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The Epigenetic Regulators ATRX and CTCF are Required for Mouse Neuroprogenitor Cell Survival and Brain Development

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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(Thesis format: Integrated Article)

by

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Abstract

Emerging evidence implicates the regulation of higher-order chromatin structure in brain development, maturation, and function. Human mutations in two important regulators of chromatin structure, *ATRX* and *CTCF,* cause microcephaly and intellectual disability and have been identified in several cancers, suggesting an important role for these proteins in the developing brain and to suppress tumourigenesis. This thesis demonstrates that chromatin structure is critical to the differentiation and survival of neural progenitor cells, and explores the mechanisms of ATRX and CTCF function in brain development. The first chapter identifies that *Atrx* deficiency induces replicative DNA damage at telomeres and pericentromeric heterochromatin, and the mutant mice display signs of premature aging, providing novel evidence that genetic damage restricted to the central nervous system can result in systemic defects that resemble aging. The second chapter demonstrates that the genome organizer CTCF is required for neural progenitor survival and to maintain the correct balance between proliferative and differentiative divisions in the mouse neocortex. The third chapter investigates the mechanism underlying p53- and PUMA-dependent apoptosis in *Ctcf*-null neural progenitor cells, focusing on a role for the protein in preventing replicative stress-induced apoptosis. Together, the findings presented here indicate that chromatin architectural proteins, such as ATRX and CTCF, are required for genomic stability to promote neural progenitor cell survival and support correct brain development.

Keywords

Co-authorship Statement

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

In chapter two, Matthew Edwards conducted Western blot experiments in Figure 2-1 and Supplementary Figure 2-4, and dissection of lenses for Figure 2-5. Yan Jiang completed the Kaplan-Meier survival curves in Figure 2-4, qRT-PCR analysis in Figure 2-6, 2-7, and Supplementary Figure 2-4, and ELISA analysis in Figure 2-6. Jennifer Ruizhe Li completed the body weight, length, and skeletal element measurements in Figure 2-4, Picrosirius red staining in Figure 2-5, and ex vivo tibial culture in Figure 2-6. Lauren Solomon performed the MicroCT analysis in Figure 2-5, and immunohistochemistry in Supplementary Figure 2-4. Some mouse husbandry was completed by Yan Jiang.

In chapter three, birth ratios in Figure 3-1 and Figure 3-2, and qRT-PCR in Figure 3-3 were completed in collaboration with Adrienne Elbert. Chromatin immunoprecipitation in Figure 3-3 was performed by Kristin Kernohan. Xu Wang and Adrienne Elbert participated in some mouse husbandry.

In chapter four, molecular combing and analysis of DNA fibers in Figure 4-3g was conducted in Dr. Grant Brown's laboratory by David Gallo.

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Make my aim true.
Give me goals to seek and the constant determination to achieve them.

\- Artemis

Abbreviations

Abbreviation	Meaning
3C:	Chromosome conformation capture
3D:	Three-dimensional
μg:	Microgram
μL:	Microliter
μm:	Micrometer
μM:	Micromolar
$^{\circ}$C:	Degrees Celsius
AA:	Amino acid
AC3:	Activated (cleaved) caspase-3
ADD:	ATRX-DNMT3-DNMT3L
AIF:	Apoptosis-inducing factor
ALT:	Alternative lengthening of telomeres
ANOVA:	Analysis of variance
AOI:	Area of interest
AP:	Apical progenitor
APB:	ALT-associated PML-NB
APE1:	Apurinic/apyrimidinic endonuclease 1
Aph:	Aphidicolin

APP:	Amyloid precursor protein
ASPM:	Abnormal spindle-like, microcephaly-associated
ATM:	Ataxia telangiectasia mutated
ATMDS:	Alpha-thalassemia myelodysplasia syndrome, somatic
ATP:	Adenosine triphosphate
ATR:	Ataxia telangiectasia mutated- and Rad3-related
ATRX:	Alpha-thalassemia mental retardation, X-linked
ATRXt:	Truncated isoform of alpha-thalassemia mental retardation, X-linked
ATR-X:	Alpha-thalassemia mental retardation, X-linked syndrome
BAX:	BCL2-associated X protein
Bbc3:	BCL2 binding component 3
Bp:	Base pairs
BP:	Basal progenitor
BDNF:	Brain derived neurotrophic factor
BER:	Base excision repair
BG:	Basal ganglia
BLM:	Bloom syndrome helicase
BMD:	Bone mineral density
BRCA:	Breast cancer, early onset

BrdU:	5'-Bromo-2'-deoxyuridine
BSA:	Bovine serum albumin
C terminus:	Carboxy terminus
CaMKII:	Calcium/calmodulin-dependent protein kinase II
CBS:	CTCF binding site
Cdc:	Cell division cycle
CDH1:	Cadherin 1
Cdk:	Cyclin-dependent kinase
Cdk5rap2:	Cyclin dependent kinase 5 regulatory subunit associated protein 2
cDNA:	Complementary DNA
CHD8:	Chromodomain helicase DNA binding protein 8
ChIA-PET:	Chromatin interaction analysis by paired-end sequencing
ChIP:	Chromatin immunoprecipitation
Chk1:	Checkpoint kinase 1
Chk2:	Checkpoint kinase 2
CKII:	Casein kinase 2
cKO:	Conditional knockout
CldU:	5'-Chloro-2'-deoxyuridine

CNS:	Central nervous system
CP:	Cortical plate
CPN:	Callosal projection neuron
CR:	Cajal-Retzius
CSK:	Cytoskeleton buffer
CTCF:	CCCTC-binding factor
CThPN:	Corticothalamic projection neuron
CTIP2:	Chicken ovalbumin upstream promoter transcription factor-interacting protein 2
Ctx:	Cerebral cortex
d:	Dermis
DAPI:	4'-6-diamidino-2-phenylindole
Daxx:	Death associated protein 6
DDR:	DNA damage response
DIV:	Days in vitro
Dlk1:	Delta-like 1 homolog
DM1:	Myotonic dystrophy type I
DMEM:	Dulbecco's Modified Eagle Medium
DNMT:	DNA (cytosine-5)-methyltransferase
DNA:	Deoxyribonucleic acid

DNA-PK$_{cs}$:	DNA-dependent protein kinase catalytic subunit
DNase:	Deoxyribonuclease
dNTP:	Deoxyribonucleotide triphosphate
DSB:	Double stranded break
dsDNA:	Double stranded DNA
DTT:	Dithiothreitol
E:	Embryonic day
ECL:	Enhanced chemiluminescence
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
Ercc:	Excision repair cross-complementation group
ESC:	Embryonic stem cell
EZH2:	Enhancer of zeste homolog 2
FACT:	Facilitates chromatin transcription
FBS:	Fetal bovine serum
FdU:	5'-Fluoro-2'-deoxyuridine
FISH:	Fluorescent in situ hybridization
FOG:	Friend of GATA
Foxg1:	Forkhead box G1
g:	Gram

G4-DNA:	G-quadruplex DNA
GABA:	Gamma-aminobutyric acid
Gapdh:	Glyceraldehyde 3-phosphate dehydrogenase
GFP:	Green fluorescent protein
GH:	Growth hormone
Ghr:	Growth hormone receptor
GluR1:	Glutamate receptor 1
GN:	Granule neuron
GnRH:	Gonadotropin-releasing hormone
Gtl2:	Gene trap locus 2
Gy:	Gray
h:	Hour
H:	Hippocampus
H&E:	Haematoxylin & eosin
H3.3:	Histone 3.3
H3K9me3:	Histone 3 lysine 9 trimethylation
HbH:	Hemoglobin H
HDAC:	Histone deacetylase
Hi-C:	Global 3C interactions
HIRA:	Histone cell cycle regulator

HP1α:	Heterochromatin protein 1 alpha
HR:	Homologous recombination
HU:	Hydroxyurea
ICF:	Immunodeficiency, centromeric heterochromatin instability, facial anomalies
ICR:	Imprinting control region
ID:	Intellectual disability
IDH1:	Isocitrate dehydrogenase 1
IdU:	5'-Iodo-2'-deoxyuridine
IGF-1:	Insulin-like growth factor 1
Igf2:	Insulin-like growth factor 2
Igfals:	Insulin-like growth factor binding protein, acid labile subunit
Igfbp1:	Insulin-like growth factor binding protein 1
IgG:	Immunoglobulin G
INCENP:	Inner centromere protein
INK4/ARF:	Inhibitor of Cdk4/Alternative reading frame
IZ:	Intermediate zone
Kb:	Kilobase
KCl:	Potassium chloride
kDa:	Kilodalton

kV:	Kilovolt
LIG:	Ligase, DNA, ATP-dependent
LTP:	Long-term potentiation
M:	Molar
mA:	Milliampere
Mb:	Megabase pair
MajSat:	Major satellite repeats
MCPH1:	Microcephalin
mm:	Millimeter
mM:	Millimolar
MeCP2:	Methyl CpG binding protein 2
MEM:	Minimum essential media
MEF:	Mouse embryonic fibroblast
$MgCl_2$:	Magnesium chloride
MicroCT:	Micro computed tomography
Min:	Minute
mL:	Milliliter
MMC:	Mitomycin C
MRE11:	Meiotic recombination 11
MRN:	MRE11-RAD50-NBS1 complex

mRNA:	Messenger RNA
MSat	Major satellite
MYC:	v-myc avian myelocytomatosis viral oncogene homolog
N terminus:	Amino terminus
NaCl:	Sodium chloride
NaCitrate:	Sodium citrate
NAD:	Nicotinamide adenine dinucleotide
NBS1:	Nijmegen breakage syndrome 1
ncRNA:	Non-coding RNA
NE:	Neuroepithelial
NER:	Nucleotide excision repair
Nf1:	Neurofibromin 1
NHEJ:	Non-homologous end joining
Nipbl:	Nipped BL
nM:	Nanomolar
NP40:	Nonyl phenoxypolyethoxylethanol
NPC:	Neuroprogenitor cell
Nrp1:	Neuropilin 1
OMIM:	Online Mendelian Inheritance in Man
oRG:	Outer radial glia

P: Post-natal day

PanNET: Pancreatic neuroendocrine tumour

PARP1: Poly(ADP-ribose) polymerase 1

PAX6: Paired box 6

PBS: Phosphate buffered saline

PCH: Pericentromeric heterochromatin

PCNA: Proliferating cell nuclear antigen

PCR: Polymerase chain reaction

PHD: Plant homeodomain

PI: Propidium iodide

PI 3: Phosphoinositide 3-kinase

PK: Proteinase K

PML-NB: Promyelocytic leukaemia nuclear body

PRC2: Polycomb repressive complex 2

Prlr: Prolactin receptor

PUMA: p53 upregulated modulator of apoptosis

qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction

Rad: Radiation mutant

Rb: Retinoblastoma

rDNA: Ribosomal deoxyribonucleic acid

Rhox5: Reproductive homeobox protein 5

RIP: RNA immunoprecipitation

RNA: Ribonucleic acid

RNAi: RNA interference

RNAPII: RNA polymerase II

RNase: Ribonuclease

ROI: Region of interest

ROS: Reactive oxygen species

RPA: Replication protein A

RTT: Rett Syndrome

s: Seconds

SA1/2: Stromal antigen 1/2

SATB2: Special AT-rich sequence-binding protein 2

SCA7: Spinocerebellar ataxia type 7

SCPN: Subcerebral projection neuron

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: Standard error of the mean

sf: Subcutaneous fat

SINE: Short interspersed element

SIRT1:	Sirtuin 1
Slc5a5:	Solute carrier family 5, member 5
SMC:	Structural maintenance of chromosomes
SNF2:	Sucrose non-fermenting 2
SNP:	Single nucleotide polymorphism
SOX2:	Sex determining region Y-box 2
SP:	Subplate
SPN:	Subplate neuron
SSB:	Single stranded break
SSC:	Saline-sodium citrate
SUMO:	Small ubiquitin-like modifier
SVZ:	Subventricular zone
SWI/SNF:	Switch/sucrose non-fermenting
T4:	Thyroxine
TAD:	Topologically associating domain
TBR1:	T-box, brain, 1
TBR2:	T-box, brain, 2
TBS:	Tris buffered saline
TBS-T:	Tris buffered saline-Tween 20
Tg:	Thyroglobulin

Tel-FISH:	Telomeric fluorescence in situ hybridization
TERRA:	Telomeric repeat-containing RNA
TERT:	Telomerase reverse transcriptase
Thrsp:	Thyroid hormone responsive
TIF:	Telomere dysfunction-induced foci
TMS:	Telomestatin
TOP1:	Topoisomerase 1
Tpo:	Thyroid peroxidase
TRF:	Telomeric repeat binding factor
TRH:	Thyroid releasing hormone
Tris:	Tris(hydroxymethyl)aminomethane
Tris-HCl:	Tris(hydroxymethyl)aminomethane-hydrogen chloride
tRNA:	Transfer ribonucleic acid
Trp53:	Tumour suppressor protein 53
TSA:	Trichostatin A
TSH:	Thyroid stimulating hormone
Tshr:	Thyroid stimulating hormone receptor
TUNEL:	Terminal deoxynucleotidyl nick end labeling
TX-100:	Triton X-100
U:	Units

UTR:	Untranslated region
UV:	Ultraviolet
V:	Volt
VNTR:	Variable nucleotide tandem repeat
VZ:	Ventricular zone
WNT:	Wingless-int
Wsb1:	WD repeat and SOCS box-containing protein 1
XLF:	XRCC4-like factor
XNP:	X-linked nuclear protein
XRCC:	X-ray repair complementing defective repair in Chinese hamster cells
YY1:	Ying Yang 1
ZF:	Zinc finger
ZNF143:	Zinc finger protein 143

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Chapter 1

1   General Introduction

Our knowledge of the genome has expanded considerably in the past decades, with the decreasing costs of high-throughput sequencing technologies and the completion of the human genome project. We now know that DNA sequence does not simply dictate function, but rather that protein factors and chemical modifications "above" the genome can dramatically alter the organization of DNA within the nucleus and constitute a major mechanism underlying transcriptional regulation, DNA replication, and DNA repair dynamics.

Sequencing of the human genome has also greatly expanded our understanding of human disease etiology, and new technologies continue to drive progress in the field. Recent advances in exome sequencing, i.e. sequencing of codon sequences that constitute 1% of the genome, has demonstrated that neuropsychiatric diseases, such as those on the autism spectrum, are primarily caused by mutations in synaptic proteins and epigenetic factors (De Rubeis et al., 2014; Pinto et al., 2014). This highlights the importance of chromatin regulation in brain development and function. Moreover, microcephaly and intellectual disability (ID) are caused by disruptions in the DNA damage response and cell cycle regulatory pathways, which coalesce at the level of chromatin regulation. However, the dynamic interplay between DNA damage signaling and chromatin structure, and how they are integrated into cell cycle regulation is not well understood, especially in the context of brain development.

The work herein describes a role for two important regulators of chromatin structure, ATRX and CTCF, in genomic stability to promote neuroprogenitor cell survival and correct brain development, and explores the functional means by which these essential proteins exert their actions.

1.1 ATRX is essential for brain development and the suppression of tumourigenesis

1.1.1 The ATRX Gene and Protein

The alpha thalassemia mental retardation, X-linked (ATRX) protein was first cloned over twenty years ago and described as a putative helicase protein due to sequence homology with the Rad54 helicase (Stayton et al., 1994). *ATRX* is comprised of 36 exons and spans approximately 300 kilobases (kb) of DNA sequence located on the long arm of the human X chromosome (Xq21.1) (Picketts et al., 1996; Villard et al., 1997). The gene is conserved between mouse and human (85% homologous), and homologs of the protein have been identified in *Drosophila melanogaster* (dATRX; 66% homologous), and *Caenorhabditis elegans* (xnp-1; 52% homologous) (Lee et al., 2007; Picketts et al., 1996; Villard et al., 1999). *In vivo* expression patterns demonstrated that ATRX is a ubiquitous nuclear protein that may play an important role in neuronal differentiation since protein levels were highest in fetal brain (Gecz et al., 1994). ATRX is a member of the SNF2 subgroup of the SWI/SNF protein superfamily that can incorporate themselves into multi-subunit complexes and utilize the energy of ATP to remodel chromatin (Picketts et al., 1996). SWI/SNF members are involved in transcriptional regulation, DNA repair, and mitotic recombination (Carlson and Laurent, 1994; Eisen et al., 1995; Picketts et al., 1996).

ATRX contains two highly conserved domains: a globular ATRX-DNMT3-DNMT3L (ADD) domain in the amino (N)-terminus, and a carboxy (C)-terminal helicase domain (Aapola et al., 2000; Argentaro et al., 2007; Picketts et al., 1998; Xie et al., 1999) (Figure 1-1a). The ADD domain is cysteine-rich and contains two types of zinc finger motifs: a plant homeodomain (PHD) finger flanked by a GATA-1-like C_2C_2 motif (Gibbons et al., 1997). The *de novo* DNA methyltransferases (DNMT) DNMT3A, DNMT3B, and DNMT3L are the only other proteins that contain this type of domain (Xie et al., 1999). ADD can bind both DNA and RNA, and has an affinity for H3K9me3-containing nucleosomes in the absence of H3K4 methylation (Argentaro et al., 2007; Dhayalan et al., 2011; Eustermann et al., 2011; Iwase et al., 2011; Mitson et al., 2011; Sarma et al., 2014) (Figure 1-1b). Furthermore, the ADD domain is one of the only known readers of

the H3K9me3-S10ph combinatorial modification (Eustermann et al., 2011; Kunowska et al., 2015; Noh et al., 2014). H3K9me3-S10ph has been shown to modulate binding of polycomb proteins during differentiation (Sabbattini et al., 2014), and H3S10ph is induced under periods of high neuronal activity at the promoter of immediate early genes (Crosio et al., 2003). The ATRX helicase domain exhibits ATPase chromatin remodeling activity that can be stimulated by nucleosomes or DNA (Tang et al., 2004; Xue et al., 2003).

Characterization of the full-length *ATRX* gene revealed that it gives rise to at least three alternative transcripts (Berube et al., 2000; Garrick et al., 2004; Picketts et al., 1996). Two of the transcripts depend on alternative splicing of exon 6, which lies upstream of the ADD domain, and generate transcripts of approximately 10.5 kb that give rise to proteins of 265 and 280 kDa, respectively (Picketts et al., 1996; Villard et al., 1997). The third isoform of ATRX results from a failure to remove intron 11, generating a premature stop codon and C-terminal truncated protein (ATRXt) of approximately 180 kDa (Berube et al., 2000; Garrick et al., 2004). The functional significance of different ATRX isoforms is not understood, however ATRXt likely does not perform identical activities as full-length ATRX since it lacks the ATPase domain.
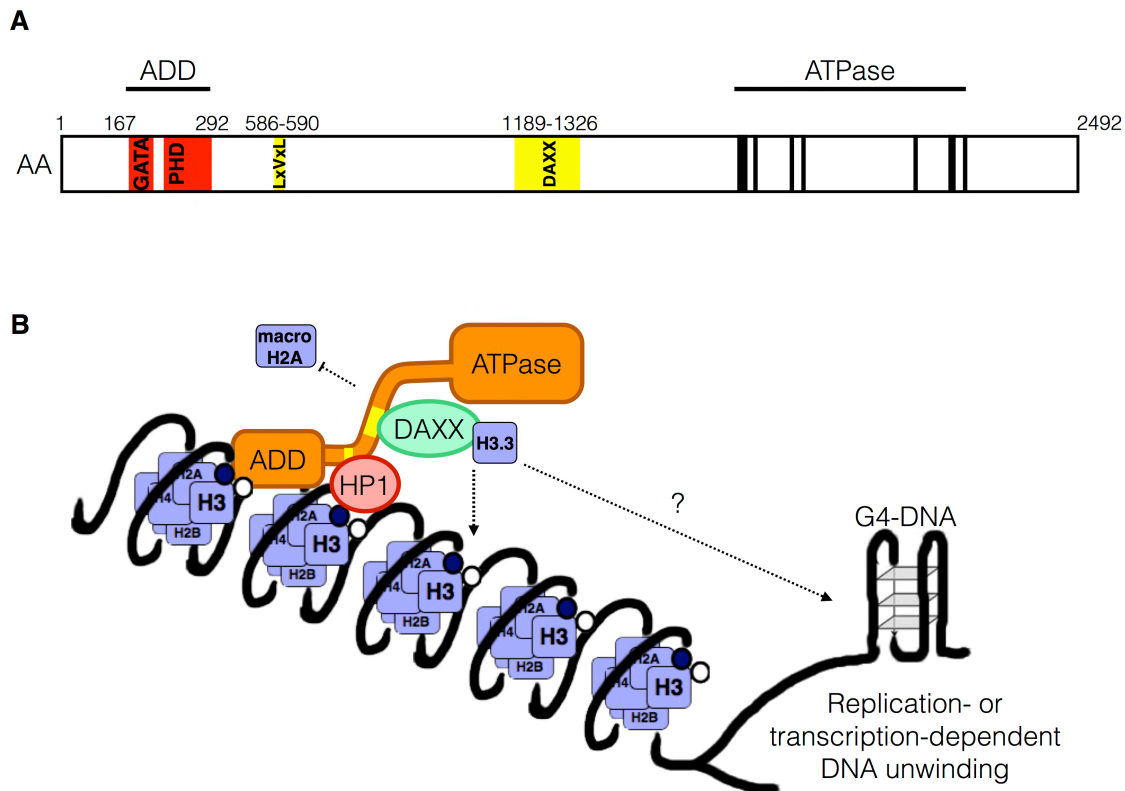
Figure 1-1 The ATRX protein and function

(A) Structure of the ATRX protein. Conserved domains and relevant protein interaction sites are indicated. (B) Model of ATRX recruitment to heterochromatin. The ADD domain of ATRX recognizes the combinatorial signature of H3K4me0 (white circle)-H3K9me3 (navy circle), the LxVxL motif within the linker domain interacts with HP1 (Lechner et al., 2005), and DAXX interacts with the ATPase domain of ATRX. ATRX-DAXX deposits H3.3 in heterochromatin (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010; Pathak et al., 2014), while restricting incorporation of macro H2A (Ratnakumar et al., 2012).

1.1.2 ATRX is a heterochromatin-associated protein

ATRX is an exclusively nuclear protein that associates with the nuclear matrix during interphase (Berube et al., 2000). At the onset of mitosis ATRX is phosphorylated and released from the nuclear matrix (Berube et al., 2000). Throughout the cell cycle ATRX localizes to pericentromeric heterochromatin (PCH) and interacts directly with heterochromatin protein 1 α (HP1α) (Berube et al., 2000; Lechner et al., 2005; McDowell et al., 1999). Depletion of ATRX has minimal effect on HP1 localization (Huh et al., 2012; Ritchie et al., 2008; Wong et al., 2010), while loss of HP1 results in abnormal targeting of ATRX to heterochromatin (Kourmouli et al., 2005), suggesting that HP1 is involved in recruitment or stabilization of ATRX. Mutation to the LxVxL HP1 interaction motif (Figure 1-1a) reduced ATRX localization at heterochromatin to a lesser extent than mutations in the ADD domain (Eustermann et al., 2011), pointing to a stabilization role for HP1 in ATRX localization rather than recruitment *per se*.

ATRX can also interact with the methyl CpG binding protein 2 (MeCP2) through its C-terminal helicase domain, and together the proteins co-localize at DAPI-bright heterochromatin bundles in the nucleus (Kernohan et al., 2010; Nan et al., 2007). MeCP2-deficient neurons display abnormal targeting of ATRX to heterochromatin (Baker et al., 2013; Nan et al., 2007). C-terminal fragments of ATRX require MeCP2 for heterochromatic localization while N-terminal fragments of the protein localize to DAPI-bright heterochromatin in a MeCP2-independent manner (Nan et al., 2007), indicating that the N-terminus contains elements required for ATRX targeting. Further support for this stems from the fact that the short isoform of ATRX (ATRXt) is capable of localizing to PCH despite lacking the C-terminal MeCP2-interaction domain (Garrick et al., 2004). Indeed, the N-terminal ADD domain of ATRX can recognize the H3K4me0-H3K9me3 repressive histone signature that is often enriched at heterochromatin (Dhayalan et al., 2011; Eustermann et al., 2011; Iwase et al., 2011; Mitson et al., 2011) (Figure 1-1b). ATRX depletion does not alter cellular H3K9me3 distribution (Ritchie et al., 2008), however more precise techniques like chromatin immunoprecipitation followed by high throughput sequencing (ChIP-sequencing) will be required to determine if ATRX is required for establishment or maintenance of this type of chromatin modification. ATRX

also localizes to the heterochromatic inactive X chromosome and can interact with the repressive histone variant macroH2A following the onset of X chromosome inactivation (Baumann and De La Fuente, 2009; Ratnakumar et al., 2012; Sarma et al., 2014).

ChIP-sequencing has demonstrated that ATRX binds intergenic regions and within gene bodies, in addition to highly repetitive DNA sequences such as telomeres, rDNA, and PCH (Law et al., 2010). Repetitive elements are susceptible to the formation of complex secondary structures such as hairpins/cruciforms, Z-DNA, triplexes, and tetraplexes, especially when the DNA is unwound during transcription or replication (Zhao et al., 2010). Telomeric DNA is particularly prone to the formation of G-quadruplex (G4) DNA structures, since the sequences of telomeres are GC-rich (Biffi et al., 2013; Lipps and Rhodes, 2009). In fact, ATRX is enriched at predicted G4-DNA-forming sequences and recombinant ATRX can interact with G4-DNA oligonucleotides *in vitro* (Law et al., 2010).

Taken together, these data indicate that ATRX targeting to heterochromatin is likely mediated through direct interaction and recognition of modifications on the histone H3 tail by the ADD domain, and that stabilization of ATRX at heterochromatin requires protein-protein interaction with MeCP2 and HP1. ATRX may also be capable of recognizing complex secondary G4-DNA structures *in vivo*, resulting in ATRX recruitment to specific sites enriched in G4-DNA, such as telomeres. However, the precise biochemical interaction between G4-DNA structures and ATRX is not well understood and deems clarification. Furthermore, the relationship between ATRX and macroH2A in the context of heterochromatin requires further characterization, as it may also play an important role in ATRX targeting.

1.1.3 ATRX forms a chromatin-remodeling complex with DAXX

An unbiased proteomic analysis to isolate ATRX interacting partners identified the death-associated protein 6 (DAXX) (Xue et al., 2003). The majority of ATRX molecules are in complex with DAXX (Xue et al., 2003), indicating that DAXX likely participates in

ATRX cellular functions. DAXX interacts with the linker region of ATRX located between the ADD and ATPase domains (Figure 1-1a,b) (Tang et al., 2004). Together, ATRX/DAXX exhibits ATP-dependent chromatin remodeling activities such as disruption of DNA-histone interactions and translocation of histones on DNA, but does not display helicase activity (Tang et al., 2004; Xue et al., 2003). However, DAXX is not required for the chromatin remodeling ability of the complex (Tang et al., 2004), which is consistent with the fact that ATRX, and not DAXX, contains an ATPase domain (Figure 1-1a). At imprinting control regions (ICRs), ATRX mediates nucleosome remodeling to facilitate recruitment of the chromatin architecture protein CCCTC-binding factor (CTCF), likely through its ATPase domain (Kernohan et al., 2014). The relationship between ATRX and CTCF will be touched upon in section 1.3.3 and 5.9.

All activities that involve the DNA template, such as transcription, replication, and repair, must be accomplished in the context of chromatin and require extensive remodeling of the nucleosome. Together, ATRX and DAXX are responsible for replication-independent deposition of the histone H3 variant H3.3 at telomeres, PCH, and rDNA (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010). DAXX acts as a chaperone through direct interaction with H3.3, and deposition requires the chromatin remodeling activities of ATRX (Drane et al., 2010; Lewis et al., 2010). The significance of ATRX/DAXX-dependent H3.3 deposition at repetitive elements is not well understood. H3.3 is typically enriched at transcriptionally active sites, which is believed to be a consequence of the inherent instability of nucleosomes containing H4-H3.3 dimers (Jin and Felsenfeld, 2007). ATRX loss results in a failure to incorporate H3.3 and induces a DNA damage response at telomeres and PCH, suggesting that H3.3 incorporation is required to maintain stability of these elements (Goldberg et al., 2010; Huh et al., 2012; Watson et al., 2013; Wong et al., 2010). However, this idea is difficult to reconcile with the inherent instability of H3.3-containing nucleosome, which is consistent with the finding that ATRX facilitates RNA polymerase elongation through intragenic deposition of H3.3 in a small subset of genes that contain predicted G4-DNA structures (Levy et al., 2014). Transcription of repetitive elements is important for maintenance of heterochromatin (Buhler et al., 2007; Schoeftner and Blasco, 2009). Despite a failure to incorporate H3.3 at telomeres and PCH in ATRX- or DAXX-depleted cells, there have been conflicting

reports of transcriptional dysregulation of pericentromeric and telomeric (TERRA) transcripts (Drane et al., 2010; Goldberg et al., 2010; Levy et al., 2014). A recent study demonstrated G_2/M-specific upregulation of TERRA in HeLa cells depleted for ATRX (Flynn et al., 2015), providing an explanation for the discrepancies in earlier findings of TERRA regulation. Together these data suggest a potential role for ATRX in resolution of G4-DNA to facilitate transcriptional elongation and replication of heterochromatin (Figure 1-1b), however future efforts will be necessary to dissect the functional significance of ATRX/DAXX-dependent H3.3 incorporation at repetitive elements and other locations.

ATRX and DAXX localize to promyelocytic leukaemia nuclear bodies (PML-NBs), which are subnuclear structures implicated in transcriptional activation, DNA replication, apoptosis, and viral infection (Bernardi and Pandolfi, 2007; Ishov et al., 1999; Ishov et al., 2004). PML-NBs can be found in most cell lines and tissue types; in a normal mammalian cell there are approximately 5-30 PML-NBs (Melnick and Licht, 1999). Over 100 proteins have been associated with PML-NBs, many of which show dynamic localization (Weidtkamp-Peters et al., 2008). In contrast to heterochromatic localization, ATRX localization at PML-NBs is DAXX-dependent (Ishov et al., 2004). It is hypothesized that PML-NBs serve as platforms for heterochromatin remodeling post replication in G_2 phase of the cell cycle, which is supported by the dynamic colocalization of ATRX/DAXX/H3.3, heterochromatin, and PML-NBs during this time (Chang et al., 2013; Ishov et al., 2004). Furthermore, PML-NBs have been suggested to be sites of H3.3 storage within the nucleus and function as a gathering point for proteins involved in replication-independent H3.3 assembly (Corpet et al., 2014). In fact, PML depletion results in dissociation of PML-NBs and decreased ATRX/H3.3 at telomeres, suggesting that the subnuclear structures are important for maintaining H3.3 at telomeres (Chang et al., 2013).

1.1.4 ATRX regulates DNA replication and cell division

Subnuclear distribution of ATRX is cell cycle-dependent. As mentioned previously, ATRX is phosphorylated at the onset of mitosis resulting in its dissociation from the nuclear matrix (Berube et al., 2000). ATRX is enriched at DAPI-bright heterochromatin throughout all stages of the cell cycle (McDowell et al., 1999; Nan et al., 2007). ATRX localization at telomeres of embryonic stem (ES) cells occurs primarily during mid-to-late S phase, which may indicate a specific requirement for ATRX in facilitating replication or remodeling of telomeric heterochromatin (Wong et al., 2010). This is consistent with its localization pattern in replicating myoblasts: pulse-labeling cells with the thymidine analog bromodeoxyuridine (BrdU) demonstrated colocalization of ATRX with large BrdU foci characteristic of late-replicating heterochromatin (Huh et al., 2012). Replication of heterochromatin and S phase progression are tightly interrelated (Quivy et al., 2008), which may explain the increased DNA damage response observed in late-replicating chromatin and S phase lengthening of *Atrx*-null cells (Clynes et al., 2014; Huh et al., 2012; Watson et al., 2013). The mechanism of ATRX function in replication is not well characterized. A prevalent hypothesis is that ATRX resolves G4-DNA to allow progression of the replication fork through regions prone to forming the complex secondary structures, such as telomeres (Clynes et al., 2013; Gibbons and Higgs, 2010), which is supported by the finding that *Atrx*-null cells are sensitive to chemically induced G4 stabilization (Watson et al., 2013). However, there is also evidence to suggest a more direct function for ATRX in replication, as *Atrx*-null cells show reduced origin firing and ATRX interacts with components of the MRE11-RAD50-NBS1 (MRN) complex that facilitates replication fork restart (Clynes et al., 2014; Leung et al., 2013). The MRN-ATRX interaction may occur in the context of telomeric chromatin, as the ataxia telangiectasia-mutated (ATM) kinase, MRN, and homologous recombination (HR) machinery are all recruited to newly replicated telomeres for reformation of the protective t-loop structure (Verdun et al., 2005; Verdun and Karlseder, 2006).

Enrichment of ATRX at pericentromeric heterochromatin (PCH) and highly condensed mitotic chromosomes suggests a function for the protein during cell division (Berube et al., 2000; McDowell et al., 1999). Indeed, studies have demonstrated a requirement for

ATRX in both meiosis and mitosis (Baumann et al., 2010; De La Fuente et al., 2004; Ritchie et al., 2008; Ritchie et al., 2014). In human HeLa cells and mouse neuroprogenitors, ATRX loss causes chromosome congression defects and a prolonged prometaphase-to-metaphase transition that ultimately results in increased mitotic defects such as anaphase bridging and micronuclei formation (Ritchie et al., 2008; Ritchie et al., 2014). In mouse oocytes, ATRX depletion disrupts alignment of chromosomes at the metaphase II spindle and causes defective chromosome cohesion, similarly resulting in micronuclei formation and centromere instability (Baumann et al., 2010; De La Fuente et al., 2004).

Replicative stress, or DNA damage that occurs during replication, can be carried over into mitosis if the damage is not severe enough to induce checkpoint activation and cell cycle arrest (Wilhelm et al., 2014). Ultimately, this can result in abnormal cell division, as under-replicated regions of chromosomes remain physically joined and eventually break or shatter to cause micronuclei or chromothripsis, respectively (Crasta et al., 2012). Therefore, it is possible that the mitotic/meiotic defects associated with ATRX loss could be a consequence of increased stress during replication. However, replicative stress and increased DNA damage can also result from abnormal cell division (Ganem and Pellman, 2012). It remains unclear whether ATRX deficiency causes replicative stress that precedes mitotic/meiotic dysfunction or vice versa. Nevertheless, there is ample evidence to conclude that ATRX is required throughout the cell cycle for both replication and cell division.

1.1.5 Inherited hypomorphic *ATRX* mutations cause syndromic and non-syndromic intellectual disability

The *ATRX* gene was originally identified through genetic linkage studies indicating that numerous mutations within the coding region of *ATRX* cause a rare mental retardation syndrome (ATR-X; OMIM#301040) characterized by a wide array of developmental abnormalities, alpha-thalassemia, and severe cognitive deficits (Gibbons, 1995). *ATRX* mutations have also been identified in patients with Chudley-Lowry (OMIM#303600),

Juberg-Marsidi, Carpenter-Waziri, and other less well-characterized X-linked intellectual disability (ID; OMIM#309580) (Abidi et al., 1999; Lossi et al., 1999; Villard et al., 1999; Villard et al., 1996). Interestingly, ATR-X syndrome is the first identified human disease caused by mutations in a chromatin remodeling factor, establishing the functional significance of epigenetic regulation in human disease pathogenesis (Gibbons et al., 1997).

Patients with *ATRX* mutations show profound intellectual disability (95% of patients), microcephaly (76%), seizures (35%), characteristic facial features (94%), skeletal abnormalities (90%), HbH inclusions (87%), and genital abnormalities (80%) (Gibbons and Higgs, 2000). Magnetic resonance imaging of ATR-X patients identified abnormalities in grey and white matter of the brain, implicating ATRX in the production of neuronal/glial cells and myelination, respectively (Wada et al., 2013). Modeling of patient mutations demonstrated that missense mutations often had little effect on protein levels, however point mutations affecting the zinc-coordinating cysteines, surface residues, and the structural core of the ADD domain resulted in a 50-90% reduction in ATRX levels, likely due to protein destabilization (Argentaro et al., 2007; McDowell et al., 1999).

Our understanding of ATR-X pathogenesis has been aided through genotype-phenotype correlations. Over 95% of identified *ATRX* mutations lie within the conserved ADD and ATPase domains of ATRX, indicating that these domains are essential for correct function (Gibbons, 2006). The lack of identified mutations outside of these domains suggests that mutations elsewhere are non-viable or that regions outside of the ADD and ATPase domains are dispensable for ATRX function. Patient mutations in the ATPase domain of ATRX result in abnormal targeting to PML-NBs, implicating the subnuclear structures in disease pathogenesis (Berube et al., 2008). Additionally, patient mutations within the ADD domain result in abnormal targeting to heterochromatin (Eustermann et al., 2011; Iwase et al., 2011; Mitson et al., 2011). Together these data emphasize the importance of ATRX targeting to PML-NBs and heterochromatin, and indicate that defects in these pathways can contribute to ATR-X pathogenesis. Truncating mutations in the C-terminus consistently result in patients with severe urogenital abnormalities,

suggesting that this region may play a key role in urogenital development (Gibbons and Higgs, 2000). While genotype-phenotype correlations have been informative, there exists phenotypic variability even in patients with the same mutation: 15 unrelated individuals with the same mutations showed variable levels of HbH inclusions (Gibbons et al., 1997). Variability in the degree α-thalassemia presentation in ATR-X patients has been correlated with the length of the $\psi\zeta$ variable nucleotide tandem repeat (VNTR) adjacent to the α-globin gene cluster that is predicted to form G4-DNA structures and is enriched for ATRX binding in normal individuals (Law et al., 2010). This finding implies that ATRX is likely targeted to this VNTR sequence due to its propensity to form non-β G4-DNA structures, and that it is required to prevent repression of nearby pathologically relevant genes. This function of ATRX may also have consequences for DNA replication and repair, as a failure to resolve G4-DNA could cause replication fork stalling and collapse into DNA double-strand breaks (DSBs) (Lipps and Rhodes, 2009). Indeed, ATR-X patient lymphocytes exhibited phosphorylation of the histone H2A variant H2AX (γH2AXS139) and of p53 (pp53S15), suggestive of an increased DNA damage response (Huh et al., 2012).

The study of X-linked intellectual disability syndromes resulting from *ATRX* mutations has been pivotal in furthering our knowledge of ATRX function. These human disorders clearly demonstrate a requirement for ATRX in development of the brain, urogenital and skeletal systems, and provide evidence that ATRX is necessary for transcriptional regulation of the α-globin gene cluster.

1.1.6 ATRX is required for mouse brain development

The overt neurological dysfunction associated with human *ATRX* mutations implies a requirement for ATRX in brain development, maturation, and/or function. Mouse models have been useful in studying the effects of genetically altering ATRX levels globally and specifically in the brain.

Overexpression of ATRX causes neurodevelopmental and craniofacial defects, suggesting that ATRX is required in correct stoichiometric amounts for normal

development (Berube et al., 2002). Forebrain-specific deletion of the full-length *Atrx* isoform causes increased p53-dependent apoptosis of neuroprogenitor cells (NPCs), resulting in decreased neonatal cortical size and hippocampal dysgenesis (Berube et al., 2005; Seah et al., 2008). ATRX is required to specify the correct number of GABAergic interneurons and hippocampal dentate granule precursors, and this function contributes to the abnormal cortical and hippocampal development of forebrain-specific mutants (Seah et al., 2008). Generation of p53/ATRX double mutant mice effectively rescued apoptosis, however failed to completely restore neonatal forebrain size, indicating that ATRX functions outside of promoting cellular survival to regulate brain size (Seah et al., 2008). Indeed, *Atrx*-null NPCs exhibit premature differentiation into neurons, resulting in early depletion of the progenitor pool and a failure to sustain birth of late-born neurons (Ritchie et al., 2014).

Atrx$^{\Delta E2}$ mice were generated to mimic an ATR-X patient mutation and study the consequence of *Atrx* exon 2 deletion in brain development and function (Nogami et al., 2011). *Atrx*$^{\Delta E2}$ mice were smaller than controls, displayed normal spatial learning and memory by the Morris water maze test, and did not display any overt differences in brain morphology (Nogami et al., 2011). However, the *Atrx*$^{\Delta E2}$ mice exhibited impaired contextual fear memory and long-term potentiation (LTP), which was attributed to aberrant activation of the LTP effectors calcium/calmodulin-dependent protein kinase II (CaMKII) and the glutamate receptor 1 (GluR1) in the hippocampus and medial prefrontal cortex, and associated with abnormal dendritic spines (Nogami et al., 2011; Shioda et al., 2011).

Collectively, animal models of ATRX dysfunction have illustrated a requirement for the protein in both brain development and maturation and provide some insight into the mechanisms underlying developmental abnormalities, such as intellectual disability and microcephaly, associated with *ATRX* mutations.

1.1.7 Somatic *ATRX* loss-of-function mutations drive cancer progression

The first clue that ATRX may be required to prevent malignancy came from the identification that alpha-thalassemia myelodysplasia syndrome, somatic (ATMDS; OMIM#300448), a rare pre-leukemic condition, is caused by acquired somatic *ATRX* mutations (Gibbons et al., 2003).

Somatic *ATRX* loss-of-function mutations have been identified in numerous tumour types by exome sequencing, such as neuroblastoma, glioma, pancreatic neuroendocrine tumours (PanNETs), and paediatric osteosarcoma (Chen et al., 2014; Jiao et al., 2012; Jiao et al., 2011; Kannan et al., 2012; Liu et al., 2012; Molenaar et al., 2012; Schwartzentruber et al., 2012). The *ATRX* mutational landscape includes point mutations scattered throughout the coding sequence, as well as large deletions of the N terminus. Mutations in *DAXX*, a protein partner of ATRX, were also reported in the tumour subtypes but never overlapped *ATRX* mutations, implying that they function in the same pathway. *ATRX/DAXX* mutations commonly occurred in conjunction with mutations in the tumour suppressors *p53* or *IDH1* (Liu et al., 2012; Schwartzentruber et al., 2012). These findings invoke a model whereby epigenetic factors like ATRX may act as "backseat drivers" in a tissue-specific manner to suppress oncogenic pathways upstream of master regulators common to a broad range to tumours, like p53 (Elsasser et al., 2011). An inverse relationship between ATRX and macroH2A levels is associated with melanoma progression, and ATRX can interact with macroH2A to negatively regulate its deposition (Kapoor et al., 2010; Qadeer et al., 2014; Ratnakumar et al., 2012). Further analysis of the full spectrum of *ATRX* mutations in cancer and the different tumour subtypes that are defined based on *ATRX/DAXX* perturbations will help to uncover the mechanism underlying tumourigenesis stemming from mutations in these epigenetic factors.

In addition to mutations in *ATRX/DAXX,* sequencing of paediatric glioma tumour samples identified K27M missense mutations in the gene encoding histone H3.3 (*H3F3A)* (Khuong-Quang et al., 2012; Schwartzentruber et al., 2012; Wu et al., 2012). This provides direct evidence that in addition to the proteins involved in the histone deposition

pathway, alterations in the histone proteins themselves can promote cancer. Analysis of the H3.3K27M mutant demonstrated alterations in genome-wide H3K27me3 levels through inhibition of the Polycomb repressive complex 2 (PRC2) enzymatic component EZH2 (Bender et al., 2013; Chan et al., 2013; Lewis et al., 2013). These findings are particularly intriguing given that ATRX is responsible for H3.3 deposition at specific genomic positions, and that ATRX interacts with EZH2 (Cardoso et al., 1998; Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010; Sarma et al., 2014). Furthermore, the K27M mutation synergizes with p53 loss in neural progenitor cells resulting in neoplastic transformation (Funato et al., 2014). Together, the data suggests that the ATRX-DAXX-H3.3 pathway may genetically interact with the PRC2-H3K27me3 pathway in cancer progression.

A common theme in tumour samples with mutations in *ATRX/DAXX* is the appearance of the alternative lengthening of telomeres (ALT) phenotype (Bower et al., 2012; Heaphy et al., 2011a; Lovejoy et al., 2012; Schwartzentruber et al., 2012). Tumourigenesis, or uncontrolled cell growth, is limited by replicative senescence that occurs as a consequence of telomere shortening every S phase (Hastie et al., 1990). Most tumour cells overcome this hurdle by hijacking telomerase to maintain telomere length and suppress senescence (Counter et al., 1994). Interestingly, telomerase reverse transcriptase (*TERT*) promoter mutations that result in increased telomerase expression are mutually exclusive with *ATRX* mutations in glioma, suggesting that the growth advantage afforded by *TERT* mutation would be equivalent to that of *ATRX* mutation (Killela et al., 2013). Approximately 5% of human cancers will instead utilize the ALT pathway to lengthen their telomeres via homologous recombination (Cesare and Reddel, 2010). ALT is prevalent in specific cancer types that can arise from mutations in *ATRX*, such as osteosarcoma and glioma (Heaphy et al., 2011b), however the mechanism by which *ATRX* mutations result in the ALT phenotype and drive cancer progression is largely unknown. Although ATRX loss has been correlated with telomere dysfunction (Flynn et al., 2015; Goldberg et al., 2010; Huh et al., 2012; Watson et al., 2013; Wong et al., 2010), its loss is not sufficient to drive ALT (Clynes et al., 2014; Flynn et al., 2015; Lovejoy et al., 2012), suggesting that additional genetic or epigenetic changes are necessary to establish the phenotype. ATRX may function rather to poise telomeres for ALT, as its

loss results in persistent association of replication protein A (RPA) with telomeric ssDNA, a key intermediate of homologous recombination (HR), as well as increased TERRA levels at telomeres (Flynn et al., 2015).

Interestingly, ALT is associated with the appearance of large PML-NBs (referred to as ALT-associated PML-NBs, or APBs) that contain telomeric DNA, the DNA repair MRN complex, replication factor A (RPA), and telomeric-repeat binding proteins TRF1 and TRF2 (Luciani et al., 2006; Wu et al., 2003; Wu et al., 2000; Yeager et al., 1999). The appearance of APBs is a robust marker for tumours that utilize ALT, and APBs rapidly assemble upon ALT induction (Costa et al., 2006; Henson et al., 2005; Perrem et al., 2001), suggesting that APBs may be involved in the mechanism underlying ALT. This is intriguing since ATRX/DAXX/H3.3 are PML-associated factors and mutations in this pathway drive cancers with a high frequency of ALT. It is possible that defects in the ATRX/DAXX pathway results in a loss of heterochromatic features at telomeres, such as H3.3 enrichment, leading to increased homologous recombination rates that are associated with ALT activity, but it remains unclear as to how PML-NBs fit into this picture exactly.

1.2 CTCF is a multifunctional and essential protein

1.2.1 The CTCF gene and protein

CCCTC-binding factor (CTCF) is a conserved zinc finger (ZF) DNA binding protein that can interact with highly divergent genomic targets through the combinatorial use of its eleven ZFs (Burcin et al., 1997; Filippova et al., 1996) (Figure 1-1a). The ZF domain of CTCF is 100% identical between human, mouse, and chicken, and the full-length protein shows 93% conservation across species (Filippova et al., 1996). CTCF is conserved in most bilaterial phyla but is absent in yeast, *Caenorhabditis elegans* and plants (Heger et al., 2012). The promoter of *CTCF* contains a binding site for the transcription factor ying-yang 1 (YY1), and elements that are conserved in cell cycle-regulated genes (Klenova et al., 1998). In fact, *CTCF* mRNA levels oscillate during the cell cycle, peaking in S/G_2 phase, and decreasing in terminally differentiated cells (Delgado et al., 1999; Klenova et

al., 1998). Exons 2 to 8 contain the ZF domains, with several individual fingers being split between intron-exon boundaries (Ohlsson et al., 2001). The stop codon resides in exon 10, followed by a long 3'UTR that likely regulates CTCF translation (Klenova et al., 1997). The first 10 ZFs are typical units of 30 residues containing a pair of cysteines invariantly separated by 12 amino acids from the pair of histidines that inserts into the major groove of DNA (el-Baradi and Pieler, 1991; Klug and Schwabe, 1995). The eleventh C-terminal ZF is structurally similar to the friend of GATA (FOG) proteins that can interact with GATA-containing proteins (Fox et al., 1999).

CTCF is a nuclear protein that is generally restricted from heterochromatin, and can interact with the nuclear matrix (Dunn et al., 2003). Unlike many other DNA-binding proteins, CTCF remains associated with chromatin during mitosis through its C-terminal domain (Burke et al., 2005), yet this appears to be locus-specific since CTCF binding is no longer detected at the *MYC* insulator element during this time (Komura et al., 2007). The molecular mass of CTCF is 82 kDa, however due to amino acid sequences in the N- and C-terminus, CTCF migrates aberrantly in SDS-PAGE gels at around 130 kDa, and truncation of the C-terminus results in a 70 kDa isoform (Klenova et al., 1997; Lobanenkov et al., 1990).

