether ATRX-null OPCs were adopting a more malleable state conducive to alternative fate specification. To answer this question, we performed immunofluorescence staining of known astrocyte markers in control and *Atrx*^{Sox10Cre} and control brain cryosections at P20. We observe increased S100 calcium-binding protein β (S100β) staining in the corpus callosum and cortex of in *Atrx*^{Sox10Cre} mice (Figure 3-8A). Cells co-expressing the endogenous Sun1GFP and S100β (by immunostaining) were counted and the results show a 19.9% increase in the number of Sun1GFP^{+} cells that co-express S100β in *Atrx*^{Sox10Cre} compared to control corpus callosum (n=3 each genotype; Student's T-test, $p<0.05$) (Figure 3-8B). However, S100β can sometimes stain OLs (Hachem, Aguirre et al. 2005) and indeed we observed a high proportion of control Sun1GFP^{+} OPCs that expressed S100β (34.2%). We thus repeated this analysis using glial fibrillary acidic protein (GFAP), another astrocyte marker. Similar to the results obtained for S100β, we observed increased GFAP staining in the cortex of *Atrx*^{Sox10Cre} mice (Figure 3-8C). Cell counts of GFAP^{+}Sun1GFP^{+} cells showed a 15.2% increase in the proportion of Sun1GFP^{+} cells that co-stain with GFAP in *Atrx*^{Sox10Cre} compared to control corpus callosum (n=3 each genotype; Student's T-test, $p<0.05$) (Figure 3-8D).

Utilizing the Ai14 reporter to assess *Atrx*^{Sox10Cre} cell morphology, we can easily see that a subset of ATRX-null Ai14^{+} cells display arborized morphology typical of protoplasmic astrocytes as previously observed in *Olig2* knockout mice (Zhu, Zuo et al. 2012, Zuo, Wood et al. 2018) (Figure 3-8E). These cells co-express GFAP (Figure 3-8E), indicating that ATRX-null OPCs have gained the ability to undergo astrocyte differentiation *in vivo.* To confirm this finding, we established primary glial cultures from control and *Atrx*^{Sox10Cre} pups after 2 days of feeding from tamoxifen-injected mothers. After just 3DIV many *Atrx*^{Sox10Cre} derived cells (Ai14^{+}) co-express GFAP which is not the case in control cells (Figure 3-8F). By 9DIV, these cells display clear astrocyte morphology, adhering to the plate unlike the rounded and raised OPC morphology in controls (Figure

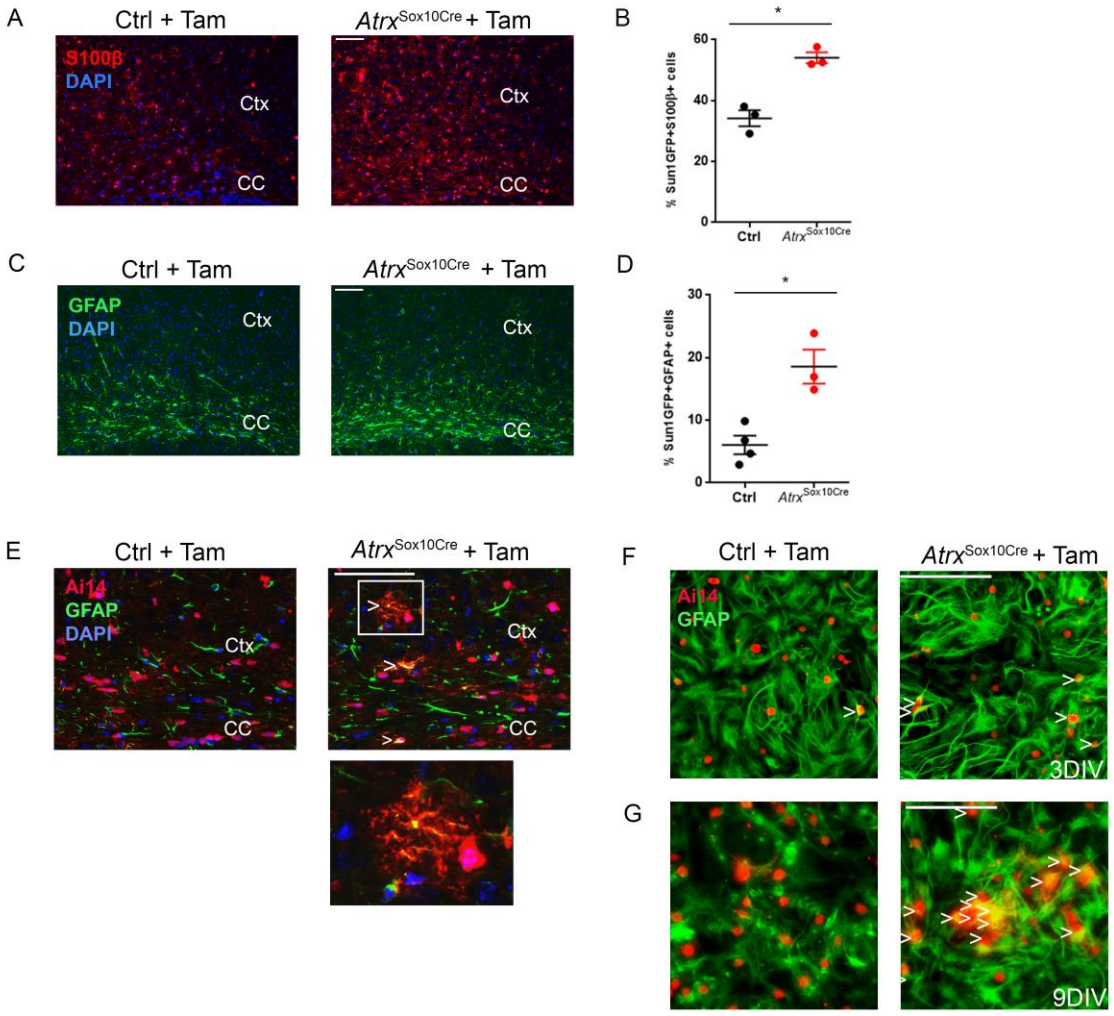


Figure 3-8 A subset of ATRX-null OPCs express astrocyte markers and adopt an astrocytic morphology *in vivo* and *in vitro*.

