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**ATM MUTANT VARIANTS AND THEIR DIFFERENTIAL
EFFECTS ON MYELIN AND CEREBROVASCULAR
PATHOLOGY IN ATAXIA TELANGIECTASIA
– GENOMIC DIAGNOSIS FOR RARE BUT CATASTROPHIC
DISEASE**

YEUNG HOI SANG

PhD

The Hong Kong Polytechnic University

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The Hong Kong Polytechnic University

Department of Health Technology and Informatics

**ATM mutant variants and their differential effects on myelin and
cerebrovascular pathology in Ataxia Telangiectasia
– genomic diagnosis for rare but catastrophic disease**

Yeung Hoi Sang

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

April 2025

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_____ (Signed)

Yeung Hoi Sang (Name of Student)

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1. Abstract

Ataxia Telangiectasia (A-T) Mutated protein (ATM) is a major kinase that responds to DNA double strand breaks. A spectrum of pathogenic mutations of ATM is known to cause this rare genetic disease with cerebellar symptoms of different severity. Myelin pathology emerges as one of the neurological deficits in young A-T patients, but the mechanism is unknown. We aim to delineate the divergent effects of different ATM mutations on oligodendrocyte (OL) lineage. The postmortem brain tissue of A-T patients were employed for histological and transcriptomic study, showing distinct pathology in A-T cerebellum and cerebral cortex. Divergent severity of A-T pathology was found among A-T patients, showing differential effect of pathogenic mutations in ATM gene. The translational study was followed with a histological investigation where OL pathology was validated through Atm deficient mice model, Atm^{Bal} (B6;129S4- $Atm^{tm1Bal/J}$), where the Atm exons was disrupted with no residual ATM activity, and Atm^{Awb} (B6.129S6- $Atm^{tm1Awb/J}$), where partial Atm mutation is being validated in a model harbouring truncated but active Atm residue. Atm deficient mice showed a significant decline of CC1 positive cell, marker of mature OLs, in both frontal and motor cortex region at 4 month-old. Region-specific effect of Atm deficiency on mature OL was consistent with the slight decline in cognitive performance of aging ATM deficient mice in behavioural test. The essential role of ATM in OL maturation was further confirmed with murine glial primary culture with intrinsic Atm mutation. In silico prediction of ATM-MBP interaction was verified with immunoprecipitation experiment, demonstrating potential mechanism of ATM affecting the

maturation process of OL. This study provided detailed investigation on A-T myelin pathology and subsequence mechanism, which also raise the potential correlation between cognitive performance and myelin pathology.

Moreover, the advantage of targeted long read sequencing on rare disease diagnosis was emphasized in this study, especially Nanopore targeted sequencing. Improved efficiency and convenience of detecting mutations in desired regions could be achieved with Nanopore sequencing. Adaptive sampling could be the further approach of targeting desire regions with Nanopore sequencing.

2. Research Output

Published

Cheng, G. W., Mok, K. K., **Yeung, S. H.**, Kofler, J., Herrup, K., & **Tse, K. H***. (2022). Apolipoprotein E ϵ 4 Mediates Myelin Breakdown by Targeting Oligodendrocytes in Sporadic Alzheimer Disease. *Journal of neuropathology and experimental neurology*, 81(9), 717–730. <https://doi.org/10.1093/jnen/nlac054>

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Submitted

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In preparation

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Yeung, S. H., Siu, K.H., **Tse, K. H***. Targeted sequencing of ATM for the genetic diagnosis of Ataxia Telangiectasia using oxford nanopore technology (In preparation)

Conference papers

Yeung, S. H., Fornage M., Herrup, K., Arai K., **Tse K.H***. Characterization of TRIM47 in the mouse brain – a GWAS-identified tripartite motif family member associated with white matter hyperintensity. The 43rd Annual Meeting of the Japan Neuroscience Society. (29th July – 1st August 2020, Kobe, Japan)

Yeung, S. H., Cheng, A., Cheng, G. W., Mok, K. K. S., Herrup, K., **Tse, K. H***. Divergent effects of ATM mutations on oligodendrocytes - the cellular basis of myelin pathology in Ataxia Telangiectasia. The 50th Annual Meeting of the Society for Neuroscience. (13th – 16th November 2021, virtual or Chicago, USA)

Yeung, S. H., **Tse, K. H***. Targeted sequencing of ATM for the genetic diagnosis of Ataxia Telangiectasia - A pilot evaluation of oxford nanopore technology. Poster Board 3495. Poster presented at: ASHG 2023 Annual Meeting. The American Society of Human Genetics; 2023 November, Washington, DC.

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4. Abbreviation

2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase)

3-methyladenine (3-MA)

4',6-diamidino-2-phenylindole (DAPI)

Acute myeloid leukemia (AML)

Adaptive sampling (AS)

Adenomatous polyposis coli (APC / CC1)

Alpha receptor for platelet-derived growth factor (PDGFR α)

Analysis of variance (ANOVA)

Anterior cingulate cortex (ACCx)

Anterior commissure (Ant Com)

Aprataxin-and-PNK-like factor (APLF)

Aspartoacylase (ASPA)

Ataxia oculomotor apraxia type 1 (AOA1)

Ataxia oculomotor apraxia type 2 (AOA2)

Ataxia telangiectasia (A-T)

Ataxia-Telangiectasia Clinical Center (ATCC)

Ataxia-telangiectasia-like disorder 2 (ATLD2)

Ataxia-telangiectasia mutated (ATM)

B6;129S4-Atm^{tm1Bal/J} (Bal)

B6.129S6-Atm^{tm1Awb/J} (Awb)

Beta-mercaptoethanol (β -ME)

Cas9-assisted targeting of chromosome segments (CATCH)

Cas9 targeted sequencing with Nanopore (nCATS)

Central nervous system (CNS)

Centralized Animal Facilities (CAF)

Cerebellum (CB)

Chondroitin sulfate proteoglycan 4 (CSPG4 / NG2)