CTCF was initially characterized as a transcription factor capable of activating or repressing gene expression in heterologous reporter assays (Baniahmad et al., 1990; Lobanenkov et al., 1990), and was later found to exhibit insulator activity (Bell et al., 1999). Insulator proteins are capable of buffering position effect variegation caused by heterochromatin spreading (barrier activity) and/or the communication between *cis* regulatory elements, such as enhancers and promoters (enhancer blocker function) (Bell et al., 2001). Recently, however, the broad properties of CTCF and its ability to mediate long-range chromatin interactions have led to its description as an architectural rather than insulator protein *per se* (Ong and Corces, 2014).
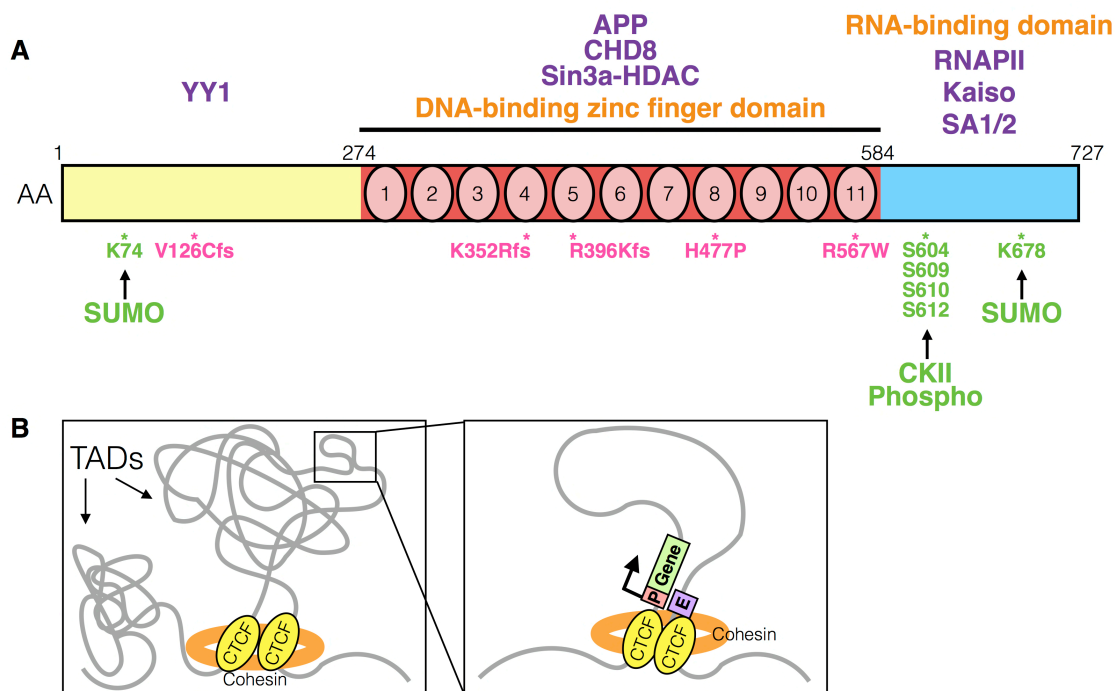
Figure 1-2 The CTCF protein, binding partners, and model of function in genome organization

(A) Structure of the CTCF protein. Nucleic acid-binding domains highlighted in orange and protein partners highlighted in purple text. Known sites of post-translational modification (SUMOylation and CKII-dependent phosphorylation) are indicated in green. Intellectual disability (ID)/autism patient mutations are indicated in pink (Gregor et al., 2013; Iossifov et al., 2014). (B) CTCF binding sites at TAD borders are potentially involved in organizing the domains (left), while binding sites within TAD interiors often colocalize with cohesin (orange ring) and facilitate gene expression by mediating interactions between distal enhancers (E) and promoters (P). Over 90% of chromatin loops are anchored by CTCF motifs in convergent orientation (Rao et al., 2014).

1.2.2 Regulation of CTCF functions

The ZFs of CTCF are capable to binding to both DNA and protein; for example, ZFs 5-7 mediate CTCF binding to the *amyloid precursor protein* (*APP*) promoter and the ZF domain also mediates CTCF interaction with the chromodomain helicase DNA binding protein 8 (CHD8) (Ishihara et al., 2006; Quitschke et al., 2000) (Figure 1-2a). Mapping of CTCF binding sites in numerous cell lines and tissues have provided functional insights: CTCF is enriched at ~55,000-65,000 sites in mammalian genomes with a genomic distribution of ~50% intergenic, ~35% intragenic, and ~15% at promoters (Chen et al., 2012; Chen et al., 2008; Kim et al., 2007b). Of CTCF binding sites, ~5,000 are highly conserved between mammalian species and tissues and correspond to high affinity sites (Schmidt et al., 2012), while 30-60% of sites are cell-type specific and tend to exhibit low occupancy of CTCF (Barski et al., 2007; Chen et al., 2008; Essien et al., 2009; Kim et al., 2007b). Paradoxically, CTCF is enriched at enhancer elements, which in stark contrast to its originally identified role as an enhancer-blocker, indicating that CTCF sites likely play different functional roles in a context-dependent manner and some sites may be involved in establishing cell type-specific transcriptional programs (DeMare et al., 2013; Song et al., 2011). Interestingly, CTCF binding sites are carried in transposable elements and evidence points to the expansion of repetitive elements as a driver of new CTCF binding events in diverse mammalian lineages (Bourque et al., 2008; Schmidt et al., 2012).

CTCF binding sites contain a 12- to 20-bp consensus motif (Bell and Felsenfeld, 2000; Kim et al., 2007b), however the basis of CTCF recruitment to genomic sites is not well understood. The CTCF consensus motif is not present in all binding sites and not all such motifs bind CTCF (Kim et al., 2007b), suggesting that CTCF recruitment is more complicated. DNA methylation plays a widespread role in modulating CTCF binding: 41% of cell type-specific CTCF binding events are linked to differential methylation concentrated at base pairs (bp) 1 and 11 of the consensus motif (Mukhopadhyay et al., 2004; Wang et al., 2012). CTCF can also directly affect DNA methylation through interaction and activation of poly(ADP-ribose) polymerase 1 (PARP1), which inhibits DNA (cytosine-5)-methyltransferase 1 (DNMT1) through poly(ADP-ribosyl)ation (Guastafierro et al., 2008; Zampieri et al., 2012). Despite this, the presence of cell-type

specific CTCF binding sites that are not differentially methylated suggests additional mechanisms that affect CTCF occupancy. CTCF is typically located in nucleosome-depleted linker regions surrounded by well-positioned nucleosomes, suggesting that nucleosome phasing is an essential upstream factor that regulates CTCF enrichment (Cuddapah et al., 2009; Fu et al., 2008). This idea is supported by evidence that CTCF recruitment or maintenance at the *H19-Igf2* and *Gtl2-Dlk1* ICRs requires local nucleosome remodeling by ATRX (Kernohan et al., 2014). Furthermore, the interaction of CTCF and CHD8 suggests that CHD8 may facilitate local chromatin remodeling to mediate CTCF binding, however this idea has not been tested experimentally (Ishihara et al., 2006). While DNA methylation and nucleosome phasing are important regulators of CTCF binding, it is likely that additional contributing mechanisms have not yet been identified.

The addition of covalent post-translational modifications has been linked to differential binding and functions of CTCF. For instance, CTCF itself can be poly(ADP-ribosyl)ated, which has been linked to its insulator (Yu et al., 2004) and barrier functions (Witcher and Emerson, 2009), as well as subnuclear localization (Guastafierro et al., 2013; Ong et al., 2013; Torrano et al., 2006). Four serines in the C-terminus of CTCF are phosphorylated by casein kinase II (CKII; Figure 1-2a), potentiating its growth suppressive functions (Klenova et al., 2001). Interestingly, mutation at this same motif present in the p53 tumour suppressor protein abrogates its growth inhibition function, and selective phosphorylation of the motif is regulated in response to genotoxic stress by the CKII-FACT (facilitates chromatin transcription) complex (Keller et al., 2001; Milne et al., 1992). The PC2 small ubiquitin-like modifier (SUMO) ligase can SUMOylate residues in the N-terminus of CTCF, contributing to its repression of *MYC* transcription without affecting its ability to bind DNA (MacPherson et al., 2009) (Figure 1-2a). Together, analysis of CTCF post-translational modifications demonstrates the complexity of CTCF regulation.

Interactions between CTCF and other proteins may also represent an additional mechanism by which the protein achieves functional diversity (Zlatanova and Caiafa, 2009). CTCF interacts with the large subunit of RNA polymerase II (RNAPII) through its

C terminal domain (Figure 1-2a) and intragenic peaks of RNAPII overlap sites of CTCF enrichment (Chernukhin et al., 2007; Wada et al., 2009). Functionally, this may be a consequence of a role for CTCF in promoting polymerase pausing, rather than recruitment *per se,* since on a global scale CTCF binding has been implicated in generating a "poised" promoter state (promoter-proximal pausing) and in the pre-mRNA processing decisions associated with alternative splicing (Paredes et al., 2013; Shukla et al., 2011). In addition to RNAPII, CTCF can interact with numerous protein partners such as zinc finger protein 143 (ZNF143), the Kaiso transcription factor, nucleophosmin, lamin A/C, ying yang 1 (YY1), and CHD8 to name a few (Zlatanova and Caiafa, 2009) (Figure 1-2a). Further complicating this issue is the recent finding that the C-terminus of CTCF can interact with RNA, which is important for its role in transcriptional regulation of *p53,* X chromosome inactivation, and in stabilizing protein-protein interactions (Kung et al., 2015; Saldana-Meyer et al., 2014; Sun et al., 2013; Yao et al., 2010) (Figure 1-2a). RNA-immunoprecipitation followed by high throughput sequencing (RIP-sequencing) identified that CTCF can interact with ~15,000 different RNA species (Kung et al., 2015; Saldana-Meyer et al., 2014), indicating that the interaction likely represents a more widespread mechanism of CTCF function.

While the aforementioned partners can influence CTCF activities at specific loci, a pervasive relationship has been established with the cohesin complex (Parelho et al., 2008; Rubio et al., 2008; Wendt et al., 2008). The cohesin complex is composed of four subunits: the structural maintenance of chromosomes 1 (SMC1) and SMC3, radiation mutant 21 (Rad21), and stromal antigen 1 or 2 (SA1 or SA2), and was initially characterized as being required for sister chromatid cohesion during mitosis (Barbero, 2011; Michaelis et al., 1997; Moser and Swedlow, 2011; Uhlmann and Nasmyth, 1998). The high degree of overlap between cohesin and CTCF binding sites genome-wide suggests a functional relationship: depending on the cell type, ~50-80% of CTCF binding sites are co-occupied by cohesin, and there is a mutualistic relationship between their binding (Parelho et al., 2008; Rubio et al., 2008; Wendt et al., 2008). By-and-large CTCF is required for cohesin recruitment, while cohesin appears to be required to stabilize most CTCF-mediated chromosomal contacts and to be essential for CTCF function at most genomic sites (Hou et al., 2010; Nativio et al., 2009). Physical interaction of the proteins

occurs between the SA1/2 subunit of cohesin and the C terminus of CTCF (Xiao et al., 2011) (Figure 1-2a). Despite this close relationship, evidence suggests that the CTCF/cohesin interaction is independent from cohesin's role in mediating sister chromatid cohesion, as CTCF-depleted cells do not exhibit cohesion defects (Parelho et al., 2008).

1.2.3 CTCF regulates higher-order chromatin organization

CTCF can mediate long-range intra- and inter-chromosomal interactions in mammalian cells (Kurukuti et al., 2006; Ling et al., 2006; Splinter et al., 2006) (Figure 1-2b). This function of CTCF is best characterized at the imprinted *H19-Igf2* locus where allele-specific binding of CTCF at the maternal *H19* ICR results in expression of *H19* and paternal expression of *Igf2* through physical interactions with the differentially methylated regions upstream of the *Igf2* promoter (Kurukuti et al., 2006; Murrell et al., 2004). CTCF is also required for interchromosomal interaction between the *H19-Igf2* and *Wsb1/Nf1* imprinted clusters (Ling et al., 2006), which is thought to help establish co-regulation of an imprinted gene network (Andrade et al., 2010; Kernohan and Berube, 2010; Varrault et al., 2006).

The discovery that CTCF interacts with the cohesin ring complex led to a model wherein long-range interactions mediated by CTCF are clamped together and stabilized by cohesin (Haering et al., 2008; Ohlsson et al., 2010b) (Figure 1-2b). This matches with the finding that CTCF binding to the DNA duplex asymmetrically bends the DNA but is unable to form a loop alone (Liu and Heermann, 2015). Indeed, cohesin is required for higher-order chromosome conformation at *H19-Igf2* (Nativio et al., 2009) and many other sites co-occupied with CTCF (Merkenschlager and Odom, 2013). Despite this, it remains unclear whether CTCF and cohesin work independently or in concert to define loop interactions; in general, cohesin depletion affects larger loops (100-200 kb interaction range), while CTCF depletion affects shorter loops (<100 kb) (Zuin et al., 2014). Moreover, CTCF depletion resulted in a gain of chromatin interactions (Zuin et

al., 2014), further complicating our understanding of the mechanisms underlying genome organization.

Hi-C maps of global chromatin interactions have identified that the genome is partitioned into large (~1 Mb) domains referred to as topologically associating domains (TADs) that are defined based on a higher interaction frequency within domains that between domains (Dixon et al., 2012; Lieberman-Aiden et al., 2009). Intriguingly, domain boundaries are enriched for housekeeping genes, short interspersed elements (SINEs), and CTCF binding sites in convergent orientation, implicating these features in the establishment of TADs (Dixon et al., 2012; Rao et al., 2014) (Figure 1-2b). CTCF depletion does not dramatically alter global domain organization, suggesting that it is likely not the sole determinant of TAD formation (Zuin et al., 2014). However, deletion of specific CTCF binding sites on the X chromosome or within the *HoxA* cluster results in structural TAD reorganization, indicating that at least in some contexts CTCF is required to delimit TAD boundaries (Nora et al., 2012). The influence of CTCF on other features regulated by TAD organization, such as replication timing (Pope et al., 2014), has yet to be characterized.

Chromatin interaction analysis by paired-end sequencing (ChIA-PET) analysis of CTCF-mediated chromatin interactions in ES cells identified ~1,800 interactions involving ~3,300 CTCF binding sites (~5-10% of total CTCF binding sites). In contrast to its characterized role as an insulator protein, the majority of CTCF/cohesin sites facilitate enhancer-promoter interactions (Handoko et al., 2011; Sanyal et al., 2012). The finding that only a small proportion of CTCF sites are involved in higher-order interactions suggests additional DNA-dependent functions for CTCF. Furthermore, the average cell contains ~200,000 CTCF molecules but only ~30,000-60,000 CTCF binding sites, perhaps indicating DNA-independent functions for the protein as well (Heath, 2007).

Together, global analysis of chromatin interactions in the context of CTCF binding suggests that enhancer blocking or barrier insulation activities of CTCF may only occur in a context-dependent manner as a consequence its primary role in mediating long-range interactions (Handoko et al., 2011; Phillips-Cremins et al., 2013; Sanyal et al., 2012)

(Figure 1-2b). Analysis of CTCF function in TAD architecture is still in its infancy, but emerging technologies to study large-scale chromatin interactions such as Hi-C and ChIA-PET will indisputably uncover the functional significance of higher-order genome organization.

1.2.4 Evidence of CTCF function during replication

In addition to its well-characterized functions in transcriptional regulation, transcription-independent roles for CTCF have also been described in the literature. For instance, CTCF regulates stability of numerous regions implicated in trinucleotide repeat expansion disorders such as spinocerebellar ataxia type 7 (SCA7) and myotonic dystrophy type I (DM1) (Filippova et al., 2001; Libby et al., 2008; Sopher et al., 2011). At the *DM1* locus, CTCF binding upstream of the repeat is required to slow DNA polymerase before replication of the repetitive tract, and mutations in the CTCF binding site results in repeat expansion likely due to strand slippage (Cleary et al., 2010). Intriguingly, *DM1* is asymmetrically replicated (Rajcan-Separovic 1998) and CTCF regulates asymmetric replication of the *H19/Igf2* imprinted domain (Bergstrom et al., 2007). Therefore, the effect of CTCF on replication may be limited to certain loci, as direct roles for the protein have only been described at asymmetrically replicated regions. CTCF has also been implicated in replication origin firing, as overexpression of the oncogenic replication licensing factor *Cdc6* causes CTCF dissociation from the promoters of the tumour-suppressor genes *CDH1* and *INK4/ARF*, resulting in reduced expression and activation of adjacent replication origins (Sideridou et al., 2011). It was suggested that because replication origins are defined by structural chromatin context rather than DNA sequence (Antequera, 2004; Cvetic and Walter, 2005), CTCF might facilitate higher-order organization of replication origins.

The CTCF partner cohesin is implicated in organizing replication factories and controlling S phase progression, however CTCF depletion had little effect on cell cycle dynamics or the overall size of replicon units identified by the DNA halo assay (Guillou et al., 2010). A more global role for CTCF in DNA replication and/or organizing replication factories has not yet been thoroughly investigated, but is of interest given the importance of CTCF in replication timing, progression, and origin firing at individual

loci. Furthermore, the finding that TADs regulate replication timing suggests that CTCF may participate in this process if indeed CTCF is involved in orchestrating TAD organization (Dixon et al., 2012; Pope et al., 2014) (Figure 1-3).
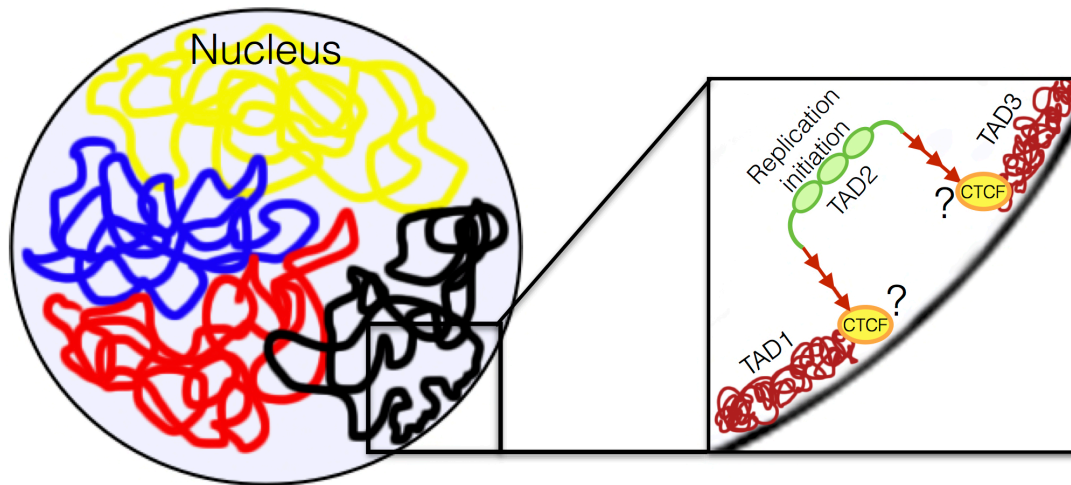
Figure 1-3 The relationship between chromosome territory organization in the nucleus, topologically associating domains (TADs), and replication timing

(Left) Chromosome territory organization within the nucleus represented by four chromosomes of different colours for simplicity. (Right) The replication domain model described in (Pope et al., 2014): TADs within the nuclear interior (green) actively fire origins and replication proceeds passively into TADs at the nuclear exterior (red). CTCF (yellow) binding at TAD borders is shown.

1.2.5 *In vivo* functions of CTCF

Most of our knowledge of CTCF activities has been derived from *in vitro* studies using immortalized cell lines (Ohlsson et al., 2010a), yet CTCF function has rarely been approached in an organismal context. Using transgenic RNA interference (RNAi), Fedoriw and colleagues demonstrated that both zygotic and maternal stores of CTCF are essential for preimplantation development (Fedoriw et al., 2004; Wan et al., 2008). These studies also indicated a requirement for CTCF in cellular survival since CTCF depletion caused meiotic and mitotic defects that culminated in apoptosis (Wan et al., 2008). Generation of a *Ctcf*-null allele (*Ctcf*$^{-/-}$) confirmed the essential nature of CTCF in early development, as homozygous mutants are lethal prior to E3.5 (Heath et al., 2008). Heterozygotes (*Ctcf*$^{+/-}$) are viable, fertile, and do not exhibit any overt phenotypes, however the decreased ratio of wild type to heterozygote mice suggests that heterozygosity of *Ctcf* does compromise survival to some extent (Heath et al., 2008).

Tissue-specific deletion of CTCF has yielded more insight into its function in development and has pointed to an essential role for the protein in cell survival and cell cycle progression. For instance, *Ctcf* deletion in the developing limb bud resulted in upregulation of the pro-apoptotic *p53 upregulated modulator of apoptosis* (*PUMA*), massive apoptosis, and a complete loss of forelimb structure (Soshnikova et al., 2010), while deletion of *Ctcf* in post-mitotic neurons did not cause apoptosis (Hirayama et al., 2012). Deletion of *Ctcf* in T cells resulted in a decreased number of cells, suggestive of increased cell death, and caused a cell cycle block due to transcriptional upregulation of *p21* and *p27* (Heath et al., 2008).

A requirement for CTCF in neuron-specific gene expression programs has recently been demonstrated (Chang et al., 2010; Hirayama et al., 2012). Brain derived neurotrophic factor (BDNF) is a critical mediator of neurogenesis, synaptic plasticity, and neuronal survival (Greenberg et al., 2009). Methylation-sensitive binding of CTCF to the *Bdnf* gene is required for its activity-dependent transcriptional activation (Chang et al., 2010). Additionally, CTCF is an important transcriptional regulator of the protocadherin cluster of genes (Hirayama et al., 2012). Protocadherins are largely expressed in the nervous system and encode cell adhesion molecules located at the pre- and post-synaptic

membrane. Cells express variable sets of protocadherin proteins, and each set may serve as a molecular barcode for neuron identity (Frank and Kemler, 2002). Therefore, at least in neurons, CTCF is required for the establishment of transcriptional programs that help to shape individual cell-specific identity (Dekker, 2012).

1.2.6 *CTCF* mutations linked to cancer and neuropsychiatric disease

Few human *CTCF* mutations have been reported, which likely reflects the essential nature of the protein (Fedoriw et al., 2004; Heath et al., 2008; Moore et al., 2012; Wan et al., 2008; Watson et al., 2014). Chromosomal deletion at 16q22.1, however, is well documented in several human cancers and is one of the most common genetic events in breast cancer (Filippova et al., 1998; Rakha et al., 2006). *CTCF* maps to 16q22.1 and has been demonstrated to function as a haploinsufficient tumour suppressor in mice, as $Ctcf^{+/-}$ mice are susceptible to spontaneous and chemical/radiation-induced tumours of epithelial, mesenchymal, and hematopoietic origin (Kemp et al., 2014). Furthermore, analysis of ~5,000 tumours across 21 cancer types revealed that CTCF was one of the most frequently mutated genes (Lawrence et al., 2014). A high proportion (21%) of *CTCF* mutations identified in human cancers were missense mutations in the ZF domain predicted to alter DNA sequence recognition, implicating this domain in the suppression of tumourigenesis (Kemp et al., 2014). Together, these data implicate a broad role for CTCF in tumour suppression, yet the mechanism of CTCF function in cancer prevention is not fully understood. Hemizygous loss of *Ctcf* destabilized DNA methylation in mouse tumours, however it remains unclear as to whether this is a direct or secondary cause of CTCF loss (Kemp et al., 2014).

Recently, *de novo* mutations in *CTCF* were identified in patients with intellectual disability (ID), microcephaly, and autistic features (Gregor et al., 2013; Iossifov et al., 2014) (Figure 1-2a) and single nucleotide polymorphisms at the *CTCF* locus have been associated with schizophrenia (Juraeva et al., 2014). This adds to the growing list of epigenetic regulators implicated in neuropsychiatric disease and suggests that CTCF function is particularly important in the developing brain, as evidence suggests that

autism and schizophrenia are developmental in origin (Cook and Scherer, 2008; Guilmatre et al., 2009; Millan, 2013; Mitchell, 2011; Pinto et al., 2014; Sebat et al., 2009). Since there have been very few patients identified with *CTCF* mutations it is difficult to generate conclusions based on genotype-phenotype correlations. However, one patient has a missense mutation (R567W) located in the eleventh ZF that does not affect mRNA or protein levels, implicating abnormal targeting of CTCF in the pathogenesis of ID (Gregor et al., 2013). Identification of the full spectrum of *CTCF* mutations in the human population will undoubtedly expand our understanding of CTCF function and the pathogenesis of ID.

1.3   The regulation of higher-order chromatin structure

1.3.1 Higher-order chromatin architecture

Nuclear architecture is a key regulator of transcription, replication, and repair (Misteli, 2007). The term nuclear architecture refers to higher-order chromatin structure, nuclear compartments, and non-random spatial genome organization (Bickmore and van Steensel, 2013). Nuclear compartmentalization has been shown to be critical in the regulation of DNA metabolism: for instance, artificial tethering of genes to the nuclear periphery results in transcriptional silencing (Finlan et al., 2008; Reddy et al., 2008), and nuclear positioning has been linked to replication timing (Gilbert, 2001; Pope et al., 2014).

The fundamental unit of chromatin is the nucleosome, which is comprised of ~147 base pairs of DNA wrapped around a (H3-H4)$_2$-(H2A-H2B)$_2$ histone octamer. The nucleosome is organized into a fiber, which is further condensed to generate chromosomes. Within the nucleus chromosomes occupy distinct territories, and chromatin folds in *cis* to mediate interactions between regulatory elements as well as bring genomic regions from long distances or different chromosomes into close spatial proximity for co-regulation (Cremer and Cremer, 2001). Large-scale genome organization was later confirmed by 3C-based experiments, demonstrating that different chromosomes display different propensity to form inter-chromosomal interactions:

chromosomes of similar size and density have a higher likelihood of interaction (Lieberman-Aiden et al., 2009; Zhang et al., 2012). Nuclear compartments differ with regards to chromatin and genic features: DNAse I hypersensitive, active, and gene-rich loci cluster together and are separate from gene-poor, transcriptionally silent chromatin (Lieberman-Aiden et al., 2009; Zhang et al., 2012). The compartments primarily appear to relate to gene expression and as such are cell-type specific.

Chromosome compartments are further organized into domains of 100 kb-1 Mb that are topologically separated from one another (TADs) and flanked by insulators (Dixon et al., 2012; Rao et al., 2014; Sexton et al., 2012). As opposed to chromosome territories, which are related to gene expression, TADs are largely conserved across cell types (Dixon et al., 2012; Nora et al., 2012). Deletion of a TAD boundary region in the X chromosome inactivation center resulted in partial fusion of the flanking TADs, suggesting that TAD boundaries are genetically defined (Nora et al., 2012). This, in conjunction with the finding that TAD boundaries are enriched for particular genomic features i.e. CTCF binding sites (Dixon et al., 2012), begs the question of whether specific elements are involved in the formation of TADs (Figure 1-3).

Global comparisons between genome topological organization and replication timing programs support a model that TADs (i.e. regions of the genome that interact with themselves more often than they interact with neighbouring regions) regulate replication timing (Dixon et al., 2012; Pope et al., 2014) (Figure 1-3). Since CTCF has been implicated in the establishment of TADs, it may play an important role in regulating the replication timing program (Dixon et al., 2012). Depletion of *Ctcf* by RNAi resulted in subtle relaxation of TAD organization and increased inter-TAD interactions (Zuin et al., 2014), however the consequences of these changes on global replication timing are unknown and the effects of complete CTCF loss rather than depletion have not been assessed. CTCF is necessary for asynchronous replication of the *H19-Igf2* imprinted domain, demonstrating that it is important for replication timing in some capacity (Bergstrom et al., 2007).

The significance of higher-order chromatin organization and TAD formation is not well understood, however the finding that compartments are correlated with transcription and that domains are units of DNA replication suggests functional importance (Markaki et al., 2010; Pope et al., 2014).

1.3.2 Interplay between CTCF, cohesin and ATRX

The first indication of a relationship between ATRX and cohesin was the finding that ATRX depletion in HeLa cells causes chromosome cohesion defects (Ritchie et al., 2008). An interaction between ATRX and the SMC1 and SMC3 subunits of cohesin can be detected by co-immunoprecipitation in neonatal mouse brain extracts (Kernohan et al., 2010), and recruitment of CTCF and cohesin to imprinting control regions is dependent on nucleosome-remodeling activities of ATRX (Kernohan et al., 2010; Kernohan et al., 2014). Preliminary analysis of ATRX-dependent CTCF recruitment indicates that ATRX is required for enrichment at a number of sites in the postnatal mouse brain (Levy et al., unpublished data), raising the intriguing possibility that ATRX may help recruit CTCF to specific sites through nucleosome remodeling and/or interaction with the cohesin complex.

1.4 Genome stability in the context of brain development

1.4.1 The response to DNA damage

The genome is constantly bombarded with sources of stress, both exogenous and endogenous, that can result in the accumulation of genetic lesions (Ciccia and Elledge, 2010). It is estimated that tens of thousands of damaging events occur daily in each cell (De Bont and van Larebeke, 2004). Chemotherapeutic agents, ultra-violet (UV) radiation from the sun, and other frequencies of radiation such as x- and gamma (γ)-rays, are all examples of exogenous sources of DNA damage, while damage can also result from endogenous sources such as replication errors and oxidative stress caused by metabolic by-products (Mirkin and Mirkin, 2007; Ward, 1988). The type of damage lesion can vary

widely from single- to double-strand breaks, interstrand cross-links, and base modifications i.e. alkylation, depurination, oxidation. The strategy for repair depends on the type and location of the lesion as well as cell cycle stage. The cell has evolved a complex DNA damage response (DDR) that is responsible not only for detection and repair of damage, but also to coordinate repair with other cellular processes such as chromatin remodeling, transcription, cell-cycle progression, and apoptosis (Jackson and Bartek, 2009). Following detection of DNA damage, genome surveillance pathways become activated to block progression through the cell cycle in order to repair damaged DNA prior to cell division and ensure that daughter cells do not inherit the damage.

The most common type of lesion is likely single-strand breaks (SSBs), which can occur during base excision repair (BER) or from abortive topoisomerase I (TOP1) reactions (Caldecott, 2008). TOP1 generates a nick, also known as a cleavage complex intermediate, to relax DNA during transcription and replication (Wang, 2002). These intermediates are usually transient and rapidly resealed by TOP1, however collision with DNA/RNA polymerases can convert them into TOP1-linked SSBs (Pommier et al., 2003). Repair of single-strand breaks requires the generation of a 3' hydroxyl and 5' phosphate compatible for ligation by a DNA ligase, such as DNA ligase 1 (LIG1). Base modifications, such as oxidation or alkylation, are another common source of DNA damage. Organs with high metabolic demand, such as the brain, are particularly vulnerable to oxidative base modifications caused by reactive oxygen species (ROS), such as 8-oxo-dG (Iyama and Wilson, 2013). Oxidative lesions are repaired by the base excision repair (BER) pathway, in which DNA glycosylases cleave the N-glycosidic bond of modified bases (Lindahl, 1974) and processing occurs through the action of APE1, DNA polymerase β, and XRCC1 (Hegde et al., 2008; Mitra et al., 2001). Structural distortions of the DNA, such as "bulky" pyrimidine dimers and DNA adducts are repaired by nucleotide excision repair (NER). NER can be broadly characterized into global-genome NER, which scans the genome for helix-distorting lesions, and transcription-coupled NER, which is activated in response to damage on the transcribed stand of active genes (de Laat et al., 1999). Both BER and NER utilize the same general program: detection, excision, gap-filling synthesis, and ligation (Kamileri et al., 2012a).

DNA damage associated with replication, commonly referred to as replication stress, requires signaling of the ATM and Rad3 related (ATR) kinase, which responds to replication protein A (RPA)-coated ssDNA found at stalled or collapsed replication forks (Cimprich and Cortez, 2008) (Figure 1-4). Interstrand cross-link repair and mismatch repair also function to overcome errors that can occur during DNA replication (Deans and West, 2011; Jiricny, 2006). Replication-associated damage and other sources of endogenous damage will be further discussed in Section 1.4.3.

The most deleterious form of DNA damage is a double-strand break (DSB) since they can result in the loss of a substantial amount of genetic information and/or cause large chromosomal rearrangements that can lead to cell death or tumourigenesis. DSBs can arise endogenously as a result of abortive topoisomerase II activity, replication fork collapse, and even neuronal activity (Gomez-Herreros et al., 2013; Nitiss, 2009; Saleh-Gohari et al., 2005; Suberbielle et al., 2013), as well as exogenous sources such as ionizing radiation and chemotherapy agents (Jekimovs et al., 2014; Ward, 1988). DSBs are detected by the MRN complex, which promotes recruitment of repair factors and activation of cell cycle checkpoints through activation of the ATM kinase (Stracker and Petrini, 2011) (Figure 1-4). The strategy of repair is dependent on cell cycle stage: while the majority of DSBs are repaired through non-homologous end joining (NHEJ), the extra copy of genetic information that exists in S/G_2-phase cells allows for homology-directed repair (also referred to as homologous recombination; HR) (Lombard et al., 2005). In NHEJ, DSB ends are recognized by Ku70/Ku80 heterodimers that recruit and activate the catalytic subunit of the DNA-dependent protein kinase (DNA-PK$_{cs}$), which facilitates recruitment of end processing proteins that prepare the DNA ends for ligation by DNA ligase IV (Waters et al., 2014). Deletions can be introduced during the end processing stage of NHEJ, making this type of repair strategy error-prone. HR, however, is virtually error-free since it utilizes the homologous sister chromatid as a template for repair. HR repair is initiated through dsDNA end resection to generate 3' ssDNA end on which the RAD51 recombinase is loaded, resulting in invasion of a homologous DNA duplex that primes DNA synthesis and DNA polymerase-mediated copying of the intact DNA molecule (Li and Heyer, 2008). Many other molecules also play key roles in HR repair, such as breast cancer 1, early onset (BRCA1) and BRCA2, Rad54, and XRCC2

(Chen et al., 1999; Liu et al., 1998). Furthermore, the cohesin complex is important for activation of DNA damage-induced intra-S phase and G_2/M checkpoints downstream of DSB signaling (Kim et al., 2002; Luo et al., 2008; Qiu et al., 2015; Watrin and Peters, 2009; Yazdi et al., 2002), and replication-associated cohesion generated by cohesin is required for post-replicative HR repair (Sjogren and Nasmyth, 2001). This role of cohesin has not been investigated with respect to its interaction with CTCF or ATRX.

The DNA damage repair and response cascade is an energy-consuming process that can in itself perturb cellular homeostasis if over activated. It is estimated that 10^4 ATP molecules are required to repair one DSB (Hoeijmakers, 2009), and PARP1 activation during the DDR can lead to nicotinamide adenine dinucleotide (NAD^+) depletion, disrupting NAD-dependent enzymes like sirtuins and/or promote cell death through the release of apoptosis-inducing factor (AIF) (Alano et al., 2010). Timely and accurate repair of DNA damage is therefore essential for cellular survival and homeostasis.

Cell cycle arrest and apoptotic pathways are largely intertwined, and the p53 protein is an upstream master regulator of both facets of tumour suppression (Lane, 1992) (Figure 1-4). DNA damage, oncogene activation, hypoxia, and oxidative stress are cellular stressors that induce activation of p53, a transcription factor that binds to target gene promoters in a sequence-specific manner (Kern et al., 1991). The cellular outcome of the p53 pathway is contingent on the target activated: *p21* is a major mediator of p53-dependent cell cycle arrest (Vogelstein et al., 2000), while *Puma* is responsible for apoptotic activation downstream of p53 (Jeffers et al., 2003; Villunger et al., 2003) (Figure 1-4). p21 induces cell cycle arrest by inhibiting cyclin D/cyclin-dependent kinase (Cdk) 4, 6 and cyclin E/Cdk2 complexes, resulting in retinoblastoma (Rb) hyperphosphorylation and arrest at the G_1/S phase transition (Stewart and Pietenpol, 2001). G_2 phase arrest can also be mediated by p21-dependent inactivation of cyclin B1 and Cdc2 (Flatt et al., 2000; Innocente et al., 1999). PUMA promotes cell death through BAX activation, resulting in apoptosis-inducing factor and cytochrome *c* release from the mitochondria and activation of the caspase cascade (Sionov and Haupt, 1999).
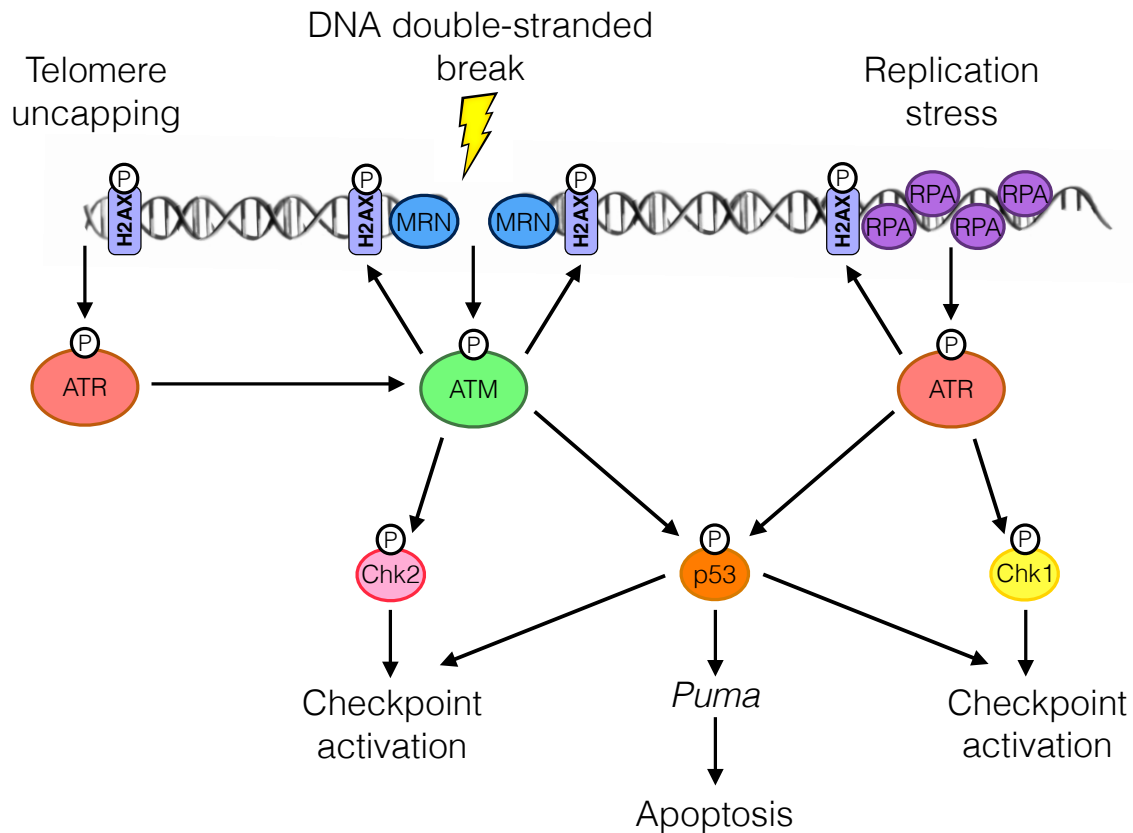
Figure 1-4 Cellular response to telomere uncapping, DNA double-strand breaks (DSBs), and replication stress

In the CNS, telomere uncapping results in ATR-to-ATM signaling, checkpoint activation and cell cycle arrest or apoptotic induction (Lee et al., 2014). DSBs cause MRN activation, resulting in ATM-dependent signaling (pATMS1981) to induce cell cycle arrest via Chk2 (pChk2T68) activation or p53-dependent apoptosis (pp53S18). Replicative stress typically causes an accumulation of RPA (pRPAS33), which activates ATR-dependent checkpoint activation via Chk1 (pChk1S317/345) or apoptotic induction via p53 (pp53S18). Genotoxic stress-induced apoptosis in neuroprogenitor cells is primarily mediated by p53-dependent transcriptional induction of *Puma* (Jeffers et al., 2003). Incorporation and phosphorylation of H2AX (S139; γH2AX) is a common event in response to many types of damage and is mediated by ATM and ATR kinases.

1.4.2 DNA damage response and repair in the context of chromatin

Since virtually all DNA damage occurs in the context of chromatin, interplay between higher-order chromatin structure and the DDR is crucial for timely detection and repair of damage. Historically, chromatin has been viewed as a barrier that has to be alleviated during repair and then restored afterwards (Smerdon, 1991). However, chromatin components have also been shown to promote DNA damage repair and signaling, resulting in the proposition of a "prime-repair-restore" model that suggests a more active role for chromatin in the DDR (Soria et al., 2012).

Several types of chromatin remodeling are important for DNA repair such as chaperone-mediated incorporation of histone variants, histone post-translational modifications, and ATP-dependent chromatin remodeling. There is also crosstalk between these mechanisms, as ATP-dependent chromatin remodeling is necessary for nucleosome eviction to allow for histone chaperone-mediated deposition of variants, underscoring the complexity of chromatin contributions to the DDR. Histone variants and modifications can function to organize and/or stabilize DNA repair machinery, and chromatin can also help to restrain transcription and signaling at the site of damage. Furthermore, higher-order 3D chromatin architecture likely plays an important role in facilitating repair, at the very least HR repair, although this has not yet been intensely studied.

The most well-characterized histone variant and modification in the context of the DDR is phosphorylation of histone H2A variant H2AX at serine 139 (γH2AX) by the PI-3 kinases ATM, ATR, or DNA-PK (Kinner et al., 2008). The FACT (facilitates chromatin during transcription) complex is responsible for deposition of H2AX (Heo et al., 2008). Immediately following induction of a DSB, γH2AX can be detected at the site of damage, which eventually spans at least a megabase of chromatin surrounding the break (Nakamura et al., 2010; Rogakou et al., 1998) (Figure 1-4). Spreading of γH2AX is a discontinuous process influenced by both transcription (Iacovoni et al., 2010) and cohesin binding (Caron et al., 2012). The DNA repair process manifests cytologically as DNA repair foci that are formed by the recruitment and accumulation of DNA repair factors at

sites of DNA damage. γH2AX is a major constituent of DNA repair foci, and serves as a beacon for the assembly of repair factors and to coordinate the DDR with other cellular functions such as cell cycle arrest (Polo and Jackson, 2011). H2AZ and macroH2A are other H2A variants with documented roles in the DDR. Replacement of H2AX with H2AZ is dynamically regulated at damage sites, stimulating DSB end resection and promoting an open chromatin conformation to help recruit repair machinery to sites of DSB (Kalocsay et al., 2009; Xu et al., 2012). MacroH2A accumulates at DNA damage sites in a PARP-dependent manner and is required for HR repair (Khurana et al., 2014). Histone H3 variant H3.3 has been shown to mediate replication fork restart and transcriptional recovery after UV-induced DNA damage in a HIRA-dependent manner (Adam et al., 2013; Frey et al., 2014), however the requirement for H3.3 incorporation at other damage lesions has not been addressed and may require distinct pathways for deposition. For instance, in distinct cellular contexts different pathways of histone deposition may be required, and it has been shown that DAXX mediates H3.3 deposition at some neuronal activity-dependent genes (Michod et al., 2012). Collectively, these data underscore the importance of chromatin dynamics in the DDR and reveal that our understanding of the contributions of histone variants to DNA damage repair and signaling is only beginning to emerge.

As mentioned in Section 1.3.1, a key feature of the nucleus is its non-random organization of chromosomes into distinct subdomains (Bickmore and van Steensel, 2013; Cremer and Cremer, 2001; Misteli, 2007). It has been well established that there are dedicated centers in the nucleus for transcription and replication, termed "factories", begging the question of whether "repair factories" exist. Low-dose irradiation in yeast leads to ~2-4 DNA repair foci, however higher doses induce larger instead of more foci, suggesting that newly formed lesions are recruited to pre-existing sites for repair (Lisby et al., 2003a; Lisby et al., 2003b; Lisby and Rothstein, 2004). This may be different in mammalian cells, where DSBs appear immobile in the nucleus (Soutoglou et al., 2007), and despite some evidence of repair center formation (Aten et al., 2004) their existence has been debated extensively (Dion and Gasser, 2013). While the relevance of higher-order chromatin structure has been thoroughly studied in the context of transcriptional regulation, we know very little of how 3D chromatin architecture influences replication

and repair. Recent evidence indicates that topologically associating domains (TADs) function as stable units of replication timing (Pope et al., 2014) (Figure 1-3). It is unknown whether DNA damage induces TAD reorganization, yet evidence that demonstrates relocation of damaged heterochromatin suggests changes to 3D structure in some capacity. Relocation of heterochromatic breaks to outside of heterochromatin domains by HR factors has been observed in *Drosophila* and is believed to prevent recombination between repetitive sequences that are often found in heterochromatin (Chiolo et al., 2011). This relocation of DSBs to the periphery of chromocentres is reminiscent of the location of major satellites replication (Guenatri et al., 2004; Quivy et al., 2004), raising the idea that a common mechanism may underlie the two processes, especially since similar proteins are implicated. DSBs in heterochromatin are characterized by slower repair kinetics than those in euchromatic regions (Goodarzi et al., 2008). Moreover, ES cells with reduced levels of the linker histone H1 display an enhanced cellular response to DSBs, which has been ascribed to the less compacted chromatin in the absence of H1 since histone deacetylase (HDAC) inhibitor trichostatin A (TSA)-induced global chromatin decompaction has a similar effect on repair efficiency (Murga et al., 2007). Collectively these results demonstrate that chromatin structure influences the repair process and that chromatin organizing proteins may help fine-tune the cellular response to DNA damage. Furthermore, the notion of cohesin mediating the spatial proximity of sister chromatids during HR implies that reorganization of higher-order chromatin structure is necessary. Studying how nuclear positioning is determined and how it may influence repair and replication will be essential to fully understand tumourigenesis and human pathology.

1.4.3 Endogenous sources of DNA damage

As mentioned in section 1.4.1, DNA lesions can arise spontaneously as a result of reactive oxygen species, abortive topoisomerase I/II reactions, or replication errors. Spontaneous replication-associated DNA damage is particularly relevant to this thesis and will therefore be the focus of this section.

Replication of genetic information is a fundamental biological process, and accurate replication is essential to maintain genome stability and suppress cancer (Allen et al., 2011). Apart from exogenous sources of damage, DNA can be damaged by replication errors that often occur at genomic regions prone to slowed replication forks, such as chromosomal fragile sites, telomeres, unusual secondary structures, DNA-RNA hybrids and repetitive sequences (Aguilera and Gomez-Gonzalez, 2008; Durkin and Glover, 2007; Mirkin and Mirkin, 2007; Sfeir et al., 2009; Szilard et al., 2010; Tuduri et al., 2009). There are a number of conserved tumour-suppressive mechanisms in place to ensure correct replication and repair of replicative errors that cause cell cycle arrest, death, or senescence. Cells are also quite vulnerable to DNA damage during replication since virtually all DNA damage blocks the replication fork, causing replication stress. Damage can therefore be a cause or consequence of replicative stress.

Although replication stress has been widely recognized as a significant hurdle to overcome in order to maintain cellular homeostasis, there is yet to be a single unifying description of the phenomenon. This is likely because replicative stress can arise from a variety of sources, and ultimately cellular indicators of replicative stress are shared with other DNA damage response/repair pathways. Typically, the persistence of ssDNA bound by RPA generates a signal to activate ATR and other replication-stress-response proteins, resulting in intra-S or G_2/M phase arrest to allow the cell to repair damaged DNA before entering mitosis (Abraham, 2001; Branzei and Foiani, 2007; MacDougall et al., 2007; Marechal and Zou, 2013; Nam and Cortez, 2011; Zou and Elledge, 2003) (Figure 1-4). ATR-dependent phosphorylation and activation of RPA (pRPAS33) and checkpoint kinase 1 (pChk1S345) and detection of ssDNA are the best-known indicators of replicative stress (Zeman and Cimprich, 2014) (Figure 1-4). However, replicative stress may occur but is not severe enough to induce ATR activation and phosphorylation of its downstream targets (Koundrioukoff et al., 2013). To date, the best detection method for replicative stress is the DNA fiber assay, which allows direct measurements of DNA synthesis through the use of thymidine analogs (Bianco et al., 2012).

1.4.4 DNA damage response in the developing central nervous system

Genesis of the central nervous system requires enormous expansion of neuroprogenitor cells (NPCs) that give rise to a diverse array of cell types. Brain development and maturation requires the generation of correct cell numbers and their integration into a complex network of neurons that are metabolically and functionally supported by astrocytes and oligodendrocytes. Before the onset of neurogenesis, NPCs (also referred to as neuroepithelial cells at this time) undergo rapid symmetric divisions to expand the progenitor pool (Figure 1-5a). At ~E11 in the neocortex, some NPCs begin to divide asymmetrically to generate one progenitor cell and one post-mitotic neuron (Figure 1-5a). Newborn neurons then migrate from the proliferative zone to their final destination where they further differentiate and become integrated into functional networks (Greig et al., 2013).