(a) Immunofluorescence staining of the astrocyte marker S100β reveals increased levels In the P20 *Atrx*^{Sox10Cre} mouse corpus callosum (CC) and cortex (Ctx) compared to controls. Scale bars,100 µm. (b) Quantification of the number of Sun1GFP^{+} OPCs that stain positive for S100β shows a significant overlap compared to control (n= 3-4 for each genotype). (c) Immunofluorescence staining of the astrocyte marker GFAP reveals increased levels In the P20 *Atrx*^{Sox10Cre} mouse corpus callosum (CC) and cortex (Ctx) compared to controls. Scale bars, 100 µm. (d) Quantification of the number of Sun1GFP^{+} OPCs that stain positive for GFAP shows a significant overlap compared to control (n=

3-4 for each genotype). (e) Immunofluorescence microscopy detects co-staining of the Cre-sensitive Ai14 reporter (red) and the astrocyte marker GFAP (green) in the AtrxSox10Cre mouse brain in vivo (white arrowheads and higher magnification image on the right. (f, g) Overlap of Ai14 (red) and GFAP (green) is also observed in *Atrx*^{Sox10Cre} OPCs at 3DIV (f) and more drastically at 9DIV (g) (white arrow heads indicate Sun1GFP^{+}GFAP^{+} cells) but not in control cells. Scale bars, 100 μm. In all graphs, error bars represent $^{+}$/-SEM and asterisks indicate significance ($p<0.05$ Student's T-test).

3-8G). These results strongly suggest that a portion of ATRX-null OPCs have gained the ability to generate astrocytes.

3.3.9 ATRX interacts with HDAC3 and occupies the *Olig2* gene locus

The histone deacetylase 3 (HDAC3) has been shown to directly promote the expression of *Olig2* in OPCs (Zhang, He et al. 2016). Deletion of *Hdac3* results in decreased *Olig2* expression, and an increased number of astrocytes at the expense of OLs (Zhang, He et al. 2016) similar to what we observed upon *Atrx* deletion in postnatal OPCs. We thus hypothesized that ATRX might directly bind and activate the *Olig2* gene in conjunction with HDAC3. Chromatin immunoprecipitation followed by quantitative PCR at various points along the *Olig2* gene (Figure 3-9A) in control cultured primary OPCs reveals occupancy of ATRX specifically at the promoter, exon 1 and 3' UTR of the gene (Figure 3-9B). Furthermore, HDAC3 and ATRX can be reproducibly co-immunoprecipitated in control forebrain extracts (n=3) (Figure 3-9C). These results indicate that ATRX directly binds to the *Olig2* gene and can interact with HDAC3, suggesting that they may co-activate *Olig2* expression to maintain OPC fate specification and suppress astrocytic fate (Figure 3-9D).

3.4 Discussion

Chromatin remodelers have emerged as critical factors in the regulation of the OL lineage progression and myelination, however, many questions remain as to the exact mechanisms by which they exert their effects. In the present study, we report key advances in our understanding of ATRX-mediated regulation of myelin development through both systemic and cell-autonomous mechanisms. Mice lacking *Atrx* in all cells of the forebrain and anterior pituitary exhibit reduced myelination that could be partially rescued by early postnatal administration of thyroxine. Cell type specific inactivation of ATRX in OPCs, but not in neurons, also led to hypomyelination. We determined that ATRX directly binds to the *Olig2* gene and interacts with HDAC3, previously identified as an *Olig2* gene activator. Consequently, Olig2 is not expressed in many ATRX-null

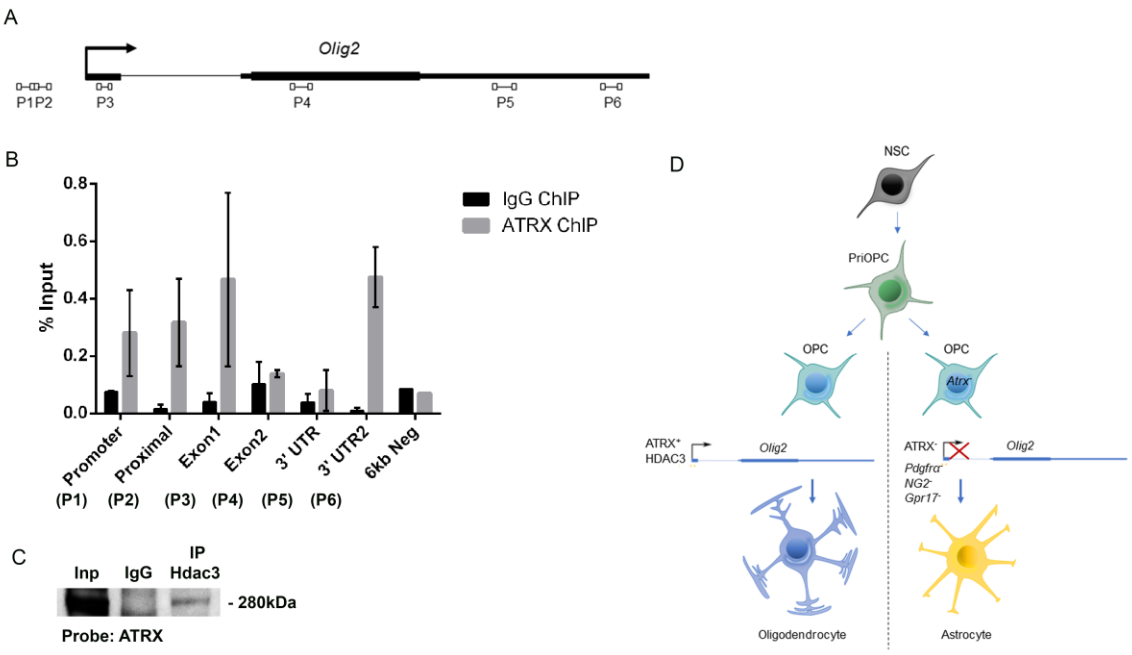


Figure 3-9 ATRX interacts with HDAC3 and occupies the *Olig2* gene locus.