Clinical Genetic Service in Department of Health (DH CGS)
Clinical Genetic Service Unit (CGSU)
Clustered regularly interspaced short palindromic repeats (CRISPR)
Complete Dulbecco's Modified Eagle Medium (cDMEM)
Copy number variants (CNVs)
Corpus callosum body (CC body)
CRISPR system-assisted dsDNA capture (CRISPR-Cap)
Deoxyribonucleotide (dNTP)
Diabetic kidney disease (DKD)
Dideoxynucleotides (ddNTPs)
Differentially expressed genes (DEGs)
Diffusion Tensor Imaging (DTI)
DNA-dependent protein kinases (DNA-PK)
Double strand break (DSB)
Dulbecco's Modified Eagle Medium (DMEM)
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12)
Familial Hemiplegic Migraine (CACNA1A & ATP1A2)
Fetal horse serum (FHS)
Fluid-attenuated inversion recovery (FLAIR)
FUSion Detection from Gene Enrichment (FUDGE)
Frontal cortex (FCx)
Genetic and Genome (G/G)
Genome Analysis Toolkit (GATK)
Guided RNA (gRNA)
Heat induced epitope retrieval (HIER)
High-grade serous ovarian cancer (HGSC)
Hippocampus (Hippo)
Hong Kong Children's Hospital (HKCH)
Hong Kong Genome Institute (HKGI)

Hong Kong Genome Project (HKGP)
Hong Kong Government (HKG)
Hong Kong Laboratory Accreditation Scheme (HOKLAS)
Hong Kong Polytechnic University (PolyU)
Hospital Authority (HA)
Individually ventilated cages (IVC)
Insertions or deletions (Indels)
Insulin-transferrin-selenium (ITS)
Intracerebral Hemorrhage Diseases (COL4)
Interstitial lung disease (ILD)
Long-term potentiation (LTP)
Magnetic resonance imaging (MRI)
Mammalian target of rapamycin (mTOR)
Mature oligodendrocyte (mOL)
Medical Laboratory Technologists (MLTs)
Motor cortex (MCx)
Multiple sclerosis (MS)
Myelin associated glycoprotein (MAG)
Myelin basic protein (MBP)
Myelin oligodendrocyte glycoprotein (MOG)
Myelin regulatory factor (MyRF)
Neural stem cells (NSCs)
Non-diabetic disease (NDKD)
Non-homologous end joining (NHEJ)
Normal donkey serum (NDS)
Oligodendrocyte (OL)
Oligodendrocyte precursor cells (OPCs)
Optimal cutting temperature (OCT)
Paraformaldehyde (PFA)

Penicillin-streptomycin (P/S)

Peripheral nervous system (PNS)

Phosphate buffer solution (PBS)

Purkinje cells (PCs)

Phosphoinositidyl 3-kinase-related protein kinase (PIKK)

Platelet derived growth factor-AA (PDGF-AA)

Poly-L-lysine (PLL)

Pulsed field gel electrophoresis (PFGE)

Prince of Wales Hospital (PWH)

Proteolipid protein (PLP)

Queen Mary Hospital (QMH)

Radioimmunoprecipitation assay (RIPA)

Rapamycin (Rapa)

Reactive oxygen species (ROS)

Retrosplenial cortex (RsCx)

Single nucleotide polymorphism (SNP)

Short tandem repeats (STR)

Standard error of mean (SEM)

S/T-Q cluster domains (SCD)

Structural illumination microscopy (SIM)

Supplementary Medical Professions Ordinance (SMPO)

Target adaptive sampling long read sequence (TAS-LRS)

Triiodothyronine (T3)

Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST)

Werner (WRN)

White matter (WM)

White matter hyperintensities (WMH)

Wildtype C57BL/6J mice (WT)

Xray cross complementing protein 4 (XRCC4)

XRCC4-like factor (XLF)

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6. Introduction

6.1 Myelination in central nervous system

6.1.1 Myelination

White matter (WM), a dichotomic anatomical description of myelin rich region in the nervous system, was discovered by Andreas Vesalius in the 16th century (Boullerne, 2016). In 1854, the term “myelin” was first introduced by German pathologist Rudolf Ludwig Virchow to describe substances filling up the volume between axis-cylinder and primitive nerve fibers (Boullerne, 2016). Myelination is the formation of myelin sheaths that wrap around the axons in both the central nervous system (CNS) and peripheral nervous system (PNS). Myelination is mediated by oligodendrocytes (OL) in CNS and mediated by Schwann cells in PNS respectively (Arroyo & Scherer, 2000). Each myelin sheath in PNS is supported by individual Schwann cell, however, each OL support multiple myelin sheath formation up to 30 or more (Salzer & Zalc, 2016).

6.1.1.1 Myelin function

White matter in healthy human brains is constituted with over 150,000 km myelinated fiber in average (Marner et al., 2003). Myelination is the process when axon of neuron is wrapped by lipid and cholesterol rich layers called myelin sheath. Myelin sheaths act as insulation cloth on electron-transmitting axons, which speed up the transmission rate and promote communication between neurons. Structurally, myelin is formed by the spiral wrapping of specialized glial cell membranes around axons, creating compact layers with periodic interruptions known as nodes of Ranvier (Simons & Nave, 2015). Without myelination, electric signals in axons propagate solely through continuous action potentials generated by sodium ion exchange across the entire

axonal membrane. In contrast, myelinated axons utilize saltatory conduction, where action potentials "jump" between nodes of Ranvier, significantly increasing the speed and efficiency of signal transmission (Huxley & Stämpfli, 1949). Unmyelinated axon could conduct electric signals in a range about 0.5 to 10 m/s, however, axons with myelin sheath could conduct signal 15 times faster at least, up to 150 m/s (Purves & Williams, 2001).

6.1.1.2 Myelin structure and composition

Myelin contains approximately 40% water by composition *in situ* (Siegel & Agranoff, 1999). To support the function of axons insulation, the dry mass of myelin is mainly composed of lipid (70-85%) that are largely nonpolar in nature. Cholesterol, galactosylceramide and ethanolamine plasmalogen are the most abundant forms of lipid in myelin. Ratio of cholesterol, phospholipids and glycosphingolipid is 40%:40%:20% in myelin sheath, which is deviated from the normal ratio of most other membrane (25%:65%:10%) (Schmitt et al., 2015).