A functioning DNA damage response (DDR) and repair system are extremely important in these early developmental stages since unrepaired lesions can greatly influence the formation of a functional nervous system (McKinnon, 2013). Overall, the nervous system has a low threshold for DNA damage, which makes sense since integrating dysfunctional cells into the neuronal network may be more detrimental than eliminating them (Lee and McKinnon, 2007), and the nervous system already utilizes apoptosis to eliminate over-produced cells during normal brain development (Nijhawan et al., 2000; Yoshida et al., 1998). Indeed, enhanced sensitivity to DNA damage following TopBP1 deletion was observed in early-born compared to later-born cortical progenitors, and irradiation of mouse brain leads to elevated levels of apoptosis in the neocortex at E11.5 compared to E14.5 (Lee et al., 2012a). These data indicate a propensity for NPCs to undergo apoptosis in response to DNA damage rather than risk expanding progenitors with abnormal DNA. The requirements for genome maintenance change substantially during the transition from neurogenesis to nervous system maturation. During neurogenesis, a primary source of damage results from replication errors due to the high proliferative index of progenitor cells (McKinnon, 2009). Differentiated neural cells are incredibly long-lived and must withstand the genetic damage throughout the entire life of an organism. Byproducts of

metabolism, such as ROS, are the primary source of damage in post-mitotic neurons due to the high energy demand of the mature brain (Harris et al., 2012). Despite these differences, there remains a constant requirement for the maintenance of genomic stability in the CNS.

DNA integrity is maintained during development by coordinated signaling pathways that pause cell proliferation to repair damage or, alternatively, activate apoptotic pathways to eliminate damaged cells and avoid acquisition of mutations. Damage signaling requires the ATM and ATR kinases, which have largely distinct roles in maintaining genome stability in the nervous system since they respond to different types of DNA damage (Herzog et al., 1998; Lee et al., 2012b) (Figure 1-4). The nervous system contains the full repertoire of DNA repair and signaling pathways that were outlined in Section 1.4.1.

The requirement of genome surveillance for proper brain development is clearly illustrated by mouse models of DDR-deficiency (Frappart and McKinnon, 2008). Mice lacking the essential HR factor XRCC2 (X-ray repair complementing defective repair in Chinese hamster cells 2) exhibit extensive apoptosis in the brain by E10.5, correlating with the period of neural progenitor proliferation, while mice deficient in the necessary NHEJ factor DNA ligase IV (LIG4) display apoptosis at E12.5 in differentiating NPCs (Orii et al., 2006). These findings indicate that the developing brain is vulnerable to endogenous DSBs and strengthen the idea that HR is the preferred method of DSB repair in proliferating cells, while post-mitotic cells default to utilizing NHEJ. However, a new finding that RNA can be used as a template for homology-directed repair demands reconsideration of this narrow view (Keskin et al., 2014), since it implies HR capabilities in all stages of the cell cycle. Nevertheless, the benchmark study by Orii et al. demonstrates that the types of DNA damage encountered in post-mitotic neurons are different than those observed in cycling neuroprogenitors and that distinct repair pathways are utilized (Orii et al., 2006). NPCs have a high proliferative index and thus encounter replication-associated damage, however post-replicative mature neurons encounter a high degree of metabolic damage, such as oxidative stress. DNA lesions in differentiated cells typically do not activate apoptosis, but can interfere with transcription if unrepaired and located in the vicinity of coding sequence (McKinnon, 2013). Along

these same lines, persistent DNA damage in mature neurons can cause redistribution of the histone deacetylase SIRT1 on chromatin, causing dysregulation of aging-associated genes (Oberdoerffer et al., 2008).

Differential susceptibility of progenitors to DNA damage may be linked to cell cycle dynamics, as cell cycle regulation of NPCs is a key aspect of cortical development and involves dynamic changes in G_1 and S phase duration (Arai et al., 2011; Dehay and Kennedy, 2007; Gotz and Huttner, 2005) (Figure 1-5b). A key difference between proliferating and neurogenic progenitors is the length of S phase; a three-fold longer S phase is associated with expanding neural progenitors compared to committed neural cells, which are characterized by a prolonged G_1 phase (Arai et al., 2011) (Figure 1-5b). This is likely due to the increased need for genome surveillance in non-committed progenitors to prevent transmission of genetic errors. Chromatin state is also an important determinant of neurogenic potential (Kishi et al., 2012), in addition to its defined roles in DNA damage response and repair (Soria et al., 2012), however our understanding of the relationship between chromatin structure, cell cycle regulation, and the DDR in the context of brain development is limited.
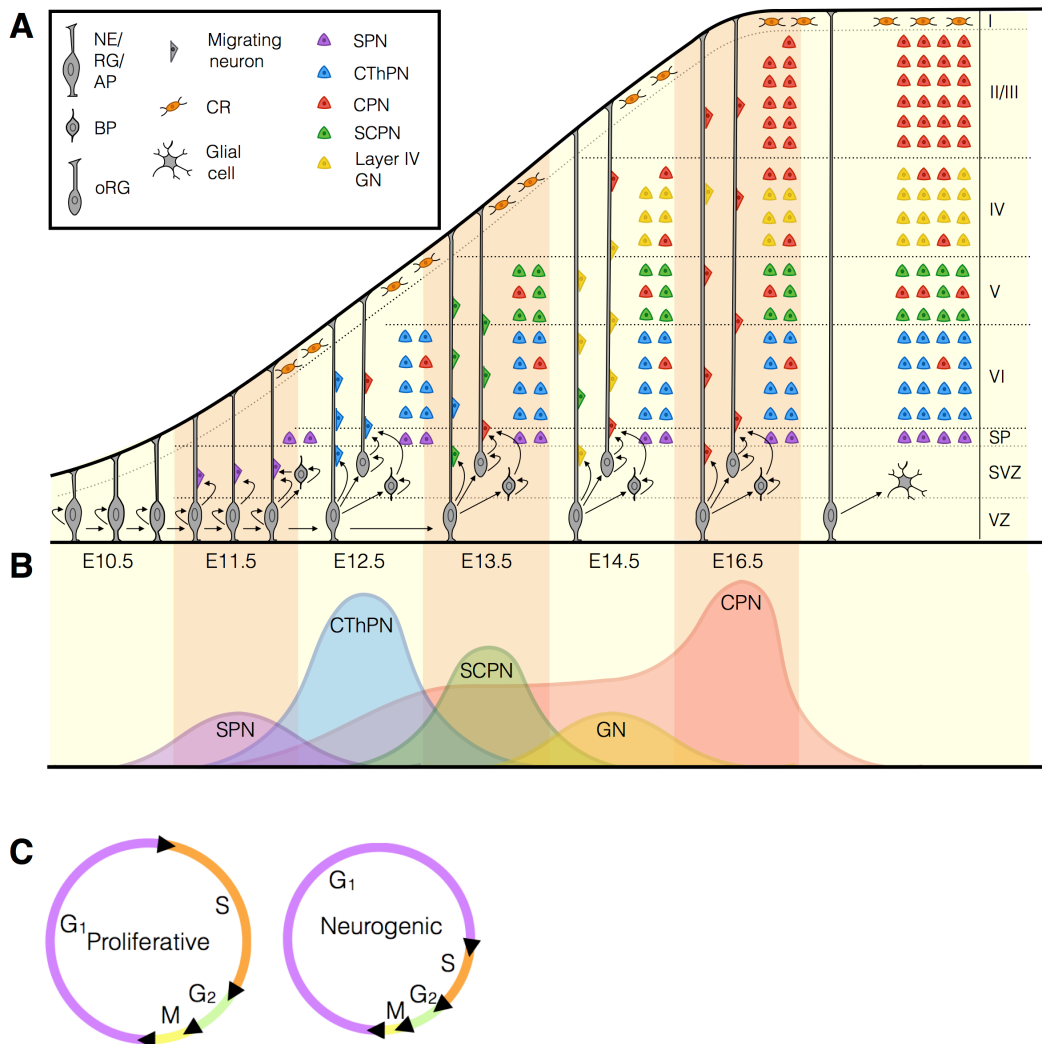
Figure 1-5 Timeline of corticogenesis and cell cycle dynamics in proliferative versus neurogenic cortical progenitors

(A) Neuroepithelial (NE) cells divide symmetrically to produce two NE cells and expand the progenitor pool before the onset of neurogenesis. In the neurogenic stage, NE cells acquire characteristics of radial glia (RG) and are termed apical progenitors (AP). APs and outer radial glia (oRG) progenitors divide asymmetrically to self-renew and generate a cell of neurogenic fate such as a postmitotic projection neuron or basal progenitor (BP). The earliest born neurons form the preplate, which eventually splits to form the subplate (SP) and marginal zone (also termed cortical layer I). The cortical plate forms in between these two layers in an inside-out fashion, with later born neurons migrating past earlier born neurons using the basal process of AP or oRG. The generation of glial cells

(oligodendrocytes and astrocytes) begins in late gestation at approximately E18. This schematic was largely reproduced using information derived from (Grieg et al., 2013). (B) Different subtypes of postmitotic neurons are born in overlapping temporal waves. Subplate neurons (SPN) are born at approximately E11.5, followed by corticothalamic projection neurons (CThPN) that are born at approximately E12.5. At the same time, callosal projection neurons (CPN) are generated throughout E12.5-E16.5. Subcerebral projection neurons (SCPN) are born at E13.5, followed by the generation of layer IV granular neurons (GN) at E14.5. (C) Proliferative and neurogenic progenitors exhibit differential cell cycle parameters, namely proliferative NPCs are characterized by a lengthened S phase (8 h versus 2.4 h) and neurogenic NPCs are characterized by a lengthened G_1 phase (17.1 h versus 12.7 h) (Arai et al., 2011). VZ: ventricular zone; SVZ: subventricular zone; CR: Cajal-Retizus.

1.4.5 Microcephaly is caused by defective cell cycle regulation, DNA damage response, and centrosome biology

Microcephaly is defined as head circumference two- to three-standard deviations below the mean, is often equivalent to microencephaly (reduced brain size), and is frequently associated with intellectual disability, or impaired cognition or adaptive functioning (Dolk, 1991). Microcephaly is a clinically heterogeneous disease and can present in the absence (primary microcephaly) of syndromic features or as a characteristic of specific syndromes, like ATR-X syndrome (Gibbons, 2006). Microcephaly is caused by abnormalities in cellular production, and as such the study of developmental microcephalies have taught us an enormous amount about how the brain generates the correct number of neurons to support brain size (Gilmore and Walsh, 2013). By and large, microcephaly-associated gene products are components of the centrosome, regulate cell cycle dynamics, or are involved in the DNA damage response. How these particular types of proteins are linked to microcephaly is still unresolved. It is possible that defective cell cycle regulation can lead to cellular proliferation deficiencies and/or apoptotic induction, which have both been observed in mouse models of microcephaly. For instance, deletion of *Cdk5rap2* causes excessive cell death (Lizarraga et al., 2010) and ASPM (abnormal spindle-like, microcephaly-associated) influences progenitor proliferation by controlling WNT signaling (Buchman et al., 2011).

Remarkably, all genes associated with primary microcephaly encode proteins associated with the centrosome or centrosomal-related activities (Bond et al., 2002; Bond et al., 2005; Guernsey et al., 2010; Jackson et al., 2002; Kalay et al., 2011; Kumar et al., 2009; Kumar et al., 2002; Nicholas et al., 2010; Yu et al., 2010b). Centrosomes are organelles with a critical role during mitosis where they associate with microtubules and are involved in formation of the mitotic spindle. How centrosomal defects cause microcephaly is not well understood. There is also crosstalk between centrosomal components, DNA damage signaling, and cell cycle regulation. For instance, the primary microcephaly gene product microcephalin (MCPH1) couples the centrosomal cycle with mitotic entry through phosphorylation of Cdc25/Chk1 (Gruber et al., 2011), and recruits the SWI/SNF complex to DNA damage sites facilitate local chromatin remodeling and

proper DDR signaling (Peng et al., 2009). This example highlights the dynamic interplay between the DNA damage response and cell cycle control, and underscores the importance of these processes in neurodevelopment since *MCPH1* mutations in humans solely affect the brain.

Microcephaly can also be caused by mutations in DNA repair pathway genes including *NBS1, XLF, DNA ligase IV,* and *MCPH1* (Ben-Omran et al., 2005; Buck et al., 2006; Carney et al., 1998; O'Driscoll et al., 2001; O'Driscoll and Jeggo, 2008; Xu et al., 2004). A question in the field is why defects in the DNA damage response pathway particularly affect brain development. During neurogenesis, replicative stress is a primary source of endogenous damage due to the rapid proliferative index of NPCs (McKinnon, 2013). Since DNA damage can lead to altered cell cycle dynamics, defects in the cellular response to or repair of damage may perturb the precise balance between NPC proliferation, differentiation and cell death. Excessive cell death during brain development would result in decreased neuronal numbers, and is one of the potential mechanisms underlying the microcephaly phenotype.

1.4.6 The DNA damage theory of aging

It has been well established that DNA damage and mutations accumulate as we age (Vijg, 2000). This fact has led to the DNA damage theory of aging, which postulates that the accumulation of genetic lesions is the major cause of the accelerated physiological decline associated with aging (Freitas and de Magalhaes, 2011; Gensler and Bernstein, 1981; Kirkwood and Holliday, 1979; Szilard, 1959). Aging is a natural process that affects most biological functions and appears to be a consequence of the cumulative action of various stressors such as telomere attrition, oxidative damage, and the decline of DNA repair (Kirkwood, 2005; Vijg and Campisi, 2008). Understanding the biological mechanisms underlying aging is of utmost importance since age is the primary risk factor for most human diseases, including neurodegeneration and cancer.

Human progeroid syndromes exhibit clinical manifestations that resemble accelerated aging, and have been linked to defects in DNA repair or processing. Genetic manipulation of DNA repair or response factors in mice result in the appearance of aging-like phenotypes, supporting the idea that DNA damage may be directly linked to aging. However, the best proof of principle would be to decrease physiological levels of DNA damage to lengthen lifespan in these models, which is difficult, if not impossible, to accomplish *in vivo*. The study of progeroid syndromes for the understanding of normal aging is controversial, since mouse models and even human progeria patients only exhibit a subset of the symptoms of normal aging and are therefore often referred to as "segmental progeria" (Miller, 2004). Nevertheless, progeroid and accelerated aging syndromes clearly demonstrate that disruption of DNA repair pathways can elicit aging phenotypes.

Hormone signaling plays a key role in the regulation of aging: in particular, the somatotroph (insulin-like growth factor 1/growth hormone) axis is a critical regulator of metabolism and longevity (Russell and Kahn, 2007) (Figure 1-6). An intriguing aspect of these longevity pathways is that they function non-cell autonomously; mutations or abnormalities in one cell type can alter the entire organism. Specifically, neuronal cells play a special role in cell non-autonomous lifespan regulation by the IGF-1 pathway in *C. elegans* (Wolkow et al., 2000). Chronic activation of the stress response is associated with human aging, and causes transcriptional changes associated with dampening of the somatotroph axis (Bartke, 2005; Lombardi et al., 2005). This molecular signature is also observed in mouse models that exhibit accelerated aging (Niedernhofer et al., 2006; van der Pluijm et al., 2007). Signaling between the hypothalamus, the pituitary gland, and the thyroid gland is essential for metabolic regulation (Figure 1-6). The hypothalamus senses low circulating levels of thyroid hormone and responds by releasing thyrotropin-releasing hormone (TRH). TRH stimulates the pituitary to produce thyroid-stimulating hormone (TSH), which in turn stimulates the thyroid to produce thyroid hormone (Dietrich et al., 2012). Thyroid hormone (thyroxine/T4) levels are also critical for the regulation of IGF-1 during prepubertal development (Xing et al., 2012) (Figure 1-6). Despite extensive evidence of a link between DNA damage and suppressed IGF-1 responses, there has not yet been satisfactory mechanistic explanation of the connection with aging.

Taken together, there is a strong relationship between DNA damage accumulation and the systemic defects associated with aging. Furthering our understanding of the complex interplay between DNA damage, metabolic signaling, and physiological decline will be necessary to solve the biological enigma of aging.
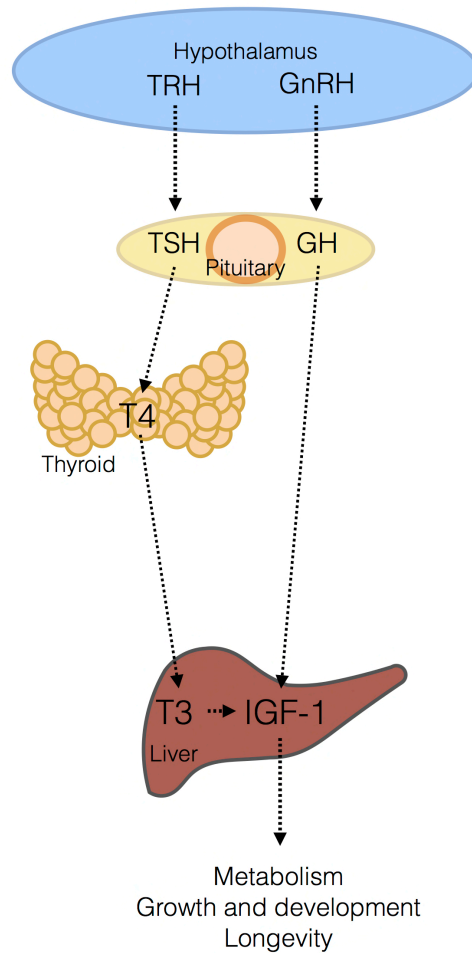
Figure 1-6 Insulin-like growth factor-1 (IGF-1) signaling controls organismal metabolism, growth and development, and longevity and can be controlled by thyroxine (T4) or growth hormone (GH)

Thyrotropin-releasing hormone (TRH)-expressing cells in the hypothalamus release TRH, which acts on cells in the anterior pituitary (yellow) to produce and release thyroid stimulating hormone (TSH). TSH acts on the thyroid gland to produce thyroxine (T4), which is converted to the biologically active T3 form that acts on the liver to induce IGF-1 production. This pathway is the major activator of IGF-1 signaling during prepubescent development (Xing et al., 2012). Alternatively, gonadotropin-releasing hormone (GnRH)-expressing neurons in the hypothalamus act on cells in the anterior pituitary to produce and release growth hormone (GH), which is released into the blood stream and increases IGF-1 levels.

1.5 Thesis Overview

The overarching goal of this thesis is to identify the molecular mechanism(s) underlying neurological disorders associated with defective chromatin structure.

1.5.1 Rationale and Hypothesis

Human mutations in *ATRX* and *CTCF* cause intellectual disability associated with microcephaly as well as various cancer subtypes, however we do not yet fully comprehend the role of ATRX or CTCF in brain development and suppression of tumourigenesis. Using mouse models deficient for *Atrx* and *Ctcf* in the embryonic brain, the work herein tests the hypothesis that chromatin regulation is critical for correct neurodevelopment by suppressing genomic instability and promoting NPC survival.

1.5.2 Chapter Two: Atrx deficiency induces telomere dysfunction, endocrine defects and reduced lifespan

This chapter describes work demonstrating that ATRX is required for NPC survival by suppressing DNA double-strand breaks at telomeric and pericentromeric heterochromatin during replication. *Atrx*-null NPCs are sensitive to telomestatin, a drug that selectively stabilizes G4-DNA structures, suggesting that ATRX may resolve G4-DNA to facilitate progression of the replicative machinery through repetitive sequences, i.e. telomeres, during late-S phase.

1.5.3 Chapter Three: Dual effect of CTCF loss on neuroprogenitor differentiation and survival

Chapter three provides evidence that CTCF is required for NPC survival, functioning to suppress p53/PUMA activation. Despite rescue of cell death in the embryonic brain, *Ctcf/Puma*-deficient mice display microcephaly, indicating that PUMA-dependent apoptosis does not completely explain the reduced brain size of *Ctcf*-deficient mice. Instead, *Ctcf*-deficient NPCs exhibit precocious differentiation, causing a depletion of the cortical progenitor pool. Together, these data indicate that CTCF functions to promote a balance between NPC proliferation/differentiation and to suppress p53/PUMA-mediated cell death for correct neurodevelopment.

1.5.4 Chapter Four: CTCF is required to prevent replicative stress and p53/PUMA-dependent apoptosis

Chapter four explores the mechanism underlying NPC death in the *Ctcf*-deficient brain and demonstrates CTCF loss results in endogenous DNA breaks that correlate with active proliferation and *Ctcf*-null NPCs exhibit characteristics of replicative stress. Moreover, *Ctcf*-null NPCs are sensitive to exogenous replicative stress, indicating that CTCF may function to prevent or repair replication-associated damage. Together, the findings described in the final chapter of this thesis identify a novel role for CTCF in preventing replicative stress and uncovers the mechanisms involved.

Overall, this thesis highlights the importance of two chromatin regulators in brain development and demonstrates that correct chromatin structure is critical to the differentiation and survival of neuroprogenitor cells. Dysfunctional chromatin induced by loss of ATRX or CTCF renders NPCs especially vulnerable to replication-associated damage and p53-dependent apoptosis. Not only do my findings provide some insight into mechanisms underlying defective brain development stemming from *ATRX* or *CTCF* mutation, it also has broad implications for the pathogenesis of complex neuropsychiatric diseases such as autism and schizophrenia.

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Chapter 2

2 *Atrx* deficiency induces telomere dysfunction, endocrine defects, and reduced lifespan

Prior to the research presented in this chapter, we identified that ATRX is required for survival of neuroprogenitor cells, and that loss of ATRX results in p53-dependent apoptosis (Berube et al., 2005; Seah et al., 2008). In this chapter, I sought to investigate the molecular mechanism underlying p53-dependent apoptosis resulting from *Atrx*-deficiency.

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2.1 Introduction

Faithful replication of DNA maintains genomic stability, limits the accumulation of cancer-promoting mutations, and can extend life span. Replication stress can occur spontaneously from stalling of the replication machinery as it encounters obstacles on the DNA template, such as DNA lesions, intricate secondary structures, or DNA-bound proteins (Branzei and Foiani, 2010). Impaired progression of the replication machinery can also occur when cells are exposed to exogenous damage or drugs that limit the nucleotide pool, or that cause interstrand crosslinking. The stabilization of paused replication forks to allow restart involves activation of checkpoint signaling to provide the cell extra time to respond (Allen et al., 2011). Failure to stabilize stalled forks causes fork collapse, a process involving dissociation of the polymerases, exposure of extended single-stranded DNA (ssDNA) regions, improper processing, and DNA breakage. Stalled replication can have serious consequences, such as genomic instability, chromosomal rearrangements from illegitimate recombination events, and reduced cell viability (Branzei and Foiani, 2010). Constitutive heterochromatin, telomeric DNA, and other DNA structures can pose particular challenges to the replication machinery. To overcome such hindrances, specialized factors are required to resolve difficult DNA structures or to promote access of restart pathway proteins to stalled forks (Buonomo, 2010).

ATRX is a Rad54-like ATP-driven DNA translocase belonging to the Swi/Snf family of chromatin remodelers (Xue et al., 2003). The biological impact of *ATRX* mutations in humans appears to vary according to the extent and timing of disruption. In humans, *ATRX* somatic frameshift and nonsense mutations that completely abolish protein function have been identified in pancreatic neuroendocrine tumours (PanNETs) (Heaphy et al., 2011a; Jiao et al., 2011; Yachida et al., 2012), pediatric and adult glioblastoma, and other cancers of the CNS (Cheung et al., 2012; Jiao et al., 2012; Kannan et al., 2012; Molenaar et al., 2012; Schwartzentruber et al., 2012), underscoring the anti-tumourigenic roles of ATRX. Tumours with *ATRX* mutations often harbor long telomeres, which are characteristic of the telomere maintenance mechanism known as alternative lengthening of telomeres (ALT) (Heaphy et al., 2011a; Jiao et al., 2011; Kannan et al., 2012; Lovejoy et al., 2012; Nguyen et al., 2013). Conversely, hypomorphic inherited mutations of the *ATRX* gene cause a rare developmental disorder, ATR-X syndrome (OMIM 301040), with diagnostic features of severe cognitive deficits, microcephaly, seizures, short stature, developmental delay, and α-thalassemia, without increased neoplasia incidence (Gibbons and Higgs, 2000; Gibbons et al., 1995a; Gibbons et al., 1995b). Thus, inactivating mutations of the *ATRX* gene during development are likely embryonic lethal, while diminished ATRX activity leads to severe developmental and cognitive abnormalities. *ATRX* somatic inactivating mutations that arise later and consequently bypass embryonic development appear to drive cancer progression, particularly in the CNS.

The cellular functions of ATRX are not completely clear; however, several lines of evidence point to a role in the maintenance of genomic integrity. Decreasing levels of ATRX by RNA interference induces spindle defects, as well as chromosomal alignment and segregation problems during mitosis and meiosis, and complete loss of ATRX in neuroprogenitor cells (NPCs) can also induce mitotic abnormalities (Baumann et al., 2010; De La Fuente et al., 2004; Ritchie et al., 2008; Seah et al., 2008). Furthermore, ATRX-depleted mouse embryonic stem cells have unstable telomeres (Wong et al., 2010). The ATRX protein can bind to specific genomic sites, including imprinting control regions and the *Rhox5* promoter (Bagheri-Fam et al., 2011; Kernohan et al., 2010), but is also highly enriched at repetitive regions, including ribosomal DNA repeats, pericentromeric heterochromatin, and telomeric chromatin, where it was proposed to

cooperate with DAXX to incorporate the histone variant H3.3 into chromatin (Baumann et al., 2008; Berube et al., 2000; Eustermann et al., 2011; Lewis et al., 2010; McDowell et al., 1999; Wong et al., 2010).

In the present report, we demonstrate that ATRX deletion in NPCs causes excessive replication–related DNA damage, which is exacerbated by loss of p53, replication stress–inducing drugs, and telomestatin (TMS), a G-quadruplex (G4) ligand. Unexpectedly, mutant mice lacking ATRX in the forebrain and pituitary had low circulating IGF-1, thyroxine (T4), and glucose levels and displayed degenerative phenotypes previously described in mouse models of progeria. Collectively, our data reveal that ATRX protects cells from replicative stress–induced DNA damage and telomeric fusions and that loss of ATRX in the embryonic CNS and anterior pituitary elicits systemic endocrine and metabolic abnormalities.

2.2 Materials and Methods

2.2.1 Mouse husbandry, genotyping, and tissue preparation

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 et al., 2005; Garrick et al., 2006). $Atrx^{loxP}$ mice, when mated to mice expressing Cre recombinase under the control of the $Foxg1$ promoter ($Foxg1^{KiCre/+}$) (Hebert and McConnell, 2000), produce male progeny ($Atrx^{loxP/Y}Foxg1^{KiCre/+}$) with $Atrx$ deficiency in the forebrain ($Atrx^{Foxg1Cre}$). To account for heterozygosity of $Foxg1$ due to knock-in of the Cre recombinase gene, Cre^+ males were used as controls. $Trp53^{tm1Tyj/J}$ mice were obtained from The Jackson Laboratory, and the mutant allele was introduced into the $Atrx^{loxP}$ and $Foxg1^{KiCre/+}$ mice. Subsequent mating of this progeny yielded mice that are $p53$-null in all tissues and $Atrx/p53$ double-null in the forebrain. For timed matings, midday of discovery of the vaginal plug was considered to be E0.5. Yolk sac or tail DNA was used for genotyping. $Atrx$, Cre, and $p53$ genotyping was performed by PCR as previously described (Berube et al., 2005; Seah et al., 2008).

2.2.2 Immunofluorescence and antibodies

Embryos were fixed overnight in 4% paraformaldehyde, washed 3 times in 1× PBS for 5 minutes each, and dehydrated in 30% sucrose PBS. Postnatal mice were perfused with 4% paraformaldehyde before PBS washes and dehydration. Brains were flash-frozen in liquid nitrogen using Cryomatrix (Thermo Scientific) cryoprotectant and sectioned as described previously (Berube et al., 2002). 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 and incubated with primary antibody overnight at 4°C. For detection of PCNA, primary antibody was incubated in 10 U/ml DNase I (Sigma-Aldrich) as previously described (Seah et al., 2008). Slides were washed in PBS and incubated with secondary antibody for 1 hour. Sections were counterstained with DAPI (Sigma-Aldrich) and mounted with Vectashield (Vector Laboratories) or SlowFade Gold (Invitrogen). For immunofluorescence staining of primary cortical culture, cells were incubated in blocking solution (PBS with 0.3% Triton-X and 5% normal goat serum) for 30 minutes and subsequently incubated with primary antibody overnight at 4°C. Cells were washed in PBS and incubated with secondary antibody for 1 hour. Cells were counterstained with DAPI and mounted with Vectashield.

The following primary antibodies were used: anti-ATRX, rabbit polyclonal (1:200; Santa Cruz Biotechnology Inc.); anti–cleaved caspase-3 (Asp175), rabbit monoclonal (1:400; Cell Signaling Technology); anti-γH2AX (Ser139), rabbit (1:100; Cell Signaling Technology); anti-γH2AX (Ser139), mouse monoclonal (1:400; Millipore); and anti-PCNA, mouse monoclonal (1:100; Santa Cruz Biotechnology Inc.). The secondary antibodies used were goat anti-rabbit–Alexa Fluor 594 (1:800 dilution; Molecular Probes) and goat anti-mouse–Alexa Fluor 488 (1:800 dilution; Molecular Probes).

2.2.3 Microscopy

Experiments demonstrating colocalization of γH2AX and PCNA, γH2AX and major satellite repeats, and γH2AX and telomeres were captured using a laser scanning confocal microscope (FV1000, Olympus). z-Stacks were obtained at 0.25-µm z intervals generally

spanning 10–20 μm. Overlapping signal was scored as a colocalization event. For colocalization of γH2AX and PCNA, only cells in which PCNA staining was characteristic of late S phase were counted. Each cell ($n = 300$) was scored for the number of γH2AX foci that overlapped with PCNA foci divided by the total number of γH2AX foci per cell, to obtain a measure of the cellular levels of DNA damage (γH2AX) at late-replicating chromatin. All other 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 and automated image capture, and processing was performed using Volocity software (PerkinElmer). For quantification of γH2AX foci per area, at least 6 serial cortical cryosections were assessed for γH2AX foci within the indicated regions. To obtain a relative measure of DNA damage, the ratio of γH2AX foci to area (mm^2) was calculated. The same method was used to quantify the ratio of cleaved capsase-3^+ and γH2AX$^+$ cells, and for this experiment a Student's t test was used to compare only the proportion of caspase-3^+γH2AX$^+$ cells between control and *Atrx*-null mice.

2.2.4 Western blot analysis

Nuclear protein was extracted from E13.5 telencephalon using a standard extraction kit (Thermo Scientific) and quantified using a Bradford assay. Protein (10 μg) was resolved on 6% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were probed with anti-ATM, rabbit monoclonal (1:500, Millipore); anti–phospho-ATM (S1981), mouse monoclonal (1:250, Rockland); anti-ATR, goat polyclonal (1:250, Santa Cruz Biotechnology Inc.); anti–phospho-ATR (S428), rabbit polyclonal (1:250, Santa Cruz Biotechnology Inc.); and anti-INCENP, rabbit polyclonal (1:10,000, Sigma-Aldrich) antibodies, followed by the appropriate horseradish peroxidase–conjugated secondary antibodies (1:4,000, GE Healthcare). The membrane was incubated in ECL before exposure to X-ray film.

2.2.5 Primary NPC cultures and manipulation

Cortical progenitor cultures were prepared as described previously (Gloster et al., 1999; Slack et al., 1998) using cortices dissected from E13.5 embryos. Cells were seeded on

polyornithine-coated (Sigma-Aldrich) plastic plates or glass coverslips. Primary cortical cultures were treated with acute γ-irradiation (0, 1, 5, and 10 Gy) at 2 days after plating using a Cobalt-60 irradiator (Theratron 60, Atomic Energy of Canada Limited) located in the London Regional Cancer Center, London, Ontario, Canada. Cells were fixed in 4% paraformaldehyde for 10 minutes, washed in PBS, and stored in PBS at 4°C at 0, 3, and 6 hours after treatment and processed for immunofluorescence. HU (Sigma-Aldrich), MMC (Sigma-Aldrich), γ-irradiation, and TMS (synthesized in the Biomedicinal Information Research Center, National Institute of Advanced Industrial Science and Technology, Tokyo, Japan) sensitivity assays in cultured NPCs were conducted 48 hours after plating cells. NPC cultures were treated with appropriate doses of HU, γ-irradiation, or TMS for the indicated length of time, and cell viability was measured using the trypan blue dye exclusion method. Cell counts were determined with a hemacytometer.

2.2.6 FISH

For DNA-FISH experiments, primary cortical cultures were permeabilized in CSK buffer with 0.5% Triton-X (Sigma-Aldrich) prior to fixation. For TelFISH, a fluorophore-labeled DNA oligonucleotide probe, TAACCC7-Alexa 488-3′ (Integrated DNA Technologies), was dissolved at 1 pmol/μl in hybridization buffer. Hybridization was performed as previously described (Zhang et al., 2009). For major satellite FISH, immunofluorescence was performed first, followed by fixation with 4% paraformaldehyde for 10 minutes and subsequent hybridization with a major satellite repeat probe as previously described (Isaac et al., 2006).

2.2.7 MEF cultures and manipulations

E13.5 embryos were minced, digested in trypsin for 45 minutes at 37°C, and resuspended in DMEM (Sigma-Aldrich) containing 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM l-glutamine. Primary $Atrx^{loxP/Y}$ and wild-type MEFs were seeded at 1.5×10^6 cells per 6-well plate and transduced with adenovirus expressing Cre recombinase and a GFP reporter gene (Ad-CreGFP) or GFP only (Ad-GFP control) approximately 12 hours later. Three days after transduction, MEFs were counted and reseeded at 1.5×10^6 cells per 6-well plate (P5/P0). HU sensitivity in MEFs was

determined by transducing 1.5×10^6 cells with adenovirus, culturing for 72 hours, and treating cells with appropriate doses of HU for 24 hours, replacing medium, and measuring viability via trypan blue dye exclusion 2 days later. Sensitivity to γ-irradiation in MEFs was assessed by transducing 1.5×10^6 cells with adenovirus, waiting 72 hours, and treating cells with appropriate doses of γ-irradiation and measuring viability via trypan blue dye exclusion 48 hours later.

2.2.8 qRT-PCR

Total RNA was obtained from control and cKO liver with the RNeasy Mini Kit (QIAGEN) and reverse transcribed into cDNA as described previously (Kernohan et al., 2010). 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, 55°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 triplicate for each sample. Conditions for amplification were as follows: 35 cycles of 95°C for 10 seconds, 55°C for 20 seconds, 72°C for 30 seconds, and a final melting curve generated in increments of 0.5°C per plate read. Standard curves were generated for each primer pair with 3-fold serial dilutions of control cDNA. Primer efficiency was calculated as: $E = (10[-1/\text{slope}] - 1) * 100\%$, where a desirable slope is –3.32 and $r^2 > 0.990$. All data were corrected against *Gapdh* as an internal control. Error bars represent SEM.

2.2.9 Alcian blue and alizarin red staining

Skinned and eviscerated mouse carcasses at P17 were fixed overnight in 95% ethanol and transferred to acetone. Fixed skeletons were stained in alizarin red and alcian blue, as described previously (Wang et al., 2007). Images were captured using an Olympus SP-57OUZ digital camera. Skeletal elements from at least 4 different littermate pairs were imaged using a Zeiss Stereo Zoom Microscope Stemi SV6, and measurements of individual bones were undertaken with a ruler accurate to 0.1 mm.

2.2.10   microCT

Mice were euthanized on P17, skinned, eviscerated, and stored in formalin. Whole skeletons were scanned on a GE Locus Ultra scanner at 120 kV and 20 mA, with a 0.154-mm^3 voxel resolution with a total of 900 slices per scan. To assess bone density, hind limbs were scanned on a GE Locus scanner at 80 kV and 0.450 mA with a 0.020-mm^3 voxel resolution with 900 slices per scan. BMD, cortical thickness, and trabecular numbers were calculated using MicroView 3D visualization and analysis software (version 2.1.2, GE Healthcare Biosciences) (Ulici et al., 2008). BMD was determined from proximal tibias using the bone analysis module within MicroView by defining the region of interest (ROI) as the area between 0.3 mm and 0.6 mm proximal to the growth plate to include the trabecular spongiosa. The ROI was a cylinder fitting within the central region of the trabecular bone, excluding the cortical bone. Cortical bone–mimicking epoxy (SB3, Gammex RMI) was used for calibration. Trabecular number was determined using the stereology function within MicroView, using the same ROI as used for BMD. Cortical thickness was determined at the tibial mid-diaphysis, defined as the distance halfway between the two ends of the tibia. Thickness of the bone was measured radially in each quadrant and averaged.

2.2.11   Picosirius red staining of bone sections

Fore- and hind limbs were dissected at P0, P7, P10, and/or P17 upon sacrifice and processed for histological analyses by 0.1% picrosirius red (Sigma-Aldrich, catalog P6744-16A) as described previously (Yan et al., 2012). Trabecular area (μm^2) within an area of interest (AOI) was measured by observers blinded to the experimental protocol using Openlab 4.0.4 software (Improvision), with the AOI set from just below the hypertrophic chondrocytes to 200 μm below the growth plate in the trabecular bone area of the mineralized zone. The AOI measurements were adapted from (O'Connor et al., 2009; Sawyer et al., 2003). Trabecular area was normalized to total AOI and expressed as a percentage. All images were taken with a Retiga EX camera (Leeds Precision Instruments Inc.) connected to a DMRA2 microscope (Leica).

2.2.12   Ex vivo tibia cultures

Tibiae were isolated from P0 mutant and control mice using a Zeiss Stereo Zoom Microscope Stemi SV6 and dissection tools, as described previously (Agoston et al., 2007). Isolated tibiae were plated in 12-well culture dishes and cultured with 1 ml per well of sterile organ culture α-MEM medium (Gibco, Life Technologies, catalog 1257063) containing ascorbic acid (0.005%, Sigma-Aldrich, A4034), β-glycerophosphate (0.02%, Sigma-Aldrich, 819830), BSA (0.2%, Fisher Scientific, 9048486), 200 nM glutamine (0.25 ml, Gibco, Life Technologies, 25030081), and penicillin streptomycin (0.4 ml, Gibco, Life Technologies, 15140122) for 7 days at 37°C, 5% CO_2. Organ culture medium was refreshed on days 2, 4, and 6. Tibia samples were fixed in 70% ethanol overnight at 4°C on day 7. Samples were processed, embedded in paraffin, and sectioned at the Molecular Pathology Core Facility, Robarts Research Institute, London, Ontario, Canada. Tibia lengths were measured on days 0 and 7 using the Zeiss Stereo Zoom Microscope Stemi SV6 with a ruler accurate to 0.1 mm.

2.2.13   Measurements of IGF-1, GH, T4, and glucose

Plasma and liver samples were collected from P17 mice. Blood was collected from the inferior vena cava. EDTA pH 7.0 was added to the blood sample and centrifuged at 21,000 g for 10 minutes at 4°C. Plasma supernatant was collected and kept frozen at − 80°C. Liver samples were collected and homogenized by 2 freeze-thaw cycles. Liver homogenate supernatant was collected and assayed immediately. Plasma and liver IGF-1 content were measured using a mouse IGF-1 ELISA kit (R&D Systems, catalog MG100). Plasma GH and T4 were assayed using Millipore (EZRMGH-45K) and Calbiotech (T4044T) ELISA kits, respectively, according to the manufacturers' instructions. Blood glucose levels were measured immediately prior to sacrifice using the OneTouch Fast*Take* Meter according to the manufacturer's instructions.

2.2.14   Cell cycle profiling

Primary NPCs were cultured for two days and pulse-labeled with cell proliferation labeling reagent [10 mM bromodeoxyuridine (BrdU) and 1 mM fluorodeoxyuridine (FdU) in H_2O] at 5 μl/ml media (GE Healthcare Life Sciences), fixed, and stained with

propidium iodide (PI). The proportion of cells in each phase of the cell cycle was determined by flow cytometry on a Beckman-Coulter Epics XL-MCL instrument, as described (Isaac et al., 2006).

2.2.15   Statistics

Statistical analysis was performed using GraphPad Prism software (4.02; 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). Statistical analyses of Kaplan-Meier survival curves were performed using the log-rank test and the Gehan-Breslow-Wilcoxon test. *P* values of 0.05 or less were considered to indicate significance.

2.2.16   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 Endogenous DNA damage accumulates in the *Atrx*-null NPCs

Atrx^{LoxP} female mice (Berube et al., 2005) were mated to *Foxg1Cre* male mice (Hebert and McConnell, 2000) to generate male progeny lacking ATRX in the embryonic forebrain starting at E8.5 (referred to as cKO mice). Since ATRX depletion can induce telomeric instability in embryonic stem cells (Wong et al., 2010), we stained brain cryosections from E13.5 embryos with antibodies for the phosphorylated histone variant H2AX (γH2AX), a canonical marker for double-stranded breaks (DSBs) (Rogakou et al., 1998). This analysis showed a high level of DNA damage throughout the cortex, hippocampal hem (future hippocampus), and basal ganglia in the cKO embryonic forebrain that was significantly increased compared with that in controls (Figure 2-1a). The level of DNA damage was appreciably lower in the neonatal brain; nevertheless, comparatively higher levels of γH2AX were observed in the neonatal hippocampus, a region that is still proliferative at this stage of development, as well as in the cortex of

mutants (Figure 2-1b). Thus, the pattern of DNA damage was generally confined to proliferative areas of the developing brain. To determine whether DNA damage incurred during the embryonic period persists in postmitotic cells, we examined γH2AX and ATRX staining patterns in P7 control and cKO forebrain cryosections. This analysis demonstrated that ATRX is indeed not expressed at the protein level and revealed that no damage was present or remained unrepaired in the absence of ATRX in the postnatal juvenile brain (Supplemental Figure 2-8a,b). Given the post-replicative state of the brain at this time, these data suggest that endogenous DNA damage due to loss of ATRX occurs primarily in proliferating cells in the embryonic and perinatal period.

2.3.2 Accumulation of DNA damage in *Atrx*-null NPCs induces ATM activation and is exacerbated by p53 deletion

Persistent DNA damage can result in the phosphorylation and upregulation of p53 by the ATR/Chk1 or ATM/Chk2 pathways, leading to cell cycle arrest or apoptosis (Roos and Kaina, 2006; Shiloh, 2001). We previously demonstrated that loss of ATRX results in a p53-dependent apoptotic response in cultured primary mouse NPCs and in vivo in the forebrain at E13.5 (Seah et al., 2008). To determine whether accumulated DNA damage could be the underlying cause of apoptosis, we performed double immunofluorescence of γH2AX and cleaved caspase-3, a marker of apoptotic cells. The majority of cells undergoing apoptosis (i.e., cleaved caspase-3$^+$ cells) in the cKO telencephalon also stained positive for γH2AX, indicating that cell death is likely a downstream consequence of DNA damage (Figure 2-1c). To substantiate this conclusion and examine the role of p53, we generated ATRX/p53 compound mutant mice and assessed levels of DNA DSBs in the brain. γH2AX signal in cKO;*p53*$^{-/-}$ embryos was increased compared with that in cKO mice, suggesting that the accumulation of DNA damage in the ATRX-deficient forebrain directly triggers a p53-dependent apoptotic response and that the absence of p53 prevents the removal of these cells by apoptosis (Figure 2-1a,b; right panels and graphs). We next explored the possible involvement of ATR and ATM signaling in the response to increased DNA damage. Western blot analysis of control and cKO E13.5 telencephalon protein extracts demonstrated similar activation of ATR in control and cKO samples. On the other hand, a clear increase in ATM activation was

observed in the cKO embryonic telencephalon (Figure 2-1d). Taken together, these findings show that loss of ATRX in the embryonic brain results in the accumulation of DNA damage, which triggers activation of ATM and p53, culminating in neuroprogenitor cell death.
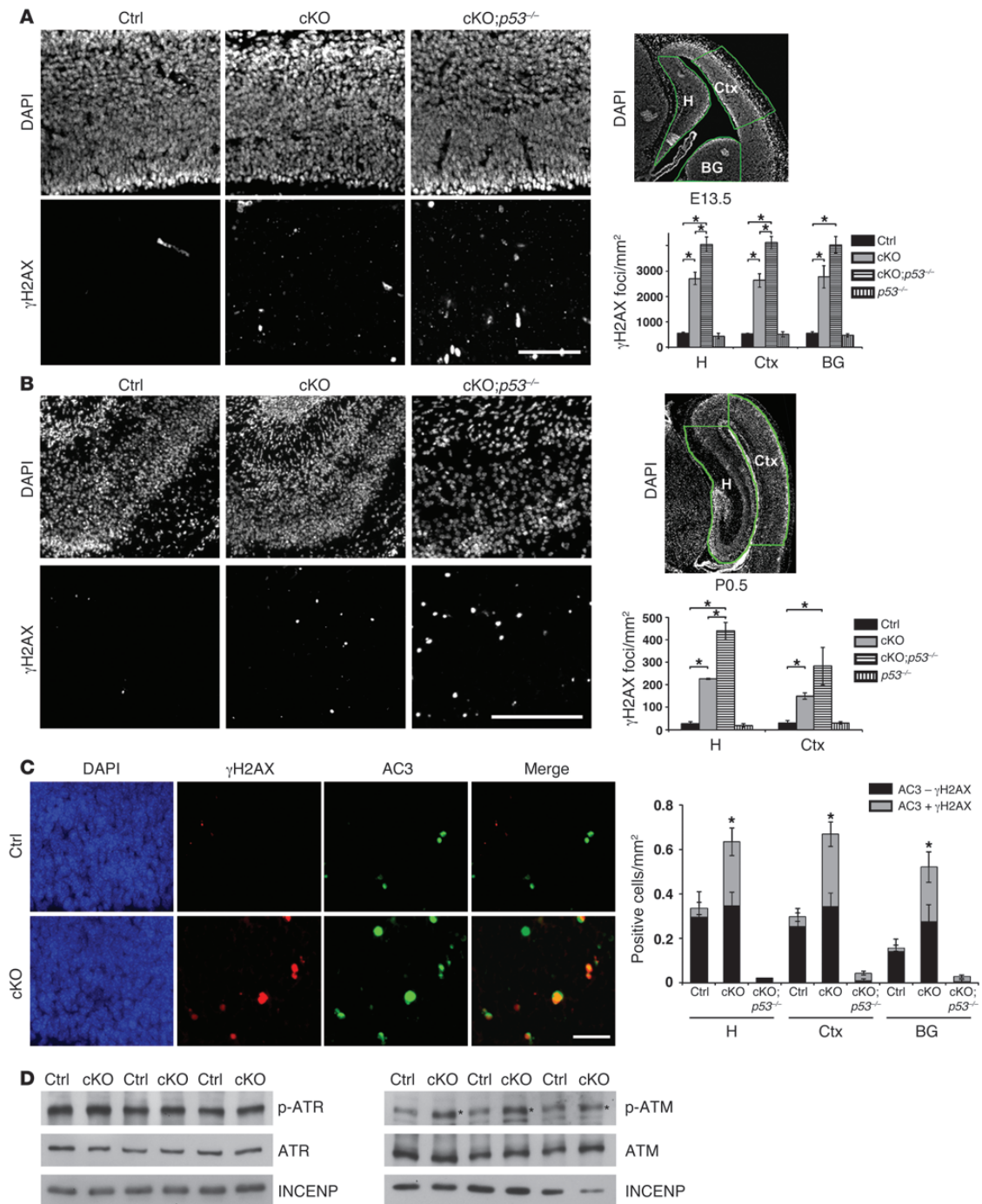
Figure 2-1 Increased DNA damage leads to ATM activation and p53-dependent apoptosis in the *Atrx*-null embryonic brain

(A) Immunostaining for γH2AX in E13.5 control (Ctrl), cKO, and cKO;*p53*−/− compound mutant cortical cryosections. Scale bar: 100 μm. DAPI staining of E13.5 forebrain highlights in green the hippocampal hem (H), cortex (Ctx), and basal ganglia

(BG) regions where γH2AX foci per unit area were scored. Control, cKO, and cKO;*p53*$^{-/-}$ ($n = 3$); *p53*$^{-/-}$ ($n = 2$). (**B**) γH2AX staining in P0.5 control, cKO, and cKO;*p53*$^{-/-}$ cortical cryosections. Scale bar: 200 μm. DAPI staining of P0.5 forebrain highlights in green the hippocampus (H) and cortex (Ctx) regions where γH2AX foci per unit area were scored. Control and cKO ($n = 3$); cKO;*p53*$^{-/-}$ and *p53*$^{-/-}$ ($n = 2$). (**C**) Co-immunofluorescence detection of γH2AX (red) and activated caspase-3 (AC3; green) in E13.5 cortical cryosections. Scale bar: 30 μm. AC3^{+} cells were scored for the presence (AC3 + γH2AX) or absence (AC3 – γH2AX) of DNA damage ($n = 3$). (**D**) Western blot analysis of nuclear protein extracts obtained from E13.5 telencephalon ($n = 3$). While levels of ATR and phospho-ATR were not increased (left panels), phospho-ATM was noticeably increased in the cKO extracts compared with controls (indicated by an asterisk). Original magnification, ×100 (**A** and **B**); ×200 (**C**). *$P < 0.05$.