(a) Schematic representation of the of the *Olig2* gene locus. (b) ATRX ChIP-qPCR analysis reveals direct binding to the *Olig2* promoter, exon 1 and 3'UTR in chromatin prepared from cultured wildtype OPCs. ChIP with IgG was performed in parallel as a control (n=2). The location of the primer pairs used for qRT-PCR (P1-P6) are indicated as boxes in (a). (c) Co-immunoprecipitation of ATRX and HDAC3 in P20 wildtype mouse forebrain extracts (n=3). (d) Proposed model of ATRX function in OPCs. Neural stem cells (NSCs) commit to the OL lineage by expressing the transcription factor Olig2 in primitive OPCs (PriOPCs). Olig2 expression is maintained in OPCs by HDAC3 and ATRX. ATRX deletion in $Sox10^{+}$ OPCs in the postnatal cortex prevents expression of Olig2 and several OPC-specific markers, leading to increased fate malleability and ectopic astrogliogenesis.

OPCs, leading to a more permissive precursor state, perhaps through modified chromatin accessibility, allowing for ectopic astrogliogenesis (Figure 3-9D).

We first demonstrated that myelination is abnormally decreased in *Atrx*^{FoxG1Cre} mice, which have low levels of circulating T4 (Watson, Solomon et al. 2013, Rowland, Jiang et al. 2018). The thyroid hormone receptor is a transcriptional activator for several myelin genes, promoting differentiation of OPCs (Farsetti, Mitsuhashi et al. 1991, Ibarrola and Rodríguez-Peña 1997, Dong, Yauk et al. 2009). Following T4 supplementation in *Atrx*^{FoxG1Cre} mice there was a significant yet partial rescue in myelin gene and protein expression. Whereas the number of APC-positive differentiated OLs increased following T4 administration, the number of PDGFRα-positive OPCs was not restored to that of controls. Furthermore, production of Olig2-expressing OLs following T4 supplementation in *Atrx*^{FoxG1Cre} mice was not observed, indicating that ATRX may be essential for OPC production or survival in a mechanism distinct from T4 regulation.

Neuronal communication with OLs is emerging as a key regulator of myelination. Neurons have been proposed to be necessary for adaptive myelination (Barres and Raff 1993, Gibson, Purger et al. 2014) and electrical activity from neurons has been shown to induce OPC proliferation and remyelination (Gautier, Evans et al. 2015). Due to the fact that ATRX is deleted in all cell types of the forebrain of *Atrx*^{FoxG1Cre} mice, it was conceivable that deletion of *Atrx* in neurons could result in a reduction of the OPC pool. Nonetheless, deletion of ATRX in neurons did not result in defects in the development of white matter, pointing to a role for ATRX in the regulation of OPCs and OLs cell-autonomously. We therefore deleted *Atrx* specifically in OPCs postnatally by administering tamoxifen to nursing mothers. Lineage tracing *in vivo* and *in vitro* revealed that ATRX-null OPCs lack Olig2 and NG2 (*Cspg4*), with a more progressive loss of PDGFR*α* expression. This might be due to the fact that *Cspg4* is directly regulated by HDAC3/p300 (Zhang, He et al. 2016) and Olig2 (Wang, Pol et al. 2014, Gotoh, Wood et al. 2018) whereas *Pdgfrα* is regulated by Sox10 (Finzsch, Stolt et al. 2008), which is activated by Olig2 (Kuspert, Hammer et al. 2011). PDGFRα is essential for OPC proliferation and its eventual loss likely contributes to the reduction in Ki67^{+} cells in

Atrx^{Sox10Cre} cultured OPCs at 9DIV. This depletion of the OPC pool explains the reduction in myelinating OLs and hypomyelination observed in *Atrx*^{Sox10Cre} mice. The absence of key fate determinants like Olig2, Sox10, NG2 and PDGFRα may allow ATRX-null OPCs to acquire an expanded fate potential as they are no longer expressing OL lineage proteins.

In support of this, we observed that ATRX-null OPCs are able to adopt an astrocytic fate that is reminiscent of the effects of *Olig2* deletion in the postnatal cortex (Zhu, Zuo et al. 2012, Zuo, Wood et al. 2018). The timing of Olig2 ablation has been shown to reflect the number of OPCs that differentiate into astrocytes. When *Olig2* is deleted constitutively in NG2 expressing cells beginning at E16.5 there is almost complete conversion of these cells to astrocytes (Zhu, Zuo et al. 2012). However, deletion of Olig2 at P2 or P18 results in a 50% and 25% conversion to astrocytes, respectively (Zhu, Bergles et al. 2008, Zuo, Wood et al. 2018). It is likely if we deleted *Atrx* earlier in development we may see an increase in OL fate conversion to the astrocyte lineage *in vivo*. Our *in vitro* system is designed for the mass production of OPCs and allows for more cell division than would normally be observed *in vivo*, especially considering proliferation capacity declines with age (Young, Psachoulia et al. 2013). Correspondingly, *Atrx*^{Sox10Cre} mixed glial cultures have a drastic increase in OPC-derived astrocytes over a period of 9DIV.