Protein portion in dry myelin ranges from 15% to 30%, which revealed a relatively lower protein to lipid ratio in myelin compared to other biological membrane (Siegel & Agranoff, 1999). This intricate arrangement, maintained by specific myelin proteins such as myelin basic protein (MBP), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP), not only facilitates rapid nerve conduction but also provides metabolic support to the underlying axons in CNS (Philips & Rothstein, 2017). Understanding the complex structure and composition of myelin is essential for comprehending its role in normal neurological function and its involvement in demyelinating disorders.

6.1.1.3 Significance of myelination in CNS

Neurogenesis is limited after birth. Magnetic resonance imaging (MRI) study showed age dependent gray matter volume decline (Bartzokis et al., 2001), which might reflect degeneration of neurons along aging. Unlike gray matter, WM volume continues increasing in frontal cortex until age around mid-forties (Bartzokis et al., 2001), while the decline of WM will then begin. Another MRI-Diffusion Tensor Imaging (DTI) study compared the intracortical myelin change with cognitive function. The result suggested a correlation between cognitive impairment and age-dependent myelin deficit (Grydeland et al., 2013). Immunohistochemistry study on non-human primate brains also supported the claim that age-dependent myelin alteration correlated with cognitive impairment (Peters & Sethares, 2002). These results showed the importance of myelination to cognitive performance, in which myelin should play an important role in postnatal cognitive development.

Due to the importance of synapse in brain plasticity, the effect of myelin remodelling on brain plasticity was often underestimated in the human CNS. Synaptic plasticity is modified by long-term potentiation (LTP), which means the persistent strengthening of synapses due to co-incidence of pre- and post- synaptic activation (Cooke & Bliss, 2006). LTP was established as the hypothesis for memory formation and learning. Recently, myelin plasticity has been recognized for CNS plasticity. Activity-dependent myelination is the mechanism that neuronal activity could regulate and modify myelination, which in turn feedback and modulate the neural network (Bonetto et al., 2020).

The thickness, length and stability of myelin sheath could modulate the axonal transmission speed of action potential (Waxman, 1997). Importantly, modulation of transmission speed in turn controls the synchrony of the action potential spike arrival time on the synapse (Figure 1). The precision of synchronous arriving timing of action potential could largely promote synaptic strength, otherwise it could be weakened by non-precise arrival of action potential in just a few

milliseconds difference (Fields, 2015). Besides, neuronal activity could modulate the *de novo* myelination through the regulation on proliferation and differentiation of OL precursor cells (OPCs) (Bonetto et al., 2020), which is the progenitor cell for myelination.

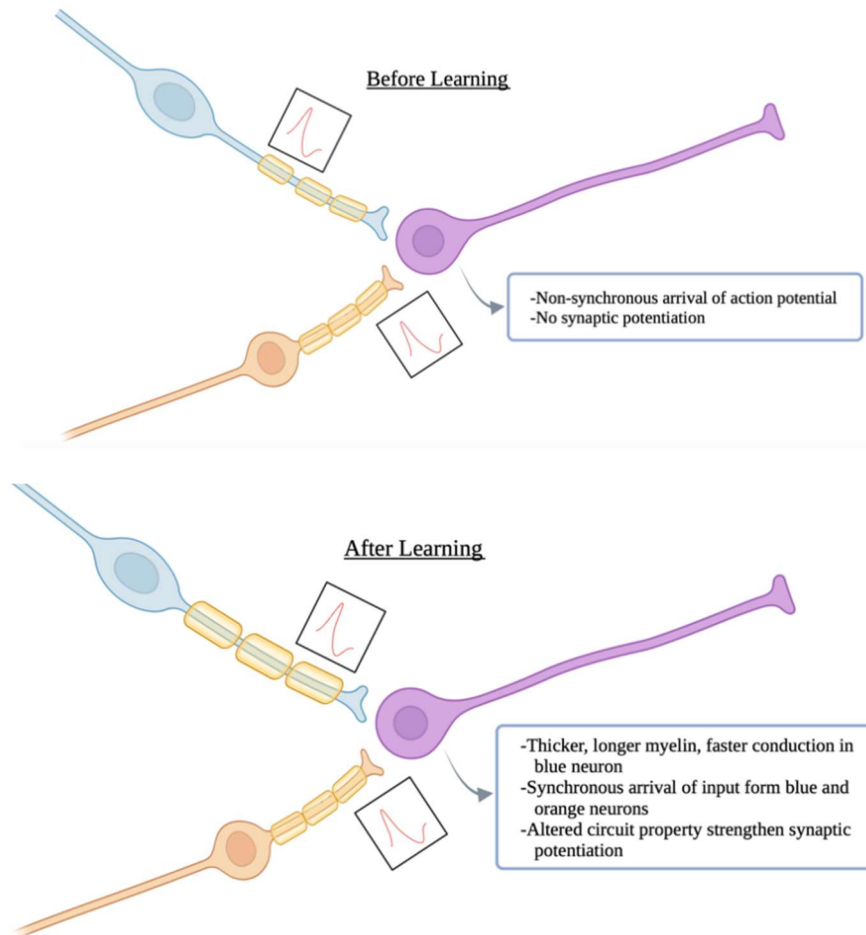


Figure 1. Activity dependent myelination effect on modulating neural circuit.

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6.1.1.4 Myelin development

Myelin sheath formation is initiated by contact between extending processes of mature OL and myelination competent axon (Simons & Nave, 2015). Each mature OL is connected to 30-60 myelin sheaths, while each mature myelin sheath has up to 160 layers of compacted lamellae (Hildebrand et al., 1993). MAG is expressed on the innermost wrap of the myelin sheath, which plays a crucial role in the axon-OL adhesion through binding towards gangliosides on the axonal surface (Schnaar & Lopez, 2009).