2.3.3 DNA damage response at telomeres and telomeric DNA end fusions

To determine the specific genomic sites of DNA damage response incurred by loss of ATRX, we examined telomeres, since ATRX enrichment has been previously reported at these genomic regions. However, since it has been proposed that ATRX associates with telomeres only in pluripotent embryonic stem cells (Wong et al., 2010), we first assessed whether ATRX protein is present at telomeres in NPCs. ATRX immunofluorescence performed in conjunction with telomere FISH (Tel-FISH) in control NPCs demonstrated colocalization of ATRX and a subset of telomeres (Figure 2-2a). Next, we used Tel-FISH in combination with γH2AX immunostaining to determine whether the DNA repair machinery is activated at telomeric chromatin in cultured cKO NPCs. Confocal imaging showed that the number of telomere dysfunction–induced foci (TIFs) per nucleus was increased in the cKO compared with control NPCs (Figure 2-2b). In fact, 66.1% of all γH2AX foci were observed at telomeres in the cKO cells (Supplemental Figure 2-9b). ATRX protein also binds pericentromeric heterochromatin, and we repeated this experiment using major satellite-specific labeled DNA probes (MSat-FISH). Again, we found that $γH2AX^+/mSAT^+$ foci were more frequent in cKO NPCs and that a large proportion of DNA damage foci (69.51% ± 1.27%) corresponded to pericentromeric heterochromatin in cKO NPCs (Supplemental Figure 2-9a,b). These numbers reflect the acrocentric nature of mouse chromosomes, with some γH2AX foci overlapping with the p arm telomere and the adjacent pericentromeric region. Our results demonstrate that heterochromatic regions of the genome are prone to DNA damage in the absence of ATRX. To further explore the function of ATRX in genome stability, we examined metaphase spreads from control and cKO NPC cultures. ATRX immunostaining of control metaphase spreads revealed strong enrichment of the protein at pericentromeric heterochromatin and at some telomeres (Supplemental Figure 2-9c). Analysis of DAPI-stained spreads revealed an increased frequency of chromosome fusions in cKO metaphase spreads compared with controls, as evidenced by overlapping chromosomes (Figure 2-2c). Centromere-to-centromere, centromere–to–q arm, and q arm–to–q arm fusions were observed. The number of chromosomes per metaphase spread was not affected in cKO NPCs (Supplemental Figure 2-9d). To further explore the nature of the

fusions, we performed TelFISH on control and cKO metaphase spreads and detected an increased incidence of telomeric DNA end fusions (Figure 2-2d). Other common defects included telomere deletions or duplications, as well as merged or bridged telomeres (Figure 2-2e). Overall, the frequency of all telomere defects was more than doubled in the cKO NPCs compared with controls, and defects involved the p and q arm telomeres at an equal frequency (Supplemental Figure 2-9e).

2.3.4 ATRX is not required for the repair of DSBs

Accumulation of DNA DSBs could occur if ATRX plays a direct role in DSB repair mechanisms. Two non–mutually exclusive pathways carry out repair of DNA DSBs: homologous recombination (HR) in proliferative cells; and non-homologous end joining (NHEJ) in postmitotic cells (Shrivastav et al., 2008). Since γH2AX foci were primarily concentrated in proliferative zones of the developing brain, we reasoned that ATRX may be specifically involved in the repair of DSBs via HR. To address this possibility, we cultured primary NPCs for 2 days in N2 neurobasal medium and exposed proliferative NPCs to γ-irradiation to induce DSBs and activate the HR pathway. The DNA damage response (i.e., γH2AX signal accumulation) was measured between 0 and 6 hours after irradiation, when damage induced by low doses of γ-irradiation should be actively resolved in control cells (Supplemental Figure 2-10). Both control and cKO NPC colonies showed a dose-dependent increase in γH2AX signal 3 hours after irradiation, which was subsequently resolved (i.e., decreased γH2AX staining) by 6 hours after treatment (Supplemental Figure 2-10). Therefore, we concluded that ATRX is not required for repair of exogenous irradiation–induced DSBs.
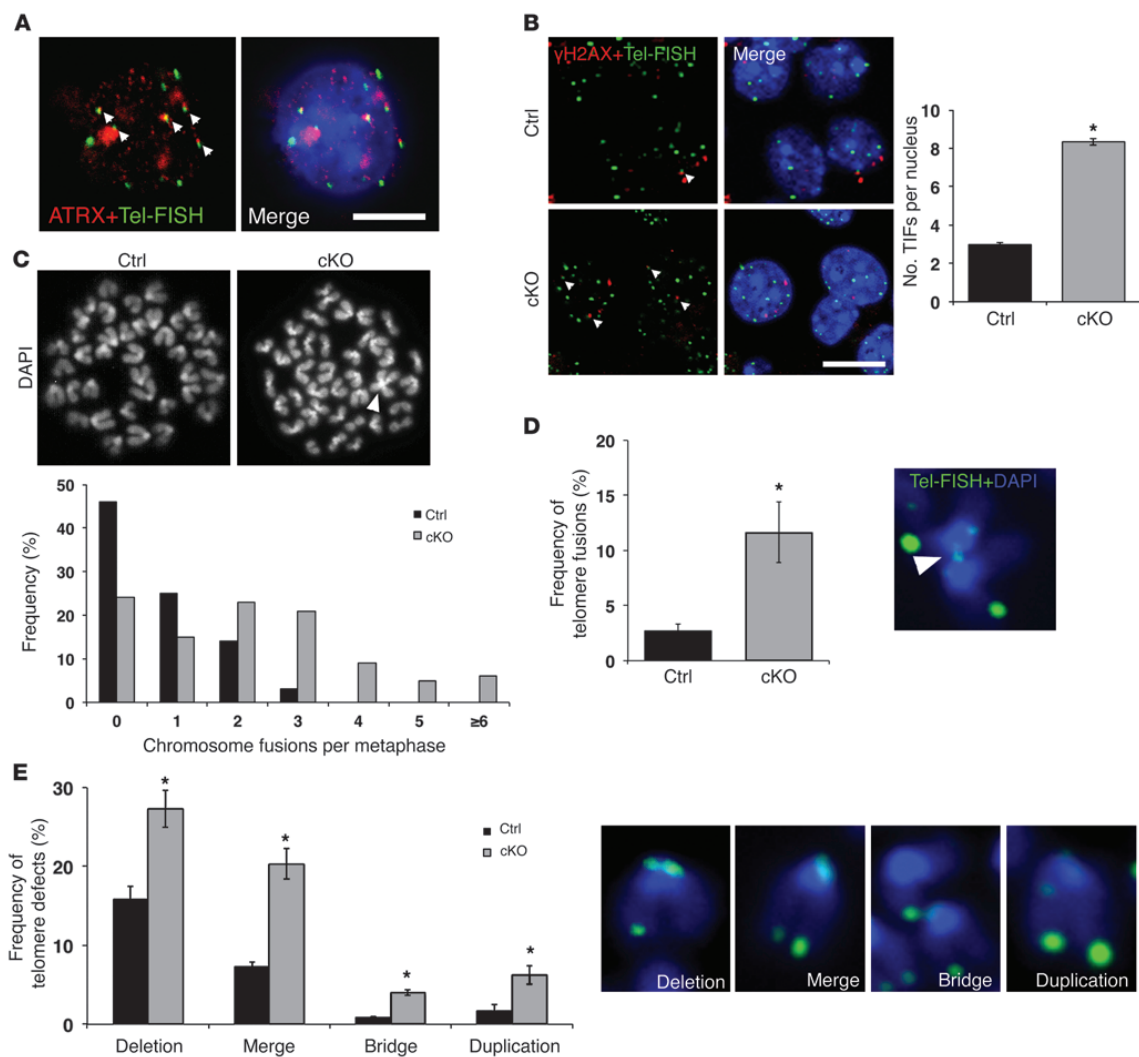
Figure 2-2 Increased DNA damage and telomere defects in cKO NPCs

(A) Confocal immuno-FISH images of ATRX (red) and telomeres (Tel-FISH; green) in NPCs demonstrates colocalization of the ATRX protein with a subset of telomeres. Scale bar: 5 μm. (B) Confocal immuno-FISH images of γH2AX (red) and telomeres (Tel-FISH; green) shows increased incidence of TIF (γH2AX/Tel-FISH colocalization) in cKO compared with control NPCs (300 nuclei counted, $n = 3$ control/cKO littermate-matched pairs). Scale bar: 10 μm. (C) DAPI staining of control and cKO metaphase spreads shows representative chromosome fusion in cKO NPC metaphase (arrowhead). Frequency of fusions per metaphase was increased in cKO metaphases compared with control (control: 88 metaphases, cKO: 108 metaphases counted, $n = 3$). (D) Tel-FISH (green) demonstrates increased telomeric fusions in cKO metaphase chromosomes compared with control (1,475 chromosomes counted; $n = 3$). (E) Telomere defects (deletion, merge, bridge, and duplication) were scored in control and cKO metaphase

chromosomes. Representative images of defects appear to the right of quantification. In all cases, cKO chromosomes showed an increase in telomeric defects compared with control (1,475 chromosomes counted; $n = 3$). Original magnification, x1,000 (A and B); x630 (C–E). *$P < 0.05$.

2.3.5 ATRX protects cells from replication stress

Since ATRX does not appear to function in the repair of DSBs, we reasoned that it might help prevent DNA damage to preserve genomic integrity. Given that damage is restricted to proliferating regions of the cKO embryonic brain, we tested the possibility that cKO NPCs are particularly sensitive to DNA damage during S phase. We inactivated ATRX in *Atrx^{loxP}* mouse embryonic fibroblasts (MEFs) with adenoviral delivery of Cre recombinase and treated them with hydroxyurea (HU), a drug that depletes deoxyribonucleotide pools, inhibits DNA synthesis, and causes replication fork stalling (Koc et al., 2004). MEFs were treated with increasing doses of HU for 24 hours, and cell viability was assessed 2 days later. MEFs lacking ATRX (Ad-CreGFP) were more sensitive to HU than untreated cells or cells treated with Ad-GFP. Ad-CreGFP treated MEFs were not more sensitive to γ-irradiation than Ad-GFP–transduced or untransduced cells (Figure 2-3a). We also treated control and cKO NPCs with HU and mitomycin C (MMC), a DNA crosslinker that also causes replication fork stalling. Both HU and MMC treatment caused decreased viability of cKO NPCs compared with control cells. As in MEFs, exposure to various doses of γ-irradiation did not alter cKO NPC viability compared with control NPCs, demonstrating a specific sensitivity to drugs that induce replication fork stalling (Figure 2-3b).

Blocked replication forks could be a source of genomic instability in the cKO cells, since they can lead to collapse of the replisome and the formation of DSBs (Cahill et al., 2006). Replicating cortical NPCs lacking ATRX protein were assessed for DNA damage (γH2AX) at replication foci using an anti-PCNA antibody and imaged using confocal microscopy (Figure 2-3c). PCNA is a component of the replication machinery and exhibits a distinct staining pattern during S phase. In late S phase, the replication foci are observed in regions of the nucleus containing heterochromatin and as such colocalize with DAPI-bright heterochromatin bundles (Takanari et al., 1994). *Atrx*-null NPCs accumulated more DNA damage at these late replication foci compared with control cells, confirming an increase in DSBs during the replication process (Figure 2-3c). We also performed FACs analysis to determine whether S phase is extended in these cells. We detected a small but significant decrease in the G1 population and a modest increase

in S phase population in the cKO NPCs at 4 days in vitro. This suggests that difficulties in replicating heterochromatin result in a slight delay in S phase progression of cKO cells (Supplemental Figure 2-10b). We conclude from these findings that loss of ATRX results in an accumulation of endogenous replicative damage in cultured NPCs, specifically at late-replicating chromatin.

2.3.6 G4-DNA stabilization exacerbates DNA damage and decreases viability of *Atrx*-null NPCs

A potential function of ATRX is to stabilize stalled forks or facilitate the replication of complex secondary DNA structures, such as G4-DNA, which are found at telomeres, among other genomic sites (Wang and Patel, 1992). To test whether ATRX facilitates the replication of G4-DNA, we treated control and *Atrx*-null NPCs with TMS, a natural product isolated from *Streptomyces anulatus* that binds with high affinity to G4 structures (Shin-ya et al., 2001). Neuroprogenitors dissected from control and cKO embryonic forebrain were cultured and treated with 20 μM TMS for 24 hours. γH2AX staining was increased (Figure 2-3d), and cell viability was decreased (Figure 2-3e) in the TMS-treated cKO NPCs compared with TMS-treated control cells. These findings suggest that ATRX deficiency in NPCs synergizes with G4-DNA stabilization to cause DNA damage and cell death.
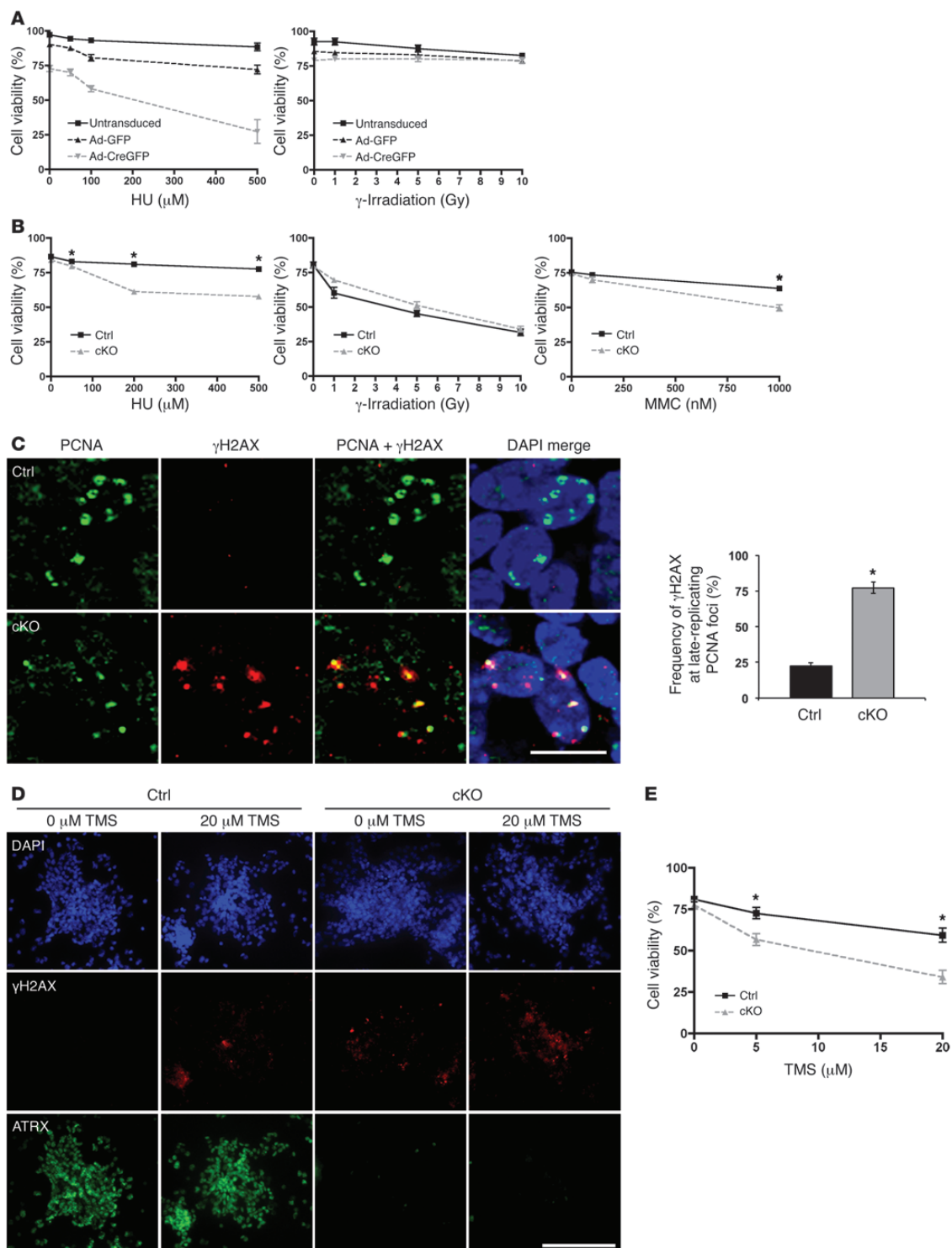
Figure 2-3 ATRX-deficient cells are hypersensitive to replication stress-inducing agents and the G4-DNA ligand TMS

(A) *Atrx^{loxP/Y}* MEFs were untransduced or transduced with adenovirus expressing Cre

recombinase fused to GFP (Ad-CreGFP) or Ad-GFP and subsequently treated with HU for 24 hours or γ-irradiated at the indicated doses. Cell viability was measured at 24 hours after HU treatment ($n = 4$) and at 6 hours after irradiation ($n = 3$) via trypan blue dye exclusion. (B) Control and cKO NPCs were treated with HU or MMC for 24 hours or γ-irradiated at the indicated doses. Cell viability was measured at 24 hours after HU and MMC treatment and at 6 hours after irradiation ($n = 3$). (C) Co-immunofluorescence detection of PCNA, a marker of replication foci, and γH2AX in control and cKO E13.5 cortical cryosections. Results were quantified by measuring the ratio of γH2AX staining that localized to late-replicating PCNA foci to total γH2AX staining per cell, to account for overall lower levels of γH2AX signal in control cells (300 nuclei counted, $n = 3$). Scale bar: 12 μm. (D) Control and cKO NPCs were treated with 20 μm TMS for 2 hours, and γH2AX signal was imaged 6 hours after treatment. Scale bar: 70 μm. (E) Control and cKO NPCs were treated with TMS for 24 hours, and cell viability was measured 24 hours after treatment ($n = 3$). Original magnification, ×600 (C); ×100 (D). *$P < 0.05$.

2.3.7 Loss of ATRX shortens life span and induces progeroid-like phenotypes

While a portion of mutant pups die in the neonatal period (Berube et al., 2005), many cKO male mice survived, but rarely longer than 30 days after birth (P30), with an average life span of 22.7 ± 1.7 days (Figure 2-4a). Moistening of food pellets to help with intake of solids did not improve life span, nor did the removal of siblings from the litter to reduce competition for breast milk (Figure 2-4a). Mutant mice at P20 had milk in the stomach, suggesting that starvation was not the cause of death or growth retardation. P17 cKO mice had severely stunted growth compared with control littermates, as determined by measurements of body weight and length, as well as length of various skeletal elements (Figure 2-4b-e). Reduced body size was apparent at birth (Berube et al., 2005), but a more dramatic effect was seen from P5 onward. The tibia, femur, humerus, radius, ulna, and foot were significantly shorter in the cKO mice at P17 (Figure 2-4e). We confirmed specific deletion of ATRX in the forebrain of cKO mice at several postnatal time points, while ATRX remained expressed in other tissues, such as the liver, heart, thymus, spleen, testes, and skeletal growth plate of cKO mice (Supplemental Figure 2-11a-d).

Microcomputed tomography (microCT) analyses showed that cKO mutant mice displayed kyphosis, an abnormal curvature of the spinal column (Figure 2-5a), as well as decreased bone mineral density (BMD), lower trabecular number, and reduced bone cortical thickness (Figure 2-5a-d). Loss of trabecular bone was confirmed by picrosirius red staining of tibia, femur, and humerus of control and cKO mice at P17 (Figure 2-5e). To determine whether this effect stemmed from a developmental problem or was due to tissue degeneration, we also assessed trabecular bone at earlier ages. We found no difference in neonatal and P7 mice and detected an intermediate phenotype at P10, suggesting that bone development occurs initially normally, with evidence of deterioration from P10 onward (Supplemental Figure 2-11a).

Extensive loss of subcutaneous fat (<25% of controls) was a constant characteristic of the mutant mice, while thickness of the dermis was not significantly affected (Figure 2-5f). Again, by measuring subcutaneous fat at various time points, we observed that this

phenotype was exacerbated at P20 compared with P10 (Supplemental Figure 2-12b,c). Mutant mice rarely opened their eyes, and analysis of the lens showed the formation of cataracts (Figure 2-5g). Accounting for decreased total body weight of the cKO mice, the spleen and liver were consistently smaller and the heart was significantly enlarged at P20 (Figure 2-5h and Supplemental Figure 2-12d). These effects were also seen at P10 but were not as dramatic, as evidenced by a lack of significant difference between control and cKO organ size (Supplemental Figure 2-12d). The phenotypes described above strongly resemble those reported in several mutant mice displaying accelerated aging, but as the deterioration and death of the mice occurred rather quickly, it is difficult to completely distinguish between developmental and progeria-like phenotypes.
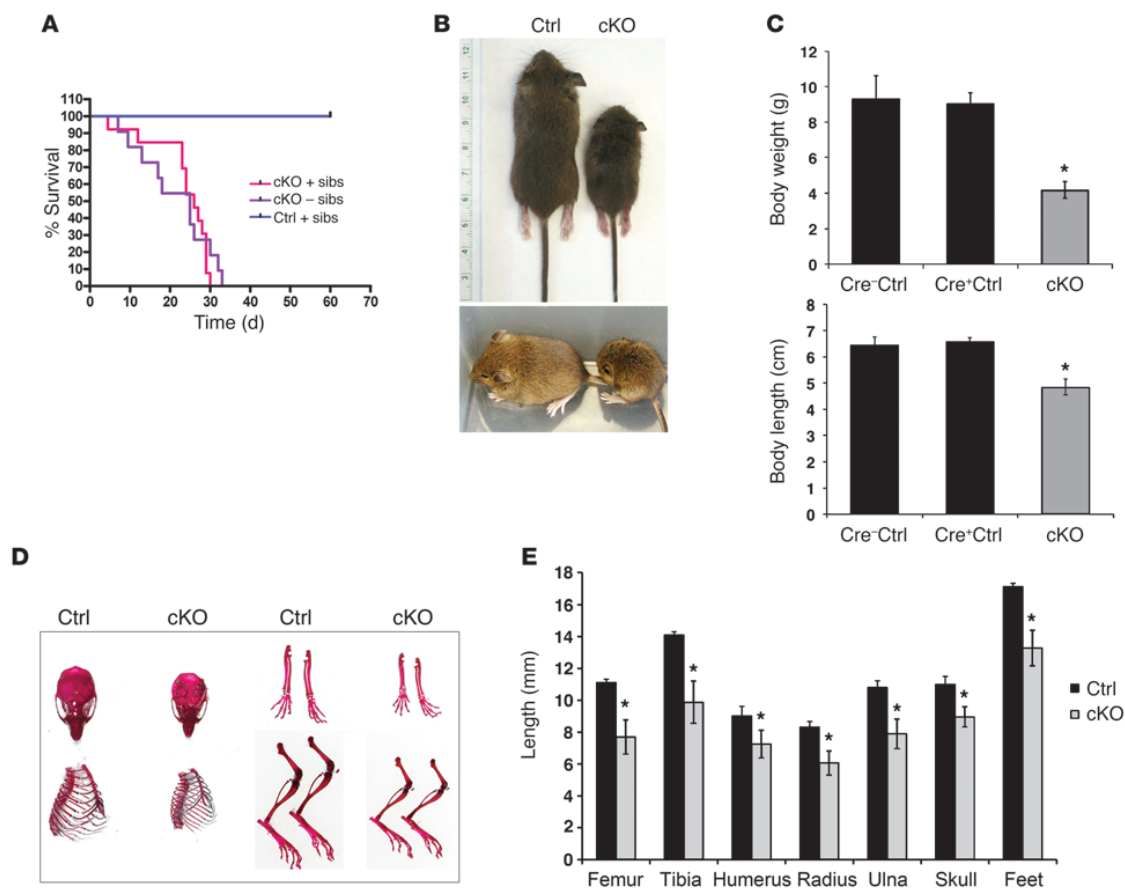
Figure 2-4 Reduced growth and life span in mice lacking ATRX in the forebrain

(A) Kaplan-Meier survival curve of Cre[+] control ($n = 12$) and cKO mice raised with (+sibs, $n = 13$) or without (–sibs, $n = 11$) siblings. Survival of cKO mice was significantly decreased compared with that of control mice ($P = 0.0001$). The survival of cKO mice was not significantly different whether they were raised with or without siblings ($P = 0.4974$). (B) Representative pictures of P17 control and cKO littermates, illustrating size difference of the mice. (C) Body weight (g) and length (cm) measurements of control (Cre[-], $n = 25$; Cre[+], $n = 8$) and cKO ($n = 24$) mice. No significant difference was observed between Cre[-] and Cre[+] control mice. (D) Skeletal elements of control and cKO mice were stained with alizarin red and alcian blue. (E) Length measurements of P17 control and cKO skeletal elements. ($n = 5$). *$P < 0.05$.
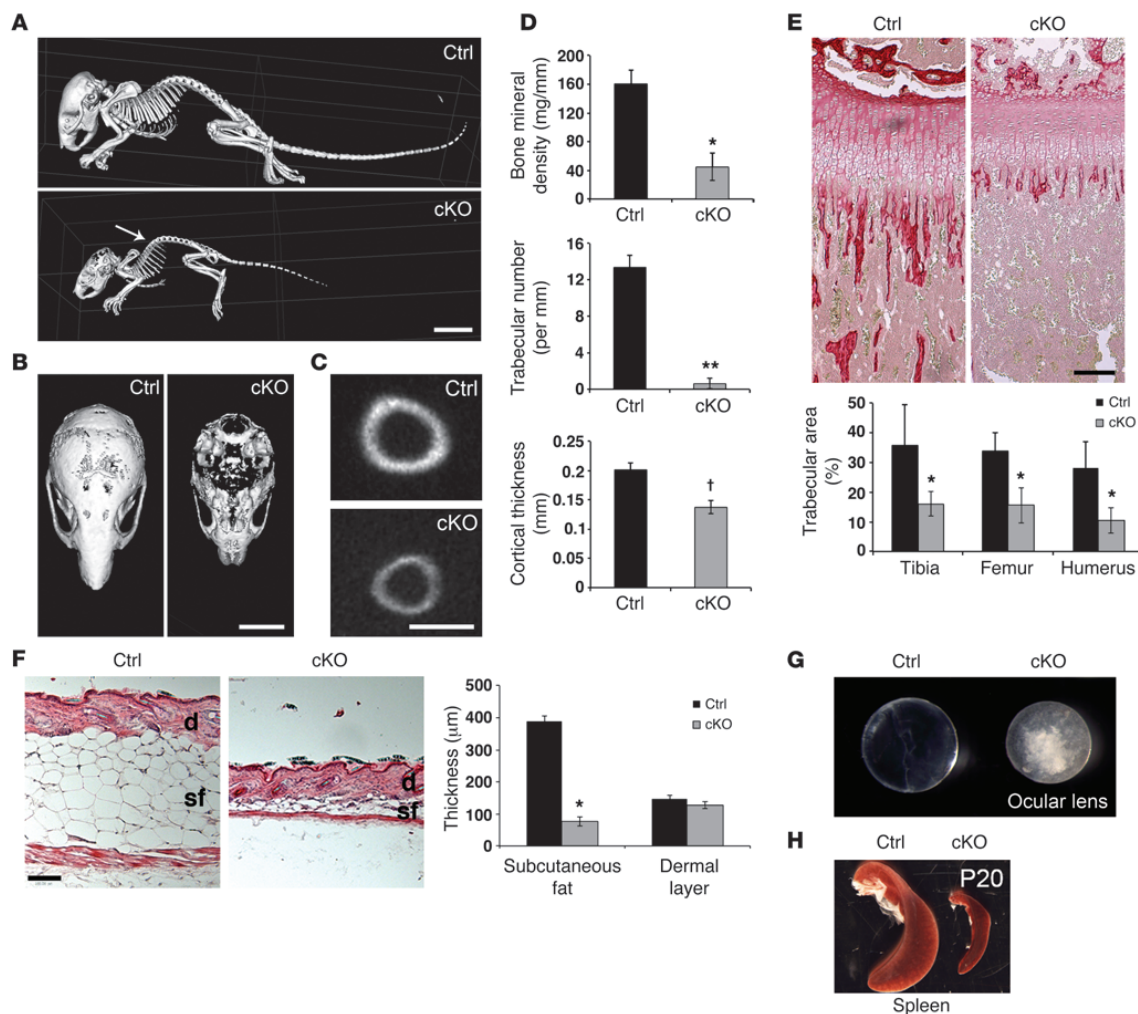
Figure 2-5 Postnatal phenotypes in *Atrx*-cKO mice

(A) Whole skeletal isosurface images of P17 control and cKO mice were generated using microCT. Arrow points to kyphosis. Scale bar: 10 mm. (B) Horizontal view of the cKO skull illustrates decreased bone mineralization. Scale bar: 5 mm. (C) Tibial cross section shows decreased cortical thickness. Scale bar: 1 mm. (D) Decreased BMD (*P = 0.002), trabecular number (**P = 0.002), and cortical thickness ($^\dagger P$ = 0.0001) in the cKO mice compared with controls. Data were obtained from hind legs using MicroView 3D software (n = 3). (E) Picrosirius red staining of P17 control and cKO tibia (representative image), femur, and humerus reveals a drastic loss of trabecular bone area in cKO compared with control mice (n = 4; *P < 0.05). Scale bar: 200 μm. (F) H&E staining of P20 skin cryosections shows loss of subcutaneous fat in cKO compared with control mice (n = 3; *P = 0.0002). Dermal thickness was not significantly different (n = 3; *P =

0.3545). Scale bar: 300 mm. sf, subcutaneous fat; d, dermis. (G) Dark field image of P20 control and cKO ocular lenses demonstrates appearance of cataracts (loss of lens transparency) in cKO compared with control. (H) Dark field image of P20 control and cKO spleen demonstrates the disproportionally smaller size of cKO spleen. Original magnification, ×50 (**E** and **F**). See also Supplemental Figure 2-12.

2.3.8 Endocrine defects in *Atrx*-cKO mice

To investigate the mechanism(s) by which ATRX disruption in the CNS could cause such drastic postnatal phenotypes, we first wanted to confirm that the skeletal growth phenotype was caused by non-cell-autonomous mechanisms. Indeed, tibiae from cKO and control mice grew at similar rates in ex vivo organ cultures and had similar growth plate length or organization (Figure 2-6a), substantiating that bone growth defects are caused by systemic alterations.

Given the small size of the cKO mice, we suspected that the endocrine system might be defective. The insulin-like growth factor 1/growth hormone (IGF-1/GH) somatotroph axis has previously been linked to growth, aging, and life span (Niedernhofer et al., 2006). We observed impairment in IGF-1 levels and signaling in the cKO mice. ELISA assays at P17 showed a dramatic reduction of serum and liver IGF-1 levels compared with those in control samples (Figure 2-6b). In addition, quantitative RT-PCR (qRT-PCR) analyses revealed marked transcriptional alterations of key genes for somatotroph signaling in the liver (Figure 2-6c).

Thyroid hormone is an important regulator of *Igf1* expression and skeletal growth during the prepubertal growth period, while GH effects are limited at that time (Xing et al., 2012). We observed that circulating thyroxine (T4) levels were dramatically decreased in the cKO mice, while the GH level was only slightly diminished, and the difference did not reach statistical significance (Figure 2-6d). In addition, expression of several thyroid hormone–responsive genes such as *Thrsp*, *Nrp1*, *Ghr*, *Prlr* was decreased in the liver (Figure 2-6e). It is important to note that *Atrx* transcript and protein levels were normal in livers of cKO mice (Supplemental Figure 2-11c and Figure 2-6e), indicating that these transcriptional effects are not due to spurious deletion of *Atrx* in the liver itself. Given that hypoglycemia is a feature of several mutant mice displaying segmental progeria (Mostoslavsky et al., 2006; van de Ven et al., 2006; van der Pluijm et al., 2007) and that thyroid hormone is essential for the maintenance of glucose homeostasis, we measured blood glucose and found that it was significantly lower in P20 cKO compared with control mice (Figure 2-6f).
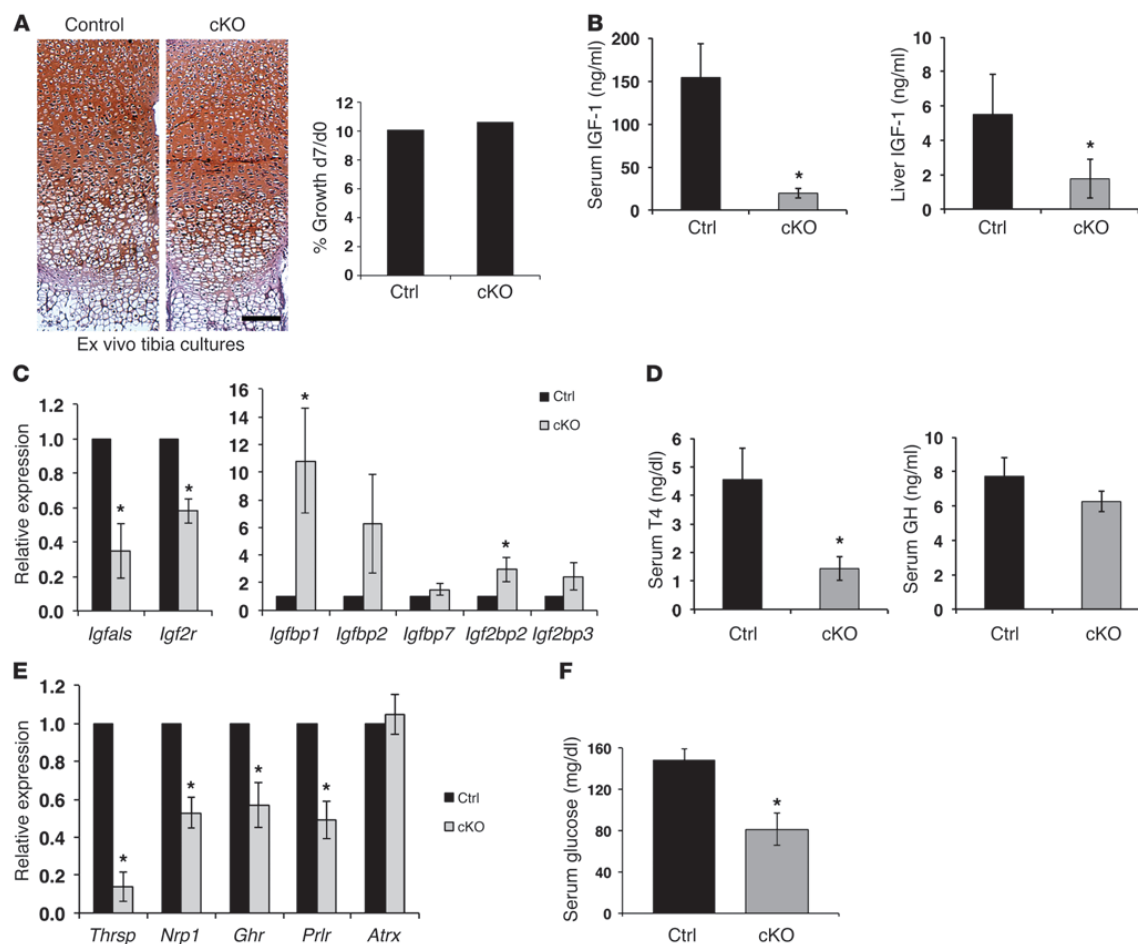
Figure 2-6 Endocrine defects and hypoglycemia in *Atrx*-cKO mice

(A) Longitudinal growth of control and cKO tibia was measured after 7 days (d7) of ex vivo culture. Results are expressed as the ratio of length at d7 to that at d0. No difference in growth was detected between control and cKO mice ($n = 3$). Scale bar: 100 μm. (B) Serum and liver IGF-1 levels are decreased in cKO mice ($n = 3$). (C) Expression of several IGF-1 pathway genes is altered in cKO liver compared with controls ($n = 3$). Real-time data were normalized to *Gapdh* expression. (D) Circulating T4 levels are significantly decreased in P20 cKO mice compared with controls, while GH levels are only mildly affected ($n = 3$). (E) Thyroid hormone target genes exhibit decreased expression in the liver of P20 cKO mice compared with controls ($n = 3$). Real-time data were normalized to *Gapdh* expression. (F) Glucose levels are reduced in P20 cKO serum compared with controls ($n = 5$). Original magnification, ×50 (A). *$P < 0.05$.

2.3.9 ATRX is deleted and causes DNA damage in the embryonic anterior pituitary

The control of thyroid hormone levels and actions is complex and relies on the production of thyroid-releasing hormone (TRH) by the hypothalamus, leading to thyroid-secreting hormone (TSH) production by the pituitary, which in turn induces T3 and T4 synthesis by the thyroid gland (Gaitonde et al., 2012). To determine the cause of low circulating T4 levels, we first investigated *Atrx* and *Tsh* expression levels in the pituitary of control and cKO mice. *Atrx* expression was decreased, as were the levels of *Tsh* transcripts (Figure 2-7a). In addition, several genes responsible for T4 production (*Tshr*, *Tpo*, *Tg*, and *Slc5a5*) were expressed at lower levels in the thyroid of cKO mice compared with control mice (Figure 2-7a). These findings suggest that *Atrx* deletion in the pituitary impairs TSH production, leading to decreased signaling to the thyroid and low T4 production. ATRX immunofluorescence of P20 pituitary cryosections confirmed ATRX expression in the pituitary of control mice and that it was absent in the anterior and intermediate pituitary of cKO mice (Figure 2-7b,c). We also observed deletion of ATRX in the embryonic anterior pituitary at E13.5 (Figure 2-7d), which corresponded to a dramatic increase in γH2AX staining (Figure 2-7e). These results suggest that ATRX is deleted early during embryonic development of the anterior pituitary of cKO mice, where it causes increased DNA damage and results in abnormal function of the thyrotrophs in postnatal cKO mice.
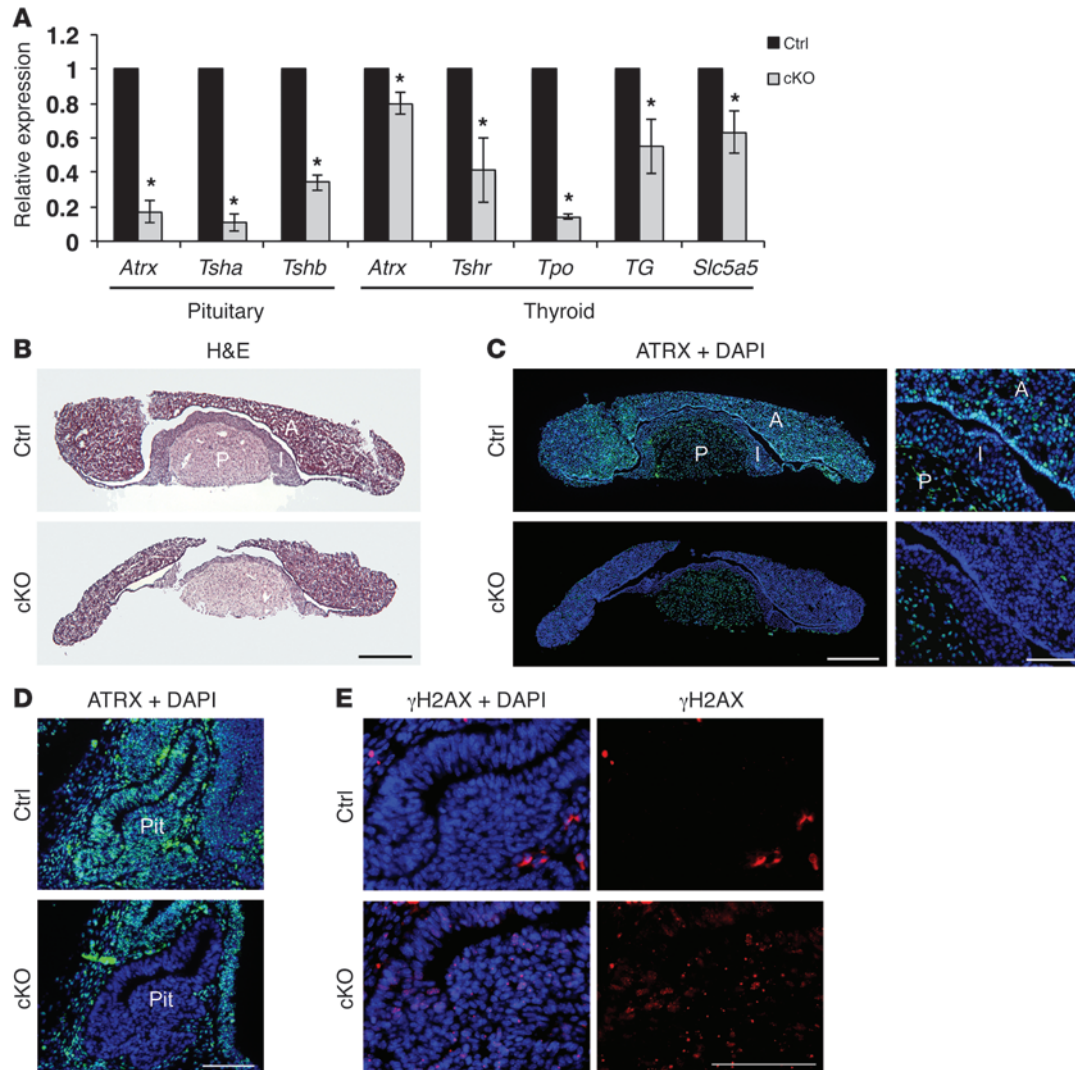
Figure 2-7 Loss of ATRX in the developing anterior pituitary causes DNA damage, reduced *Tsh* expression, and altered thyroid function

(A) Quantitative RT-PCR analysis of P23 control and cKO shows loss of *Atrx* expression in the pituitary (5.9-fold decrease) and thyroid (1.25-fold decrease). *Tsha* and *Tshb* subunits showed decreased expression in the pituitary, and a number of downstream targets of TSH showed decreased expression in the thyroid of cKO mice compared with controls (*n* = 3). (B) H&E staining of P23 control and cKO pituitary. Scale bar: 500 μm. (C) Immunofluorescence detection of ATRX expression in P23 control and cKO pituitary demonstrates specific loss of ATRX in the anterior and intermediate pituitary. Scale bars: 500 μm (left panels) and 50 μm (right panels). A, anterior; I, intermediate; P, posterior

pituitary. (D) Immunofluorescence detection of ATRX in E13.5 control and cKO sagittal embryonic pituitary cryosections shows loss of ATRX expression. Scale bar: 100 μm. Pit, pituitary. (E) Immunofluorescence detection of γH2AX in E13.5 sagittal embryonic pituitary cryosections shows increased DNA damage in cKO embryonic pituitary compared with control. Scale bar: 100 μm. Original magnification, ×25 (C, left panels); ×100 (C, right panels, and E); ×50 (D). *$P < 0.05$.

2.4 Discussion

We have shown that ATRX deficiency causes replicative DNA damage at telomeres and pericentromeric heterochromatin and increases the incidence of telomeric fusions. Moreover, deletion of the *Atrx* gene in the embryonic nervous system and anterior pituitary attenuates postnatal endocrine and metabolic signaling with concurrent growth reduction and progressive progeria-like tissue deterioration. Control experiments confirmed that loss of ATRX is restricted to the forebrain and anterior pituitary. Furthermore, our previous studies have shown that specific and efficient inactivation of the *Atrx* gene in cartilage does not cause any of the skeletal and growth phenotypes described here (Solomon et al., 2009), providing further evidence that these defects are non-cell-autonomous.

We do not yet fully comprehend the mechanisms responsible for the accumulation of DNA damage observed in the *Atrx*-null embryonic forebrain and pituitary. We established that loss of ATRX does not prevent DNA repair, since γH2AX foci did no persist in the postnatal brain at P7 and *Atrx*-null cultured NPCs could resolve DSBs induced by γ-irradiation. However, we provide evidence that the accumulation of damage is partly due to replication stress linked to G4-DNA stability. Telomeres are nucleoprotein complexes that protect chromosome ends from degradation and end fusions. They consist of repetitive DNA high in guanine and cytosine nucleotide residues and have the ability to form G4s when in the single-stranded form (Wang and Patel, 1992). The presence of these bulky DNA adducts can potentially impede DNA replication. We demonstrate that *Atrx*-null NPCs are hypersensitive to the potent G4 ligand TMS, suggesting that in the absence of ATRX, cells have difficulty in resolving G4-DNA. This conclusion is supported by the demonstration that the ATRX protein can bind G4-DNA structures in vitro (Law et al., 2010). Since G4-DNA structures form at telomeres, the γH2AX signals we observed at telomeres in *Atrx*-null NPCs may result from a failure to properly resolve G4 structures, leading to telomere uncapping and initiation of the DNA damage response. This in turn may explain the increase in telomere fusions observed in *Atrx*-null NPCs due to repeating break-fusion-break cycles. In contrast, heterochromatic regions flanking centromeres are AT rich, and a role for ATRX

in facilitating replication of G4-DNA structures cannot explain the increased genetic damage at these sites. ATRX potentially plays a more general role in heterochromatin remodeling/replication during late S phase or may be required to facilitate replication of other secondary structures. ATRX is associated with the DAXX histone chaperone and was proposed to participate in the incorporation of histone variant H3.3 at highly repetitive regions, including pericentric heterochromatin and telomeric chromatin (Drane et al., 2010; Lewis et al., 2010). There is evidence that promyelocytic nuclear bodies (PML-NBs) are involved in facilitating heterochromatin remodeling/replication in late S/G2 phase (Luciani et al., 2006). ATR-X syndrome patient mutations have been shown to alter ATRX targeting to PML-NBs, suggesting an important requirement for ATRX at these subnuclear domains (Berube et al., 2008). ATRX and DAXX localize to PML-NBs during S phase and may be required for remodeling of heterochromatin during replication, a process likely to involve nucleosome remodeling and histone variant deposition or ejection (Ishov et al., 2004).

An important and unexpected finding from our studies is that specific loss of ATRX early during embryonic brain and pituitary development can influence postnatal health and life span. Systemic loss or mutation of several factors required for telomeric stability, response to replication stress, or nucleotide excision repair (NER) cause phenotypes that resemble premature aging in mice (de Boer et al., 2002; Harada et al., 1999; Jaarsma et al., 2011; Murga et al., 2009; Niedernhofer, 2008a, b; O'Driscoll, 2009; van de Ven et al., 2006; Weeda et al., 1997) and segmental progeria syndromes in humans, including *XPF/ERCC1* in xeroderma pigmentosum, *ERCC6/8* in Cockayne syndrome, and *ATR* in Seckel syndrome (Neveling et al., 2007).

The link between excessive DNA damage and a suppressed IGF-1 response, although reported in many progeria models, has not yet been satisfactorily explained. Here, we provide evidence that DNA damage in the embryonic pituitary leads to decreased expression of *Tsh*, with the predictable outcome of low thyroxine production by the thyroid. Recent reports have demonstrated that thyroid hormone is more critical than growth hormone in the regulation of IGF-1 levels at prepubertal stages of development (Wang et al., 2010). Genetically modified mice that are deficient in thyroid hormone

show greater than 50% reduction in *Igf1* expression in liver and bone, and T4 treatment can reverse this effect (Wang et al., 2010). In addition, thyroid hormone and its receptor can bind intron 1 of the *Igf1* gene and stimulate its expression (Wang et al., 2010). In line with this model, circulating thyroid hormone levels were decreased in the *Atrx*-cKO mice, and several thyroid hormone–responsive genes were decreased in the liver. We also detected altered expression of several genes involved in IGF-1 signaling in the liver, including reduced *Igfals* and increased *Igfbp1* expression. Notably, $Ercc1^{-/-}$ mice exhibit a quite rapid postnatal degeneration phenotype similar to that of the *Atrx*-cKO mice, and *Thrsp* is the most downregulated gene in the *Ercc1*-null liver, exhibiting a 15-fold decrease (Kamileri et al., 2012b). This suggests that diminished thyroid hormone action might be implicated in both models, and it will be important in the future to examine thyroxine levels in other models of progeria. Taken together, our findings suggest that DNA damage incurred in the embryonic anterior pituitary leads to defective expression of *Tsh* postnatally, causing hypothyroidism, decreased IGF-1 signaling, and hypoglycemia. Decreased IGF-1 in the serum can negatively impact skeletal growth development, trabecular content, and subsequent mineralization, as seen in our cKO mice (Mohan and Kesavan, 2012). It should be noted that thyroid hormone can also act directly on target tissues such as growth plate chondrocytes, independent of liver-derived IGF-1. Reduced bone growth, and possibly other phenotypes observed in our mutant mice, might therefore be caused by a combination of reduced circulating IGF-1 and reduced thyroid hormone receptor activation in the target tissue (Wit and Camacho-Hubner, 2011; Wojcicka et al., 2013).

Hyperactivity of the p53 tumour suppressor gene shortens life span and accelerates aging in mice (Tyner et al., 2002). We had previously reported that p53 is activated in the absence of ATRX in the embryonic brain and that loss of p53 rescued cell death in the ATRX-deficient mouse brain (Seah et al., 2008). Thus, p53 is required for the removal of cells with excessive DNA damage in the *Atrx*-null brain, which explains the enhanced accumulation of DNA damage we observed upon simultaneous loss of ATRX and p53. Decreased telomere stability and length are also key determinants of life span and have been reported in cancer as well as several types of segmental progeria syndromes, such as Werner syndrome, Cockayne syndrome, dyskeratosis congenita, and Hutchinson-Gilford

progeria (Batenburg et al., 2012; Benson et al., 2010; Chang et al., 2004; Crabbe et al., 2007; Damerla et al., 2012; Mitchell et al., 1999; Opresko et al., 2004; Shay and Wright, 1999). If telomeric damage is the key driver of life span, our findings would then suggest that telomeric abnormalities confined to specific brain and/or pituitary cells are in themselves sufficient to induce several aging-like phenotypes prematurely, a provocative idea that would have to be confirmed or dismissed in future work.