The OL to astrocyte fate change could potentially be mediated by changes in the chromatin landscape. It was shown that deletion of HDAC3 leads to a reduction in Olig2 expression, a loss of OLs and a concomitant increase in the number of astrocytes (Zhang, He et al. 2016). In addition to direct activation of *Olig2* with the histone acetyltransferase (HAT) p300, HDAC3 also inhibits transcription of astrocyte genes when not bound to p300 (Zhang, He et al. 2016). When HDAC3 is deleted, p300 is able to bind Stat3 to activate genes in the astrocyte lineage (Zhang, He et al. 2016). We show that ATRX can bind to both HDAC3 and the *Olig2* gene locus indicating that ATRX may work in conjunction with HDAC3 and p300 to activate *Olig2* expression. This was unexpected as histone deacetylation usually results in chromatin compaction and gene repression (Ruthenburg, Li et al. 2007). Unconventionally, HATS and HDACs are found together, primarily at actively transcribed genes and very rarely at repressed genes (Wang, Zang et

al. 2009). It is possible that at the *Olig2* gene, HDAC3 functions to remove acetyl groups added by p300 during transcriptional initiation and elongation to help maintain an appropriate level of acetylation and to reset chromatin modifications after gene activation.

The finding that OPCs lacking ATRX aberrantly differentiate into astrocytes could also have important implications in our understanding of ATRX loss of function in astrocytomas (Abedalthagafi, Phillips et al. 2013). In fact, a recent report described a patient with an oligodendroglioma that transitioned into astrocytoma upon loss of ATRX (Kim, Jang et al. 2019). Additionally, this is the first report investigating the regulation of myelination by ATRX linking hypomyelination to intellectual disability in ATR-X syndrome patient. In addition to potential benefits of thyroxine administration, our study highlights a novel role for ATRX in the oligodendrocyte-astrocyte lineage choice during postnatal cortical development.

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Chapter 4

4 Discussion and Future Directions

4.1 Thesis Summary

This thesis describes the role of ATRX in the prevention of premature aging phenotypes by the maintenance of cell type specific gene expression programs, which is partially mediated by activated thyroid hormone receptor. Aging is known to be caused by DNA damage and telomere abnormalities (López-Otín, Blasco et al. 2013) both of which are observed following *Atrx* deletion in neuroprogenitor cells (Watson, Solomon et al. 2013). We can now add transcriptional regulation of *Igf1* in the liver and *Olig2* in OPCs to the list of evidence to support that ATRX is necessary for the prevention of premature aging phenotypes.

In chapter two I built upon previous work that demonstrated mice lacking ATRX in all cells of the forebrain and anterior pituitary (*Atrx*^{FoxG1Cre}) exhibit premature aging-like phenotypes (Watson, Solomon et al. 2013) (Figure 4-1). Aging phenotypes were accompanied by low circulating levels of IGF-1 and T4 (Watson, Solomon et al. 2013). Based on evidence that T4 activates expression of *Igf1* (Xing, Govoni et al. 2012), T4 was administered to *Atrx*^{FoxG1cre} mice during the prepubertal period. Despite restoration of normal serum T4 levels, circulating IGF-1 was not recovered, nor was expression of *Igf1* and several other thyroid hormone responsive genes. Unexpected Cre-mediated *Atrx* inactivation in the liver likely explains the inefficacy of T4 treatment, indicating *Atrx* plays a role in the activation of some thyroid hormone responsive genes, like *Igf1*.

Chapter 3 focuses on a more in-depth investigation of the only aging phenotype that was ameliorated by T4 treatment, hypomyelination (Figure 4-1). I found that *Atrx*^{FoxG1Cre} mice exhibit hypomyelination and T4 treatment led to increased OL differentiation and partial rescue of myelination. However, the number of OPCs was still reduced, suggesting additional, T4-independet mechanisms for ATRX in myelin production. Specific