After the initial contact, the wrapping of myelin membrane was found under microscope, which was driven by the expansion of the inner tongue of myelin sheath (Snaidero et al., 2014). The extension of inner tongue membrane was mediated by F-actin disassembly and redistribution in the leading edge of membrane (Nawaz et al., 2015). Formation of large impermeable molecular sieve by MBP and PLP expression on lipid-rich myelin-membrane sheets cause extrusion of cytoplasmic component between the membrane layers, eventually lead to intracellular myelin compaction (Aggarwal et al., 2011). The significance of myelin compaction was found with the tendency of myelin degradation without MBP mediated compaction of myelin (Weil et al., 2016).

Nodes of Ranvier formation is followed by myelin compaction, which depends on interdependence mechanism between OL and axon. Clustering of sodium channels is demonstrated at both axonal initial segment and node of Ranvier, which was found initiated by contact-dependent mechanism and soluble factor secreted by OL (Rasband & Peles, 2015). McKenzie et al. (2022) demonstrated that the half-life of existing compact myelin was around 20 weeks, which implied the need of continuous myelin synthesis for maintenance of myelin integrity.

6.1.1.5 Myelin pathology in aging brain

The vulnerability of myelin and OL increased with age due to progressive accumulation of the toxicity in the intracortical environment (Bartzokis, 2004). The increasing trend of myelin deficit against age and cognitive decline was then found associated with accumulated DNA damage in OL (Tse et al., 2018). Cell death was then followed by aberrant re-expression of cell cycle protein in well differentiated OL (Tse et al., 2018). Previous research also revealed minimal neuronal pathology with significant OL abnormality in frontal cortex of dementia

patients, which might indicate the potential sequential pathology of neurodegeneration in frontal cortex (Tse et al., 2018).

6.1.2 Oligodendrocyte

6.1.2.1 Oligodendrocyte lineage

Myelination in CNS is exclusively dependent on OL which is mainly responsible for myelin production and maintenance. During development, lineage of oligodendrocyte begins with neural stem cells (NSCs) in subventricular zone, after exiting self-renewing cycle, they gradually differentiate into OPCs in gliogenic stage (Naruse et al., 2017). Chondroitin sulfate proteoglycan 4 (CSPG4, also known as NG2) and alpha receptor for platelet-derived growth factor (PDGFR α) are the well known antigens for OPC lineage (Kang et al., 2010). Transcription factors Olig2 and SOX10 are expressed in all stage of oligodendrocyte lineage, which can be used as the pan-oligodendrocytic marker. Mature oligodendrocyte (mOL) could be characterized by expression of myelin regulatory factor (MyRF), as it is the master transcription factor of myelin-related protein, such as MBP, PLP and 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) (Figure 2) (Pepper et al., 2018). In histological studies, aspartoacylase (ASPA) and adenomatous polyposis coli (APC, antibody clone as CC1) are commonly used as markers to label mOL (Chakraborty et al., 2001). After differentiation into mOL, compact layers of myelin sheath would be formed on neuronal axons with the expression of myelin-related proteins.

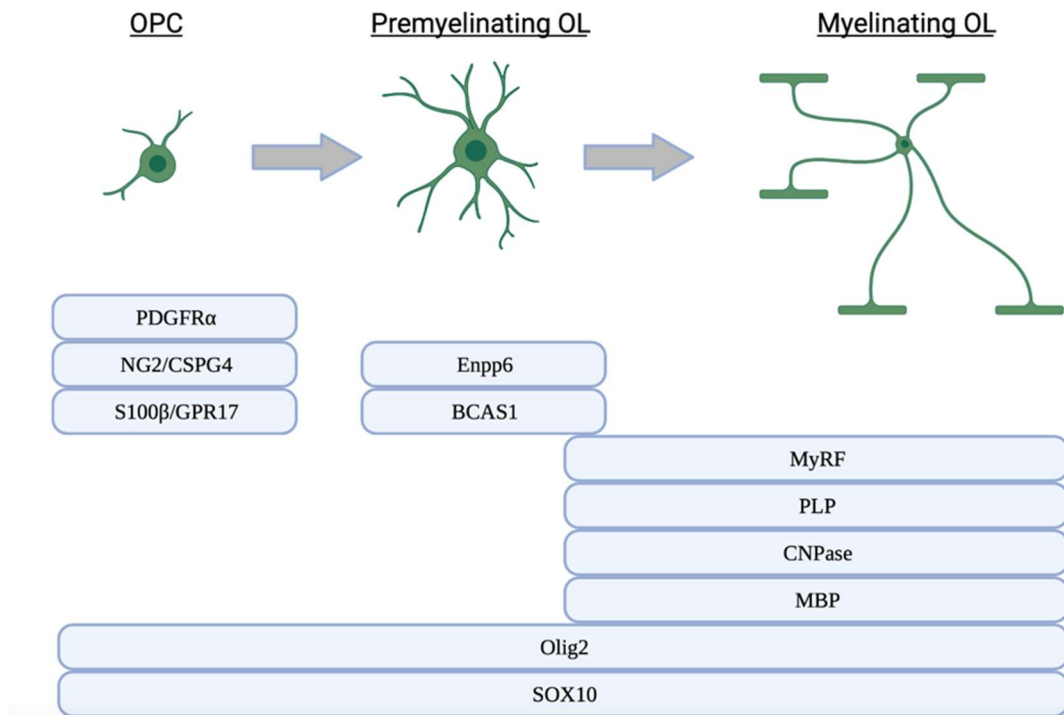


Figure 2. Oligodendrocyte lineage identification.