Problems in replication have been shown to lead to chromosome segregation failures in ensuing mitoses (Chan et al., 2009). Similarly, several G4-DNA ligands have been demonstrated to cause telomeric fusions and telomere aggregate formation that eventually lead to chromosomal instability, anaphase bridges, and mitotic catastrophe (Gauthier et al., 2012; Hampel et al., 2013; Incles et al., 2004; Kim et al., 2003; Tahara et al., 2006). It is conceivable that replicative damage is the underlying cause of mitotic defects that we previously reported in ATRX-deficient human cells, such as chromosome missegregation, anaphase bridges, and micronucleus formation (Ritchie et al., 2008). Functions for ATRX in restricting replicative stress, telomere fusions, and mitotic defects may also help explain the tumour-suppressive roles recently ascribed to the ATRX protein. Mutations in the *ATRX* gene were identified in several types of cancers, including pediatric brain tumours (Cheung et al., 2012; Heaphy et al., 2011a; Jiao et al., 2011; Schwartzentruber et al., 2012; Yachida et al., 2012; Yuan et al., 2014). A common denominator in the majority of *Atrx*-null tumours was the frequent appearance of large telomeric foci, a hallmark of alternative lengthening of telomeres (ALT), providing further evidence that ATRX function is intimately linked to telomere biology, possibly as a suppressor of illegitimate recombination events. The hypersensitivity of *Atrx*-null neuroprogenitors to TMS is an important finding in that regard, as it indicates that *Atrx*-null tumours may be susceptible to treatment with G4-DNA binding ligands.

2.5 Supplementary Figures

DAPI γH2AX **B** DAPI ATRX

Ctrl

Ctrl

cKO

cKO
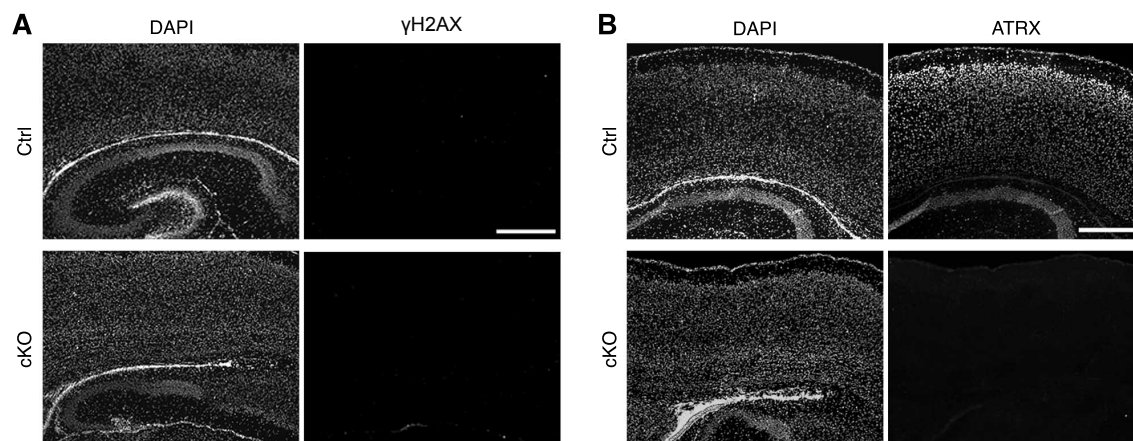
Figure 2-8 DNA damage is not detected in the ATRX-null postnatal brain

(A) Immunostaining for γH2AX in P7 control and cKO cortical cryosections. Scale bar: 200 μm. (B) Immunostaining for ATRX in P7 control and cKO cortical cryosections. Scale bar: 200 μm. Original magnification, x50 (A and B).
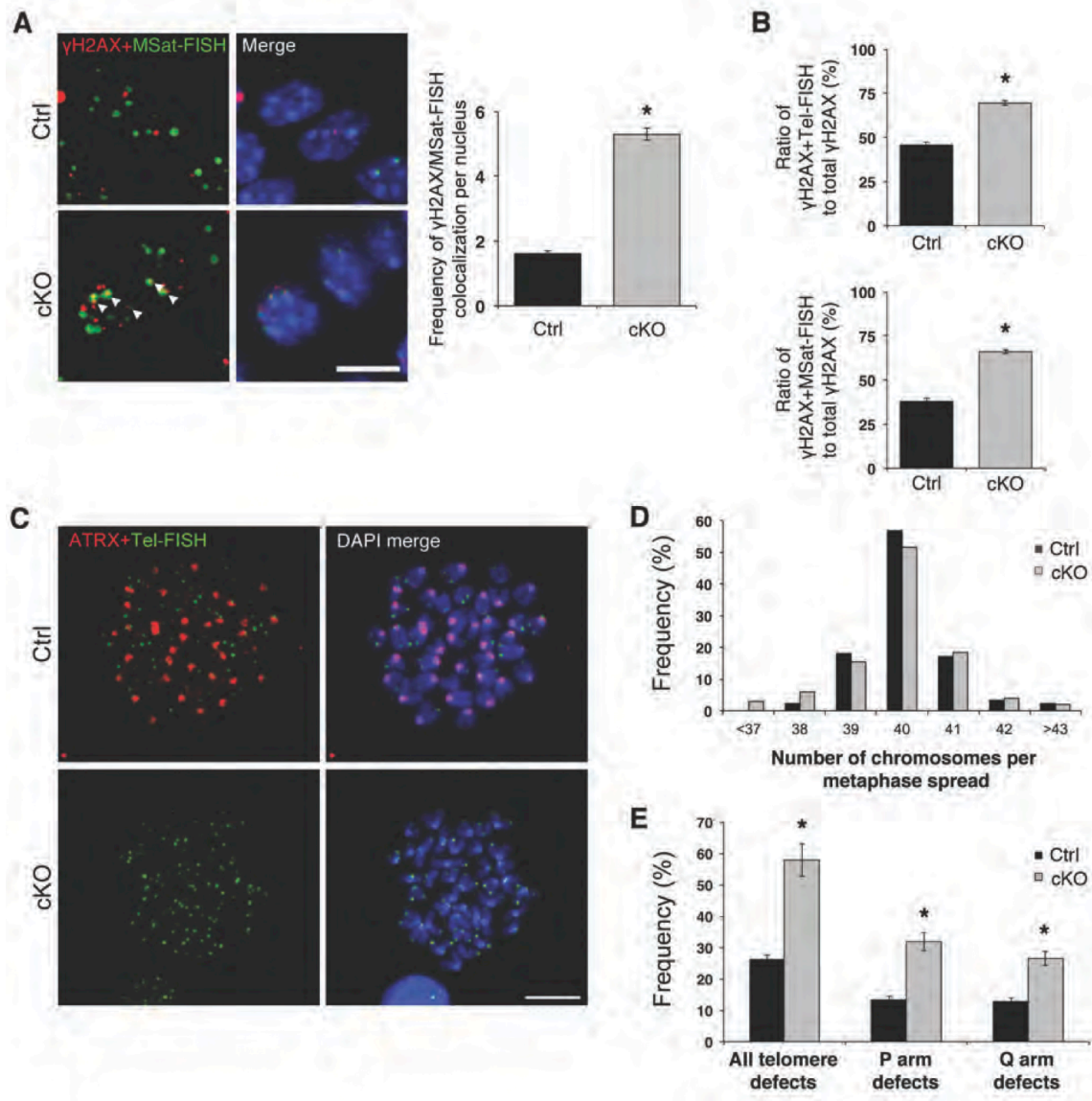
Figure 2-9 DNA damage occurs at major satellite repeats and telomeric repeats in ATRX-null NPCs, however there is no difference in chromosome number and telomere defects are not restricted to the p or q arms of cKO chromosomes

(A) Confocal immunoFISH images of γH2AX (red) and major satellite repeats (MajSat-FISH; green) shows that DNA damage occurs at PCH more frequently in cKO compared to control NPCs (γH2AX/MSat-FISH colocalization; 300 nuclei counted, $n = 3$). (B) Since endogenous γH2AX levels are higher in cKO NPCs compared to control, the ratio of γH2AX and Tel-FISH or MajSat-FISH colocalization vs. total γH2AX foci per cell was calculated to obtain a relative measure of the percentage of γH2AX foci per nucleus

that occurred at either telomeres or major satellite repeats (300 nuclei counted, $n = 3$). Scale bar: 10 μm. (C) ImmunoFISH staining of ATRX (red) and telomeres (Tel-FISH; green) in control and cKO NPCs demonstrates loss of ATRX staining in cKO NPCs. Scale bar: 10 μm. (D) Chromosome number per metaphase spread did not differ between control and cKO NPCs (control: 88 metaphases and cKO 108 metaphases, $n = 3$). (E) Telomeric defects quantified in Figure 2-2e were scored for occurring on the P or Q arm of chromosomes. Frequency of defects on either arm did not differ in control and cKO NPCs (1475 chromosomes counted, $n = 3$). Original magnification, x1000 (A); x630 (C).
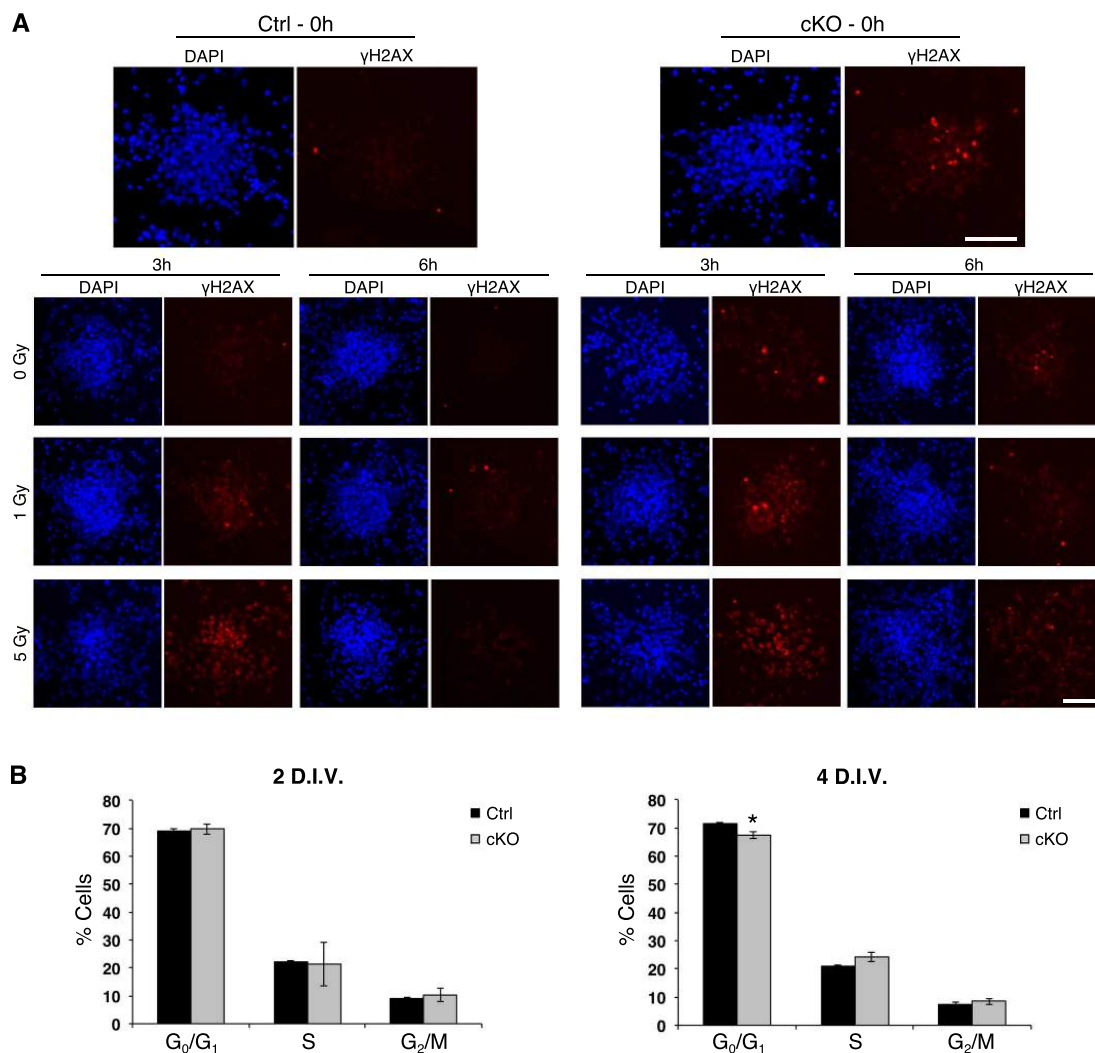
Figure 2-10 ATRX deficiency does not influence repair of DSBS, however causes a slight decrease in G_0/G_1-phase NPCs with a concomitant increase in S-phase cells at 4 days *in vitro* (D.I.V.)

(A) Control and cKO NPCs were exposed to 0, 1 and 10 Gy doses of γ-irradiation to induce DSBs. γH2AX signal was assessed at 0h, 3h and 6h post-irradiation. DSBs induced by γ-irradiation were largely repaired by 6h post-treatment in both control and cKO NPCs, as evidenced by the resolution of γH2AX foci. Scale bars: 100 μm. (B) Actively proliferating control and cKO NPCs were pulse-labeled with BrdU at 2 and 4 D.I.V., processed for flow cytometry, and analyzed for propidium iodide (PI) and BrdU staining. The proportion of cells in each phase of the cell cycle is indicated ($n = 3$). Original magnification, x200 and x100 (A).
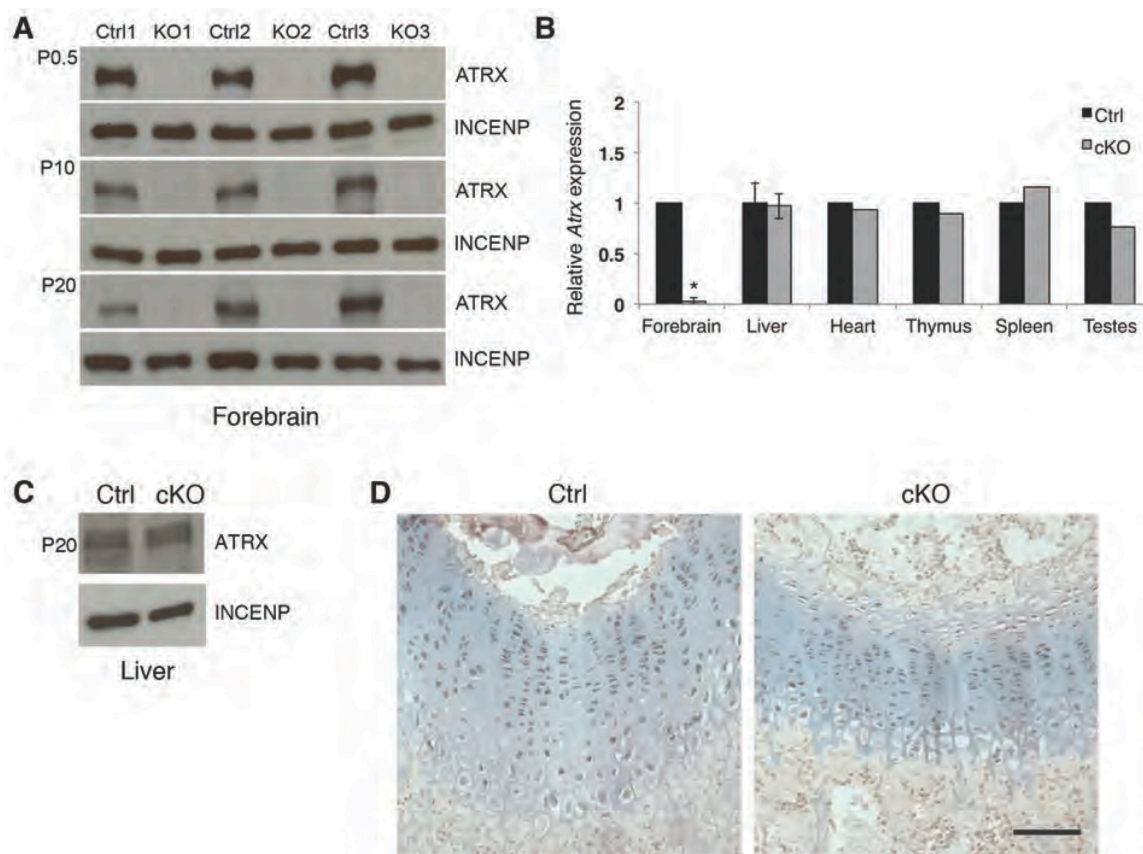
Figure 2-11 ATRX is specifically deleted in the forebrain

(A) Western blot analysis of ATRX expression using nuclear protein extracts obtained from P0.5, P10, and P20 control and cKO telencephalon ($n = 3$). (B) Quantitative RT-PCR analysis of *Atrx* expression in P20 control and cKO organs. Forebrain and liver ($n = 3$); heart, thymus, spleen, and testes ($n = 1$). Real-time data is normalized to *Gapdh* expression. (C) Western blot analysis of ATRX expression using nuclear protein extracts obtained from P20 control and cKO liver. (D) Immunohistochemistry detection of ATRX in P20 control and cKO tibia paraffin-embedded sections. Scale bar: 100 μm. Original magnification, x50 (D).

*

*

P7   P10

Thickness (µm)

500
400
300
200 *
100
0
         Ctrl   cKO

Thickness (µm)

300
250
200
150
100
50
0
         Ctrl   cKO

Thickness (µm)

cKO

P20
Ctrl   cKO
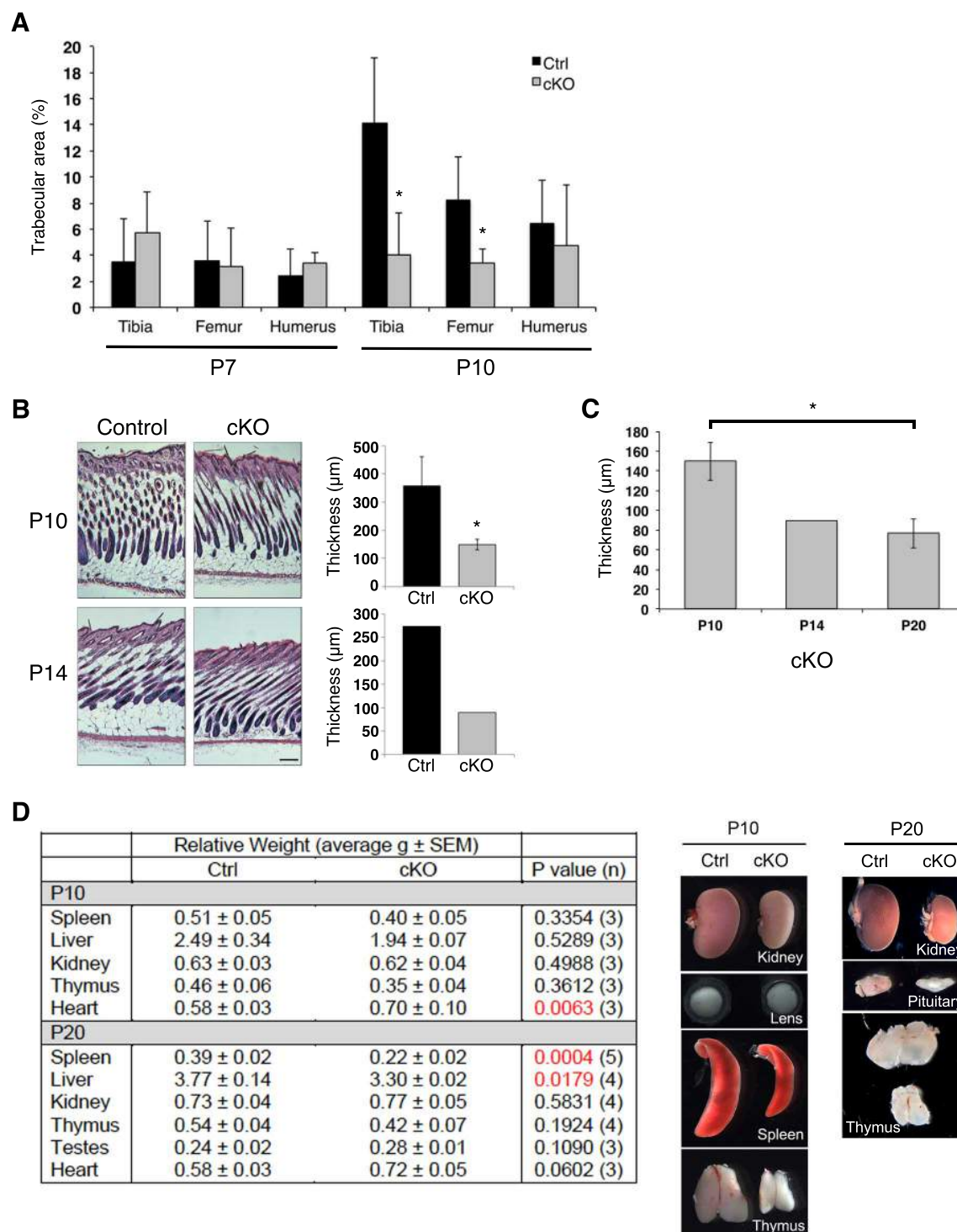
Figure 2-12 Worsening postnatal phenotypes in ATRX cKO mice

(A) Quantification of Picrosirius Red staining in tibia, femur and humerus reveals trabecular bone area is not significantly different between control and cKO until approximately P10 ($n = 3$ at both time points). (B) H&E staining of skin cryosections from P10 and P14 shows decreased subcutaneous fat thickness as early as P10 in cKO mice compared to controls (P10, $n = 3$; P14, $n = 1$). Scale bar: 200 μm. (C) Subcutaneous fat thickness in the *Atrx* cKO at different developmental time points shows cKO mice develop subcutaneous fat (P10), which is gradually reduced in thickness by P20 ($n = 3$ at P10 and P20; *$P = 0.0409$). (D) Organ weight (g) relative to total body weight at P10 and P20. Representative dark field images of organs from control and cKO mice at the indicated time points. Original magnification, x50 (B).

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Chapter 3

3 Dual effect of CTCF loss on neuroprogenitor differentiation and survival

This study was the first to investigate the functions of CTCF in brain development. Prior to this study virtually nothing was known regarding CTCF activities in the brain, or in a general physiological context. This chapter investigates the consequences of CTCF loss-of-function to embryonic brain development by primarily focusing on neocortical neurogenesis, which is particularly relevant to understanding the significance of human *CTCF* mutations that cause intellectual disability.

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3.1 Introduction

CTCF is a multifunctional DNA binding protein that regulates higher-order chromatin structure to influence transcriptional regulation, genomic imprinting, X chromosome inactivation, and chromatin insulation (Holwerda and de Laat, 2013). It binds to a variety of highly divergent target sequences throughout the genome using a combination of its 11 zinc finger motifs (Nakahashi et al., 2013). CTCF partners with a number of chromatin-related proteins such as the cohesin complex (Parelho et al., 2008; Wendt and Peters, 2009), nucleophosmin, and CTCF itself (Yusufzai and Felsenfeld, 2004; Yusufzai et al., 2004). These interactions may allow CTCF sites to contact one another and/or to be tethered to subnuclear domains. CTCF-mediated chromatin interactions detected by Chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) correlate with approximately 10% of all CTCF binding sites (CBSs), however, indicating that CTCF likely plays additional roles within the cell (Handoko et al., 2011).

De novo mutations in *CTCF* have been identified previously in patients with varying degrees of intellectual disability and microcephaly (Gregor et al., 2013), highlighting the importance of chromatin organization for the normal development and function of the CNS. Whereas CTCF function has been studied extensively in cell culture systems, its

function in an *in vivo* context remains to be completely resolved (Ohlsson et al., 2010a). Ubiquitous deletion of CTCF in the mouse leads to lethality before embryonic day 3.5 (E3.5), suggesting that it is essential for early developmental processes (Fedoriw et al., 2004; Heath et al., 2008; Moore et al., 2012). Conditional deletion of *Ctcf* in specific tissues causes either reduced proliferation or apoptotic cell death, depending on the tissue targeted for *Cre* recombination. For example, deletion of *Ctcf* in thymocytes resulted in increased *p21* and *p27* expression and cell cycle arrest, whereas reduced CTCF in mouse oocytes induced meiotic and mitotic defects and apoptotic cell death before the blastocyst stage (Fedoriw et al., 2004; Heath et al., 2008; Wan et al., 2008). Deletion of *Ctcf* in the developing limb bud resulted in massive apoptosis and near-complete loss of limb structures, accompanied by increased p53 upregulated modulator of apoptosis (PUMA), a known activator of caspase-mediated apoptosis (Nakano and Vousden, 2001; Soshnikova et al., 2010; Yu et al., 2001). In human cancer cells, CTCF binds to the *Puma* gene, and its depletion results in increased *Puma* transcript and rapid apoptosis, indicating that *Puma* transcription can be directly influenced by the presence or absence of CTCF (Gomes and Espinosa, 2010a, b).

Given the deleterious effects of *CTCF* mutations in the human CNS, we specifically inactivated *Ctcf* in the developing mouse brain. CTCF loss of function using two different *Cre* driver lines in mice triggered apoptosis in dividing neuroprogenitor cells (NPCs) of the forebrain. Despite prevention of apoptosis by *Puma* deletion, rescued *Ctcf/Puma* double-null apical and outer radial glia (oRG) progenitors exhibited decreased proliferative capacity. Furthermore, loss of CTCF caused premature neurogenesis, resulting in depletion of the progenitor pool and a microcephaly phenotype at birth. These findings highlight the complexity of CTCF activities during neurogenesis.

3.2 Materials and Methods

3.2.1 Mouse husbandry and genotyping

Mice were exposed to 12 h light/dark cycles and fed tap water and regular chow *ad libitum*. The *Ctcf^{loxP}* mice, in which *loxP* sites flank exons 3–12, have been described previously (Heath et al., 2008). Mice conditionally deficient in CTCF were generated by

crossing *Ctcf^{loxP/+}* females (C57BL/6 background) with heterozygous *Foxg1Cre* knock-in male mice (129/sv background) or with *NestinCre* heterozygous male mice (C57BL/6 background; (Berube et al., 2005; Hebert and McConnell, 2000). To account for decreased *Foxg1* expression due to knock-in of the Cre recombinase gene, *Cre^+* males were used as controls (*Ctcf^{+/+}Foxg1-cre^{+/−}*) unless stated otherwise. Using the *NestinCre* driver line, *Ctcf^{loxP/loxP}* mice were crossed with *Ctcf^{loxP/+};Nestin^+* mice to generate *Ctcf^{loxP/loxP}* or *Ctcf^{loxP/+}* (controls) and *Ctcf^{loxP/loxP};Nestin^+* (*Ctcf^{Nes-cre}*). *Puma^{−/−}* (*Bbc3^{tm1Ast}*) mice were obtained from The Jackson Laboratory (stock #011067; (Villunger et al., 2003). DNA from tail biopsies of newborn pups or yolk sac from embryos was genotyped by PCR. Primer sequences are provided in Table 1.

3.2.2 Immunostaining and histology

For immunofluorescence staining, cryosections were incubated with the primary antibody overnight at 4°C, washed in PBS/0.3% Triton-X 100, and incubated with the secondary antibody for 1 h. Sections were counterstained with DAPI (Sigma-Aldrich) and mounted in SlowFade Gold (Invitrogen). Primary antibodies used were as follows: rabbit anti-CTCF (1:400; Cell Signaling Technology), rabbit anti-cleaved caspase 3 (AC3; Asp175; 1:400; Cell Signaling Technology), mouse anti-BrdU (1:50; BD Biosciences), rabbit anti-PUMA (1:200; Cell Signaling Technology), rabbit anti-TBR2 (1:200; Abcam), goat anti-SOX2 (1:400; Santa Cruz Biotechnology), goat anti-PAX6 (1:200; Santa Cruz Biotechnology), rabbit anti-SOX2 (1:100; Millipore Bioscience Research Reagents), rabbit anti-Ki67 (1:200; Abcam), rabbit anti-TBR1 (1:200; Abcam), mouse anti-SATB2 (1:200; Abcam), and rabbit anti-CTIP2 (1:200; Abcam). Secondary antibodies used were as follows: goat-anti-rabbit Alexa 594 (1:800; Invitrogen), goat-anti-mouse Alexa 488 (1:800; Invitrogen), donkey-anti-sheep Alexa 594 (1:800; Invitrogen), and donkey-anti-mouse Alexa 488 (1:800; Invitrogen). Sections were subjected to antigen retrieval (incubated in 0.1 mM sodium citrate, pH 6.0, heated to ~95°C and microwaved on low for 10 min) before overnight incubation (for BrdU, SOX2, PAX6, TBR2, STAB2, TBR1, and CTIP2). Terminal deoxynucleotidyl nick end labeling (TUNEL) was performed according to the manufacturer's instructions (Roche). For histological studies, slides were stained with hematoxylin and eosin (H&E).

3.2.3 BrdU labeling

Pregnant mice were injected intraperitoneally with cell proliferation labeling reagent [10 mM bromodeoxyuridine (BrdU) and 1 mM fluorodeoxyuridine (FdU) in H_2O] at 1 ml/100 g body weight, or 0.3 mg/g body weight (GE Healthcare Life Sciences). Animals were killed after 1 h by CO_2 asphyxiation, and the embryos were recovered in ice-cold PBS, pH 7.4, and were fixed in 4% paraformaldehyde. Tissue was equilibrated in 30% sucrose/PBS and frozen in OCT (Tissue Tek). For cell cycle exit analysis, pregnant female mice (E13) were injected with cell proliferation labeling reagent at 1 ml/100 g body weight (GE Healthcare Life Sciences), and embryos were collected and processed for immunofluorescence analysis 24 h later. Before immunofluorescence analysis, cryosections (8 μm) were treated with 2N HCl to denature the DNA, and neutralized with 0.1 M $Na_2B_4O_7$, pH 8.5.

3.2.4 Primary NPC cultures and manipulation

Cortical progenitor cultures were prepared as described previously (Gloster et al., 1999; Slack et al., 1998; Watson et al., 2013) using cortices dissected from E12.5 embryos. Cells were seeded on polyornithine-coated (Sigma-Aldrich) plastic plates or glass coverslips. Cells were fixed in 4% paraformaldehyde for 10 min, washed in PBS, and processed for immunofluorescence. Cell viability was measured using the trypan blue dye exclusion method. Cell counts were determined with a hemacytometer.

3.2.5 Western blot analysis

Nuclear protein was extracted from the E16.5 telencephalon using a standard extraction kit (Thermo Scientific) and quantified using the Bradford assay. Protein (20 μg) was resolved on a 6% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was probed with rabbit anti-p53 (1:500; Santa Cruz Biotechnology) and rabbit anti-inner centromere protein (INCENP) (1:10000; Sigma Aldrich) antibodies followed by the appropriate horseradish peroxidase-conjugated secondary antibodies (1:4000; GE Healthcare Life Sciences). The membrane was incubated in ECL before exposure to x-ray film. Densitometry analysis was performed using ImageJ software (version 1.47).

3.2.6 Real-time PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen). First-strand cDNA was synthesized from 1 μg of total RNA using the SuperScriptTM II Reverse Transcriptase kit (Invitrogen) with deoxyribonucleotide triphosphates (dNTPs) (1 mM final concentration; GE Healthcare), porcine RNAguard (GE Healthcare), and 0.3 μg random primers (GE Healthcare). Amplification was performed using a Chromo-4 Continuous Fluorescence Detector (Bio-Rad) in the presence of iQ SYBR Green supermix (Bio-Rad) and recorded using Opticon Monitor 3 software (Bio-Rad). Results were normalized to β-actin expression, and relative gene expression levels were calculated using GeneX software (Bio-Rad). Samples were amplified as follows: 95°C for 10 s, annealed for 20 s, 72°C for 30 s. After amplification, a melting curve was generated, and samples were resolved on a 1.5% agarose gel (75 V for 1 h) to verify amplicon purity. Primer oligomers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and were obtained from Integrated DNA Technologies. Sequences are provided in Table 1.

3.2.7 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as described previously (Kernohan et al., 2010). Briefly, mouse forebrain tissue was dissected and processed to single-cell suspension. Cells were cross-linked in 1% formaldehyde, lysed in SDS buffer and sonicated. Immunoprecipitation was performed with a rabbit anti-CTCF antibody (Cell Signaling Technology) and a rabbit anti-p53 antibody (Santa Cruz Biotechnology). Input samples represent 1/25 of total chromatin input. ChIP products were amplified in duplicate with iQ SYBR Green Master Mix (Bio-Rad) on a Chromo-4 thermocycler under the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, 55°C for 20 s, 72°C for 30 s, and a final melting curve generated from 55°C to 95°C in increments of 1°C per plate read. Fold change and percent input formulas were adapted from (Mukhopadhyay et al., 2004) as follows: percent input = 100 * [2(ΔC_tInput − ΔC_tInput) − (ΔC_tInput − ΔC_tAb)]/25. Error bars represent the SEM. Primer sequences are provided in Table 1.

3.2.8 Microscopy

Images were captured with a digital camera (ORCA-ER; Hamamatsu) using an inverted microscope (DMI 6000b; Leica). Openlab imaging software (PerkinElmer) was used for manual image capture, and processing was performed using Volocity software (PerkinElmer). For quantification of $AC3^+$ cells per area, $AC3^+$ cells were counted in a defined area in at least six serial cortical cryosections, and the ratio of $AC3^+$ cells to area (square millimeters) was calculated. For BrdU, SOX2, TBR2, BrdU/Ki67, TBR1, SATB2, and CTIP2 quantification, at least two serial cortical cryosections were assessed for positive cells within the indicated regions per embryo. DAPI morphology was used to bin cortex into the ventricular/subventricular zone (VZ/SVZ), intermediate zone (IZ), and cortical plate (CP).

3.2.9 Statistical analyses

Statistical analysis was performed using GraphPad Prism software (GraphPad Software; version 4.02), and all results are expressed as the mean ± SEM. Unless indicated otherwise, p values were generated using Student's t test (unpaired, two-tailed) to compare between two independent data sets. Genotype ratios (Figure 3-1a, 3-2a) were compared using a χ^2 test. To compare cell viability data (see Figure 3-4e), a one-way ANOVA was used with Dunnett's multiple comparison post-test to compare the cell viability of each genotype ($Ctcf^{Nes\text{-}cre}$, $Ctcf^{Nes\text{-}cre};Puma^{-/-}$, and $Puma^{-/-}$) to control.

3.2.10 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.

Primer Name	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
Ctcf	CTAGGAGTGTAGTTCAGTGAGGCC	GCTCTAAAGAAGGTTGTGAGTTC
NestinCre	TGACCAGAGTCATCCTTAGCG	AATGCTTCTGTCCGTTTGCC
Sry	GCAGGTGGAAAAGCCTTACA	AAGCTTTGCTGGTTTTTGGA
Puma genotyping WT	AGGCTGTCCCTGCGGTCATCC	GGACTGTCGCGGGCTAGACCCTCTA
Puma genotyping KO	AGGCTGTCCCTGCGGTCATCC	ACCGCGGGCTCCGAGTAGC
Ctcf expression	CGATATGCTCTCATCCAGCA	TCCCACACTTGGAACAGACA
Puma expression	CGTGTGGAGGAGGAGGAGT	GGAGGAGTCCCATGAAGAGA
β-actin expression	CTGTCGAGTCGCGTCCACCC	ACATGCCGGAGCCGTTGTCG
5′ 5 kb *Puma* CBS	ACCCTCGTGTTTGGAGTGAC	CTCCTGCCTTGTGCCTAAAG
Puma CBS1	GCTCCTCCCAGGTCTCACTA	CAGCTTTCATCACTGGGACT
Puma CBS2	AGGAATGGATCTGCTGGATG	GTTGCTGACTCACCGGCTAT
3′ 5 kb *Puma* CBS	GAGCCCCTGCCTAGTAGGAT	TAGTCCCTGTGTGTGCTTGC
5′ 1 kb p53BS	AAAAATGGGCTTGGAGAGC	CCACCACTGTCCAGCTTGTT
Puma p53BS	CTGTCCCCACGCTGC	GCTTGCTTGCTGGTGTCG
3′ 1 kb p53BS	AGCCAGGGCTACACAGAGAA	CTGAGCCATCTCTCCAGTCC

KO, Knock-out; WT, wild type.

Table 1 List of primers used for genotyping, gene expression, and qChIP analyses

3.3 Results

3.3.1 *Ctcf*$^{Foxg1\text{-}cre}$ mice exhibit widespread apoptosis and profound loss of telencephalic and anterior retinal tissue

To study the function of CTCF in the embryonic brain, *Ctcf* floxed mice were intercrossed with the previously characterized *Foxg1Cre* mice (Berube et al., 2005; Heath et al., 2008; Hebert and McConnell, 2000). *Foxg1* expression is first detected between E8 and E9 in the telencephalic neuroepithelium, the basal ganglia, the olfactory bulbs, and the anterior retina (Dou et al., 1999). *Foxg1-cre*$^{+}$ *Ctcf*$^{loxP/loxP}$ progeny resulting from this cross are *Ctcf* null in the anterior retina and forebrain, and will be referred to as *Ctcf*$^{Foxg1\text{-}cre}$ throughout this text.

We failed to recover live *Ctcf*$^{Foxg1\text{-}cre}$ pups at birth (Figure 3-1a). At E13.5, the size of telencephalic and retinal structures was greatly diminished in mutant embryos (Figure 3-1b,c). At E11.5, the telencephalon of mutant embryos was already noticeably smaller compared to littermate-matched controls (Figure 3-1d, hatched circle). CTCF immunostaining demonstrated nuclear expression of the protein throughout the forebrain in control tissue and its absence in mutant embryos (Figure 3-1e). To determine the underlying cause of cell loss, we measured levels of proliferation and cell death in E11.5 *Ctcf*$^{Foxg1\text{-}cre}$ and control embryos. Acute (1 h) BrdU labeling of control and *Ctcf*$^{Foxg1\text{-}cre}$ embryos revealed that the percentage of cells in S phase does not vary significantly between mutant and control forebrain tissue (Figure 3-1f,g). To measure apoptosis, we stained sections with an antibody against activated caspase-3 as a marker of cell death (Figure 3-1h). Quantification of the results revealed a large increase in the proportion of cells undergoing apoptotic cell death in *Ctcf*$^{Foxg1\text{-}cre}$ embryos compared to littermate-matched controls (Figure 3-1i). TUNEL also showed increased apoptosis in *Ctcf*$^{Foxg1\text{-}cre}$ embryos compared to control (Figure 3-1j). Since we already observed profound cell loss by E11.5, we wanted to analyze whether a similar mechanism was occurring earlier in this system. We observed increased AC3 immunostaining in *Ctcf*$^{Foxg1\text{-}cre}$ forebrain cryosections at E10.5, confirming that cell death occurs at this earlier time point (data not shown). We conclude that deletion of *Ctcf* in the mouse forebrain at approximately E8.5

causes extensive apoptosis resulting in profound loss of telencephalic and anterior retinal tissue by E13.5.
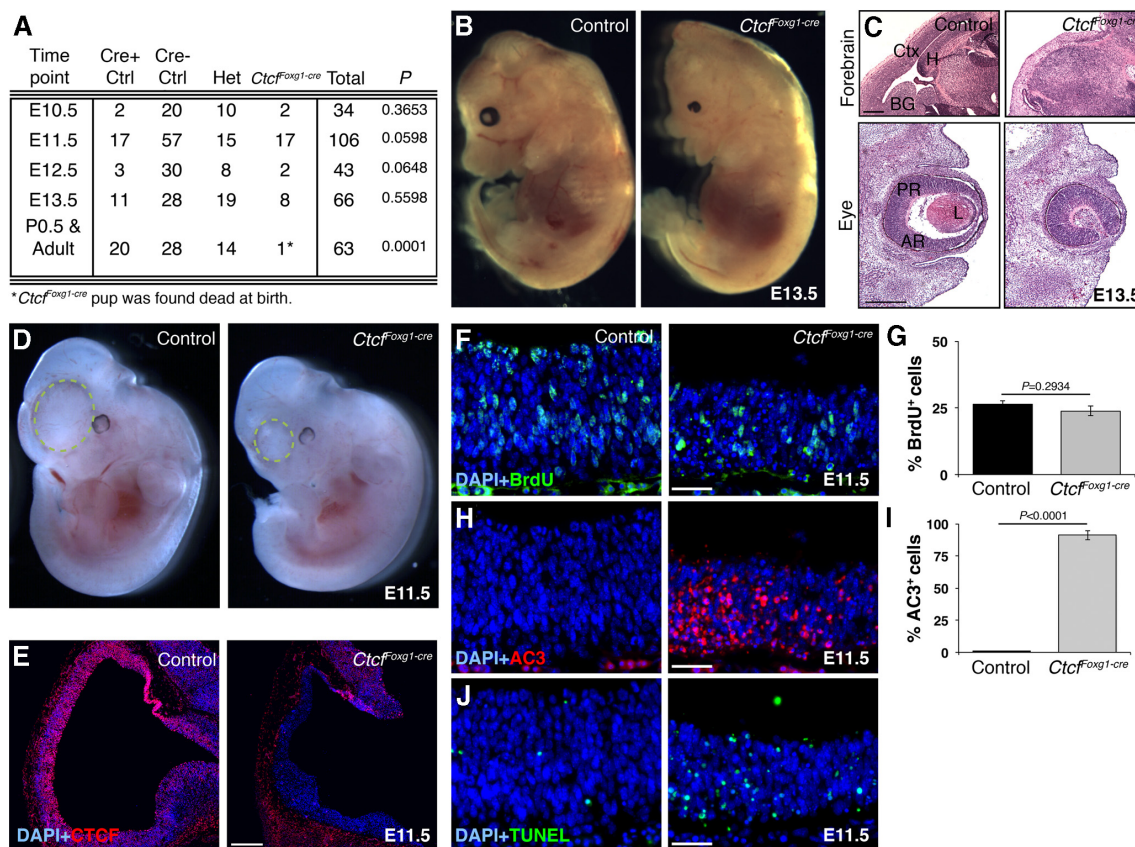
Figure 3-1 _Foxg1Cre_-mediated deletion of _Ctcf_ results in a massive increase in apoptosis

(A) Table of genotypes obtained during the embryonic and postnatal periods. Ratios at each time point were analyzed using a χ^2 test. Het, $Ctcf^{flox/WT};Foxg1-cre^{+/-}$. (B) Dark-field images of control and $Ctcf^{Foxg1-cre}$ embryos at E13.5. (Please note limbs were taken for genotyping). (C) H&E staining of E13.5 sagittal cryosections demonstrates complete loss of cortex (Ctx), hippocampal hem (H), basal ganglia (BG), lens (L), and anterior retina (AR), but not the posterior retina (PR), in $Ctcf^{Foxg1-cre}$ embryos. (D) Dark-field images of control and $Ctcf^{Foxg1-cre}$ embryos at E11.5. The dashed circle outlines the telencephalon, which is visibly reduced in size in the $Ctcf^{Foxg1-cre}$ embryos compared to littermate controls. (E) Immunodetection of CTCF (red) in E11.5 sagittal cryosections confirms specific loss of CTCF expression in the forebrain neuroepithelium of $Ctcf^{Foxg1-cre}$ embryos. (F) Pregnant females were subjected to a 1 h BrdU pulse before being killed. Immunodetection of BrdU in E11.5 control and $Ctcf^{Foxg1-cre}$ cortical neuroepithelium is shown. (G) BrdU^{+} cells were counted and expressed as a percentage of the total number

of DAPI$^+$ cells ($n = 3$). (H) Immunodetection of activated caspase-3 (red) in control and *Ctcf$^{Foxg1-cre}$* cortical neuroepithelium at E11.5. (I) AC3$^+$ cells were counted and expressed as a percentage of the total number of DAPI$^+$ cells ($n = 3$). (J) TUNEL (green) detection in E11.5 control and *Ctcf$^{Foxg1-cre}$* cortical neuroepithelium. Error bars represent the SEM. Original magnification: (C) x25; (E) x50; (F, H, J) x200. Scale bars: (C) top, 1 mm; bottom, 400 μm; (E) 200 μm; (F, H) 50 μm; (J) 100 μm.

3.3.2 *NestinCre*-driven inactivation of *Ctcf* decreases cell survival

To determine the outcome of *Ctcf* deletion at a later embryonic time point in the telencephalon, we mated *Ctcf*$^{flox/flox}$ mice to the previously characterized *NestinCre* transgenic mice (Berube et al., 2005; Heath et al., 2008). The resulting mutant is hereafter referred to as *Ctcf*$^{Nes-cre}$ for simplicity. *Cre* is expressed after preplate formation in this system, resulting in specific deletion of *Ctcf* in neural progenitor cells at approximately E11 (Bérubé et al., 2005). We obtained the expected ratio of *Ctcf*$^{Nes-cre}$ mice at birth; however, almost all mutant pups were already dead and blue in color (Figure 3-2a). One live *Ctcf*$^{Nes-cre}$ pup was found struggling to breathe, appeared weak, and was killed. We conclude that neonatal lethality of *Ctcf*$^{Nes-cre}$ mice is likely due to asphyxiation at birth.

We first established that the CTCF protein was lost in the basal ganglia and many cells of the cortex at E12.5 (Figure 3-2b), and expression was undetectable in the E14 *Ctcf*$^{Nes-cre}$ cortex, basal ganglia, and hippocampal hem (Figure 3-2c). To determine whether *NestinCre*-mediated deletion of *Ctcf* also induces apoptosis, we analyzed *Ctcf*$^{Nes-cre}$ forebrain cryosections at E12.5–E15.5 for evidence of AC3. At E12.5, we observed no increase in apoptotic cells in the mutant telencephalon (Figure 3-2d). However, at E14, we detected an increase in AC3^{+} cells in the *Ctcf*$^{Nes-cre}$ cortex, basal ganglia, and hippocampal hem compared to controls (Figure 3-2d). The increase in caspase activation was also observed at E15.5 in all three forebrain regions, with the highest level of AC3^{+} cells in the basal ganglia (Figure 3-2d,e). Increased apoptotic cell death was also observed *in vitro* in primary cortical progenitor cultures established from E12.5 control and *Ctcf*-null telencephalon. Levels of AC3 staining were low in both control and *Ctcf*-null NPCs after 2 d *in vitro* (DIV), and we only observed a detectable increase in staining at 4 DIV (Figure 3-2f). Together, the data suggest that when using the *NestinCre* driver line of mice, *Ctcf* deficiency does trigger the activation of caspase-mediated apoptosis in NPCs, but the effect is delayed and less severe than that observed in the *Ctcf*$^{Foxg1-cre}$ embryos, suggesting that early neuroepithelial cells are more sensitive to CTCF loss than the neuroprogenitors present slightly later in development.

A

Time point	Control	Het	*Ctcf*^{Nes-cre}	Total	*P* value
E12.5	64	27	32	123	0.7371
E13.5	53	57	39	122	0.1803
E14	152	84	89	325	0.4700
E15.5	35	25	24	84	0.3077
E16.5	77	34	38	148	0.8220
E17.5	30	19	22	71	0.3757
P0.5	42	26	15*	83	0.2313

*14 of 15 *Ctcf*$^{Nes-cre}$ pups were found dead at birth.
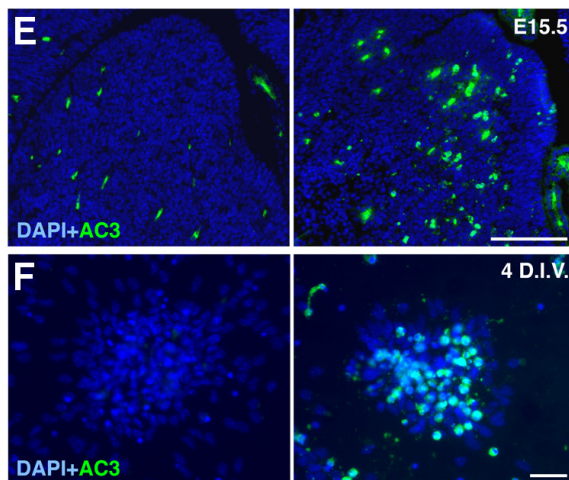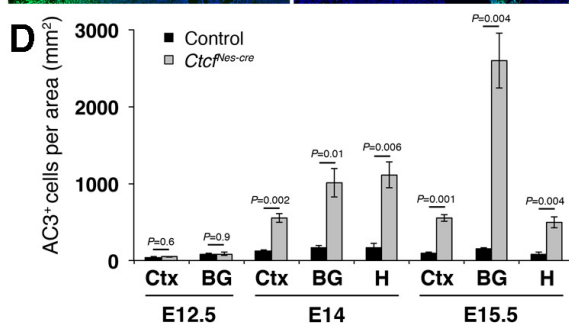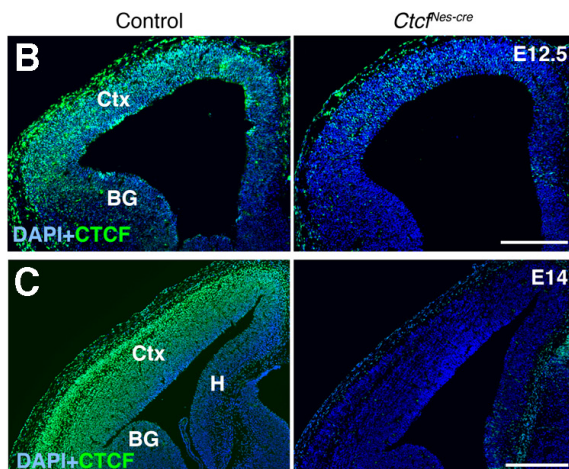
Figure 3-2 *NestinCre*-mediated deletion of CTCF results in activation of caspase-mediated apoptosis

(A) Table of genotype ratios obtained during the embryonic period. Ratios at each time point were analyzed by a χ^2 test. (B) Immunodetection of CTCF in E12.5 control and *Ctcf*$^{Nes-cre}$ coronal forebrain sections. Scale bar: 220 μm. (C) Immunodetection of CTCF in E14 control and *Ctcf*$^{Nes-cre}$ coronal forebrain sections. Scale bar: 300 μm. (D) Quantification of AC3 immunostaining in E12.5, E14, and E15.5 forebrain tissue ($n = 3$). AC3$^+$ cells were counted and expressed per unit area (square millimeter). (E) Immunodetection of AC3 in E15.5 control and *Ctcf*$^{Nes-cre}$ basal ganglia. Scale bar: 100 μm (F) Immunodetection of AC3 in control and *Ctcf*$^{Nes-cre}$ at 4 DIV. Scale bar: 25 μm. Het, *Ctcf*$^{flox/WT}$*; Nestin-cre*$^+$; Ctx, cortex; BG, basal ganglia; H, hippocampal hem. Error bars represent the SEM. Original magnification: (B, C) x50; (D) x100; (E) x200.