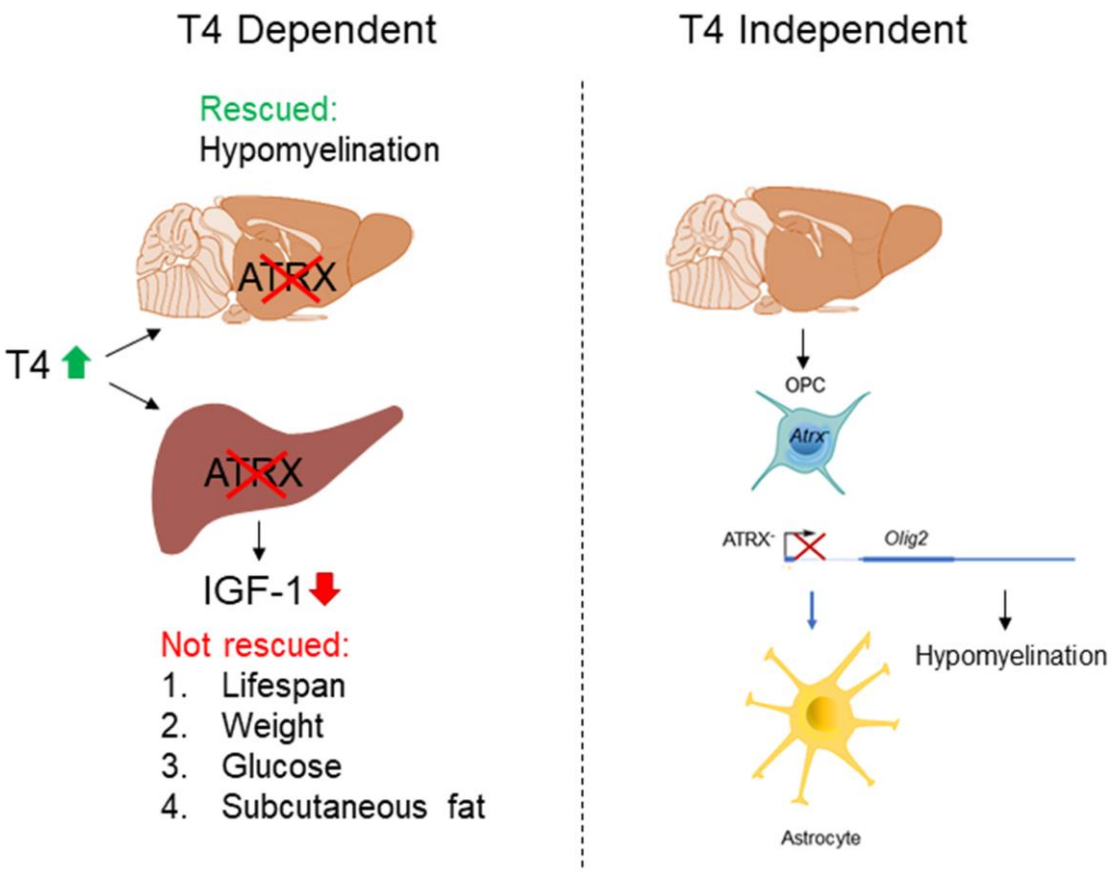

Figure 4-1 Thyroxine-Dependent and -Independent Effects on Premature Aging Phenotypes in ATRX Mutant Mice.

Despite restoration of normal serum T4 levels in *Atrx*^{FoxG1Cre} mice, circulating IGF-1 was not recovered, nor were several phenotypes of premature aging (left). *Atrx*^{FoxG1Cre} mice also display the premature aging phenotype hypomyelination and T4 treatment led to a partial rescue of myelination (left). However, myelination rescue was partial suggesting additional, T4-independet mechanisms for ATRX in myelin production. Specific inactivation of *Atrx* in postnatal OPCs results in a failure to induce *Olig2* expression, ectopic astrogliogenesis and consequently, hypomyelination (right).

inactivation of *Atrx* in postnatal OPCs results in ectopic astrogliogenesis, a reduction in the number of OLs and consequently, hypomyelination. ATRX associates with HDAC3 to directly activate expression of the fate specifying transcription factor *Olig2*.

Taken together, this thesis highlights the role of ATRX maintaining normal gene expression in the liver and the OL lineage (Figure 4-1). It also expands upon many controversial mechanisms in the literature, including *Igf1* regulation, OPC multipotency and HDAC-mediated gene activation.

4.2 The role of ATRX in IGF-1 signaling and the prevention of premature aging phenotypes

Watson et al. previously described the $Atrx^{FoxG1Cre}$ mice with *Atrx* deficiency in the forebrain and anterior pituitary resulting in low serum T4 and IGF-1 (Watson, Solomon et al. 2013). $Atrx^{FoxG1Cre}$ mice display many phenotypes of premature aging, including short lifespan and growth abnormalities (Watson, Solomon et al. 2013). Several lines of evidence led us to attempt T4 supplementation to reverse phenotypes in $Atrx^{FoxG1Cre}$ mice. First, the features of $Atrx^{FoxG1Cre}$ mice are comparable to mice deficient for the *Zmpste* gene important for the processing of Lamin A (Mariño, Ugalde et al. 2010). Supplementing $Zmpste^{-/-}$ mice with IGF-1 restored their somatotrope axis and drastically improved phenotypes of aging and extending longevity (Mariño, Ugalde et al. 2010). Second, mice lacking the receptor for TSH ($Tshr^{-/-}$), have low T4, IGF-1 and growth abnormalities, all of which were rescued following prepubertal T4 treatment (Xing, Govoni et al. 2012). Third, several reports revealed that thyroid hormone is able to control *Igf1* expression in the early postnatal stages of life (O'Shea, Bassett et al. 2005, Wang, Shao et al. 2010, Xing, Govoni et al. 2012). Thus, we hypothesized that the low T4 observed in $Atrx^{FoxG1Cre}$ mice may be directly responsible for the reduction in serum IGF-1 and subsequent premature aging phenotypes, and that T4 injections would reverse these effects.

Contrary to our expectations, prepubertal T4 administration did not restore hepatic *Igf1* transcription, serum IGF-1 levels or phenotypes of premature aging in $Atrx^{FoxG1Cre}$ mice

(except for hypomyelination, discussed below). Upon further investigation, we found that *Atrx* is unexpectedly absent in a subset of hepatocytes due to off-target Cre expression from the *Foxg1Cre* allele. Moreover, it appears that ATRX is actually required in hepatocytes for the expression of a subset of thyroid hormone responsive genes in the liver, including *Igf1*, thus explaining the ineffectiveness of T4 treatment. ATRX is known to bind another nuclear hormone receptor in the testes, the androgen receptor, to facilitate transcription of its target genes (Bagheri-Fam, Argentaro et al. 2011). It is possible that ATRX is able to bind TRs in a similar fashion, to facilitate transcription of its target genes. With that said, we were unable to co-immunoprecipitate ATRX and Trβ in control liver extracts (data not shown) and further studies are needed to determine if this interaction does indeed occur.

The role of ATRX in IGF-1 signaling, could be clarified by generating mice with targeted inactivation of *Atrx* in hepatocytes, the cell type that produces and secretes IGF-1 into the serum. Hepatocyte-specific deletion of *Igf1* results in an 80% reduction in serum IGF-1, however, body weight and length were unaffected in the mice (Yakar, Liu et al. 1999), unlike what is observed in whole body knockout of *Igf1* (Liu, Baker et al. 1993, Powell-Braxton, Hollingshead et al. 1993) and in our multiple organ ATRX-deficient mice. The absence of deleterious effects on growth following liver deletion of *Igf1* emphasizes the importance of IGF-1 production at the tissue level. In light of this, it is possible that *Atrx* deletion in hepatocytes would not be sufficient to elicit an aging phenotype.