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6.1.2.2 Morphology and function of oligodendrocyte in CNS

At early stage of OL lineage, NG2 expressing still remain its cell multipotency proliferating into other glial cells, such as astrocyte (Michalski & Kothary, 2015). OPC has branched processes and highly dynamic protrusions with trajectory projection at averaged speed $0.3 \pm 0.01 \mu\text{m min}^{-1}$ recorded under time-lapse two-photon microscopy (Hughes et al., 2013). In the developmental stage of CNS, OPC competed for limited axonal survival factors, about 20% OPCs degenerated in cerebral cortex without forming compact myelin on axons (Trapp et al., 1997). Some of the OPC population remain undifferentiated throughout adult CNS. The ratio of OPCs to myelinating mOL was found 1:1 in spinal cord and 1:4 in forehead grey matter (Dawson et al., 2003). OPCs population in adult brain function as a reservoir of ready-to-differentiate OL responding to OL loss in both healthy and disease brain (Hughes et al., 2013; Young et al., 2013). Previous study also revealed that adult OPCs were capable of differentiating into myelinating OL in response to altered neuronal activity (Gibson et al., 2014).

Mature OLs extend multiple processes upon axonal contact, forming compact myelin sheath on axons from different neurons for accelerated action potential propagation and saltatory conduction (Czopka et al., 2013). Moreover, myelinating OL promotes axonal survival through secretory factors such as CNP1, GDNF, KIF5A and KIF1B (Sherman & Brophy, 2005).

6.1.2.3 Plasticity and adaptation of OL

Adaptive myelination describes the activity-dependent and plastic changes on myelin sheath in response to altered neuronal activity (Bechler et al., 2018). Stimulation of neurons in cortical layer was found to result in regional upregulated oligodendrogenesis and increased myelin thickness (Gibson et al., 2014). Moreover, the substance released from synaptic vesicle also promotes the myelin production ability of OL (Mensch et al., 2015). Indeed, the plasticity of OL upon neuronal activity grants OL the ability to remodel existing myelin structure, which can be illustrated by the re-positioning of the node of Ranvier and alteration of internode length (Young et al., 2013).

6.1.2.4 Oligodendrocyte pathology in demyelinating disease

Oxidative stress is the main source of DNA damage accumulated in aging brain, which progressively lead to genomic instability of brain cells (Tse & Herrup, 2017). DNA damage is consistently found in OLs located in demyelinating lesions, and likely to be part of the cellular pathology for the loss of myelination (Tse et al., 2018). Indeed, unrepaired DNA damage could accumulate in the genome of postmitotic mOL in brain of patient suffering from multiple sclerosis (MS) and cause apoptosis (Haider et al., 2011). MS is a demyelinating disease associated with CNS chronic inflammation and demyelination, which hints a link between oxidative DNA damage in mOL and demyelination. The source of oxidative stress might be attributed to chronic effect of the immune response, such as microglia, macrophage and T cell

expressions, in MS patients. Transcriptome profiling study on human prefrontal cortex showed accelerated DNA damage after age 40, which reduce expression of gene related to learning and memory (Lu et al., 2004). The source of oxidative stress in aging might varies, such as UV radiation, PM particles and heavy metal in the environmental pollution (Aseervatham et al., 2013), but the trend showing accelerated DNA damage meets the decline of myelination at around age 40s. Moreover, there is an emerging role of oxidative stress that disrupt OPC differentiation with interference in its transcriptional and metabolic mechanisms (Spaas et al., 2021).

6.2 Ataxia Telangiectasia

6.2.1 Cause and epidemiology

Ataxia telangiectasia (A-T) is an autosomal recessive genetic disease which was first named by Elena Boder and Robert P. Sedgwick in 1957 (Rothblum-Oviatt et al., 2016). It was named after its hallmark syndromes, progressive cerebellar ataxia and oculocutaneous telangiectasia. Besides, ataxia-telangiectasia mutated (ATM) gene was first identified and sequenced in 1995, which was found always mutated in A-T patients (K. Savitsky et al., 1995). As it is an autosomal recessive degenerative genetic disease, the occurrence of A-T is low, approximately 1:40000 to 1:100000 (Amirifar et al., 2019). Indeed, patient with null mutation only have about 50% survival rate in their twenties (Micol et al., 2011), and a high mortality rate in postnatal development. Although it is not affecting most population, A-T patients suffer from a wide range of catastrophic clinical symptoms with different severity. In general, there are two clinical forms of A-T dependent on their severity. The one with more severe manifestation would be described as “classic”, “typical”, “early onset” or “childhood onset”, otherwise the less severe form would be described as “variant”, “atypical”, “later onset” or “adult onset” (Rothblum-Oviatt et al., 2016).

6.2.2 Clinical manifestation

Different clinical symptoms were reported in A-T patients, but severity among A-T patients varies with their mutation status. In general, cerebellar degeneration, ocular telangiectasia, immunological, pulmonary disorder, and cancer predisposition are the most common symptoms of A-T disease.

6.2.2.1 Neurological symptoms

Cerebellar ataxia is the most obvious neurological manifestation occurred in the childhood of patient with classic A-T. Ataxia of gait and stance of children is the clinical hallmark for cerebellar degeneration which is shown in many A-T patients (Hoche et al., 2012), especially for the classic A-T patients. Indeed, the neurological effect of A-T children is difficult to notice until they reach 5-year-old. After that, the neurological symptom deteriorates rapidly, and most of the children completely lost their walking ability since eight-years-old (Nissenkorn et al., 2011). Hence, A-T was classified as neurodegenerative disease (Herr et al., 2024). Cerebellar degeneration also causes dysarthria in around 80% A-T patient (Nissenkorn & Ben-Zeev, 2015). Impaired motor speech function caused by muscle degeneration could greatly inhibit the development of normal oral communication skills. Moreover, dysmetria is the next most frequent symptom shown up in over 75% A-T children (Hoche et al., 2012). This symptom is presented by the inability to coordinate limbic movement, especially not able to determine the actual distance, speed or other parameter needed for motor function. Coinciding with cerebellar degeneration, cognitive impairment was presented in A-T in early stage, which was found worsen progressively (Hoche et al., 2014).

6.2.2.2 Telangiectasia

Ocular telangiectasia is another hallmark symptom of A-T, which is presented in over 97% A-T patients (Nissenkorn & Ben-Zeev, 2015). Dilated capillaries appeared as fine red lines near the surface of the mucous membrane of conjunctiva, but it does not bleed or itch. Different from allergy, the visible blood vessel appears constantly in A-T. The abnormalities have no direct effect on the vision (Farr et al., 2002). Yet, dilation of veins and hyalinization of arterioles were reported in A-T brain meninges (Kamiya et al., 2001).