3.3.3 Increased p53 and PUMA levels in the *Ctcf*-null telencephalon

Both the *Foxg1Cre* and *NestinCre* models of CTCF loss exhibit increased levels of apoptosis, although the timing of onset differs between the two mutants. To investigate the molecular mechanism responsible for neuronal cell death due to CTCF loss, we assessed activation of the p53/PUMA pathway. Using Western blot analysis, we found that neuronal cell death correlated with an increase in p53 protein levels in the *Ctcf*^{Nes-cre} forebrain compared to control, suggesting activation and stabilization of the protein (Fig. 3*A*). We next investigated the levels of PUMA, which has been demonstrated previously to mediate NPC death downstream of p53 (Jeffers et al., 2003). Moreover, *Puma* is one of the most upregulated genes in response to CTCF loss in the limb bud (Soshnikova et al., 2010). CTCF binding sites in the *Puma* gene demarcate an intragenic chromatin boundary that is abolished upon CTCF knockdown in human cells, resulting in increased PUMA expression (Gomes and Espinosa, 2010b). To determine whether PUMA is involved caspase-mediated cell death in the *Ctcf* mutant embryos, we examined PUMA protein levels in the *Ctcf*^{Foxg1-cre} and *Ctcf*^{Nes-cre} mice. In the E11.5 *Ctcf*^{Foxg1-cre} forebrain, we observed an increase in PUMA immunostaining compared to littermate controls, corresponding to the high levels of AC3 staining in these embryos (Figure 3-3b). In the *Ctcf*^{Nes-cre} embryos, PUMA was increased in the E15.5 basal ganglia, again correlating with the highest levels of cell death (Figure 3-3c). In primary cultures, *Ctcf*-null NPCs exhibited increased PUMA immunostaining at 4 DIV when compared to control NPCs obtained from littermate embryos (Figure 3-3d). We also quantified *Puma* transcript levels by quantitative reverse-transcriptase PCR and observed a significant increase in the E16.5 *Ctcf*^{Nes-cre} forebrain compared to controls (Figure 3-3e). These observations demonstrate that increased PUMA transcript and proteins levels occur as a consequence of CTCF loss in NPCs and correlate with p53 activation and the onset of caspase-mediated cell death.

PUMA upregulation in the *Ctcf*-deficient embryonic brain could result from decreased CTCF occupancy within the *Puma* gene body, or because of p53-dependent activation of the gene. To investigate these possibilities further, we performed quantitative CTCF and p53 ChIP using E16.5 telencephalon isolated from control and *Ctcf*^{Nes-cre} embryos (Figure

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3-3f-h). ChIP analysis confirmed CTCF binding sites found downstream of *Puma* exon 1 (CBS1) and exon 3 (CBS2) at the *Puma* gene in control tissue, similar to the binding profile of CTCF in human cancer cells (Figure 3-3g; (Gomes and Espinosa, 2010a). As expected, CTCF binding was diminished in the *Ctcf*^Nes-cre^ tissue (Figure 3-3g). ChIP for p53 demonstrated specific enrichment of the protein at the *Puma* promoter in *Ctcf*^Nes-cre^ forebrain compared to control in E16.5 tissue (Figure 3-3h). Together, loss of CTCF in NPCs causes increased *Puma* transcription and protein levels due to increased p53-dependent transcriptional activation, likely combined with the loss of CTCF-dependent repression.
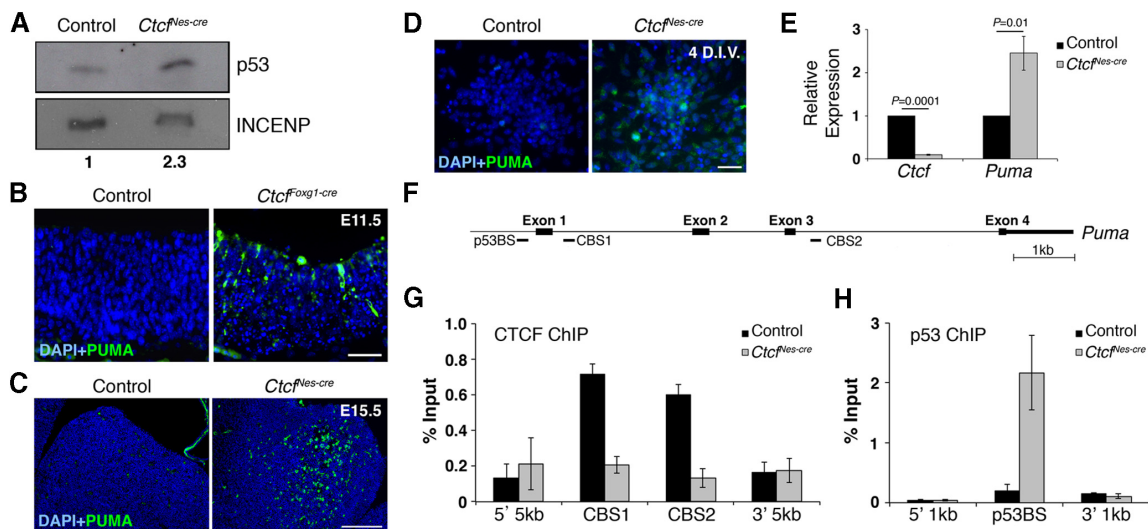
Figure 3-3 Loss of CTCF causes p53-dependent transcriptional activation of *Puma*

(A) Western blot analysis of nuclear protein extracts obtained from E16.5 control and *Ctcf*^*Nes-cre*^ telencephalon. Densitometry analysis of blots revealed a 2.3-fold increase in *Ctcf*^*Nes-cre*^ p53 levels compared to control. INCENP was used as a loading control. (B, C) Immunodetection of PUMA in E11.5 control and *Ctcf*^*Foxg1-cre*^ cortical neuroepithelium (B), E15.5 control and *Ctcf*^*Nes-cre*^ forebrain (C), and control and *Ctcf*-null NPCs (D) at 4 DIV. Scale bars: 50 μm (B, C); 25 μm (D). (E) Quantitative real-time PCR analysis of *Ctcf* and *Puma* expression in control and *Ctcf*^*Nes-cre*^ E16.5 forebrain using primers spanning *Puma* exons 3– 4 (*n* = 3). β-actin was used as an internal control. (F) Schematic representation of the mouse *Puma* gene. PCR products used to detect sites of CTCF binding (CBS1 and CBS2) and p53 binding (p53BS) are shown. (G) Quantitative chromatin immunoprecipitation of CTCF at the *Puma* gene in E16.5 control and *Ctcf*^*Nes-cre*^ telencephalon tissue (*n* = 3). Primer pairs 5 kb upstream and downstream of binding sites were used as negative controls. (H) Quantitative chromatin immunoprecipitation of p53 at the *Puma* gene in E16.5 control and *Ctcf*^*Nes-cre*^ telencephalon tissue shows increased p53 occupancy at the *Puma* promoter in the *Ctcf*^*Nes-cre*^ telencephalon (*n* = 3). Primer pairs 1 kb upstream and downstream of the binding site were used as negative controls. Error bars represent SEM. Original magnification: (A, C) x200; (B) x100.

3.3.4 Deletion of *Puma* in a *Ctcf*-null context rescues cell death, but does not improve hypocellularity at birth

To investigate whether increased PUMA levels cause cell death in the *Ctcf*-deficient embryonic brain, we introduced a mutant *Puma* allele (*Bbc3^{tm1Ast}*) in the *Ctcf^{loxP}* and *NestinCre* mice to generate mice that lack both *Ctcf* and *Puma* expression in the brain (hereafter referred to as *Ctcf$^{Nes-cre}$;Puma$^{-/-}$*). Histological analysis of E16.5 *Ctcf$^{Nes-cre}$* cryosections showed thinning of the VZ/SVZ, hypocellularity of the intermediate zone, and a dramatic reduction in the size of the hippocampal hem (Figure 3-4a). *Ctcf$^{Nes-cre}$;Puma$^{-/-}$* embryos at E16.5 showed rescue in the VZ/SVZ thickness, as well as hippocampal size (Figure 3-4a). We confirmed these observations by partitioning the E16.5 cortex into the CP, IZ, and VZ/SVZ based on cytoarchitecture and quantifying the number of DAPI$^+$ cells per region (Figure 3-4b,c). We observed a restoration in the number of IZ cells and partial restoration in the number of VZ/SVZ cells in the *Ctcf$^{Nes-cre}$; Puma$^{-/-}$* compared to *Ctcf$^{Nes-cre}$* cortex (Figure 3-4c). There was no significant difference in the number of CP cells between genotypes at E16.5 (Figure 3-4c).

We performed AC3 immunostaining in E16.5 brain sections to investigate whether apoptotic cell death was rescued in the double mutant brain. AC3$^+$ cells were elevated in the *Ctcf$^{Nes-cre}$* cortex, basal ganglia, and hippocampal hem compared to control (Figure 3-4b,d). AC3$^+$ cells were largely localized to the ventricular zone of the neocortex, indicating that *Ctcf*-null proliferating cells are more susceptible to caspase-dependent cell death than differentiated cells of the cortical plate. Deletion of *Puma* in the *Ctcf$^{Nes-cre}$* mouse was sufficient to abolish apoptotic cell death, indicating that PUMA mediates increased caspase-dependent cell death in *Ctcf*-null neuroprogenitor cells (Figure 3-4b,d). Together, these data indicate a specific decrease in intermediate zone cellularity in the E16.5 *Ctcf$^{Nes-cre}$* cortex that is fully restored upon inhibition of cell death. The number of cells in the ventricular/subventricular zone is only partially restored, suggesting that loss of cells cannot completely be explained by increased cell death.

To further confirm that cell viability is rescued by deletion of *Puma* in a *Ctcf*-null context, we established primary cortical progenitor cultures from E12.5 control, *Ctcf$^{Nes-cre}$*, *Ctcf$^{Nes-cre}$;Puma$^{-/-}$*, and *Puma$^{-/-}$* telencephalon and measured viability via trypan blue

dye exclusion at 2–5 DIV. Each time point was analyzed by a one-way ANOVA followed by Dunnett's multiple comparisons post-test to determine which genotypes had a mean percentage of viability that was significantly different from control. No difference in viability was observed at 2 and 3 DIV; however, at 4 and 5 DIV, $Ctcf^{Nes\text{-}cre}$ NPCs exhibited a marked reduction in viability, correlating with increased levels of AC3 (Figure 3-4e). NPCs obtained from $Ctcf^{Nes\text{-}cre};Puma^{-/-}$ embryos did not show a significant decrease in viability at any of the time points analyzed (Figure 3-4e).

Since we observed a rescue in caspase-mediated cell death in the embryonic $Ctcf^{Nes\text{-}cre};Puma^{-/-}$ brain, we predicted that the brain size at birth would also be recovered and that the mice would survive into the postnatal period, allowing for more extended analyses. Surprisingly, we failed to recover live $Ctcf^{Nes\text{-}cre};Puma^{-/-}$ mice at birth. Histological staining of postnatal day 0.5 (P0.5) $Ctcf^{Nes\text{-}cre};Puma^{-/-}$ sections demonstrated severe hypocellularity and disorganization of the cortical plate and hippocampus, and an overall similarity to the $Ctcf^{Nes\text{-}cre}$ neonatal brain (Figure 3-5a). Histological cell counts confirmed that there are fewer cells in $Ctcf^{Nes\text{-}cre}$ and $Ctcf^{Nes\text{-}cre};Puma^{-/-}$ cortical plate compared to controls; however, no significant difference in cell number was detected between $Ctcf^{Nes\text{-}cre}$ and $Ctcf^{Nes\text{-}cre};Puma^{-/-}$ (Figure 3-5b). These findings indicate that although deletion of *Puma* prevents *Ctcf*-null cells from undergoing apoptosis, other factors come into play to prevent cortical size expansion in the double mutant brain.
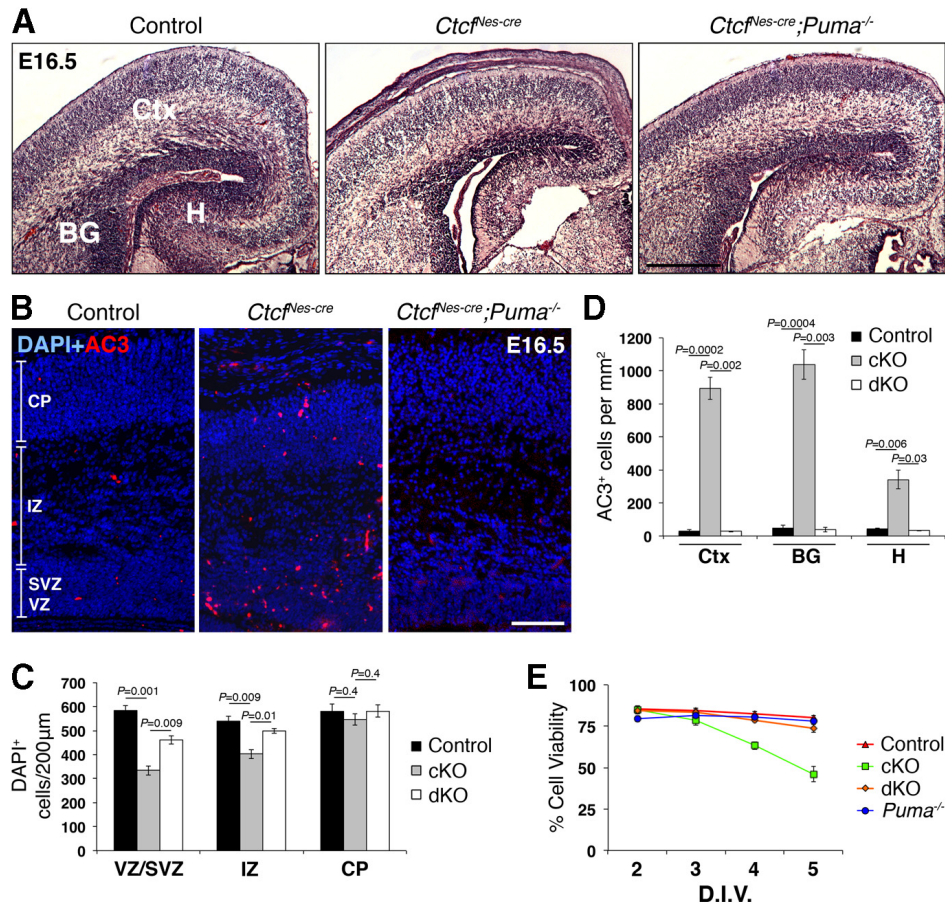
Figure 3-4 Apoptosis is abolished upon deletion of PUMA in the *Ctcf*-deficient embryonic brain

(A) H&E staining of E16.5 control, *Ctcf^Nes-cre* and *Ctcf^Nes-cre;Puma^-/-* coronal cortical cryosections. Skin and skull cap were not dissected from *Ctcf^Nes-cre* brain. Scale bar: 200 μm. (B) AC3 immunostaining in E16.5 control, *Ctcf^Nes-cre*, and *Ctcf^Nes-cre;Puma^-/-* neocortex. Sclae bar: 100 μm. (C) DAPI$^+$ cells were quantified in 200 μm-wide regions of the control, *Ctcf^Nes-cre* (conditional knock-out), and *Ctcf^Nes-cre;Puma^-/-* (double knock-out) CP, IZ, and VZ/SVZ ($n = 3$). (D) AC3$^+$ cells were quantified per unit area in the cortex (Ctx), basal ganglia (BG), and hippocampal hem (H; $n = 3$). (E) Cell viability of NPC cultures was measured by trypan blue dye exclusion after 2–5 DIV ($n = 3$). Data were analyzed by a one-way ANOVA followed by Dunnett's multiple comparisons test to determine which means were significantly different from the control. No significant difference in cell viability was found at 2 and 3 DIV (P = 0.2122 and 0.2062,

respectively); however, viability was significantly different between *Ctcf*^{Nes-cre} and control at 3 and 4 DIV ($P = 0.01$), but not between *Ctcf*^{Nes-cre}*;Puma*^{-/-} and control or *Puma*^{-/-} and control ($P > 0.05$). Error bars represent SEM. Original magnification: (A) **x**100; (C) x50.
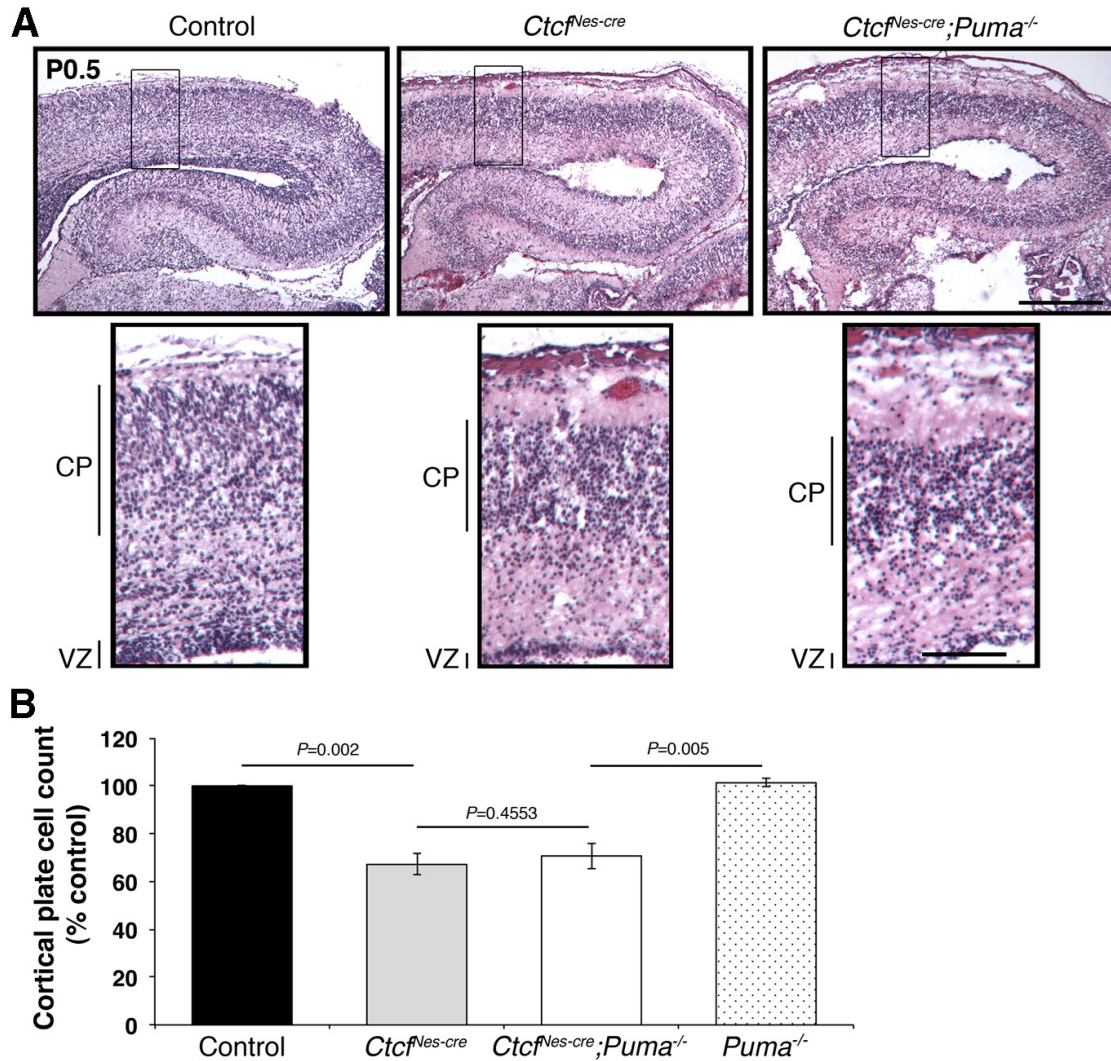
Figure 3-5 *Puma* deletion in the *Ctcf*-deficient brain fails to restore hippocampal size or hypocellularity of the cortical plate

(A) H&E staining of neonatal (P0.5) control, $Ctcf^{Nes-cre}$, and $Ctcf^{Nes-cre};Puma^{-/-}$ cortex and hippocampus. Scale bars: top, 800 μm; bottom, 200 μm. (B) Graph depicting cortical plate cell counts as a percentage of control ($n = 3$). Error bars represent SEM. Original magnification, (A) x25.

3.3.5 *Ctcf*-null apical and oRG progenitors rescued from death by *Puma* deletion fail to proliferate

Given that cell death at E16.5 was rescued in the double mutant embryos but the brain hypocellularity at birth was not, we speculated that the cells rescued from apoptosis might become arrested in the cell cycle, resulting in decreased proliferation. To test this hypothesis, we performed acute BrdU labeling at E16.5 and quantified the proportion of cells in S phase by BrdU immunostaining. To compare the cortical distribution and number of NPCs in S phase, the E16.5 cortex was partitioned into the CP, IZ, and VZ/SVZ based on cytoarchitecture (Figure 3-6a). The number of BrdU$^+$ cells was significantly reduced in the *Ctcf$^{Nes-cre}$* and *Ctcf$^{Nes-cre}$;Puma$^{-/-}$* intermediate zones and ventricular zone/subventricular zones compared to control (Figure 3-6a,b). Despite substantial rescue in cellularity of the *Ctcf$^{Nes-cre}$;Puma$^{-/-}$* cortex compared to *Ctcf$^{Nes-cre}$* at E16.5, the proliferative capacity of progenitors was not significantly different (Figure 3-6a,b). Our results demonstrate that *Puma* deletion rescues cell death in the *Ctcf*-null brain, but that the rescued cells display reduced proliferative capacity.
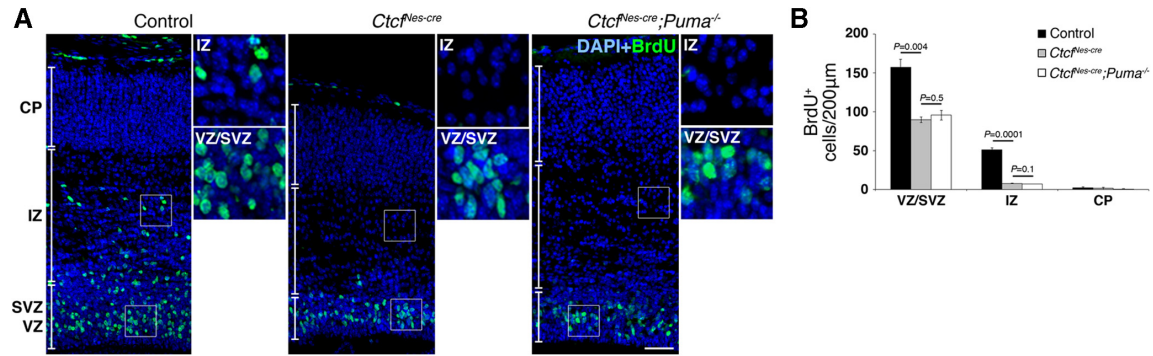
Figure 3-6 *Ctcf* deficient cells that are rescued from apoptotic death display reduced proliferative capacity

Pregnant females were subjected to a 1 h BrdU pulse before being killed. (A) BrdU (green) immunostaining of E16.5 control, *Ctcf^Nes-cre*, and *Ctcf^Nes-cre;Puma^-/-* cortical cryosections. The inset demonstrates fewer BrdU$^+$ cells in the *Ctcf^Nes-cre* and *Ctcf^Nes-cre;Puma^-/-* IZ compared with control. Scale bar, 50 μm. Original magnification, x100. (B) The number of cells in S phase was quantified by counting BrdU$^+$ cells in 200-μm-wide cortical images (*n* = 3). Error bars represent SEM. Original magnification, (A) x100.

Three NPC subtypes exist in the embryonic cortex: apical radial glia (apical progenitors), basal progenitors, and outer radial glia. Apical progenitors can be identified by their expression of SOX2 and PAX6 transcription factors (Bani-Yaghoub et al., 2006; Gotz et al., 1998; Shitamukai and Matsuzaki, 2012; Tarabykin et al., 2001). They undergo interkinetic nuclear migration and divide at the ventricular surface to either self-renew or differentiate into basal progenitors, outer radial glia, or cortical neurons (Shitamukai et al., 2011; Shitamukai and Matsuzaki, 2012). Basal progenitors reside in the subventricular zone and uniquely express the T-box transcription factor TBR2 (Englund et al., 2005). They are reported to have the potential to self-renew; however, the majority of their divisions are neurogenic (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Outer radial glia are similar to radial glia in that they have a basal process and express SOX2 and PAX6; however, oRG cells are located outside of the VZ in the outer SVZ. They are able to divide asymmetrically, producing one oRG and one neuron-committed cell with each division (Reillo et al., 2011; Shitamukai et al., 2011; Shitamukai and Matsuzaki, 2012; Wang et al., 2011a; Wang et al., 2011b). We performed acute BrdU-labeling for 1 h and coimmunostained E16.5 cortical sections with antibodies against BrdU and TBR2 or SOX2 to examine the behavior of different progenitor subtypes after loss of CTCF (Figure 3-7,8).

The total number of TBR2$^+$ basal progenitor cells was reduced in the $Ctcf^{Nes\text{-}cre}$ cortex compared to control (Figure 3-7a,b). The number of basal progenitors was restored to control levels in the $Ctcf^{Nes\text{-}cre};Puma^{-/-}$ cortex, and most rescued cells were still able to enter S phase (Figure 3-7a,c). Despite the correct overall number of TBR2$^+$ cells in the $Ctcf^{Nes\text{-}cre};Puma^{-/-}$ cortex, the localization of basal progenitors appeared to be shifted apically compared to control (Figure 3-7a,b). This is perhaps due to an inability of the rescued basal progenitors to correctly delaminate from the ventricular surface.
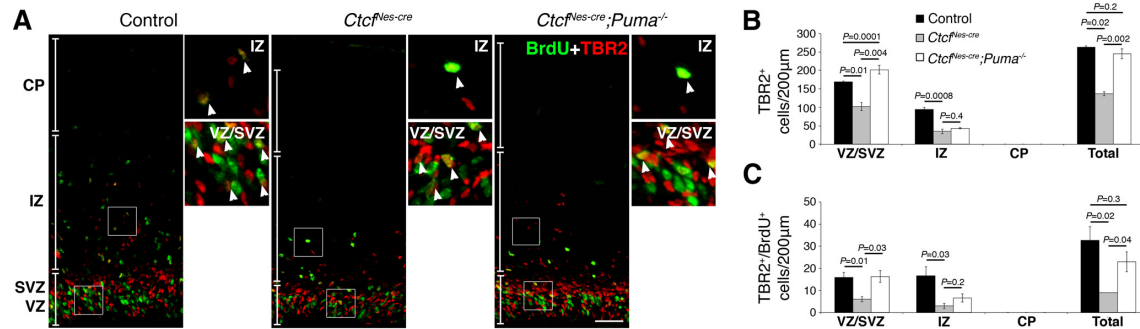
Figure 3-7 *Ctcf*-deficiency results in PUMA-dependent apoptosis of basal progenitor cells

Pregnant females were subjected to a 1 h BrdU pulse before being killed. (A) TBR2 (red) and BrdU (green) coimmunostaining of E16.5 cortical cryosections. Arrowheads indicate TBR2$^+$/BrdU$^+$ cells in the IZ and VZ/SVZ. Scale bar, 50 μm. Original magnification, x100. (B) TBR2$^+$ cells were quantified in 200-μm-wide cortical images ($n = 3$). (C) TBR2$^+$/BrdU$^+$ cells were quantified in 200-μm-wide cortical images ($n = 3$). Error bars represent SEM.

Quantification of SOX2$^+$ cells indicated a dramatic reduction in the number of apical progenitors and oRGs in the *Ctcf*$^{Nes-cre}$ cortex (Figure 3-8a,b). The number of SOX2$^+$ apical progenitors was only partially rescued in the *Ctcf*$^{Nes-cre}$*;Puma*$^{-/-}$ cortex, indicating that CTCF may control the size of the apical progenitor pool independently of apoptosis (Figure 3-8a,b). Conversely, oRG (SOX2$^+$ cells in the intermediate zone) numbers were restored to control levels in the *Ctcf*$^{Nes-cre}$*;Puma*$^{-/-}$ cortex, indicating that these cells are lost via *Puma*-mediated apoptotic cell death upon deletion of *Ctcf* (Figure 3-8a,b). The proliferative capacity of apical progenitors (SOX2$^+$/BrdU$^+$ cells in the VZ/SVZ) was severely diminished in the *Ctcf*$^{Nes-cre}$ cortex compared to control, and was not restored in the *Ctcf*$^{Nes-cre}$*;Puma*$^{-/-}$ cortex (Figure 3-8a,c). Similarly, BrdU incorporation in the rescued *Ctcf*$^{Nes-cre}$*;Puma*$^{-/-}$ oRG cells was extremely low, indicating that they are likely arrested in the cell cycle and fail to correctly enter S phase (Figure 3-8a,c). To confirm that these cells are oRG progenitors, we performed SOX2 and PAX6 coimmunostaining and indeed observed a decreased number of SOX2$^+$PAX6$^+$ cells in the intermediate zone of *Ctcf*$^{Nes-cre}$ embryos compared to control and *Ctcf*$^{Nes-cre}$*;Puma*$^{-/-}$ at E16.5 (Figure 3-8d).
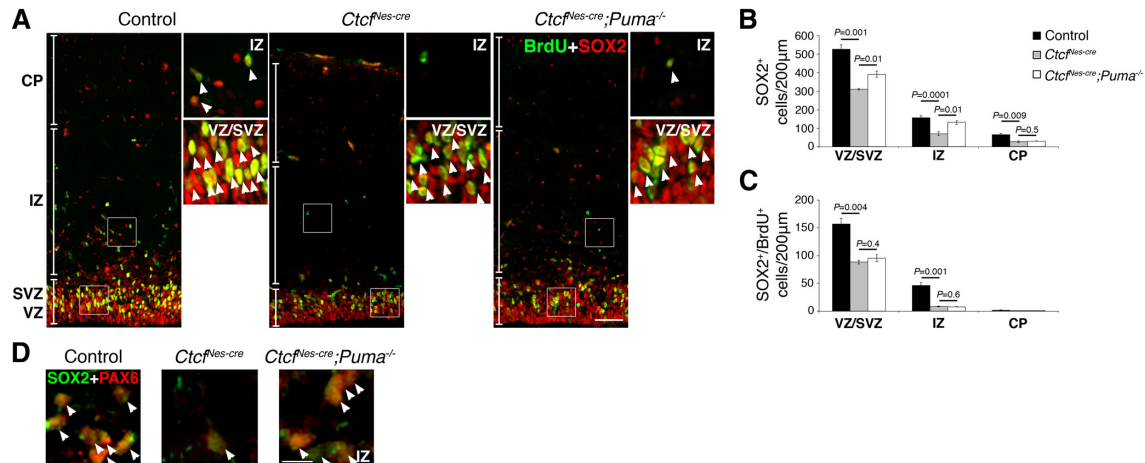
Figure 3-8 *Ctcf*-deficiency results in PUMA-dependent apoptosis of apical and outer radial glia progenitors

Apical and outer radial glia progenitors that are rescued from apoptotic death fail to proliferate. Pregnant females were subjected to a 1 h BrdU pulse before being killed. *A*, SOX2 (red) and BrdU (green) coimmunostaining of E16.5 cortical cryosections. The inset demonstrates fewer SOX2$^+$ cells in the *Ctcf*$^{Nes-cre}$ IZ than control or *Ctcf*$^{Nes-cre}$*;Puma$^{-/-}$*. Scale bar: 50 μm. (B) SOX2$^+$ cells were quantified in 200-μm-wide cortical images (*n* = 3). (C) SOX2$^+$/BrdU$^+$ cells were quantified in 200-μm-wide cortical images (*n* = 3). (D) Immunodetection of SOX2 (green) and PAX6 (red) in the E16.5 cortical IZ demonstrates restoration of oRG progenitors in *Ctcf*$^{Nes-cre}$*;Puma$^{-/-}$* cortex compared to *Ctcf*$^{Nes-cre}$. Scale bar: 10 μm. Arrowheads indicate SOX2$^+$/PAX6$^+$ oRG cells. Error bars represent SEM. Original magnification: (A, D) x100.

3.3.6 *Ctcf* loss causes premature differentiation of apical progenitors

Incomplete restoration of the apical progenitor population in the $Ctcf^{Nes-cre};Puma^{-/-}$ cortex suggests that loss of CTCF leads to an apoptosis-independent reduction of these cells (Figure 3-8a,b). To identify the cause of reduced apical cell numbers, we analyzed the progenitor pool composition, cell cycle exit indices, and postmitotic projection neuron subtypes in control and $Ctcf^{Nes-cre}$ cortex at E14.

Apical progenitors begin to produce projection neurons at approximately E11.5 (Figure 1-5a). At the same time, they generate neuron-committed basal progenitors and oRG progenitors (Figure 1-5a). Since the majority of basal progenitor divisions are terminal and result in the production of two neurons, this would result in increased generation of neurons at the expense of the progenitor pool (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Watson et al., 2014). The relative abundance of TBR2$^+$ basal progenitors was significantly increased in the E14 $Ctcf^{Nes-cre}$ cortex compared to control, whereas the number of SOX2$^+$ apical progenitors was not affected (Figure 3-9a,b), suggesting that differentiation of apical progenitors into basal progenitors is increased. Next, we measured the proportion of cells exiting the cell cycle by labeling embryos with BrdU for 24 h and analyzing Ki67$^+$ and BrdU$^+$ cells in E14 control and $Ctcf^{Nes-cre}$ cortex. Cell cycle exit (BrdU$^+$Ki67$^-$ cells/total BrdU$^+$ cells) was significantly higher in $Ctcf^{Nes-cre}$ cortex compared to controls (Figure 3-9c,d).

The cortical plate is established by projection neurons that organize themselves in an "inside-out" manner, such that early-born neurons populate deeper cortical layers (layer VI, then layer V), and late-born neurons migrate past the early-born neurons to populate the more superficial layers of the cortex (layer IV, then layer II/III; (Greig et al., 2013) (Figure 1-5a). Increased cell cycle exit and differentiation at E13–E14 is predicted to result in an increased number of layer VI corticothalamic projection neurons (TBR1$^+$), layer V subcerebral projection neurons (CTIP2$^+$), and callosal projection neurons (SATB2$^+$) (Figure 1-5a). We found that the relative generation of these neuronal subtypes was increased in the $Ctcf^{Nes-cre}$ cortex compared to control (Figure 3-9e-g), confirming premature differentiation of *Ctcf*-null apical progenitors, which is predicted to result in a reduced number of this progenitor pool by late neurogenesis.
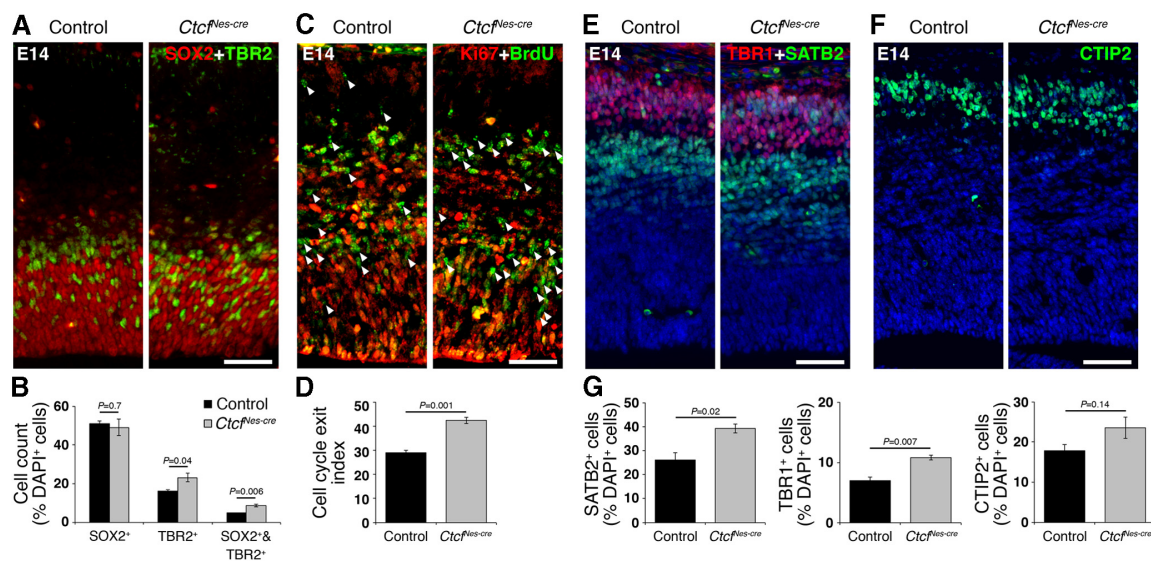
Figure 3-9 *Ctcf*-deficiency causes premature neurogenesis

(A) Immunodetection of SOX2 (red) and TBR2 (green) in E14 control and *Ctcf*[Nes-cre] cortex. Scale bar: 50 μm. (B) SOX2$^+$, TBR2$^+$, and SOX2$^+$/TBR2$^+$ cells were quantified in 150-μm-wide cortical images and expressed as a percentage of total DAPI$^+$ cells (*n* = 3). (C) Pregnant female mice were subjected to a 24 h BrdU pulse before being killed. Ki67 (red) and BrdU (green) immunostaining was used to determine the percentage of cells exiting the cell cycle in control and *Ctcf*[Nes-cre] E14 cortex. Arrowheads indicate BrdU$^+$Ki67$^+$ cells that have exited the cell cycle. Scale bar: 50 μm. (D) Cell cycle exit indices were calculated by measuring the ratio of BrdU$^+$Ki67$^-$ cells to total BrdU$^+$ cells in control and *Ctcf*[Nes-cre] cortex at E14 (*n* = 3). (E) Immunodetection of TBR1 (red) and SATB2 (green) in E14 control and *Ctcf*[Nes-cre] cortex. Scale bar: 50 μm. (F) Immunodetection of CTIP2 (green) in E14 control and *Ctcf*[Nes-cre] cortex. Scale bar: 50 μm. (G) SATB2$^+$, TBR1$^+$, and CTIP2$^+$ cells were quantified and expressed as a percentage of DAPI$^+$ cells (*n* = 3). Error bars represent SEM. Original magnification: (A, C, E, F) x100.

3.4 Discussion

This study provides evidence that CTCF is required at very early stages of telencephalon development for the maintenance and survival of neuroprogenitor cells. We found that ablation of CTCF leads to *Puma*-dependent apoptosis of NPCs using two different conditional deletion strategies. Increased apoptosis correlated with p53-dependent *Puma* transcription, suggesting that CTCF loss results in p53 stabilization and transcriptional activation of its downstream targets. CTCF loss might also result in a more open chromatin environment at the *Puma* gene, facilitating p53-dependent activation of transcription and elongation of RNA polymerase II (Gomes and Espinosa, 2010a, b). *Ctcf* inactivation in postmitotic cortical and hippocampal neurons does not induce apoptosis (Hirayama et al., 2012), pointing to a specific survival role for CTCF in proliferating cells. However, CTCF loss of function may not induce apoptosis in all types of proliferating cells *in vivo*, as *Ctcf* deletion in thymocytes was shown previously to induce cell cycle arrest without induction of apoptosis (Heath et al., 2008). It is possible that the outcome of CTCF deficiency leads to either p53-dependent cell cycle arrest or apoptosis, depending on varying cell-specific or temporal cues. This could be similar to the outcomes described upon *Nbs1* (*Nijmegen breakage syndrome 1/Nibrin*) deletion in the CNS, which leads to p53-dependent apoptosis in the cerebellum, but causes p53-dependent cell cycle arrest in the neocortex (Li et al., 2012).

In the present report, we demonstrate that deletion of *Puma* effectively rescues apoptotic cell death observed at E16.5 in the *Ctcf*$^{Nes-cre}$ brain (Figure 3-4). Despite this apparent recovery at E16.5, the double mutant brain at birth appeared hypocellular and was nearly undistinguishable histologically from the *Ctcf*$^{Nes-cre}$ brain (Figure 3-5). We showed that *Ctcf*$^{Nes-cre}$*;Puma*$^{-/-}$ apical and oRG progenitors that cannot activate the apoptotic pathway fail to incorporate BrdU, which might be explained by a p53-mediated cell cycle arrest. Further investigations into the mechanism underlying increased p53 levels in the *Ctcf*-null brain will be important to fully elucidate the role of CTCF in progenitor cell survival. Given that oRG cells are implicated in neocortical expansion in humans, their reduced ability to proliferate in the double mutant cortex might partly explain the failure to recover cortical size at birth, and may be relevant to microcephaly caused by *CTCF*

mutations in humans (Hansen et al., 2011; Lui et al., 2011; Reillo et al., 2011).

A key observation is that deletion of *Puma* did not completely restore the number of apical cells in the *Ctcf*$^{Nes-cre}$*;Puma*$^{-/-}$ cortex, suggesting that while a proportion of this progenitor population undergoes *Puma*-dependent death in the absence of CTCF, apical cells exhibit an independent defect. We found that *Ctcf*-null apical progenitors differentiate prematurely, causing an initial increase in the production of basal progenitors and early-born postmitotic neurons (Figure 3-9). However, the increased number of basal progenitors is later counteracted by a reduction of the progenitor pool from which they are derived, and exacerbated by increased levels of apoptosis. Given that CTCF colocalizes with the cohesin complex and is required for cohesin localization to specific genomic sites to influence gene expression (Parelho et al., 2008; Wendt and Peters, 2009), it is conceivable that premature neurogenesis in the *Ctcf*$^{Nes-cre}$ cortex results from dysregulation of CTCF target genes.

The number of basal progenitor cells marked by TBR2 expression was completely restored in the double mutant brain, indicating that PUMA activation causes basal progenitor cell death in the absence of CTCF. Simultaneous labeling of brain sections with TBR2 and BrdU showed that rescued basal progenitors are still able to proliferate, unlike rescued apical and oRG cells. Despite the overall equivalent number of TBR2^{+} cells in the control and double mutant cortex, several rescued basal progenitors did not move basally out of the ventricular zone, perhaps due to a defect in apical radial glia or an inability to correctly delaminate from the apical surface of the *Ctcf*-deficient cortex.

In summary, we demonstrated that CTCF is required in the early developing mouse brain for neuroprogenitor cell survival and that its deletion induces p53- and PUMA-dependent apoptosis. Independent from its role in promoting cell survival, CTCF is required for the correct balance of proliferative versus differentiative divisions and maintenance of the apical progenitor pool. Together, these functions of CTCF contribute to the normal development of the mammalian neocortex.

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Chapter 4

4 CTCF is required to prevent replication-associated DNA damage in neuroprogenitor cells

This chapter aims to address the molecular mechanism underlying p53/PUMA-dependent apoptosis downstream of CTCF loss by investigating a role for CTCF in genomic stability. Several reports have identified that CTCF is and important regulator of replication (Bergstrom et al., 2007; Cleary et al., 2010), and one study reported telomere dysfunction as a consequence of CTCF depletion (Deng et al., 2012a), however the exact roles for CTCF in replication are not well understood. This is the first study to identify that CTCF loss causes replication-associated damage.

4.1 Introduction

The CCCTC-binding factor (CTCF) is implicated in higher-order genome organization through its ability to mediate intra- and inter-chromosomal contacts, bringing disparate regions of the genome into close proximity (Kurukuti et al., 2006; Ling et al., 2006; Splinter et al., 2006). This function of CTCF has been primarily studied in the context of transcriptional regulation, whereby CTCF controls enhancer-promoter interactions. Additionally, transcription-independent roles for CTCF have been described in the literature: CTCF regulates stability of trinucleotide repeats implicated in expansion disorders such as spinocerebellar ataxia type 7 (SCA7) and myotonic dystrophy type I (DM1) (Filippova et al., 2001; Libby et al., 2008; Sopher et al., 2011). At the *DM1* locus, CTCF binding upstream of the repeat is required to slow DNA polymerase before replication of the repetitive tract, and mutations in the CTCF binding site results in repeat expansion likely due to strand slippage (Cleary et al., 2010). Intriguingly, DM1 is asymmetrically replicated (Rajcan-Separovic 1998) and CTCF regulates asymmetric replication of the *H19/Igf2* imprinted domain (Bergstrom et al., 2007). CTCF is thus an important factor for DNA replication, although its actions may be limited to certain loci as direct roles for the protein have only been described at asymmetrically replicated regions. CTCF has also been implicated in replication origin firing. Overexpression of the oncogenic replication licensing factor *Cdc6* causes CTCF dissociation from the

promoters of the tumour-suppressor genes *CDH1* and *INK4/ARF*, resulting in reduced expression and activation of adjacent replication origins (Sideridou et al., 2011). It was suggested that because replication origins are defined by structural chromatin context rather than DNA sequence CTCF might facilitate higher-order organization of replication origins (Antequera, 2004; Cvetic and Walter, 2005).

Whereas the CTCF partner cohesin is implicated in organizing replication factories and controlling S phase progression, CTCF depletion had little effect on cell cycle dynamics or the overall size of replicon units identified by the DNA halo assay (Guillou et al., 2010). A more global role for CTCF in DNA replication have not yet been thoroughly investigated, but is of interest given the importance of CTCF for replication timing, progression, and origin firing at individual loci. Furthermore, the finding that TADs regulate replication timing suggests that CTCF may participate in this process if indeed CTCF is involved in orchestrating TAD organization (Dixon et al., 2012; Pope et al., 2014).

Human mutations in *CTCF* cause intellectual disability associated with microcephaly and autistic features (Gregor et al., 2013) and single nucleotide polymorphisms (SNPs) at the *CTCF* locus are associated with schizophrenia (Juraeva et al., 2014). Deletion at 16q22.1, the genomic locus of *CTCF,* is one of the most frequent genetic events in breast cancer and is well documented in several other cancers (Filippova et al., 1998; Rakha et al., 2006). Together, this suggests that CTCF is important for correct brain development and to suppress tumourigenesis, however the molecular underpinnings of these associations are unknown.

CTCF is an essential factor for cellular survival (Fedoriw et al., 2004; Heath et al., 2008; Moore et al., 2012; Soshnikova et al., 2010; Wan et al., 2008; Watson et al., 2014). However, the mechanisms responsible have not been characterized. We previously reported that CTCF loss in neuroprogenitor cells (NPCs) causes p53 stabilization, *Puma* upregulation, and PUMA-dependent apoptosis (Watson et al., 2014). Here, we provide novel evidence that CTCF is required to safeguard the genome through S phase during early stages of fate restriction in NPCs. CTCF localizes to damage sites under conditions

of replicative stress but it does not appear to be required for repair of exogenous replication-associated damage, perhaps reflecting a more general role for CTCF in organizing chromatin domains during replication to facilitate replication and prevent genetic instability.

4.2 Materials and Methods

4.2.1 Mouse husbandry and genotyping

Mice were exposed to 12 h light/dark cycles and fed tap water and regular chow *ad libitum*. The *Ctcf^loxP* mice, in which *loxP* sites flank exons 3–12, have been described previously (Heath et al., 2008). Mice conditionally deficient in CTCF were generated by crossing *Ctcf^loxP/+* females (C57BL/6 background) with heterozygous *Foxg1Cre* knock-in male mice (129/sv background) or with *NestinCre* heterozygous male mice (C57BL/6 background; (Berube et al., 2005; Hebert and McConnell, 2000). To account for decreased *Foxg1* expression due to knock-in of the Cre recombinase gene, *Cre^+* males were used as controls (*Ctcf^+/+Foxg1-cre^+/−*) unless stated otherwise. Using the *NestinCre* driver line, *Ctcf^loxP/loxP* mice were crossed with *Ctcf^loxP/+;Nestin^+* mice to generate *Ctcf^lox/P/loxP* or *Ctcf^loxP/+* (controls) and *Ctcf^loxP/loxP;Nestin^+* (*Ctcf^Nes-cre*). DNA from tail biopsies of newborn pups or yolk sac from embryos was genotyped by PCR, as previously described (Watson et al., 2014).