The NER protein ERCC1 plays a key role in DNA repair, and has been linked to both progeria in humans (Niedernhofer, Garinis et al. 2006) and in mice (McWhir, Selfridge et al. 1993, Weeda, Donker et al. 1997, Dolle, Kuiper et al. 2011). It is therefore quite intriguing that ERCC1 colocalizes with ATRX, CTCF and cohesin to repress imprinted genes in the liver (Chatzinikolaou, Apostolou et al. 2017). The authors propose that in hepatocytes lacking *Ercc1*, persistent DNA damage elicits changes to chromatin structure, mediated by CTCF and ATRX, which adversely affect gene expression in the liver (Chatzinikolaou, Apostolou et al. 2017). We have reported a similar role for ATRX in the silencing of imprinted genes in the brain, although without the link to ERCC1

(Kernohan, Jiang et al. 2010, Kernohan, Vernimmen et al. 2014). It is plausible that ATRX works with ERCC1 in multiple tissue types to maintain chromatin structure as well as normal gene expression programs, and that dysfunction in either protein results in premature aging-like phenotypes.

4.3 The role of ATRX in myelination

A common symptom of aging is the gradual decline in white matter (Bartzokis, Beckson et al. 2001, Bartzokis, Cummings et al. 2003). The structure of the aged myelin sheath has dense cytoplasm, vesicular inclusions, multilamellar myelin fragments and membrane ballooning, all indicative of axon degeneration (Stadelmann, Timmler et al. 2019). OLs are highly susceptible to DNA damage and the latter is often associated with hypomyelination (Tse and Herrup 2017). Additionally, myelin defects have been observed in both patients (Koob, Laugel et al. 2010, James and Jose 2017) and mouse models of Cockayne Syndrome (Revet, Feeney et al. 2012) further implicating impaired genomic maintenance to the age-related decline in myelination. Neonatal hypothyroidism has been shown to cause hypomyelination which can be reversed with thyroxine treatment (Jagannathan, Tandon et al. 1998). Lastly, ATR-X syndrome patients display abnormal myelination (Wada, Ban et al. 2013, Lee, Lee et al. 2015) but it has not been reported whether patients display low levels of thyroid hormone or IGF-1. While thyroxine treatment did not improve IGF-1 dependent aging-related phenotypes, it did ameliorate myelination in the *Atrx*^{FoxG1Cre} mice due to the direct transcriptional effects of ligand-bound TRs on OL differentiation (Farsetti, Mitsuhashi et al. 1991, Billon, Tokumoto et al. 2001, Billon, Jolicoeur et al. 2002) and myelin gene expression (Farsetti, Mitsuhashi et al. 1991, Rodriguez-Peña, Ibarrola et al. 1993, Dong, Yauk et al. 2009). In spite of this, myelin restoration in *Atrx*^{FoxG1Cre} mice by thyroid hormone was partial, indicating that ATRX is required independently of circulating T4 levels to control myelin production.

To address the potential requirement for ATRX in cells of the nervous system, we characterized myelination in two mouse models where ATRX is conditionally ablated directly in OPCs, or in neurons. The observation that *Atrx* deletion in neurons does not

result in hypomyelination during the developmental period does not rule out the possibility that ATRX is required in neurons for the maintenance of the myelin sheath. Axons are very important for maintaining myelination throughout life although the exact mechanisms by which this occurs in the CNS have yet to be deciphered (Bercury and Macklin 2015). For example, in diseases like Niemann-Pick type C disease (Yu and Lieberman 2013) and Charcot–Marie–Tooth disease (Juárez and Palau 2012), mutations in genes essential for neuron function and survival result in demyelinating neuropathy. Recently, we demonstrated that deletion of *Atrx* in adult forebrain glutamatergic neurons using the CaMKII-Cre driver results in a reduction of corpus callosum volume as observed by magnetic resonance imaging in 9-month-old mice (data not shown). It is plausible that $Atrx^{NexCre}$ mice may exhibit similar phenotypes of myelin deterioration later in life. Additionally, we cannot rule out that myelin defects in $Atrx^{FoxG1Cre}$ mice are in part due to deletion of *Atrx* in astrocytes or microglia causing non cell-autonomous effects on OPCs and/or OLs. This idea could be tested in the future to better differentiate the role of ATRX in each cell type with respect to myelin development.

4.4 The role of ATRX in the OPC-to-astrocyte fate switch

The investigation of the cell-autonomous effects of ATRX deletion on myelination was especially insightful, leading to the discovery that ATRX is important in the OL and astrocyte fate choice. We found that ATRX directly controls expression of the OL fate specifying transcription factor *Olig2,* potentially in concert with HDAC3. Consequently, the loss of ATRX in OPCs leads to ectopic astrogliogenesis, a reduction in both OPCs and OLs, with subsequent hypomyelination. It is well documented that OLs can become astrocytes *in vitro* (Raff, Miller et al. 1983) and research is ongoing to determine the degree of plasticity that OPCs exhibit under normal conditions *in vivo* (Rivers, Young et al. 2008, Kang, Fukaya et al. 2010). Under normal *in vivo* conditions, OPCs have been shown to become astrocytes with high prevalence in the ventral forebrain (Zhu, Bergles et al. 2008). While a small number of OPCs can become astrocytes in the dorsal cortex, this is restricted to areas of grey matter and has not been observed at all in areas of white matter (Zhu, Bergles et al. 2008). OL to astrocyte fate switch is documented in areas of

grey matter of the dorsal cortex following deletion of *Olig2* (Zhu, Zuo et al. 2012, Zuo, Wood et al. 2018) or *Hdac3* (Zhang, He et al. 2016). Following deletion of *Atrx*, we provide compelling evidence that ectopic astrocyte production can occur in the corpus callosum, the largest white matter tract in the brain.