6.2.2.3 Immunodeficiency

Humoral immunodeficiency is common among A-T patients. According to a study on 100 A-T patients from the Johns Hopkins Ataxia-Telangiectasia Clinical Center (ATCC), 65% of patients showed IgG4 deficiency, 63% showed IgA deficiency, 48% showed IgG2 deficiency, 23% showed IgE deficiency, and 18% showed IgG deficiency (Nowak-Wegrzyn et al., 2004). Indeed, ATM deficiency would compromise the V(D)J recombination, which is essential for immunoglobulin variable region exons assembly (Zha et al., 2011). Furthermore, lymphocyte deficiency occurred in over 70% of A-T patients from ATCC, for instance CD19 B lymphocytes and CD4 T lymphocytes were decreased in around 70% A-T patients (Nowak-Wegrzyn et al., 2004). As a result, around 80% of A-T patients are estimated to suffer from infections (Amirifar et al., 2019).

On top of the effect on immunoglobulin recombination, ATM deficiency disrupts the autophagy-lysosomal pathway in neuron (Cheng et al., 2021). Aggregation of lysosome was found in mice cerebellar neuron of Atm-deficient mice (Barlow et al., 2000). There is no direct evidence of autophagy disruption or immunodeficiency on OL in A-T patients. However, autophagy plays an important role in OL differentiation and myelin repairment (Bankston et al., 2019).

6.2.2.4 Respiratory dysfunction

Over 70% A-T patient suffered from pulmonary manifestations and 50% of them eventually die from respiratory failure in their adolescence (Nowak-Wegrzyn et al., 2004). High mortality rate of A-T is largely attributed to respiratory disease, such as sinopulmonary infections, pulmonary fibrosis, bronchiectasis, and interstitial lung disease (ILD). Although the pathological mechanism of pulmonary disorder in A-T disease is not confirmed, it could be the

secondary effect attributed to immunodeficiency, premature aging, inflammation, and oxidative stress.

6.2.2.5 Cancer predisposition

In a retrospective cohort study of 240 A-T patients from 1954 to 2005, 53 patients (22.1%) developed cancer, which is the main contributor of mortality (Micol et al., 2011). As ATM plays an important role in cell cycle checkpoints, which serves as a break to arrest the replication cycle in cell with DNA double strand break (DSB), it could suppress the potential malignancy development (Iyama & Wilson, 2013). Moreover, in response to DNA DSB, ATM promotes p53 and other tumour suppressor genes to initiate programmed cell death in cancer suspected cell (Shiloh & Ziv, 2013). Leukemia and lymphoma account for over 85% malignancy in the childhood of A-T patient with age below 20 (Taylor et al., 1996). When the A-T patients get older, they might further develop solid tumors, such as breast cancer and ovarian cancer (Olsen et al., 2001). Associations between ATM variants and different kinds of CNS tumors, including glioma, medulloblastoma and meningioma, were also revealed in various tumor research (Estiar & Mehdipour, 2018).

6.2.3 Diagnosis

6.2.3.1 Clinical Diagnosis

Clinical diagnosis is made before any further diagnosis. Generally, A-T is suspected in children with progressive ataxia before their age of five (Nissenkorn & Ben-Zeev, 2015). Another hallmark symptom, telangiectasia normally appears after the age of six. However, A-T is a rare genetic disease with specific gene mutation, matching the symptoms of A-T should not be the only judgement for diagnosis. Before molecular diagnosis, laboratory tests including serum

alpha fetoprotein level measurement and X-ray induced chromosomal breakage detection in cultured cell were usually made for detecting A-T disease. However, clinical diagnosis is not accurate enough to judge if the patient gets A-T or other A-T like disorders, especially in some milder adult-onset cases, symptoms could be developed at a later age.

6.2.3.2 Molecular diagnosis

Molecular diagnosis provides a variety of specific laboratory tests that would be required before confirming a real A-T patient. Otherwise, the diagnosis might be confused with other childhood onset ataxia, such as ataxia-telangiectasia-like disorder 2 (ATLD2), ataxia oculomotor apraxia type 1 (AOA1), and ataxia oculomotor apraxia type 2 (AOA2) (Amirifar et al., 2019). There are two molecular tests for confirming A-T in the laboratory. The first method is confirming the presence of ATM protein or ATM kinase function from skin biopsies or lymphocytes cell culture originated from suspected patient (Rothblum-Oviatt et al., 2016). The second method is protein analysis. Tissue or cell lysate collected from biopsy or lymphocyte would be electrophoresed in SDS-PAGE gel for western blotting. Significant drop in ATM expression might reveal a defective function in ATM production (Chun et al., 2003).

6.2.4 Genetic mutations in A-T

A-T is a rare familial disease that is inherited through an autosomal recessive gene localized at chromosome 11 (11q22-23). Although A-T is caused by mutation on ATM gene on chromosome 11q23.1, the ATM mutation sites are not consistent in every A-T patient. In fact, human ATM is a large gene that comprise 66 exons with over 9000 nucleotides (Choi et al., 2016; Kinneret Savitsky et al., 1995). The long length of ATM gene could attribute to the heterogeneity of A-T clinical symptom since different sites of mutation could result in different

effect on ATM protein expression. Over 600 sites of mutation had been already identified as the disease-causing mutation of A-T, with the majority of them are missense or nonsense variations (Stenson et al., 2003). Indeed, different clinical reports had also reflected the mutation-site-dependent severity of A-T manifestation. According to clinical data collected from A-T patients with different ethnicity (Supplementary Table 1), variation in ATM mutation sites alters the ATM protein formation (Amirifar et al., 2021a; Huang et al., 2013; Podralska et al., 2014). Based on these observations, almost all reported A-T patients presented ataxia manifestation (98.7%), most of them also presented another hallmark of A-T, telangiectasia (77.9%), but only around half of them showed immunodeficiency. The clinical variation among patients could be attributed to the difference in ATM mutation site. Some of the ATM mutations involve frameshifts, which result in a truncation of ATM protein that would largely suppress the protein activity.