4.2.2 Immunostaining, histology, and antibodies

For immunofluorescence staining, cryosections and cells were incubated with the primary antibody overnight at 4°C, washed in PBS/0.3% Triton-X 100, and incubated with the secondary antibody for 1 h at room temperature. Sections and cells were counterstained with DAPI (Sigma-Aldrich) and mounted in SlowFade Gold (Invitrogen). Primary antibodies used were as follows: rabbit anti-CTCF (1:400; Cell Signaling Technology), rabbit anti-γH2AX (S139; 1:100; Cell Signaling Technology), mouse anti-PCNA (1:400; Santa Cruz Biotechnology), mouse anti-BrdU (1:50; BD Biosciences), rabbit anti-phosphorylated H3 (S10; 1:500; Millipore). Secondary antibodies used were as follows: goat-anti-rabbit Alexa 594 (1:800; Invitrogen), goat-anti-mouse Alexa 488 (1:800; Invitrogen), donkey-anti-sheep Alexa 594 (1:800; Invitrogen), and donkey-anti-mouse

Alexa 488 (1:800; Invitrogen). Sections were subjected to antigen retrieval (incubated in 0.1 mM sodium citrate, pH 6.0, heated to ~95°C and microwaved on low for 10 min) before overnight incubation. For histological studies, slides were stained with hematoxylin and eosin (H&E).

4.2.3 BrdU labeling

Pregnant mice were injected intraperitoneally with cell proliferation labeling reagent [10 mM bromodeoxyuridine (BrdU) and 1 mM fluorodeoxyuridine (FdU) in H_2O] at 1 ml/100 g body weight, or 0.3 mg/g body weight (GE Healthcare Life Sciences). Animals were killed after 1 h by CO_2 asphyxiation, and the embryos were recovered in ice-cold PBS, pH 7.4, and were fixed in 4% paraformaldehyde. Tissue was equilibrated in 30% sucrose/PBS and frozen in OCT (Tissue Tek). Before immunofluorescence analysis, cryosections (8 μm) were treated with 2N HCl to denature the DNA, and neutralized with 0.1 M $Na_2B_4O_7$, pH 8.5.

4.2.4 Primary NPC cultures and manipulation

Cortical progenitor cultures were prepared as described previously (Gloster et al., 1999; Slack et al., 1998; Watson et al., 2013) using cortices dissected from E12.5 embryos. Cells were seeded on polyornithine-coated (Sigma-Aldrich) plastic plates or glass coverslips. Cells were fixed in 4% paraformaldehyde for 10 min, washed in PBS, and processed for immunofluorescence. Cell viability was measured using the trypan blue dye exclusion method. Cell counts were determined with a hemacytometer.

4.2.5 Western blot analysis

Total protein was extracted using RIPA buffer and quantified using the Bradford assay. Protein (20 μg) was resolved on a 6% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was probed with primary antibodies followed by the appropriate horseradish peroxidase-conjugated secondary antibodies (1:4000; GE Healthcare Life Sciences). The membrane was incubated in ECL before exposure to x-ray film or on a ChemiDoc™ MP (BioRad). Densitometry analysis was performed using ImageJ software (version 1.47).

4.2.6 Cell cycle profiling

Primary NPCs were cultured for two days and pulse-labeled with cell proliferation labeling reagent [10 mM bromodeoxyuridine (BrdU) and 1 mM fluorodeoxyuridine (FdU) in H_2O] at 5 μl/ml media (GE Healthcare Life Sciences), fixed, and stained with propidium iodide (PI). The proportion of cells in each phase of the cell cycle was determined by flow cytometry on a Beckman-Coulter Epics XL-MCL instrument, as described (Isaac et al., 2006).

4.2.7 Immunoprecipitation

Total cell lysates were obtained from primary NPC cultures using RIPA buffer for 30 minutes on ice. 500 μg of protein was treated with 100U/ml DNase I (Sigma) for 10 minutes at 37°C, followed by incubation with 1 μg mouse anti-PCNA antibody (Santa Cruz Biotechnologies) while rotating overnight at 4°C. Normal mouse IgG (Santa Cruz Biotechnologies) was used as a negative control. Samples were then incubated with Dynabeads protein G (Invitrogen) for 2 h at 4°C. Immunoprecipitates were washed three times with 1XPBS/0.2% Tween-20, eluted at 70°C for 10 minutes, and resolved on 8-12% SDS-PAGE. Western blot analysis was carried out with a rabbit anti-CTCF (1:1,000; Cell Signaling) and mouse anti-PCNA (1:500; Santa Cruz Biotechnology).

4.2.8 Molecular Combing

Primary NPCs were cultured for two days and pulse-labeled with CldU (25μM) for 30 minutes, followed by washing with 1XPBS, and IdU (125μM) labeling for 30 minutes. Cells were trypsinized and embedded into 1% low melt-grade agarose plugs (Bioshop) to a final concentration of $1x10^6$ cells/ml. The plugs were incubated in 1% *N*-lauryl sarcosyl plus 1mg/ml proteinase K until processing. Plugs were washed, immunostained, and analyzed as previously described (Yang et al., 2012).

4.2.9 Microscopy

Experiments demonstrating co-localization of γH2AX/PCNA and repair capacity (resolution of γH2AX foci) were captured using a laser scanning confocal microscope (FV1000, Olympus). Stacks were obtained at 0.25-μm *z* intervals generally spanning 10–

20 μm. Overlapping signal was scored as a co-localization event. All other images were captured with a digital camera (ORCA-ER; Hamamatsu) using an inverted microscope (DMI 6000b; Leica). Openlab imaging software (PerkinElmer) was used for manual image capture, and processing was performed using Volocity software (PerkinElmer). For quantification of γH2AX foci, nuclei were counted in a defined area in at least three serial cortical cryosections, and the ratio of γH2AX foci to area (mm^2) was calculated. For BrdU and phosphorylated histone H3 (S10) quantification, at least three serial cortical cryosections were quantified in 150μm-wide sections per embryo.

4.2.10   Statistical analyses

Statistical analysis was performed using GraphPad Prism software (GraphPad Software; version 4.02), and all results are expressed as the mean ± SEM. Unless indicated otherwise, *p* values were generated using Student's *t* test (unpaired, two-tailed) to compare between two independent data sets. For replication fork rates, *P* values were determined using the Mann-Whitney U-test for non-parametric data.

4.3 Results

4.3.1 CTCF loss causes an accumulation of endogenous DNA damage in the embryonic brain

CTCF loss in NPCs causes p53 stabilization, *Puma* upregulation, and PUMA-dependent apoptosis (Watson et al., 2014). Given the essential nature of CTCF for cellular survival (Fedoriw et al., 2004; Heath et al., 2008; Moore et al., 2012; Soshnikova et al., 2010; Watson et al., 2014) and aspects of chromatin organization (Zuin et al., 2014) we hypothesized that CTCF may be required to prevent genomic instability, an upstream activator of p53/PUMA signaling in NPCs (Jeffers et al., 2003).

To test this hypothesis we evaluated the nuclear pattern and levels of γH2AX, a marker of DNA double-strand breaks (DSBs) in two models of embryonic brain-specific *Ctcf* deficiency (*Ctcf*$^{Foxg1-cre}$ and *Ctcf*$^{Nes-cre}$). We observed that in both models of *Ctcf* deficiency, the number of γH2AX foci were elevated, indicating that endogenous DNA

damage accumulation is likely responsible for activation of p53/PUMA-dependent apoptosis in the two systems (Figure 4-1a-c). We observed that γH2AX-positive cells were primarily localized to the ventricular/subventricular zone (VZ/SVZ) of the embryonic $Ctcf^{Nes-cre}$ neocortex. Moreover, γH2AX foci were rarely present in post-mitotic cells of the cortical plate (CP) (Figure 4-1b). These observations indicate that damage accumulation in $Ctcf$-deficient embryonic brain might be linked to proliferative state.
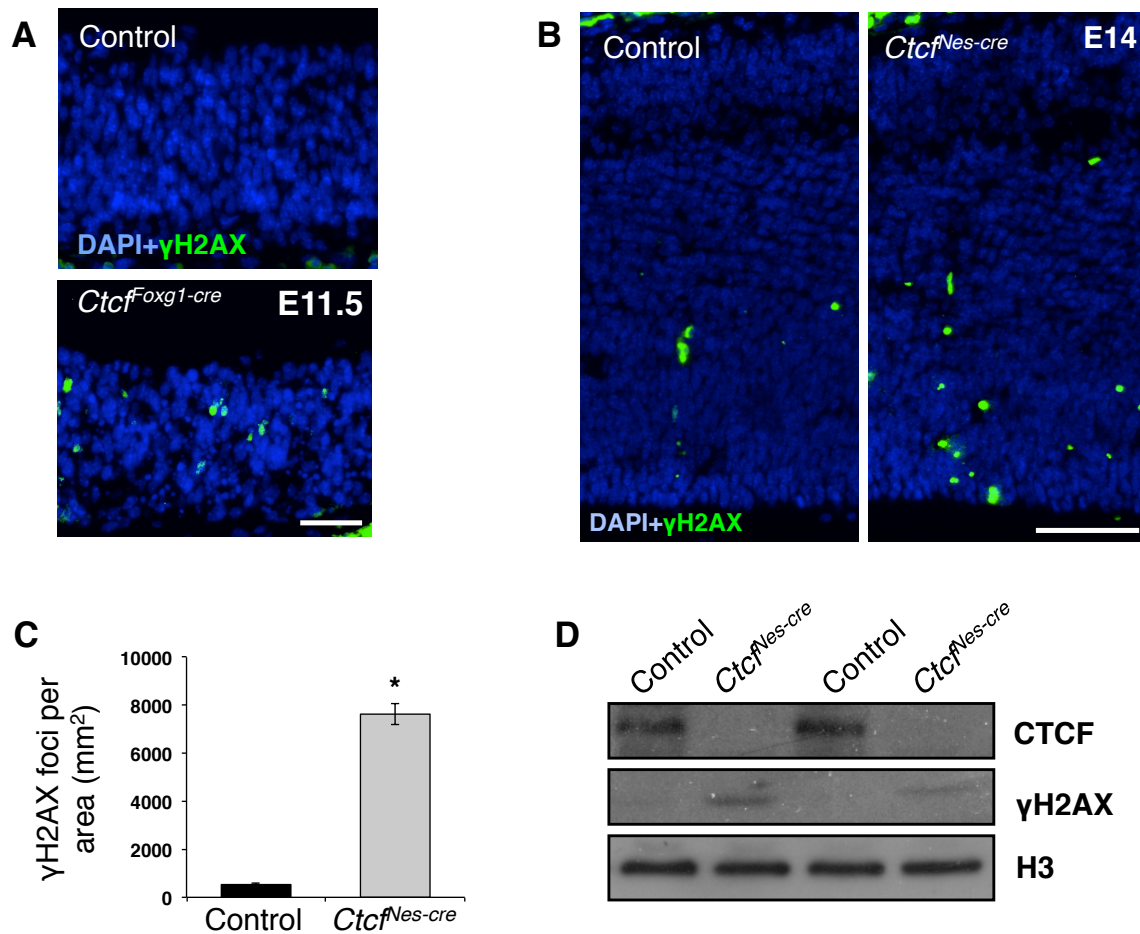
Figure 4-1 CTCF loss in the developing mouse brain is associated with increased endogenous DNA damage

(A) γH2AX (green) immunostaining in E11.5 control and *Foxg1*-cKO cortical neuroepithelium. Scale bar: 50μm. Nuclei were counterstained with DAPI (blue). (B) γH2AX (green) immunostaining in E14 control and *Nestin*-cKO neocortex. Scale bar: 100μm. Nuclei were counterstained with DAPI (blue). (C) Quantification of γH2AX foci per area (mm²) in E14 control and *Nestin*-cKO neocortex, *n* = 3; *P* <0.0001. Data is represented as mean ± standard error of the mean. (D) Western blot analysis of E16.5 control and *Nestin*-cKO forebrain extracts probed for CTCF and γH2AX. Histone H3 was used as a loading control.

4.3.2 CTCF protects against endogenous replicative stress

To investigate the relationship between damage accumulation and proliferative state, we assessed PCNA and γH2AX immunoreactivity in control and $Ctcf^{Nes\text{-}cre}$ neocortex (Figure 4-2). PCNA is a cell cycle marker present at high levels during late-G_1, appears in a punctate pattern during mid/late-S phase, and is downregulated in G_2 before the onset of mitosis (Arai et al., 2011; Bravo and Macdonald-Bravo, 1987). PCNA-positive *Ctcf*-deficient NPCs exhibited an elevated number of γH2AX foci per cell than PCNA-negative cells, and both populations had a significant increase in the number of γH2AX foci over control cells (Figure 4-2b). Additionally, the percentage of γH2AX foci that co-localize with punctate PCNA foci was increased in $Ctcf^{Nes\text{-}cre}$ cells (Figure 4-2c), despite the finding that CTCF loss was associated with a reduced number of PCNA foci (Figure 4-2b,d). Since punctate PCNA staining is typically observed in mid/late-S phase, when heterochromatin is replicated, *Ctcf*-null cells may have difficulties in replicating heterochromatin. Together, these data point to a link between damage accumulation and DNA replication sites during S phase in *Ctcf*-deficient NPCs.
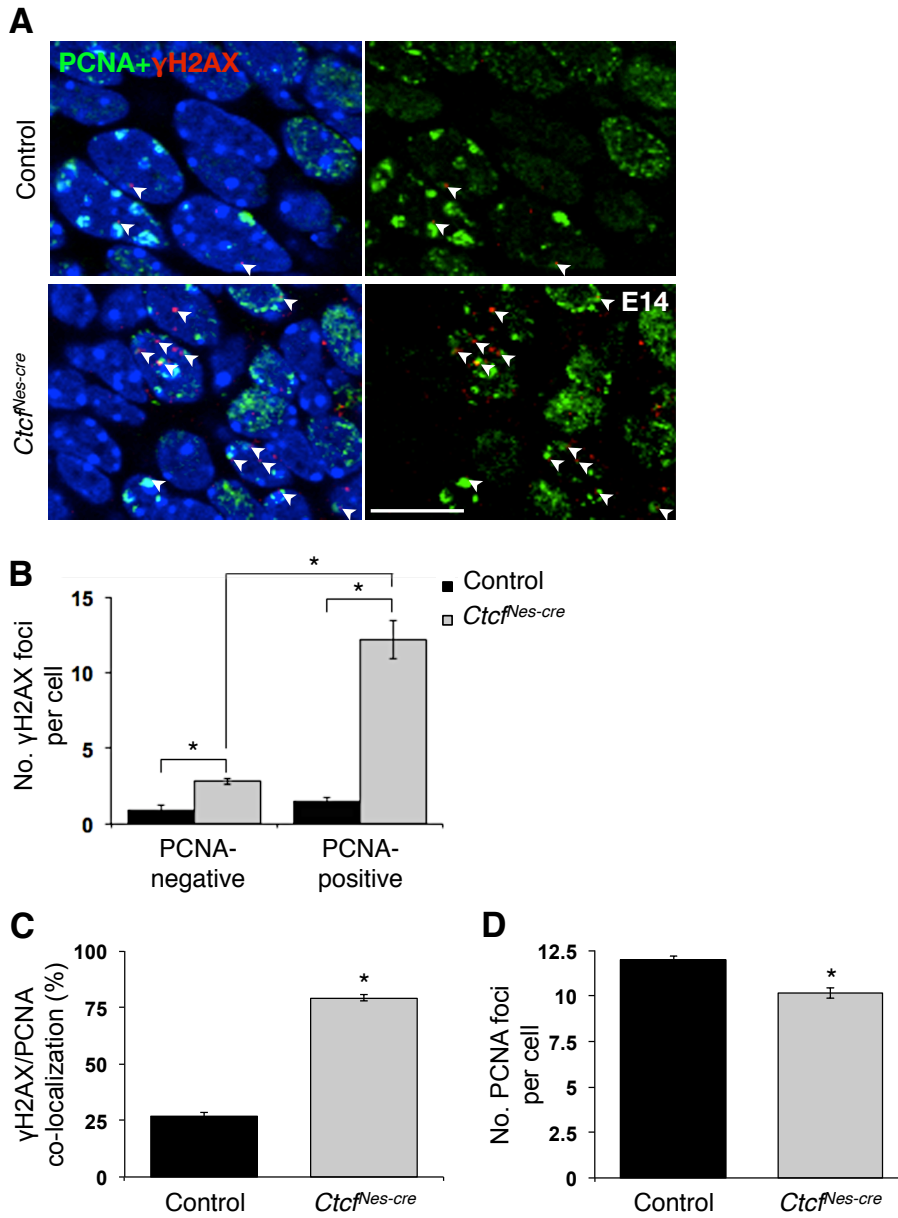
Figure 4-2 CTCF loss induces replication-associated damage in NPCs

(A) PCNA and γH2AX immunostaining in E14 control and $Ctcf^{Nes-cre}$ neocortex. Scale bar: 20μm. (B) Quantification of γH2AX foci per cell in PCNA-positive and PCNA-negative populations, $n = 3$; 100 cells counted per genotype and population; $*P < 0.05$. (C) Percentage of γH2AX foci that co-localize with PCNA foci, $n = 3$; 100 cells counted per genotype and population; $*P < 0.05$. (D) Number of PCNA foci per cell, $n = 3$; 100 cells counted per genotype and population; $*P < 0.05$.

To gain more insight into the type of DNA damage incurred in the absence of CTCF, NPCs were subjected to a variety of exogenous damaging agents. *Ctcf*-null NPCs were hypersensitive to the DNA polymerase inhibitor aphidicolin (Berger et al., 1979), the ribonucleotide reductase inhibitor hydroxyurea (Young and Hodas, 1964) and the interstrand crosslinking agent mitomycin C (Nakata et al., 1961), but not γ-irradiation or the topoisomerase II inhibitor etoposide (Minocha and Long, 1984) (Figure 4-3a-e). Collectively, these findings implicate CTCF in either the response to, or repair of, DNA replication-related damage.

Replicative stress has been broadly defined as any event that alters fulfillment of the DNA replication program (Magdalou et al., 2014; Zeman and Cimprich, 2014). Replication fork stalling results in a build-up of single stranded DNA (ssDNA), accumulation and phosphorylation of replication associated protein A (pRPAS33), recruitment of the PI-3 kinase ataxia telangiectasia and Rad3 related (ATR), and activation of the checkpoint kinase Chk1 (pChk1S345) (Zeman and Cimprich, 2014). Replication stress can also occur if the replication fork encounters barriers to its progression i.e. transcriptional complexes, unresolved DNA secondary structures, or double-stranded breaks (DSBs) (Allen et al., 2011; Mirkin and Mirkin, 2007). Replication stress can itself result in DSB formation, as prolonged replication fork stalling can result in collapse of the fork (Zeman and Cimprich, 2014). Western blot analysis of control and *Ctcf*[Nes-cre] NPCs established from E12.5 telencephalon and cultured for two days *in vitro* demonstrated modest increases in pRPAS33, pChk1S345 and γH2AX levels under basal conditions, suggesting that CTCF loss induces a replicative stress response (Figure 4-3f,g). Induction of exogenous replicative stress by the addition of aphidicolin resulted in a similar increases in pRPAS33, pChk1S345 and γH2AX levels in *Ctcf*[Nes-cre] compared to control NPCs (Figure 4-3f,g). These results indicate that CTCF is not required for signaling downstream of replicative stress through these molecules (Figure 4-3f,g). A more detailed analysis of replication fork dynamics by molecular combing revealed decreased replication fork speed in *Ctcf*[Nes-cre] NPCs relative to control, suggesting that CTCF is required for normal replication fork progression (Figure 4-3h). Together, these experiments demonstrate that CTCF is required to prevent replication-associated damage and a replicative stress response in NPCs.
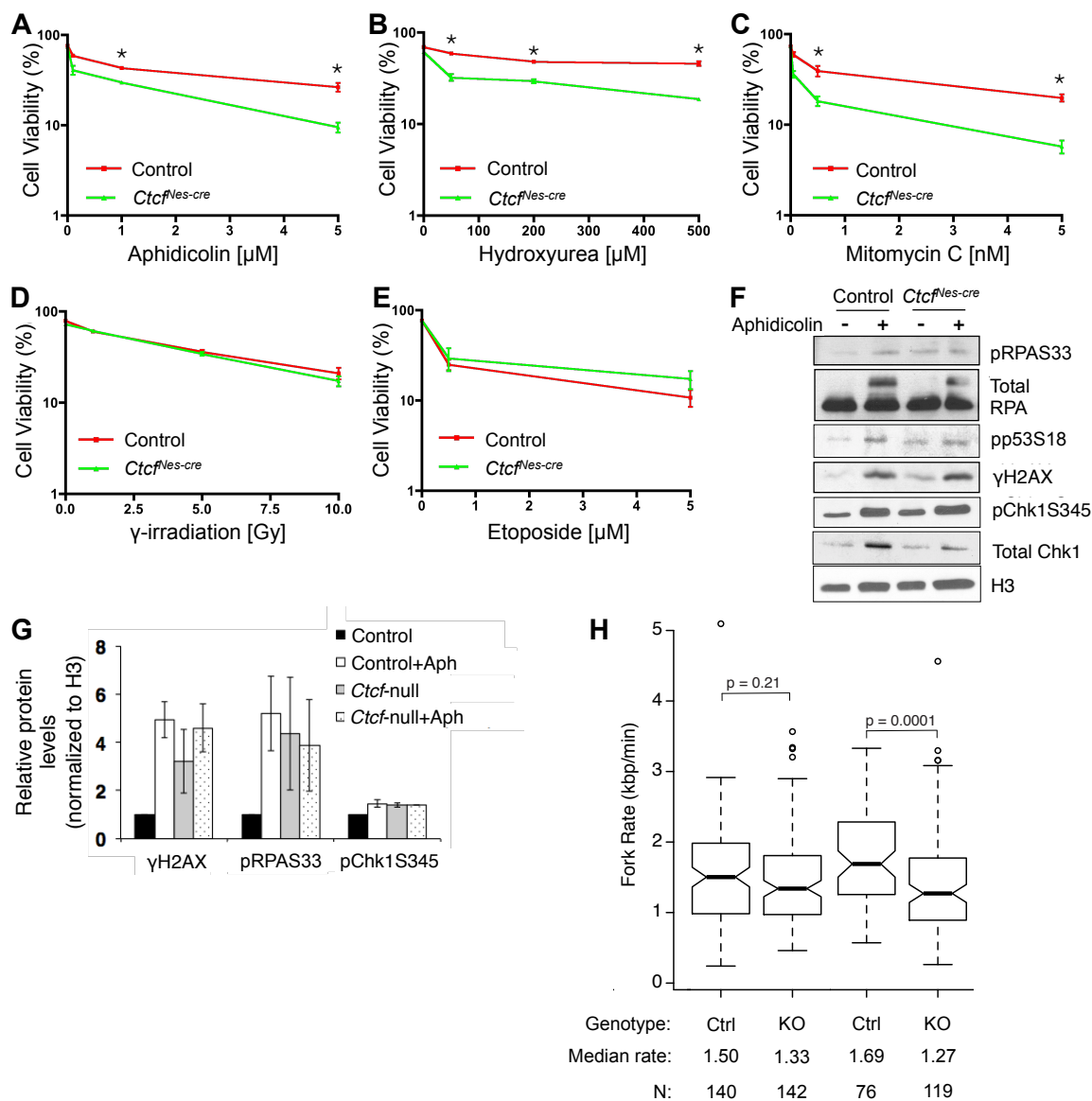
Figure 4-3 *Ctcf*-null NPCs exhibit evidence of replicative stress

(A-E) Cells were treated with a variety of DNA damaging agents for 24h (except for acute γ-irradiation) and viability was measured 24h later, *n* = 4; *P < 0.05. (F) Control and *Ctcf*$^{Nes-cre}$ NPCs were cultured in the presence or absence of aphidicolin (Aph; 10μM, 6h) and lysates were probed for pRPAS33, total RPA, γH2AX, and pChk1S345. H3 was used as a loading control. Densitometry analysis of γH2AX, pRPAS33, and pChk1S345 relative to H3 is depicted in (G), *n* = 2. Graphical data is represented as mean ± standard error of the mean. (H) The distribution of replication fork progression rates in control and *Ctcf*$^{Nes-cre}$ (KO) NPCs are represented in a box plot. Track lengths were converted

into kilobase pairs using a conversion factor for λ DNA and the median fork rate for each biological replicate is shown. *P* values were determined using the Mann-Whitney U-test for non-parametric data to compare the distributions of fork rates (kb/min) between two samples.

Activation of the replicative stress response can result in intra-S or G_2/M arrest (Feijoo et al., 2001; Walworth et al., 1993). Flow cytometry was performed to determine the effect of CTCF loss on cell cycle progression of NPCs established from E12.5 telencephalon and cultured for two days *in vitro* (Figure 4-4a). Analysis of BrdU/propidium iodide incorporation indicated a modest increase in the proportion of *Ctcf*-null NPCs in S phase, with a concomitant decrease in G_0/G_1 cells (Figure 4-4a). This trend was not statistically significant, likely owing to variability between biological replicates. *In vivo* immunofluorescence analysis of E14 cryosections using a G_2/M marker (pH3S10) in conjunction BrdU (1h injection of pregnant dam prior to sacrifice) indicated no significant differences between the proportion of cells in S phase (BrdU$^+$/pH3$^-$) or G_2 phase (BrdU$^+$/pH3$^+$) in control and *Ctcf*$^{Nes\text{-}cre}$ neocortex (Figure 4-4b-d). There was a slight increase in the number of mitoses (pH3$^+$/BrdU$^-$) in the basal SVZ, while there was no difference in the number of apical mitoses along the VZ (Figure 4-4e). This data fits with our previous results showing an increase in the population of TBR2$^+$ basal progenitor cells, which undergo mitosis in the SVZ (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Watson et al., 2014). In summary, despite evidence that supports activation of a replicative stress response as measured by phosphorylation of RPA (pRPAS33) and Chk1 (pChk1S345), cell cycle profiles are similar between control and C*tcf*-null NPCs (Figure 4-4).
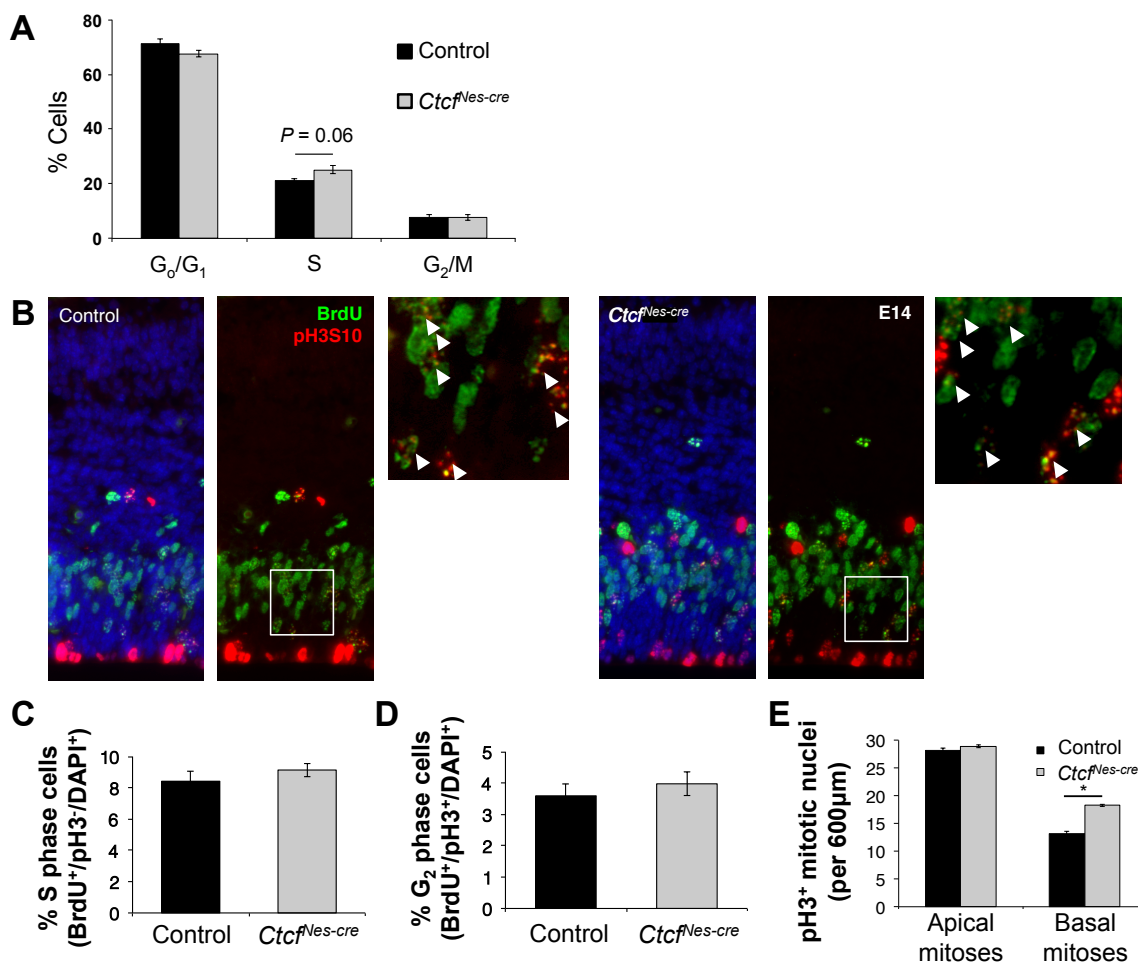
Figure 4-4 Cell cycle profiles of control and *Ctcf^Nes-cre* NPCs are similar both *in vitro* and *in vivo*

(A) Control and *Ctcf*-null NPCs were cultured for three days and labeled with BrdU for 1.5h. Cell cycle profiles were generated by BrdU and propidium iodide labeling followed by flow cytometry, $n = 4$. (B) BrdU (1h; green) and phosphorylated histone H3S10 (pH3S10; red) immunostaining in E14 control and *Ctcf^Nes-cre* neocortex. White box indicates region of higher magnification; arrowheads point to BrdU$^+$/pH3$^+$ cells presumed to be in G$_2$ phase. The percentage of cells in S (BrdU$^+$/pH3$^-$; C) and G$_2$ (BrdU$^+$/pH3$^+$; D) phase were quantified in 150μm-wide sections, $n = 4$. (E) The number of solid-staining pH3$^+$ cells were quantified in 600μm-wide sections of E14 control and *Ctcf^Nes-cre* neocortex within 20μm of the ventricle (apical mitoses) and in the SVZ (basal mitoses), $n = 2$. Graphical data is represented as mean ± standard error of the mean.

4.3.3 An interaction between CTCF and PCNA is DNA-mediated

An important role for CTCF in DNA replication has been supported by the finding that CTCF is important for asynchronous replication of the *H19/Igf2* imprinted domain, and that CTCF binding upstream of the DM1 repetitive tract in the myotonic dystrophy gene is required to slow the replication fork before encountering repetitive DNA to ensure replicative fidelity (Bergstrom et al., 2007; Cleary et al., 2010; Libby et al., 2008). Additionally, mass spectrometry analysis of CTCF binding partners in embryonic stem cells revealed numerous binding partners that participate in DNA replication such as PCNA, RCF1, RCF2, RCF4, Orc1, and DNA polymerase delta (van de Nobelen, 2008). We validated the interaction between CTCF and PCNA by immunoprecipitation of PCNA in control NPCs, followed by immunoblotting for CTCF (Figure 4-5a). Pretreatment of the protein lysates with DNAse I prior to immunoprecipitation demonstrated a diminished interaction between CTCF and PCNA, indicating an indirect interaction mediated by DNA but supporting a close association between the two proteins (Figure 4-5a).

4.3.4 CTCF is not required for timely repair of exogenous replication-associated damage

We next considered that the increased endogenous replication-associated damage observed in *Ctcf*-deficient cells might result from a requirement for CTCF in DNA repair. Indeed, NPCs encounter a high degree of replicative stress due to their high proliferative index (McKinnon, 2013), and CTCF localizes to replicative stress-induced damage foci in NPCs (Figure 4-5b). Given the close relationship between CTCF and the cohesin complex (Parelho et al., 2008; Rubio et al., 2008; Wendt et al., 2008), and the fact that cohesin is required for post-replicative repair by homologous recombination (Sjogren and Nasmyth, 2001), this became an intriguing hypothesis. To test this, we subjected control and *Ctcf*-null NPCs to aphidicolin treatment and measured repair kinetics by assessing γH2AX levels over time (Figure 4-5b,c). Control NPCs exhibited a 6-fold increase in γH2AX foci 6 h after aphidicolin treatment that was reduced by 24 h post treatment (Figure 4-5b,c). The number of γH2AX foci in *Ctcf*-null NPCs followed a similar trend, despite the basal level of γH2AX foci (Figure 4-5c,d). This experiment provides evidence

that CTCF is not required for resolution of γH2AX foci after the induction of exogenous replicative stress. Interestingly, the DNA-mediated interaction between CTCF and PCNA was enhanced under conditions of replicative stress (10μM aphidicolin, 6h; Figure 4-5a), however the functional significance of this relationship is not yet understood.
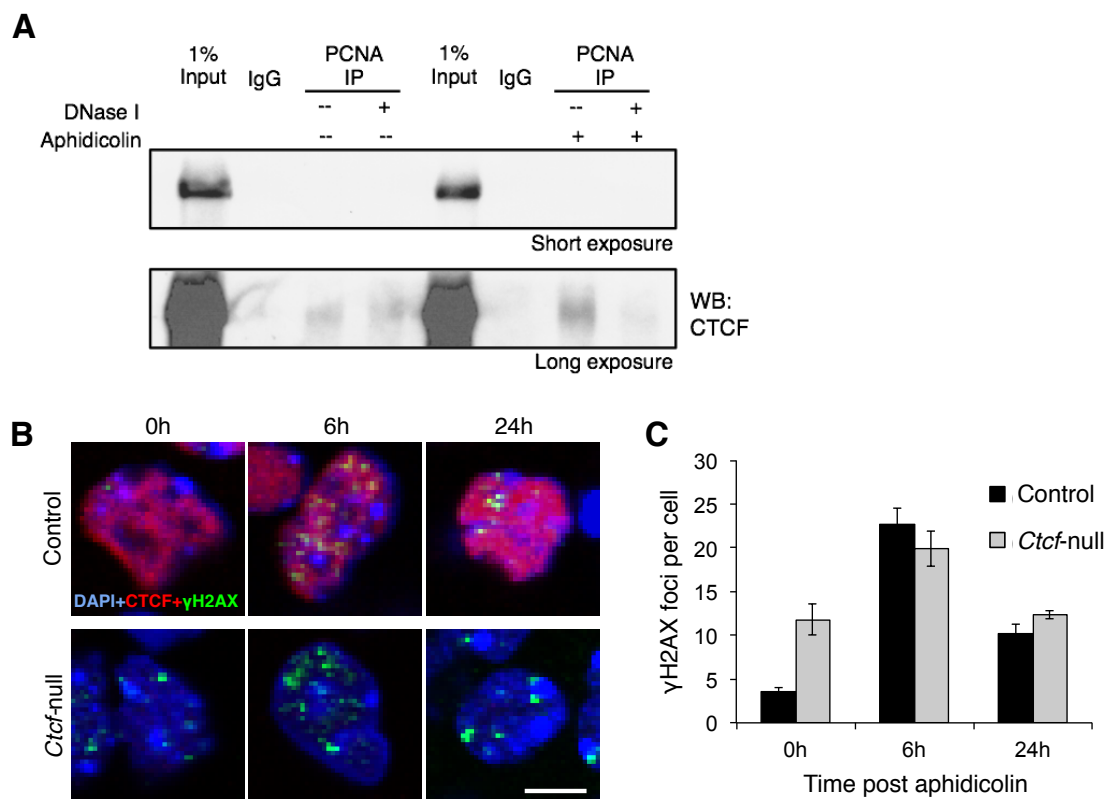
Figure 4-5 An indirect interaction between CTCF and PCNA is enhanced under conditions of replicative stress, however CTCF is not required for repair of replication-associated damage

(A) Control NPCs were cultured in the presence or absence of 10μM aphidicolin for 6h and subjected to PCNA immunoprecipitation (IP) followed by immunoblotting for CTCF. Lysates were pretreated with Dnase I prior to IP where indicated. (B) Confocal images of γH2AX immunostaining in control and *Ctcf*-null NPCs post 10 μM aphidicolin for the length of time indicated. Scale bar: 5 μm. (C) The number of γH2AX foci per DAPI$^+$ nucleus was quantified at 0, 6, and 24 hours post 10 μM aphidicolin treatment, $n = 4$; 50-100 nuclei counted per treatment. Graphical data is represented as mean ± standard error of the mean.

4.4 Discussion

We, and others, have previously demonstrated that CTCF is an essential factor for cellular survival (Fedoriw et al., 2004; Heath et al., 2008; Moore et al., 2012; Soshnikova et al., 2010; Wan et al., 2008; Watson et al., 2014). Despite this important role in the cell, the mechanism underlying apoptosis of *Ctcf*-deficient cells is largely unknown. Here, we provide evidence that CTCF is required to prevent replication-associated DNA damage in NPCs.

Mice in which *Ctcf* was deleted from early forebrain progenitors using *Foxg1-cre* exhibited massive apoptosis resulting in near-complete ablation of telencephalic structures (Watson et al., 2014). Remarkably, when *Ctcf* was deleted a few days later using *Nestin-cre*, cortical progenitor loss was reduced (Watson et al., 2014). Both models of CTCF loss exhibited elevated DNA damage concurrent with apoptosis. This is reminiscent of models of *Topbp1* deletion, where levels of apoptosis were higher upon deletion of *Topbp1* in early progenitors (using *Emx1-cre*) compared to later progenitors (using *Nestin-cre*) despite similar levels of DNA strand breaks in the two models (Lee et al., 2012a). The results presented here support the idea that earlier NPCs have a lower threshold for DNA damage-induced apoptosis compared with progenitors generated at a later developmental stage.

In response to DNA damage, p53 activates cell cycle arrest and/or apoptosis (Amundson et al., 1998). *Ctcf*-deficient NPCs have increased levels of γH2AX, exhibit replicative stress, and are sensitive to exogenous agents that perturb replication. However, the increased replication-associated DNA damage was not sufficient to dramatically alter cell cycle progression, perhaps indicating that DNA damage levels only exceeded a threshold sufficient to activate cell cycle arrest in a proportion of mutant cells. This idea is supported by the modest increases observed in activation of the effector proteins involved in replicative stress-associated cell cycle arrest, and a subtle trend towards increased *Ctcf*-deficient S phase cells *in vitro* and *in vivo* (Figures 4-3f, 4-4a,c). Since neurogenic progenitors have a lengthened G_1 phase, and postmitotic neurons behave like cells in G_1 in their DNA content and failure to incorporate BrdU, then the increased basal progenitor population and increased generation of postmitotic neurons observed in the *Ctcf*-null

neocortex would result in an artificially high G_1 population by flow cytometry. Since flow cytometry is a population-based measure, the proportion of cells in other phases would be compromised. Analysis of strictly proliferative progenitors by analyzing *Tis21*-negative cells (Calegari and Huttner, 2003) would more accurately identify S phase populations.

Our experiments provide evidence that CTCF is not required for repair of exogenous replication-associated DNA damage since *Ctcf*-null NPCs exhibited similar capacity to resolve γH2AX foci relative to control cells after aphidicolin treatment (Figure 4-5a,b). This result must be interpreted with caution, however, since different types of stress elicit different cellular responses. More sensitive assays to measure repair kinetics such as the comet assay to directly measure single- and double-stranded breaks without relying on a cellular marker like γH2AX will provide more insight into the ability of *Ctcf*-null cells to repair DNA damage. The finding that CTCF is not required for repair of aphidicolin-induced damage but that *Ctcf*-null cells are sensitive to the drug indicates that additional replicative stress in the absence of CTCF likely exceeds the threshold for apoptosis. Interestingly, CTCF is enriched at aphidicolin-induced damage foci and indirectly interacts with PCNA and γH2AX under conditions of replicative stress. While CTCF may not be required for repair of aphidicolin-induced damage *per se*, it may play a role in organizing higher-order chromatin structure surrounding DNA breaks to help facilitate timely repair.

Genome-wide maps of chromatin interaction revealed that the genome is organized into specific domains within the nucleus, termed topologically associating domains (TADs), whose borders are enriched for CTCF binding sites, suggesting a role for CTCF in establishment of this type of genome organization (Dixon et al., 2012). Recently, it was demonstrated that chromatin loops bound by CTCF demarcate TAD borders (Rao et al., 2014) and that TADs show a striking correlation with replication timing domains (Pope et al., 2014). Thus, if CTCF is important for TAD organization, it may also be important for the organization of replication timing. Indeed, RNAi-mediated CTCF knockdown results in the loss of some TAD boundary activity (Zuin et al., 2014). Furthermore, large changes to the replication-timing program can result in replicative stress, and thus CTCF

may function to organize TADs and by extension replication timing domains to suppress this type of damage (Buonomo et al., 2009; Cornacchia et al., 2012). In light of our findings, it will be important to test this hypothesis by assessing genome-wide replication timing profiles of control and *Ctcf*-null NPCs in the future.

Collectively, our findings provide insight into the mechanism underlying CTCF function in cellular survival. CTCF is required to prevent replication-associated damage, p53 activation, and apoptotic induction. It remains unclear whether CTCF is required directly for repair of endogenously occurring replicative stress, or rather if increased DNA damage resulting from CTCF loss are a secondary consequence of its functions in chromatin organization. Future studies addressing the contributions of CTCF function to DNA replication and repair will be critical to further our understanding of the requirements for CTCF in cellular survival.

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Chapter 5

5 Discussion and Future Directions

The body of work presented in this thesis provides molecular evidence that epigenetic regulators, such as those involved in organizing higher-order chromatin structure, are necessary for NPC survival and growth by promoting genomic stability to support correct brain development. This data aligns with the finding that intellectual disability, microcephaly, and/or neuropsychiatric disorders are enriched for mutations in epigenetic regulators (De Rubeis et al., 2014; Gibbons et al., 1995a; Gregor et al., 2013; Iossifov et al., 2014; Pinto et al., 2014; Ronemus et al., 2014), and that human cancers are enriched for alterations in epigenetic pathways (Elsasser et al., 2011; Filippova et al., 1996; Huether et al., 2014; Jiao et al., 2011; Schwartzentruber et al., 2012).

In chapter two, I demonstrate that ATRX is required for stability of repetitive elements, such as telomeres and pericentromeric heterochromatin, particularly during DNA replication. I began exploring a mechanism underlying genomic instability in the absence of ATRX and found that *Atrx*-null NPCs are sensitive to G4-DNA stabilization, suggesting that ATRX may resolve G4-DNA structures that have the propensity to form at telomeric sequences. More globally, ATRX may aid in the replication of repetitive sequences through incorporation of H3.3, explaining the instability observed at pericentromeric heterochromatin, which is AT- rather than GC-rich. Systemically, loss of ATRX in the forebrain and anterior pituitary resulted in aging-associated phenotypes, endocrine dysregulation, and reduced lifespan. Together, these findings implicate ATRX in suppressing genome instability, which when restricted to the forebrain and anterior pituitary can cause systemic defects that resemble accelerated aging.

Chapter three and four focus on characterizing the role of CTCF in embryonic brain development. I found that CTCF is required for NPC survival by suppressing replication-associated DNA damage and p53/PUMA-dependent apoptotic signaling. Additionally, CTCF controls the balance between NPC proliferation and differentiation to support correct brain growth and size. Taken together, these studies identify CTCF as an

important regulator of brain development through controlling cell cycle dynamics and cellular survival.

Collectively, the work presented in this thesis highlights the dynamic interplay between chromatin structure, genomic stability, and brain development by revealing that ATRX and CTCF are essential genomic stability factors in NPCs. The findings also begin to dissect the mechanism underlying neurodevelopmental abnormalities stemming from loss of ATRX or CTCF by identifying that loss of either factor causes replication-associated DNA damage, highlighting the requirement of safeguarding the genome for correct development of the central nervous system.

5.1 ATRX is a critical regulator of genomic stability

At the outset of this study, ATRX had not been implicated in promoting genomic stability. In fact, it was believed that although ATRX is a homolog of the DNA repair protein Rad54, it was unlikely to play a role in maintaining stability of the genome since ATR-X patient cells do not display UV sensitivity (Gibbons et al., 1995b). My work clearly demonstrates a requirement for ATRX in promoting genomic stability, providing an explanation for p53-dependent apoptosis of *Atrx*-null NPCs (Seah et al., 2008). Other studies have since shown that ATRX is required to prevent DNA damage during DNA replication in myoblasts, HCT116 human colon cancer cells, limb bud cells, and ES cells (Clynes et al., 2014; Conte et al., 2012; Huh et al., 2012; Leung et al., 2013; Solomon et al., 2013).

ATRX-mediated genomic stability is critical for cellular survival, however it is not yet known whether accumulation of DNA damage in *Atrx*-null cells is primary or secondary to defects in replication. This remains difficult to distinguish since replication defects can cause DNA damage and vice versa. For instance, DNA DSBs can impede the replication fork to cause replication stalling and S phase lengthening. At the same time it is equally possible that problems during replication, like fork slowing or failure to resolve complex secondary structures, could cause stalling and collapse of the replication fork into DSBs.

Several studies have demonstrated a role for ATRX in correct cell division (Baumann et al., 2010; De La Fuente et al., 2004; Ritchie et al., 2008; Ritchie et al., 2014). Given the close relationship between DNA replication, cell division, and cell cycle progression, it has been difficult to decipher whether meiotic/mitotic defects stemming from ATRX loss are primary or secondary to replicative stress. Issues during replication, such as under-replication of DNA sequences, failure to repair DSBs, or telomere uncapping can manifest as anaphase bridges and/or micronuclei (Burrell et al., 2013; Gauthier et al., 2012; Hampel et al., 2013; Incles et al., 2004; Kim et al., 2003; Sofueva et al., 2011; Tahara et al., 2006). At the same time it is possible to envision mitotic defects, such as congression and cohesion failure, to be capable of inducing DNA double-strand breaks and issues during replication. For instance, mutation- or drug-induced mitotic spindle disruption can induce DSBs that are found in centromere-containing micronuclei (Dalton et al., 2007; Guerrero et al., 2010; Quignon et al., 2007). Moreover, chromosomal instability can result from chromosome missegregation during mitosis (Dobles et al., 2000).

Current evidence in the literature supports a model wherein ATRX functions to promote replication fork processivity through notoriously difficult-to-replicate repetitive genomic regions, thereby preventing fork stalling and collapse into DSBs that can aberrantly affect cell division (Figure 5-1). This is substantiated by the findings that ATRX targets repetitive DNA (Law et al., 2010), that *Atrx*-null cells exhibit replication defects (Clynes et al., 2014; Leung et al., 2013), the particular sensitivity of *Atrx*-null cells to replicative stress-inducing drugs (Clynes et al., 2014; Conte et al., 2012; Leung et al., 2013; Watson et al., 2013), and that DNA damage foci in *Atrx*-null cells colocalize with late-replicating heterochromatin (Huh et al., 2012; Watson et al., 2013). Further evidence for this model of ATRX function is presented below.

ATRX genomic binding sites are predicted to adopt non-B form secondary structures (Law et al., 2010), which have been shown to impede replication fork progression leading to fork stalling or collapse into DSBs (Paeschke et al., 2011; Sarkies et al., 2012; Sarkies et al., 2010). It is tempting to speculate that ATRX may facilitate replication by resolving G4-DNA structures, an idea supported by the particular sensitivity of *Atrx*-null cells to

replication stressors and the G4 ligand telomestatin (TMS) (Watson et al., 2013). However, although recombinant ATRX can interact with G4-DNA oligonucleotides *in vitro* (Law et al., 2010), it performed poorly in a G4-DNA unwinding assay compared to the Bloom Syndrome helicase protein BLM (Clynes et al., 2014), suggesting that ATRX does not directly unwind G4-DNA. ATRX may, however, indirectly overcome these secondary structures by incorporating H3.3 into chromatin to maintain B-form DNA, or alternatively through template switching. The ATPase domain of ATRX is similar to the homologous recombination (HR) protein Rad54 (Picketts et al., 1998), which stabilizes Rad51 onto presynaptic filaments to enable HR repair (Alexiadis and Kadonaga, 2002; Golub et al., 1997; Tan et al., 1999) and to resolve stalled replication forks (Maher et al., 2011). *Atrx*-deficient cells have decreased Rad51 levels (Huh et al., 2012), suggesting ATRX may also function to stabilize Rad51 in some capacity.