The brain is a heterogenous tissue and is comprised of several highly interconnected cell types. With this in mind, it is difficult to perform molecular experiments that are cell-type specific. To evaluate the transcriptional effects of ATRX loss in OPCs, we employed FANS to specifically isolate Sun1GFP^{+} control Sun1GFP+ *Atrx*-null nuclei. Using this strategy, we could detect decreases expression of several OPC-specific genes (*Olig2, Pdgfrα, Cspg4, Gpr17*) in ATRX-null OPCs, however, we were unable to detect a difference in expression of astrocyte-related genes (*Gfap*, *Nfia*). This may be due to the fact that there is a relatively small proportion of ectopic astrocytes (18.6%), making gene expression changes difficult to detect. Single-cell RNA sequencing may be a more effective way to determine gene expression changes in both the OPC, astrocyte and potentially unknown populations of nuclei that are generated following ATRX deletion in OPCs.

It is unclear whether *Atrx*-null, OPC-derived astrocytes are functional and indistinguishable from control astrocytes. In mixed glial culture, OPCs proliferate on top of an astrocyte monolayer which facilitate their growth by secretion of several growth factors and signaling molecule. PDGF and other growth factor levels could be measured from the media of both *Atrx*^{Sox10Cre} and control cultures to determine if there is discrepancy in growth factor secretion by ATRX-null OPC-derived astrocytes.

In *Atrx*^{Sox10Cre} mice, around 50% of ATRX-null OPCs in the corpus callosum still express Olig2 and go on to produce normal, myelinating OLs. In addition to this, in the adult brain OPCs make up 5% of the total cells (Dawson, Polito et al. 2003). Resident adult OPCs proliferate and differentiate following myelin injury or neurodegeneration (Kang, Fukaya et al. 2010), contributing to remyelination in the CNS (Gautier, Evans et al. 2015). The next step will be to determine if the hypomyelination observed at P20 in

Atrx^{Sox10Cre} mice persists into adulthood or if remyelination occurs due to the large number of OPCs still present. Regardless of the potential for remyelination, the behaviour of these mice can be evaluated at different points in development to determine if hypomyelination affects cognitive functions. Deletion of *Olig2* in differentiated but immature OLs leads to memory deficits, elevated glutamate levels and anxiety-like behaviours (Chen, Zhang et al. 2015, Chen, Wang et al. 2019). If these aberrant behaviours are observed in our model, it may be indicative of intellectual disability and autistic-like features observed in ATR-X syndrome patients.

4.5 The proposed mechanisms by which ATRX promotes *Olig2* expression in OPCs

The discovery that ATRX may work together with HDAC3 to control *Olig2* expression was unexpected, as histone deacetylation usually results in chromatin compaction and gene repression (Ruthenburg, Li et al. 2007). Nevertheless, HATs and HDACs are often found together at actively transcribed genes although the role of HDACs in gene activation is still under investigation (Wang, Zang et al. 2009). It has it has been proposed that, following gene activation, the transcriptional machinery alters chromatin structure and that acetylation needs to be reset to allow for gene reactivation (Wang, Kurdistani et al. 2002). It appears the role of HDACs at active genes is to maintain the proper level of acetylation to promote gene expression (Wang, Zang et al. 2009), however, more research is needed to confirm this hypothesis.

What is a potential mechanism by which ATRX, HDAC3 and p300 may control the expression of certain genes like *Olig2*? In OPCs, HDAC3 was shown to only activate genes like *Olig2* when bound with the HAT p300 (Zhang, He et al. 2016). The exact role of H3.3 at enhancers and active genes is still under investigation. In a recent report, it was shown that deletion of H3.3 negatively impacts p300 enzymatic activity and that phosphorylation of H3.3S31, allows H3.3 to become a nucleosomal cofactor for p300 to promote enhancer acetylation (Martire, Gogate et al. 2019). Deletion of ATRX could potentially result in a failure to deposit H3.3 which is predicted to cause a reduction in

p300 activity and a subsequent decrease in *Olig2* gene expression due to presence of HDAC3 without HAT p300. This mechanism provides an explanation for the controversial ability of OPCs to become astrocytes only in certain conditions.

It will be important to understand if the recruitment of p300, HDAC3 and H3.3 is perturbed by deletion of *Atrx* in OPCs. Our ATRX ChIP experiment was performed on control OPCs physically purified from mixed glial cultures. ATRX-null OPCs that take on an astrocyte fate, change morphology and adhere to the surface of the culture dish and can no longer be separated from control astrocytes. A method to investigate the role of ATRX in p300, HDAC3 and H3.3 recruitment, would be to use FANS to obtain purified nuclei in control and $Atrx^{Sox10Cre}$ brain extracts. It will also be valuable to investigate chromatin accessibility using assay for transposase-accessible chromatin using sequencing (ATAC-seq). If HDAC3 activity is enhanced and/or p300 activity is attenuated at *Olig2* following ATRX deletion, this will correspond to less accessible chromatin and fewer reads using ATAC-seq.