One of the MRI studies even showed manifestations in the extracerebellar region of brain (Kieslich et al., 2010). Cerebellar degeneration is one of well-known A-T symptoms so most of the A-T patient showed atrophy in cerebellum or hyperintense and lesion on cerebellum. According to the Table 1 , 10 out of 11 patients showed moderate to severe level of hyperintensities in cerebellar vermis (Table 1). However, only three patients were diagnosed with cerebral white matter hyperintensities and white matter lesions. Differences in mutation sites might be a key to the heterogeneity of A-T pathology.

Patient ID	1	2	3	4	5	6	7	8	9	10	11
Gender	F	M	M	M	M	F	F	M	M	F	F
Age	26	20	11	10	9	25	26	20	20	18	8
ATM mutation	NM_000051.3(ATM): c.7788G>A	790delT/9, 920- 1G→T(Int9)	n.d.	5972insA/42 heterozygote	c.5932G>T Heterozygote	5441insT/38	5441insT/38	n.d.	n.d.	n.d.	n.d.
Protein mutation	p.Glu2596=	p.Tyr264fs	n.d.	p.E1991fs	p.E1978X	R2032K	R2032K	n.d.	n.d.	n.d.	
AT-score	30	27	26	21	12	23	23	27	21	28	19
Clinical symptoms	ASA, ABA	ASA, ABA	ASA	ASA, ABA	None	ASA	ASA	EPM	EPM	ASA	None
MRI lesions	WM, MA, PA, CHA, CVA	WM, MA, PA, CHA, CVA	WM, CHA, MA, CVA	CHA, CVA	None	MA, CHA, CVA	CHA, CVA	BG, MA,PA, CHA, CVA	MA, PA, CHA, CVA	CHA,CVA, PA, MA	MA, PA, CHA, CVA
Astasia	+	+	+	+	-	+	+	+	+	+	-
Abasia	+	+	-	+	-	-	-	+	+	-	-
Extrapyramidal symptom	-	-	-	-	-	-	-	Dystonia	Choreoathetosis	-	-
Cerebellar vermis	+++	+++	++	++	-	++	++	+++	++	+++	+++
Cerebellar hemispheres	+++	++	+	+	-	+	+	+++	+	++	++
Pontine atrophy	+	+	-	-	-	-	-	+	+	++	+
Spinal atrophy	+	++	-	-	-	+	-	++	+	++	+
Basal ganglia	-	-	-	-	-	-	-	+	-	-	-
Cerebral white matter	+++	++	+	-	-	-	-	-	-	-	-
α-fetoprotein (ng/ml)	360	960	222	129	283	153	139	n.d.	389	190	156

Table 1. Synopsis of the A-T patients with of semi-quantitatively assessed neurodegeneration and prevalence of extrapyramidal and cerebellar disease symptoms.

Note. This table was modified and adapted from Kieslich K. in 2010, describing the extracerebellar MRI abnormalities in A-T patients. From “Extracerebellar MRI-lesions in ataxia telangiectasia go along with deficiency of the GH/IGF-1 axis, markedly reduced body weight, high ataxia scores and advanced age.”, by Kieslich K., 2010 Cerebellum, 9(2), p. 190-197.

Abbreviation: IGF1: insulin-like growth factor-1, IGF-BP3: insulin-like growth factor binding protein 3; BMI body mass index; EPM: extra-pyramidal symptoms, AST: astasia, ABA: abasia; BG: basal ganglia, WM: white matter, MA: myelon atrophy, PA: pontine atrophy, CHA: cerebellar hemispheric atrophy, CVA: cerebellar vermis atrophy; -: indiscernible, +: obvious, ++: moderate, +++: severe

6.2.5 CNS pathology of A-T

6.2.5.1 Cerebellar pathology

Cerebellar atrophy is the most significant phenotype in classic A-T, which could be identified with non-invasive neuroimaging method. MRI studies supported the pathological findings in the cerebellar degeneration with significant atrophy (Sahama et al., 2014). Other neurodegenerative symptoms, such as decreased mobility, weakened muscle, sensory deficit and absent of peripheral reflexes (Taylor et al., 2015), show a chronic decline in neural functions. Postmortem studies on the cerebellum of A-T patient reveal a great loss of Purkinje and granule neurons (Meshulam et al., 2012). Progressive loss of Purkinje cells and granular cells accounts for the degeneration of cerebellum. The presence of empty basket cells in cerebellar cortex also provide evidence of Purkinje cell loss. Moreover, the dendritic processes are reduced in the remaining Purkinje cells, and the mislocation of Purkinje cells might point towards the abnormal migration during the neurodevelopmental progress (Nissenkorn & Ben-Zeev, 2015). It is hypothesized that cerebellar pathology begins with the vulnerability of Purkinje cell and granule neurons. The attenuated DNA repairing functions of ATM could be the critical cause for substantial death of Purkinje cell and granule neurons (Rothblum-Oviatt et al., 2016).

Cerebellar dysfunction is the hallmark of A-T disease, some patients were identified with cerebrocerebellar pathology (Hoche et al., 2014). Hoche and her collaborators also showed the significant cognitive impairment in this group of A-T patient with cerebrocerebellar symptom. Therefore, the sign of neurological degeneration is not only limited in the cerebellum, but also in the cerebrum. As a result, muscle coordination would be significantly disrupted, and the A-T patient eventually develops motor function deficits. Presence of lewy bodies, plaques and neurofibrillary tangles were reported in some older A-T patients (Verhagen et al., 2012). However, the effect of cognitive impairment is still debatable in A-T. Some clinical data

showed normal cognitive function of infants with A-T (Hoche et al., 2012), but other clinical data showed a delayed judgement duration in children and adolescents with A-T (Mostofsky et al., 2000), which provided an insight of age dependent effect on cognition.