We now know that ATRX interacts with the MRN complex (Clynes et al., 2014; Leung et al., 2013), although the functional implications of this interaction are unclear. MRN is required for DNA damage detection, signaling, and repair (Dinkelmann et al., 2009; Rass et al., 2009; Taylor et al., 2010; Xie et al., 2009), as well as for promoting fork restart after replicative stress (Bryant et al., 2009; Falck et al., 2012). MRN localizes to telomeres in S and G_2 phase, Mre11 can interact with G4-DNA in yeast (Ghosal and Muniyappa, 2005; Leung et al., 2013; Verdun and Karlseder, 2006; Zhu et al., 2000), and MRN is implicated in the alternative lengthening of telomeres (ALT) phenotype, providing additional parallels with ATRX functions (Jiang et al., 2005; Zhong et al., 2007). Human mutations in MRN components cause a spectrum of disease. For instance, *MRE11* mutations cause ataxia telangiectasia-like disorder (A-TLD) characterized by ataxia and neurodegeneration, which shares similarities to A-T resulting from mutations in *ATM* (Gatti et al., 1988; Stewart et al., 1999), and mutations in *NBS1* cause Nijmegen breakage syndrome (NBS) characterized by microcephaly, immunodeficiency, and cancer predisposition (Carney et al., 1998; Rodrigues et al., 2013; Varon et al., 1998). The parallels between ATR-X and NBS, like microcephaly, are intriguing since ATRX interaction with MRN appears to be mediated by NBS1 (Leung et al., 2013), suggesting that the overlapping phenotype may result from cooperative functions of the proteins.

Chapter two of this thesis describes a requirement for ATRX in preventing accumulation of endogenous DNA damage in the embryonic brain. The first indication that genomic instability was linked to cellular proliferation came with the finding that the post-replicative juvenile *Atrx*-null mouse brain does not exhibit elevated DNA damage (Figure 2-9). However, *Atrx*-null cells do not display a typical response to replicative stress in that mutant cells exhibit increased activation of ATM (pATMS1981) rather than ATR (pATRS428) (Figure 2-1). A mouse model of Seckel syndrome carrying a hypomorphic *Atr* mutation demonstrated that replicative stress activates an ATM-dependent DNA damage response resulting in increased γH2AX levels (Murga et al., 2009). There is also extensive cross-talk among the ATM and ATR kinases in NPCs (McKinnon, 2013). In fact, telomere dysfunction in NPCs results in ATR-to-ATM-dependent signaling (Lee et al., 2014), evoking the possibility that ATM and ATR may both be activated in *Atrx*-null cells. Since ATR is phosphorylated on a number of different residues to mediate its activation, it will be worthwhile to assess activation status of downstream targets of ATR-dependent signaling like Chk1 and RPA in *Atrx*-deficient NPCs (Durocher and Jackson, 2001; Shiloh, 2001). In human HCT116 cells, deletion of ATRX resulted in reduced phosphorylation of Chk1 on serine 317 (Leung et al., 2013), suggesting ATRX is required for correct checkpoint signaling downstream of ATR. Therefore, our observation of increased ATM signaling in *Atrx*-null NPCs may result from defective ATR signaling in response to the replicative stress induced by ATRX loss. The exact molecular details of ATRX function in DNA damage signaling are clearly not completely understood and require further examination.

The novel role for ATRX in genomic stability requires a thorough evaluation of the DNA damage response in patient cells. Indeed, ATR-X patient lymphocytes exhibited increased γH2AX and p53 phosphorylation suggestive of an increased DNA damage response (Huh et al., 2012). However, it remains unclear whether patient cells exhibit evidence of replicative stress or increased occurrence of unresolved secondary structures like G4-DNA. With the recent generation of a G4-DNA-specific antibody (Biffi et al., 2013) it will be possible to test this hypothesis in cellular models of *Atrx* deficiency as well as in ATR-X patient cells.
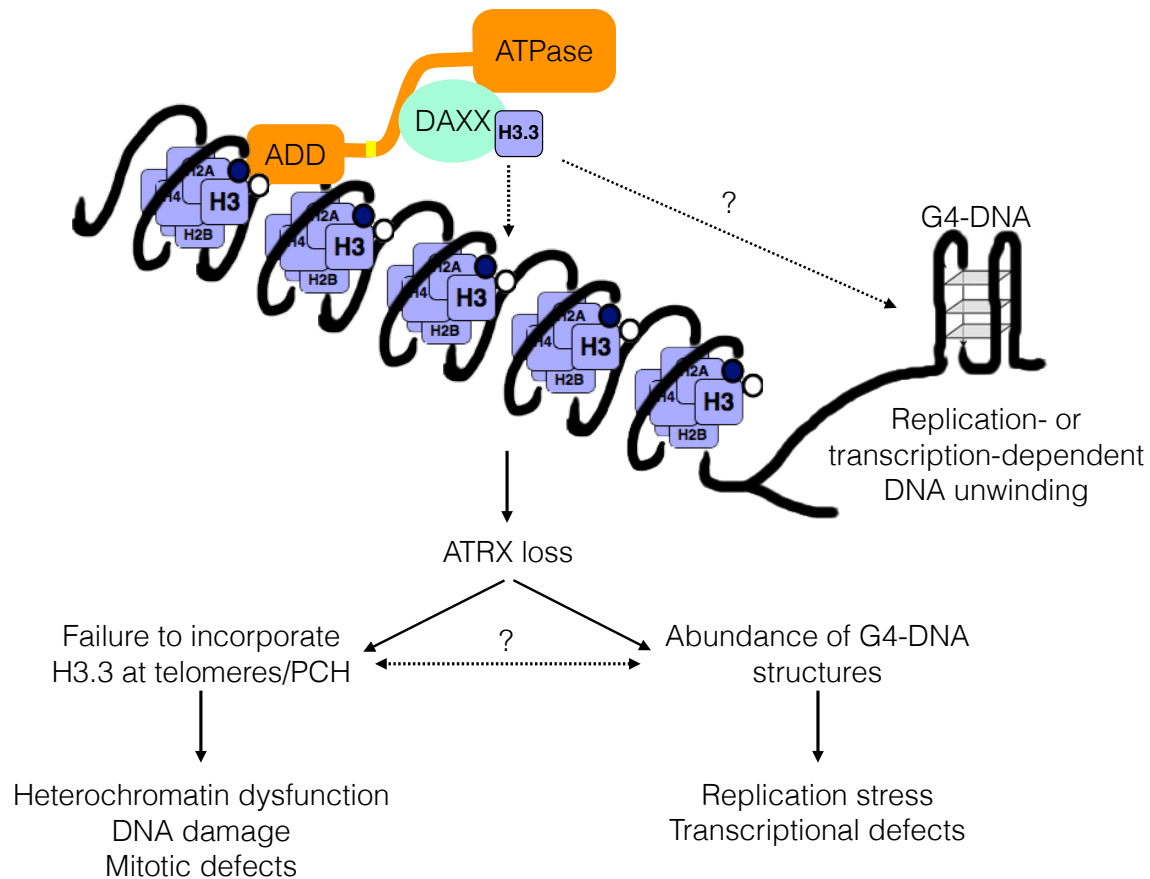
Figure 5-1 Model of ATRX function in genomic stability

ATRX is critical for stability of heterochromatin, presumably via incorporation of histone H3.3 to maintain heterochromatin function. H3.3 incorporation may also help resolve G4-DNA structures, which have the propensity to form at telomeres and GC-rich ssDNA exposed during replication or transcription. The consequences of ATRX loss is (1) failure to incorporate H3.3 at heterochromatin, resulting in dysfunction, DNA damage, and mitotic defects and (2) failure to resolve G4-DNA structures, resulting in their over-abundance that could cause replication stress and transcriptional dysregulation. The relationship between H3.3 incorporation and G4-DNA resolution is not well characterized.

5.2 ATRX is a novel telomere-binding protein

This study is one of many to identify ATRX as an important component of telomeres (Goldberg et al., 2010; Huh et al., 2012; Watson et al., 2013; Wong et al., 2010). In 2010, two groups demonstrated that ATRX is required for H3.3 deposition at telomeric sequences (Goldberg et al., 2010; Wong et al., 2010). The functional significance of H3.3 incorporation at telomeres remains unclear, especially in light of conflicting findings regarding the effect of ATRX loss on transcription of the non-coding telomeric transcript TERRA (Goldberg et al., 2010; Levy et al., 2014) and given that H3.3 enrichment was generally observed in regions of active transcription (Hake et al., 2006). ATRX is also involved in restricting the repressive variant macroH2A incorporation at telomeres (Ratnakumar et al., 2012). Therefore, ATRX may be required to balance active and repressive modifications at chromosome ends to promote their stability, a phenomenon that has been observed at centromeres wherein a dual open and closed chromatin state is necessary for stability (Chueh et al., 2009; Nakano et al., 2008; Wong et al., 2007). Loss of this balancing act may render telomeres hypercondensed, leading to defects in TERRA transcription or DNA replication. Furthermore features intrinsic to repetitive elements, such as DNA bending and the propensity to form of G4 structures, can adversely affect nucleosome occupancy and telomeres have decreased tendency to form nucleosomes *in vitro* (Cacchione et al., 1997). The ATRX-DAXX complex may function to re-establish H3.3 containing nucleosomes at these elements to help maintain proper nucleosome density for heterochromatin formation, serving as a specialized chromatin assembly pathway for repetitive regions such as telomeres, centromeres, and other regions of constitutive heterochromatin. Defects in nucleosome assembly pathways, like the ATRX-DAXX pathway, may therefore lead to increased DNA damage and genomic instability. Telomere damage and mitotic fusions imply disintegration of the shelterin complex that protects telomere ends from being recognized as DSBs (de Lange, 2005), therefore it would be interesting to assess telomeres in *Atrx*-deficient cells for the presence of the protective components.

This thesis also describes a role for ATRX in PCH stability. PCH is AT-rich and therefore not enriched for G4-forming sequence, suggesting that the mechanism underlying destabilization of the major satellite repeats in an ATRX-deficient context differs from that of telomeric repeats. The common link between telomeric and pericentromeric heterochromatin is enrichment of H3K4me0-K9me3 and the requirement for ATRX in H3.3 deposition (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010). Therefore the mechanism underlying ATRX-dependent stability of repetitive elements may more broadly involve H3.3 deposition rather than specific resolution of G4-DNA structures. The recent discovery that ATRX can tolerate the histone H3K9me3-S10ph modification is intriguing since other H3K9me3 readers, such as HP1, are unable to bind this signature and become displaced (Kunowska et al., 2015). H3S10ph is induced during late G_2 and remains associated with chromatin during mitosis (Jeong et al., 2010; Nigg, 2001). It is possible that ATRX functions at heterochromatin are particularly relevant in actively cycling cells due to its ability to remain bound to H3K9me3-S10ph residues, when other proteins required for heterochromatin stability are displaced. Telomeres and PCH also express non-coding RNA transcripts that act as structural components implicated in heterochromatin formation and maintenance (Azzalin et al., 2007; Bulut-Karslioglu et al., 2012; Nergadze et al., 2009; Schoeftner and Blasco, 2008). Given that ATRX can interact with RNA (Sarma et al., 2014), the non-coding transcripts originating from telomeres and PCH may play a role in ATRX targeting and/or suppression of the DDR at these structures in the absence of ATRX. Indeed, dysregulation of PCH transcripts results in mitotic dysfunction (Bulut-Karslioglu et al., 2012) and alterations in TERRA levels causes activation of the DDR at telomeres (Deng et al., 2009; Deng et al., 2012b).

The mechanism of ATRX recruitment to telomeres is unknown. Telomeric chromatin is enriched for inactive histone post-translational modifications recognized by the ATRX ADD domain, such as H3K9me3 (Dhayalan et al., 2011; Eustermann et al., 2011; Iwase et al., 2011; Mitson et al., 2011), providing one explanation for ATRX recruitment to the genomic structures. ATRX interaction with G4-DNA, at least *in vitro* (Law et al., 2010), provides another explanation. Moreover, ATRX recruitment may also be mediated or stabilized by protein-protein or protein-RNA interactions, as ATRX was recently

identified as an RNA-binding protein (Sarma et al., 2014), and interacts with telomere-enriched proteins such as the MRN complex (Verdun et al., 2005), HP1 (Fanti et al., 1998; Fanti and Pimpinelli, 2008), the cohesin complex (Remeseiro et al., 2012). Moreover, the ATRX partner MeCP2 interacts with TERRA RNA (Deng et al., 2009), indicating the possibility that ATRX recruitment may occur via MeCP2. Future studies aimed at identifying the ATRX protein domains required for targeting to telomeres will be pivotal in furthering our understanding of ATRX contributions to telomere biology and genomic stability.

5.3 ATRX functions as a tumour-suppressor protein

Somatic loss-of-function mutations in *ATRX* were first described in pancreatic neuroendocrine tumours (PanNETs) and later in CNS tumours such as glioma and astrocytoma (Chen et al., 2014; Jiao et al., 2012; Jiao et al., 2011; Kannan et al., 2012; Liu et al., 2012; Molenaar et al., 2012; Schwartzentruber et al., 2012). Genomic instability is a hallmark of the early stages of tumourigenesis (Negrini et al., 2010). Exacerbation of the DNA damage phenotype in cells lacking ATRX and p53 highlights the tumour suppressive functions of p53 in mediating cell death to protect against acquisition of genomic instability (Seah et al., 2008; Watson et al., 2013). Moreover, the data fit well with the finding that *ATRX/DAXX* mutations commonly occur in conjunction with mutations in the tumour suppressors *p53* or *IDH1* (Liu et al., 2012; Schwartzentruber et al., 2012) and support a model wherein epigenetic factors like ATRX may act as "backseat drivers" in a tissue-specific manner to suppress oncogenic pathways upstream of master regulators common to a broad range to tumours, like p53 (Elsasser et al., 2011). Studying mice and human cells lacking ATRX and p53 will improve our understanding of the mechanisms underlying *ATRX* mutations promote tumourigenesis.

ATR-X syndrome patients do not display increased cancer susceptibility (Gibbons, 2005). This is likely due to the differential mutational landscape observed in ATR-X syndrome versus *ATRX*-null tumours: ATR-X syndrome mutations are hypomorphic and result in reduced activity or levels of the protein (Argentaro et al., 2007; Higgs et al.,

2005), while mutations identified in cancers render the protein non-functional. Perhaps decreased quantity or activity of ATRX protein renders specific cell types, like NPCs and myoblasts (Huh et al., 2012; Watson et al., 2013), susceptible to DNA damage-induced apoptosis during development, while cancer progression requires additional mutations in tumour suppressors like p53 to prevent apoptosis and/or drive tumourigenesis.

Tumours harboring *ATRX/DAXX* mutations often utilize the alternative lengthening of telomeres (ALT) pathway to confer immortality (Bower et al., 2012; Heaphy et al., 2011a; Lovejoy et al., 2012; Schwartzentruber et al., 2012). The mechanism by which *ATRX* mutations result in the ALT phenotype and drive cancer progression is largely unknown. While my data demonstrates that ATRX loss causes telomere dysfunction, deletion of ATRX alone is not sufficient to drive ALT (Clynes et al., 2014; Flynn et al., 2015; Lovejoy et al., 2012), suggesting that additional genetic or epigenetic changes are necessary to establish the phenotype. Accordingly, ATRX loss in NPCs did not dramatically affect telomere FISH signal intensity (a surrogate for telomere length), although this was not assessed in ATRX/p53 mutant cells.

Interestingly, ALT is associated with the appearance of large PML-NBs (referred to as ALT-associated PML-NBs, or APBs) that contain telomeric DNA, the DNA repair MRN complex, replication factor A (RPA), and telomeric-repeat binding proteins TRF1 and TRF2 (Luciani et al., 2006; Wu et al., 2003; Wu et al., 2000; Yeager et al., 1999). The appearance of APBs is a robust marker for tumours that utilize ALT, and APBs rapidly assemble upon ALT induction (Costa et al., 2006; Henson et al., 2005; Perrem et al., 2001), suggesting that APBs may be involved in the mechanism underlying ALT. PML or MRN knockdown results in disassembly of ALT-associated PML-NBs, leading to inhibition of telomere elongation in ALT cells (Jiang et al., 2005; Jiang et al., 2007; Wu et al., 2000; Yu et al., 2010a). This is intriguing since ATRX/DAXX/H3.3 are PML-associated factors (Chang et al., 2013; Ishov et al., 2004; Luciani et al., 2006), ATRX interacts with the MRN complex (Clynes et al., 2014; Leung et al., 2013), and mutations in this pathway drive cancers with a high frequency of ALT. It is possible that defects in the ATRX/DAXX pathway results in a loss of heterochromatic features at telomeres, such as H3.3 enrichment, increased TERRA transcription, and persistent RPA-ssDNA,

leading to increased homologous recombination rates that are associated with ALT activity. However it remains unclear as to how PML-NBs fit into this picture exactly.

5.4 Future Directions: ATRX Functions in Genomic Stability

Several questions remain unanswered with regards to ATRX function in promoting cellular survival through genomic stability. These questions along with strategies to address them are discussed below.

First, where exactly does DNA damage localize in *Atrx*-null cells? Is it concentrated at telomeres and PCH, or does it also occur genome-wide? While my data provides evidence that damage occurs at repetitive elements like telomeres and PCH, it is unclear if these are the only regions susceptible to DNA damage in the absence of ATRX. Evaluation of γH2AX occupancy genome-wide by ChIP-sequencing would identify the landscape of DNA damage accumulation in *Atrx*-null cells. Given that ATRX targets repetitive DNA (Law et al., 2010), the prediction would be that damage is enriched at these sites if indeed ATRX plays a direct role in preventing γH2AX accumulation.

Next, what role, if any, does H3.3 incorporation play in the DNA damage phenotype stemming from ATRX loss? While heavily speculated upon, the role for H3.3 incorporation in ATRX-dependent genomic stability is largely unknown. HIRA-dependent H3.3 deposition is required for replication fork restart after UV damage (Adam et al., 2013). It is possible that other pathways of H3.3 deposition, like ATRX-DAXX, are responsible for replication fork restart after endogenous replicative stress. Although H3.3 is only unique in five amino acids relative to H3.1 (Maze et al., 2014), the recent generation of H3.3-specific antibodies allows for analysis of H3.3 enrichment genome-wide. Our group recently demonstrated a requirement for ATRX in H3.3 deposition within gene bodies to promote transcriptional elongation (Levy et al., 2014). Therefore it would be worthwhile to test whether ATRX is required for H3.3 incorporation at specific sites of damage, which can be induced using lasers or rare-cutting endonucleases (Jasin, 1996; Kim et al., 2007a). An additional hypothesis that has gained much attention is that ATRX resolves G4-DNA structures to facilitate transcription and replication (Clynes and Gibbons, 2013; Clynes et al., 2013). The recent

generation of a G4-DNA antibody demonstrated the *in vivo* occurrence of these structures (Biffi et al., 2013), and provides a useful tool in testing the aforementioned hypothesis.

5.5 Hormone signaling from the CNS: Implications for understanding aging?

A surprising finding from chapter two was that *Foxg1Cre*-mediated deletion of ATRX from the forebrain and anterior pituitary causes systemic defects manifested by decreased body weight and length, kyphosis, degeneration of subcutaneous fat, cloudy lenses that resemble cataracts, altered relative organ sizes, and decreased bone mineral density. We determined that the systemic defects correlated with altered endocrine signaling, as the mutant mice displayed decreased thyroxine (T4) and insulin-like growth factor 1 (IGF-1) levels compared to age- and littermate-matched controls (Figure 2-6). Moreover, the mutant mice exhibited reduced lifespan even when controlling for competition amongst littermates (Figure 2-4). Overall, the systemic phenotypes we described for the conditional knockout (cKO) animals were characteristic of aged mice and resembled mouse models of accelerated aging.

Numerous accelerated aging syndromes are caused by defects in the cellular response to DNA damage, including Cockayne syndrome, Werner syndrome, and ataxia telangiectasia (Hasty et al., 2003). Most of these syndromes are segmental, in that they do not fully recapitulate all aspects of normal human aging, making the relevancy of these disorders to human aging controversial (Kipling et al., 2004). However, the segmental nature of the syndromes is congruent with the DNA damage theory of aging that posits that aging results from DNA damage accumulation (Gensler and Bernstein, 1981; Kirkwood and Holliday, 1979; Szilard, 1959), since damage is stochastic and each tissue has different requirement for repair mechanisms and damage thresholds (Kirkwood, 2005).

The most consistent determinant of lifespan is the IGF-1 pathway (Bartke, 2005; Kenyon, 2005). IGF-1 is typically regulated by the action of growth hormone (GH), however T4 has been demonstrated to regulate IGF-1 during prepubescent development, particularly in the context of bone development (Xing et al., 2012). The regulation of IGF-1 by T4

was especially relevant in our study, since we did not observe changes in GH that could explain the dramatic reduction in IGF-1 levels. This study was one of the first to acknowledge a potential link between T4 and aging-like phenotypes. Moreover, thyroxine is critical for development and maturation of the CNS, particularly neuronal differentiation, migration, and myelination (de Escobar et al., 2004; Oppenheimer and Schwartz, 1997; Porterfield and Hendrich, 1993). It is therefore possible that many of the congenital neurological defects resulting from ATRX mutation or deletion in humans and mice could be caused by thyroxine deficiency. This intriguing concept could be tested by administering thyroxine to mouse models of *Atrx*-deficiency, as well as by measuring thyroxine levels in ATR-X patients. Intriguingly, ATR-X patients display kyphosis, muscle hypotonia, and delayed ambulation (Gibbons, 2006), however it is currently unknown whether this is relevant to the endocrine defects observed in the cKO mice described herein.

A key observation is that the somatotroph axis is suppressed in the ATRX cKO mice, which correlates with increased DNA damage. This is consistent with genetic deletion of the nucleotide excision repair factor ERCC1 (Niedernhofer et al., 2006), SIRT6 (Mostoslavsky et al., 2006), and overexpression of the short isoform of p53 (Maier et al., 2004). Furthermore, chronic exposure of wild-type mice to genotoxic stress can also dampen the somatotroph axis (Niedernhofer et al., 2006). Collectively, these studies provide strong evidence of a link between genome maintenance and somatotroph axis signaling. Our findings are unique in that deletion of ATRX, resulting in DNA damage accumulation, occurred only in the forebrain and anterior pituitary. This suggests that damage restricted to these regions can result in defective endocrine signaling and promotion of systemic aging-like characteristics. Furthermore, telomeres are heavily implicated in aging and cellular attrition (Sousounis et al., 2014), and this study links telomeric dysfunction with specific features of aging. However, it remains unclear exactly how telomere dysfunction fits into the systemic defects observed in ATRX cKO mice. Short telomeres have been linked to premature aging-like phenotypes, which can be rescued by reintroduction of telomerase (Samper et al., 2001). If the systemic defects in the cKO mice are due to abnormal telomere shortening, then overexpression of telomerase may constitute a means to rescue those phenotypes.

5.6 CTCF regulates NPC proliferation, differentiation, and survival

Prior to my studies, virtually nothing was known regarding CTCF functions in the context of brain development. In fact, CTCF activities had rarely been approached in an organismal context. Given the critical role for CTCF in development (Fedoriw et al., 2004; Heath et al., 2008; Moore et al., 2012; Soshnikova et al., 2010; Splinter et al., 2006; Wan et al., 2008; Watson et al., 2014) and the finding that human *CTCF* mutations cause autism and schizophrenia (Gregor et al., 2013; Iossifov et al., 2014; Juraeva et al., 2014), CTCF has emerged as a critical regulator of brain development. In particular, my data indicates that CTCF regulates brain size by balancing NPC proliferation, differentiation, and survival.

CTCF deletion in mouse NPCs results in microcephaly. I discovered that the microcephaly phenotype of mutant mice could not be fully explained by increased cell death in the absence of CTCF, since rescue of caspase-mediated apoptosis through *Puma* deletion failed to restore brain size at birth. A contributing factor to the microcephaly phenotype is the fact that CTCF influences timing of NPCs differentiation. *Ctcf* deletion causes precocious differentiation of NPCs into early-born deep layer cortical neurons, resulting in premature depletion of the progenitor pool and a failure to sustain the correct number of later-born superficial neurons. Together, aberrant differentiation timing and increased apoptosis of NPCs likely accounts for the neurodevelopmental abnormalities observed in *NestinCre*-mediated *Ctcf* deletion. The phenomenon of a premature switch form symmetric to asymmetric NPC divisions has been observed in many mouse models of microcephaly, and more recently induced pluripotent stem cell (iPSC)-based 3D neuronal culture models (Gilmore and Walsh, 2013; Lancaster et al., 2013), indicating that alterations in division mode can affect brain size.

An important distinction from the work presented in this thesis is that mutations identified in intellectual disability/microcephaly patients are *de novo* and only affect one copy of the *CTCF* gene (Gregor et al., 2013), as opposed to complete loss of CTCF. Detailed analysis of the consequences of three patient mutations indicated that frameshift

mutations resulted in an approximately 50% reduction in *CTCF* levels, while a missense mutation in the ZF domain did not affect transcript or protein levels (Gregor et al., 2013). This suggests that both the reduction of CTCF levels and alterations in its targeting or functions can cause neurological abnormalities. Further investigations into the functional consequences of human *CTCF* mutations will be necessary to fully resolve CTCF contributions to brain development and function.

Fate determination of NPCs is orchestrated by intrinsic and extrinsic mechanisms. Many studies indicate an essential role for centrosomes in neurogenesis (Doxsey et al., 2005; Higginbotham and Gleeson, 2007; Knoblich, 2008; Wang et al., 2009), functioning to maintain the neuroprogenitor pool while inducing the neuronal fate. CTCF has been positioned at the centrosome in HeLa cells (Rosa-Garrido et al., 2012; Zhang et al., 2004), raising the intriguing possibility that centrosomal activities of CTCF may regulate neurogenesis. However my analysis of CTCF localization in NPCs indicated enrichment at the mitotic spindle rather than at centrosomes *per se* (data not shown). Future experiments aimed at uncovering CTCF activities at the mitotic spindle and/or centrosomes will likely provide important insights into fate determination of NPCs.

Given that CTCF is a well-documented transcriptional regulator, it is also possible that altered NPC fate results from dysregulation of cell cycle regulators or other genes that play important roles in fate determination. Preliminary transcriptional profiling of the *Ctcf*-deficient embryonic brain has indicated altered expression of several cell cycle genes such as decreased expression of *Cdkn2a* and upregulation of *cyclin D1* and *Cdk6,* which is predicted to promote the G_1 to S phase transition and fits with the slight increase in the proportion of *Ctcf*-null S phase NPCs (Figure 4-4a). The *Ctcf*-null neocortex was characterized by an increase in the basal progenitor (BP) population, similar to what is observed after overexpression of *cyclinD1* in neural stem cells (Lange et al., 2009). *CDK6* mutations cause microcephaly in humans, and were shown to affect progenitor cell proliferation (Hussain et al., 2013). Furthermore, alterations in the retinoblastoma (Rb) and Cdkn1a (p21) cell cycle regulators cause neurological phenotypes that resemble CTCF loss (McClellan and Slack, 2006; Seoane, 2004; Slack et al., 1998). Global transcriptional analysis failed to detect differences in the mRNA levels of *Rb* or *p21,*

however these genes have been documented as direct CTCF targets in the literature (De La Rosa-Velazquez et al., 2007; Filippova, 2008; Qi et al., 2003). *In vivo* analysis of cell cycle progression failed to indicate any overt differences between control and *Ctcf*-null cells (Figure 4-4), but a more detailed investigation of cell cycle parameters including BrdU pulse-chase experiments and calculations of individual phase lengths may indicate subtle differences in *Ctcf*-null NPCs that could have dramatic consequences on brain development and growth.

Microcephaly can also result from increased cell death during brain development. Indeed, CTCF deletion using two separate Cre driver lines caused increased NPC death. Investigation of the mechanism underlying cell death indicated an increase in *Puma* transcript and protein levels. PUMA is a potent activator of the mitochondrial cell death pathway (Nakano and Vousden, 2001). *Ctcf* deletion in the developing limb bud also caused increased *Puma* levels (Soshnikova et al., 2010), and analysis of *Puma* regulation in HCT116 colon carcinoma cells indicated a p53-independent role for CTCF in suppressing *Puma* transcription (Gomes and Espinosa, 2010a, b). This led to the hypothesis that loss of CTCF-specific *Puma* repression causes ectopic induction of apoptosis in a p53-independent manner. Analysis of p53 activation as well as generation of *Ctcf/Puma* double mutant mice demonstrated that this hypothesis does not accurately describe the complexity of apoptotic induction downstream of CTCF loss. *Ctcf*-deficiency resulted in evidence of p53 activation such as increased total p53 protein levels and its translocation to the *Puma* promoter (Figure 3-3). Caspase-mediated apoptosis is PUMA-dependent in *Ctcf*-null NPCs, as caspase activation was inhibited in the *Ctcf/Puma*-deficient forebrain. However, the microcephaly phenotype at birth was not restored in the double mutant brain, presumably due to upstream activation of p53. Generation of *Ctcf/p53* double mutant mice would be helpful in delineating apoptotic from cell cycle defects downstream of CTCF loss.

5.7 Towards understanding CTCF functions in cellular survival

Genotoxic stress triggers activation of p53/PUMA-dependent signaling in NPCs (Jeffers et al., 2003). Since CTCF is regarded as an important organizer of global chromatin structure (Ong and Corces, 2014), and *Ctcf*-null NPCs activate the p53/PUMA-dependent signaling axis, I hypothesized that CTCF may regulate genomic stability to promote NPC survival. Indeed, *Ctcf* mutant cells display elevated endogenous DSB levels and sensitivity to exogenous stressors, particularly replicative stress-inducing agents. Further analysis indicated a correlation between endogenous damage and active proliferation, suggesting CTCF may protect cells from replicative stress. Despite this relationship, the exact mechanism of CTCF function in preventing damage accumulation remains enigmatic. My data suggests that CTCF is not involved in replicative stress signaling or repair, however more thorough analysis is required to substantiate this claim. Given the existing links between CTCF and replication (Cleary et al., 2002; Cleary et al., 2010; Libby et al., 2008), the protein may play a more indirect role in preventing genomic instability by facilitating DNA replication or the organization of replication timing domains.

CTCF binding upstream of repetitive tracts is hypothesized to slow the replication fork before progressing through difficult-to-replicate regions (Cleary et al., 2010). This idea is similar to the proposed role for CTCF in alternative splicing regulation, wherein CTCF binding downstream of weak exons promotes RNAPII pausing and exon inclusion (Shukla et al., 2011). Therefore CTCF may be required globally to slow polymerase progression, which in the context of replication may have important consequences for repeat stability. In particular, CTCF depletion causes a telomere dysfunction phenotype in U2OS cells (Deng et al., 2012a), indicating that at the very least CTCF is required for telomeric stability.

The downstream consequences of *Ctcf* deletion using two different Cre driver lines were remarkably distinct. In both instances, CTCF loss in NPCs resulted in elevated endogenous DNA damage levels, *Puma* upregulation, and apoptotic induction. However, deletion of CTCF from early progenitors at approximately E8.5 via *Foxg1-cre* was more severe than deletion at approximately E11 using *Nestin-cre*. This result is reminiscent of

inactivation of the replicative stress-signaling protein TopBP1. Deletion of TopBP1 in early progenitors using *Emx1-cre* results in profound loss of forebrain structure, while deletion in progenitors later in development using *Nestin-cre* causes a milder apoptotic phenotype (Lee et al., 2012a). Moreover, irradiation-induced DNA damage results in similar levels of DNA breaks in early and later progenitors, however apoptosis is elevated in response to radiation in early progenitors versus later-born progenitors (Lee et al., 2012a). Together, these data indicate that NPCs early in development trigger apoptosis to prevent the propagation of damaged genetic material to daughter cells. Furthermore, it provides a mechanism underlying the disparate phenotypes associated with CTCF loss using two different Cre driver lines.

DNA lesions activate PARP1, triggering the synthesis of ADP-ribose and PARylation of DNA repair proteins (Davies et al., 1978; Durkacz et al., 1980). Furthermore, PARP1 activity is required for efficient reversal of stalled replication forks (Ray Chaudhuri et al., 2012). CTCF is poly(ADP-ribosyl)ated by PARP1 (Yu et al., 2004) and activates PARP1 by acting as a link between DNA and poly(ADP-ribosyl)ation (Farrar et al., 2010). However, the CTCF and PARP1 interaction has primarily been studied in the context of chromatin insulation and transcriptional regulation. My findings suggest a reevaluation of the interaction between CTCF and PARP1 in the DDR and during replication fork restart.

The *CTCF* gene is located on chromosome 16q22.1, a deletion region well documented in several human cancers (Filippova et al., 1998; Rakha et al., 2006). *Ctcf* functions as a haploinsufficient tumour suppressor protein in mice (Kemp et al., 2014), strengthening the link between CTCF loss and cancer progression. $Ctcf^{+/-}$ tumours exhibited widespread DNA methylation changes, however it was unclear whether this effect was a primary or secondary cause of CTCF loss, and if it is the direct upstream source of $Ctcf^{+/-}$ tumour formation. My findings provide an additional avenue by which CTCF loss may promote tumourigenesis. Indeed, genomic instability and replicative stress are potent mediators of cancer progression, suggesting that replication-associated DNA damage may drive tumour formation in the absence of CTCF. Furthermore, there are existing links between DNA methylation status and genomic instability (O'Hagan et al., 2008; Robertson and Jones, 1997), suggesting that altered methylation profiles could explain damage

accumulation in the absence of CTCF. It is possible to test this by profiling global DNA methylation in *Ctcf*-null NPCs by next-generation bisulfite mutagenesis sequencing. However, it will be important to assess genomic stability in cells heterozygous for *Ctcf* as well as in tumours harboring *CTCF* mutations.

5.8 Future Directions: CTCF Functions in Genomic Stability

The body of work described in chapters three and four of this thesis demonstrates that CTCF is required for NPC survival through suppression of genomic instability. CTCF impinges on the p53 signaling network to mediate survival and likely to control cellular proliferation (Figure 5-2a). Several questions remain unanswered regarding CTCF activities in preventing endogenous DNA damage accumulation, namely, is CTCF directly or indirectly involved in the DDR?

Preliminary evidence suggests that CTCF is not required for timely repair of replication-associated damage, as *Ctcf*-null NPCs were able to resolve aphidicolin-induced γH2AX foci to baseline levels 24 hours post treatment (Figure 4-5). A more detailed analysis of DNA repair capabilities in the absence of CTCF will be required to exclude a role for CTCF in repair, especially given its localization at damage foci. The DR-GFP HR assay would be an excellent method to assess whether *Ctcf*-null NPCs are repair-competent, especially since replication-associated damage is typically repaired via HR (Jasin, 1996; Pierce et al., 1999). DR-GFP consists of two expression plasmids: (1) containing two tandem *GFP* genes, the first is a full-length *GFP* gene mutated with the 18 bp SceI endonuclease recognition sequence and the second is a truncated *GFP* that can be utilized as a template for repair, and (2) an SceI expression plasmid. Expressing both of the plasmids in cells results in a SceI-mediated DSB in the full-length *GFP* gene. If cells are capable of HR repair, they will utilize the truncated *GFP* for repair and will express the *GFP* reporter (Pierce et al., 1999). It would also be worthwhile to induce damage with different types of stressors, such as γ-irradiation or mitomycin C, to test whether CTCF is required for the repair specific types of lesions.

An equally plausible scenario is that CTCF loss causes global chromatin dysregulation, resulting in endogenous DNA damage. Global analysis of DNA damage sites via γH2AX

ChIP-sequencing in mutant cells will be informative in understanding the role of CTCF in suppressing endogenous damage accumulation. CTCF depletion causes global chromosome compaction (Tark-Dame et al., 2014). Intriguingly, chromatin condensation can lead to DDR signaling (Burgess et al., 2014), raising the distinct possibility that global changes in chromatin organization artificially signal activation of the DDR downstream of CTCF loss. Alternatively, changes to global chromosome architecture or subnuclear domain organization may cause changes to the replication timing program that can induce replicative stress (Cornacchia et al., 2012; Pope et al., 2014) (Figure 1-3), a possibility that can be tested by profiling replication timing in *Ctcf*-deficient cells.

CTCF may instead function more directly during DNA replication, which is supported by the finding that CTCF binding upstream of the repetitive *DM1* locus is required for its efficient and correct replication (Cleary et al., 2010) (Figure 5-2b) and my findings of altered replication dynamics in NPCs (Figure 4-3h). Moreover, the work herein demonstrates an interaction between CTCF and PCNA, albeit indirect, which would support the model that CTCF binding upstream of repetitive elements is required for polymerase fidelity (Figure 5-2b). Alternatively, CTCF may be involved in organizing replication factories with cohesin (Figure 5-2c), which is supported by the interaction between CTCF and the cohesin complex, namely the SA1 subunit that has been implicated in telomere replication and stability (Remeseiro et al., 2012). Evidence also points to a role for CTCF in organizing TAD boundaries (Dixon et al., 2012; Narendra et al., 2015; Nora et al., 2012), which have recently been shown to regulate replication timing (Pope et al., 2014). Profiling the timing (early and late-replicating) of control and *Ctcf*-null cell DNA replication is the most direct method to test whether CTCF is required for replication timing (Ryba et al., 2011) (Figure 5-2d). Together, my data suggests that CTCF is required to prevent replication-associated damage in NPCs, however the upstream molecular mechanism is currently unknown. The interaction between CTCF and PCNA requires further examination, especially since the interaction appeared to be enhanced under conditions of replicative stress. This finding may indicate a requirement for the partnership between CTCF and cohesin in postreplicative repair (Figure 5-3e), as cohesin is a well-established postreplicative repair factor (Sjogren and Nasmyth, 2001).
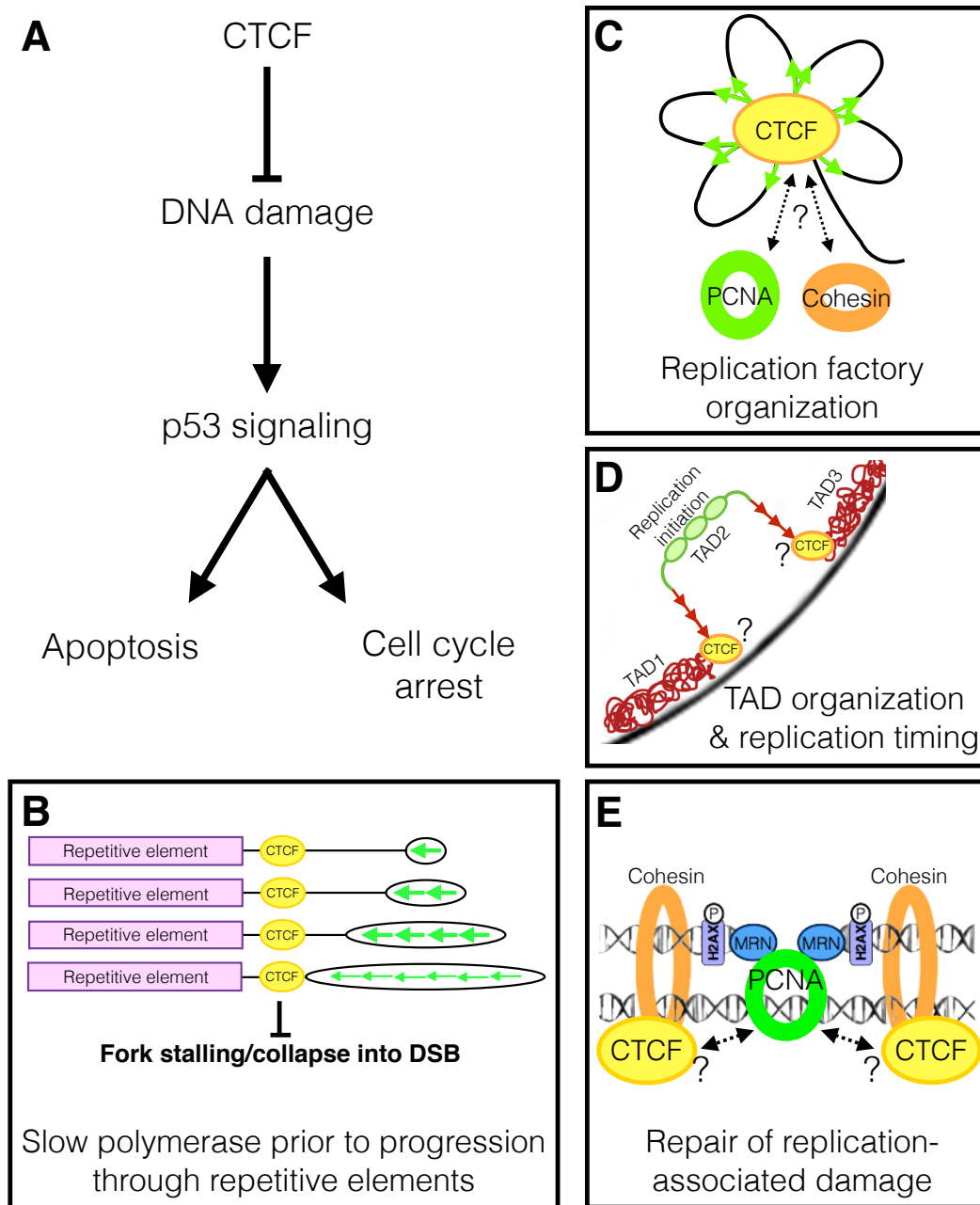
Figure 5-2 Possible mechanisms underlying CTCF-dependent suppression of DNA damage and p53-dependent signaling

(A) In NPCs, CTCF is required to suppress DNA damage and p53-dependent signaling. Potential mechanisms of CTCF-dependent DNA damage suppression supported by the literature and this work are presented in panels B-E. (B) CTCF binding upstream of repetitive elements such as the *DM1* locus (Cleary et al., 2010) and telomeres (Deng et al., 2012a) is required for fork/polymerase slowing, as represented by green arrows, prior

to progression through the elements to prevent fork slippage and collapse. (C) CTCF may help organize replication factories through its ability to mediate higher-order chromatin organization and interact with the cohesin complex and PCNA. (D) CTCF may be required for the establishment of TADs, which play important roles in regulating genome-wide replication timing domains (Pope et al., 2014). The diagram highlights CTCF binding at TAD borders, which demarcate the boundaries between early-replicating active euchromatin (green) within the nuclear interior and passive replicating regions (red arrows) that represent the transition regions between early- and late-replicating (heterochromatin found at the nuclear exterior; red) domains. (E) CTCF may interact with cohesin and assist with postreplicative repair of DNA damage acquired during S phase.

5.9 Implications of Aberrant Chromatin Structure to Brain Development

A key finding of this work is that proteins that regulate higher-order chromatin structure are essential for correct brain development through the balance of NPC proliferation, differentiation, and survival. Deficiency of either *Atrx* or *Ctcf* in the embryonic brain causes profound neurodevelopmental abnormalities, providing some insights into the pathologies stemming from human *ATRX* and *CTCF* mutations.

The physiological ramifications of aberrant chromatin structure are still widely unknown, however alterations in epigenetic regulators appear to particularly affect brain development and cognition (Kleefstra et al., 2014). A high proportion of ID disorders are caused by heterozygous *de novo* mutations in epigenetic regulators, suggesting that gene dosage is critically important for correct brain development and functioning. Mutations in *ATRX* and *CTCF* both cause intellectual disability disorders characterized by microcephaly and autistic features (Gibbons et al., 1995b; Gregor et al., 2013; Iossifov et al., 2014). Phenotypic similarities observed in these disorders may reflect common molecular pathways affected by mutations in different genes.

The overlapping phenotype associated with *Atrx*- and *Ctcf*-deficiency, namely accumulation of endogenous replication-associated DNA damage, indicates that the proteins may function together to regulate NPC survival. Given that ATRX is required for nucleosome remodeling and CTCF recruitment to imprinting control regions (ICRs) (Kernohan et al., 2014), it may be worthwhile to assess whether ATRX is required for CTCF localization in NPCs. Interestingly, CTCF sites are enriched for H3.3 (Jin et al., 2009) and H3.3 deposition at CTCF sites facilitates H3K27me3 removal in HeLa cells (Weth et al., 2014). Moreover, CTCF binds to human and mouse subtelomeric sequences, and its depletion results in decreased TERRA transcription and telomere dysfunction in U2OS cells (Deng et al., 2012a). ATRX may therefore facilitate CTCF recruitment to subtelomeric regions to promote telomere stability. ATRX loss is correlated with increased TERRA levels, however, suggesting that the relationship between ATRX, CTCF, and telomere stability is not straightforward. It is also important to interpret telomeric data derived from U2OS studies with caution since the cells do not express

ATRX and exhibit ALT (Lovejoy et al., 2012). Nevertheless, investigations of telomeric stability in *Ctcf*-null NPCs will provide insight into the genomic regions that acquire damage in the absence of CTCF and may point to the underlying molecular mechanism responsible for endogenous DNA damage downstream of CTCF loss. Unlike ATRX, however, CTCF binds to numerous genomic locations (Kim et al., 2007b), suggesting that CTCF function in preventing endogenous DNA damage is likely not telomere-specific. More global approaches, such as γH2AX ChIP-sequencing, will be required to determine if specific DNA sequences are more susceptible to damage acquisition in the absence of CTCF.

The finding that both *Atrx* and *Ctcf* deficient cells exhibit replication-associated damage may simply be a consequence of the loss of genomic stability factors in rapidly proliferating cortical NPCs. In other words, the genomic instability observed in *Atrx*- and *Ctcf*-null cells may be enhanced by the highly proliferative nature of NPCs. This idea is supported by the differences in cell viability after deletion of the genes using a *Foxg1-cre* or *Nestin-cre* driver line (Berube et al., 2005; Watson et al., 2014), which likely reflects the differential susceptibility of early and late NPCs to DNA damage (Lee et al., 2012a). However, it is not supported by the selective sensitivity of *Atrx*- and *Ctcf*-null NPCs to replicative stress-inducing agents. Furthermore, it is not supported by the finding that ATRX localizes to late-replicating heterochromatin foci, the majority of DNA foci in *Atrx*-null NPCs localizes to late-replicating foci, and the proposed model of ATRX functions in remodeling heterochromatin in G_2. It is also not supported by the finding that *Ctcf*-null NPCs in the same regions of the embryonic cortex exhibit cell cycle-dependent DNA damage that correlates with PCNA staining. In conclusion, ATRX and CTCF may not be involved in preventing replicative stress *per se* but rather are critical to suppress DNA damage; the consequence of loss of these proteins being p53-dependent apoptosis in the quickly dividing NPC population. Alternatively, the phenotypes observed downstream of ATRX and CTCF loss may indicate critical cell cycle-dependent roles for these proteins. Future experiments aimed at uncovering the exact biochemical mechanisms of function for these proteins in different phases of the cell cycle will be instrumental in dissecting their true actions in protecting the genome from DNA damage.

Moreover, deficiency of either *Atrx* or *Ctcf* in NPCs causes precocious differentiation early in corticogenesis, resulting in depletion of the progenitor pool and a failure to sustain generation of late-born neurons (Ritchie et al., 2014; Watson et al., 2014). Selective lengthening of the cell cycle, particularly the G_1 phase, is hypothesized to causally contribute to progenitor switching from proliferative to neuron-generating divisions (Calegari et al., 2005; Calegari and Huttner, 2003). Replication errors or unresolved replication intermediates generated during S/G_2 phase can be converted into DSBs during mitosis and are repaired in the subsequent G_1 (Lukas et al., 2011), which can induce G_1 lengthening. If G_1 lengthening is causal in promoting neurogenic divisions of progenitors, then G_1 lengthening may be responsible for the precocious differentiation observed in the *Atrx*- and *Ctcf*-null neocortex (Figure 5-3).

The most common disease linked to replication stress is cancer (Bartek et al., 2012). This fact is currently being exploited to induce synthetic lethality in tumour cells. For example, PARP inhibitors have been used to block repair of ssDNA breaks that are processed into DSBs during S phase, and results in apoptotic induction of HR-deficient tumour cells (Jackson and Bartek, 2009). Along the same lines, ALT-positive tumour cells, such as those with inactivating *ATRX* mutations, are sensitive to ATR inhibition (Flynn et al., 2015). Further investigation of the exact mechanism underlying tumourigenesis downstream of *CTCF* mutation will be necessary to advance cancer therapeutics, especially given the high occurrence of 16q22.1 deletions in various human cancers (Filippova et al., 1998; Rakha et al., 2004).

Replicative stress has also been associated with human neurological diseases such as Seckel (OMIM# 210600), Aicardi-Goutieres (OMIM# 610333), Meier-Gorlin (OMIM# 224690), Nijmegen breakage (OMIM# 613078), and Wold-Hirschhorn (OMIM# 194190) syndromes (Zeman and Cimprich, 2014). These syndromes feature overlapping characteristics with ATR-X syndrome and patients with mutations in *CTCF* such as intellectual disability and microcephaly, suggesting similar molecular mechanisms underlying disease pathogenesis. However, analysis of patient cells and/or generation of effective animal models of the disorders will be necessary to confirm or refute this claim.

The findings documented in this thesis broadly illustrate the importance of higher-order chromatin regulation for correct brain development. They also support the genome-wide association studies indicating that epigenetic pathway alterations play a major role in the pathogenesis of neurodevelopmental and neuropsychiatric disease (De Rubeis et al., 2014; Pinto et al., 2014). Collectively, the data positions two important epigenetic regulators, ATRX and CTCF, at the intersection between chromatin structure and genomic stability, and demonstrates the importance of higher-order chromatin organization in safeguarding the genome to support correct brain development.

5.10 References

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Appendix 3 Statement of permission for the use of animals for experimental research

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

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