4.6 ATRX, HDAC3 and p300 may work together to maintain the balance of gene activation and repression in several tissues

The data in this thesis are provocative and provide key new information regarding ATRX function, bringing to the front new ideas and hypotheses. For example, there may be a common mechanism by which ATRX, HDAC3 and p300 function to regulate the development not only of OPCs, but also of thyrotropes in the pituitary and hepatocytes in the liver. We previously reported perturbed gene expression of both *Atrx* and *Tshβ* in the pituitary as well as serum levels of TSH in $Atrx^{FoxG1Cre}$ mice (Watson, Solomon et al. 2013). Diminished TSHβ serum levels are predicted to reduce circulating T4 in $Atrx^{FoxG1Cre}$ mice because TSHβ activates T4 production in the thyroid. Unliganded TRs repress target genes through the recruitment of NCoR co-repressor protein which exerts its effects in complex with HDAC3 to enhance deacetylation and gene repression (Li, Wang et al. 2000). HDAC3-NCoR have been shown to repress expression of *Pit1* (Xu, Lavinsky et al. 1998), the transcription factor responsible for the production of

thyrotropes in the pituitary and expression of *Tshβ* (Scully and Rosenfeld 2002). NCoR and unliganded TR have also been shown to repress transcription of several thyroid hormone responsive genes in the liver (Astapova, Lee et al. 2008). Lastly, HDAC3 has been shown to induce expression of *Olig2* when coupled with p300 (Zhang, He et al. 2016). With this in mind, one could envision a scenario in which ATRX is in complex with HDAC3 and p300 to activate genes in multiple tissues. In the absence of ATRX, p300 activity is reduced and HDAC3 deacetylation activity is enhanced, leading to abnormal gene inhibition in the pituitary (*Pit1*), the liver (*Igf1, Ghr, Prlr*) and in the brain (*Olig2*), ultimately causing premature aging phenotypes.

4.7 Thesis conclusions

Premature aging research has focused on elucidating the mechanisms behind evolutionarily conserved genetic and biochemical pathways that control the aging process to ultimately extend lifespan. The insulin and IGF-1 signaling pathway has been broadly studied in both premature aging and normal aging and its manipulation can either reduce or extend lifespan in several model organisms (López-Otín, Blasco et al. 2013). On the other hand, although hypothyroidism has been observed in aging and premature aging models, the exact reason for this is still under investigation (Gussekloo, van Exel et al. 2004, Atzmon, Barzilai et al. 2009, Over, Mannan et al. 2010, Visser, Bombardieri et al. 2016).

To assess the reported role of thyroid hormone mediated activation of *Igf1* expression and rescue of premature aging phenotypes (Xing, Govoni et al. 2012), I directly injected mice with T4. Despite restoration of T4 levels to that of controls, *Atrx*$^{\text{FoxG1Cre}}$ mice still displayed several premature aging phenotypes. The lack of rescue was due to a failure to recover IGF-1 serum levels and *Igf1* transcript levels in the liver. To determine if thyroid hormone signaling was intact in the *Atrx*$^{\text{FoxG1Cre}}$ liver, the main site of IGF-1 production, I assessed transcript levels of several genes normally activated by ligand-bound TR. Results showed that although T4 injections was able to activate transcription of several genes that were not expressed in *Atrx*$^{\text{FoxG1Cre}}$ livers, other genes were still

transcriptionally repressed, including *Igf1*. This unexpected result prompted investigation into the liver and the discovery that ATRX was absent from a subset of hepatocytes in the *Atrx*^{FoxG1Cre} liver particularly at P14. This result was quite unexpected as ATRX levels were reported to be that of control in *Atrx*^{FoxG1Cre} livers at P20 (Watson, Solomon et al. 2013) and likely reflects proliferation or infiltration of ATRX^{+} immune cells in the liver that occurs sometime between P14 and P20. This suggests that ATRX may be required in the liver to co-activate *Igf1*, potentially by binding liganded TR, similar to the reported mechanism with AR in the testes (Bagheri-Fam, Argentaro et al. 2011). By understanding the molecular mechanisms that control premature aging, we can begin to determine what causes normal aging and ultimately ameliorate symptoms in progeroid syndromes and in normal aging.

Both Cockayne syndrome patients (Koob, Laugel et al. 2010) and ATR-X syndrome patients (Wada, Ban et al. 2013, Lee, Lee et al. 2015) display hypomyelination which could contribute to cognitive deficits seen in these syndromes. Additionally, ligand-bound thyroid hormone has been shown to directly activate MBP and MAG expression as well as induce differentiation of OLs independently of IGF-1 (Farsetti, Mitsuhashi et al. 1991). Thyroxine administration did improve hypomyelination and increase the number of differentiated OLs in *Atrx*^{FoxG1Cre} mice. T4 supplementation is a conceivable treatment for ATR-X syndrome patients displaying hypomyelination and as more patient data becomes available, we will be able to determine if they exhibit low serum T4. Due to the fact that the rescue in myelination was partial, I hypothesized ATRX may be required cell-autonomously in the brain to promote OL development. *Atrx* deletion in OPCs resulted in *Olig2* downregulation allowing for astrocyte differentiation at the expense of the OL production. Although it is well established that OPCs have the potential to become astrocytes *in vitro* (Raff, Miller et al. 1983, Franklin, Bayley et al. 1995), there have been very few cases this has been demonstrated *in vivo* (Zhu, Bergles et al. 2008, Zhu, Zuo et al. 2012, Zhang, He et al. 2016, Zuo, Wood et al. 2018). The fact that ATRX-null OPCs can become astrocytes provides evidence that OPCs are indeed able to commit to the astrocyte lineage under certain conditions. ATRX is also able to directly bind the *Olig2* locus potentially in conjunction with HDAC3 to activate its expression,

promote the development of the OL lineage and drive CNS myelination. The discovery that ATRX is essential for *Olig2* transcription has implications not only for ATR-X syndrome patients but also patients with astrocytoma as a recent report described an oligodendroglioma that converted to astrocytoma upon loss of ATRX (Kim, Jang et al. 2019). One potential explanation is that the cell of origin for many ATRX-null astrocytomas might be OPCs that acquire an astrocytic fate.

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