In general, the brainstem is not greatly affected in A-T, but some pathological studies showed varying neuronal loss in cranial nerves and axonal atrophy in the medulla oblongata tegmentum (Sahama et al., 2014). Interestingly, degeneration of anterior horn cell in spinal cord was revealed in A-T patients with milder phenotype that could survive into their thirties (Verhagen et al., 2012). It revealed the heterogeneity of A-T disease and potential chronological effect of atypical A-T. Moreover, WM degeneration in corticospinal tract was revealed in a diffusion MRI study (Sahama et al., 2015).

6.2.5.2 Cerebrovascular pathology

Telangiectasia, defined as the visibility of small linear red blood vessel, is the other highlighted phenotype of the A-T patients, however, not much research focused on the pathogenesis of the vessel dilation in telangiectasia. Ocular sclerae is the primary telangiectasia site for classic A-T patient, which mainly appears before 10 years old (Amirifar et al., 2019). Being exposed to ionizing radiation, such as UV light, human endothelial cell could be induced apoptosis as well as senescence (Wang et al., 2016). In a pericyte-endothelial *in vitro* model, hydrogen-peroxide-induced senescence on primary cultured mouse endothelial cell caused impairment of the barrier integrity as well as reduction of the tight junction protein expression in the endothelial cell (Yamazaki et al., 2016). This report provided an insight that vasculature disruption in A-T patient might be resulted from senescent endothelial cell, while oxidative stress induced endothelial cell senescence was associated with ATM-dependent Akt/p53/p21 signaling pathway (Zhan et al., 2010). Therefore, the vascular pathology of A-T might be highly related to vascular senescence.

Apart from ocular telangiectasia, intracerebral telangiectasia was identified in 60% adult cerebrum with A-T after CNS irradiation in a brain MRI study (Lin et al., 2014). Indeed, patchy degeneration of neuron or glial cell is usually found around the ischemic cerebral blood vessels (Kamiya et al., 2001). As massive necrosis is expected to occur in the region with ischemia, cerebrovascular abnormalities could contribute to neuropathological effect in A-T. Vein dilation and arterioles hyalinization were discovered many times in the meninges of A-T patients (Kamiya et al., 2001). Abnormalities in the brain parenchyma showed an impact of ATM deficiency on brain vasculature malformation and permeabilization.

Atm-deficient mice model also showed a decreased tight junction protein occludin expression, leading to increase in vascular permeability (Raz-Prag et al., 2011). Otherwise, promoting ATM activity in mice model could help maintaining insulin resistance and decelerates atherosclerosis, vice versa (Schneider et al., 2006). This experimental result showed a potential role of ATM on preventing vascular senescence.

White matter hyperintensities (WMH) were discovered in A-T patient with cerebral microbleeds under fluid-attenuated inversion recovery (FLAIR) sequences (Tiet et al., 2021). TRIM47, a genome-wide association studies identified top genetic risk factor of WMH, demonstrated its regulatory function on autophagy in brain endothelial cell (Yeung et al., 2024). The results revealed the potential correlation between WMH and telangiectasia pathology of A-T.

6.2.5.3 Cerebrum white matter pathology

Importance of A-T myelin pathology was emerging in postmortem immunohistological studies and MRI studies. Lesions of WM were also observed in cerebrum, which is consistent with the occurrence pattern of scattered calcified region and MRI-identified space- occupying area (Kamiya et al., 2001). Multiple hypointense foci were discovered in WM from A-T patient

(Ciemins & Horowitz, 2000), which might represent abnormal myelin structure. Moreover, significant WM degeneration was revealed in MRI-DTI study in terms of corticomotor, corticospinal and somatosensory WM tracts (Sahama et al., 2015). Based on a recent single nucleus transcriptomic analysis on A-T patients, OL was significantly reduced without significant neuronal loss in A-T prefrontal cortex, which was highly correlated with the clinical finding of cortical WM degeneration in A-T patients (Lai et al., 2024).

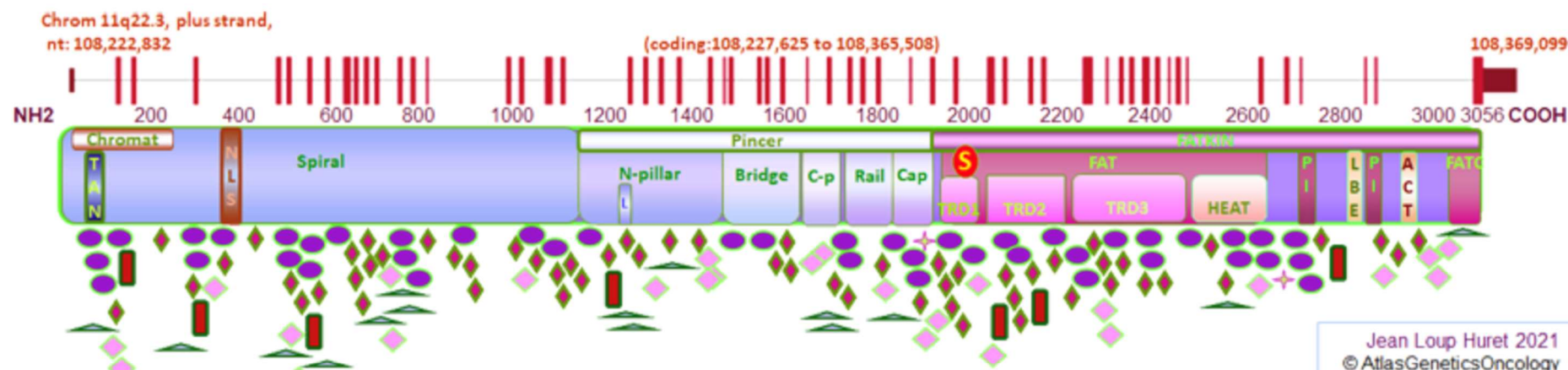
Interestingly, myelin abnormalities might be correlated with cerebrovascular pathology. Severe loss of myelin and oligodendrocyte was found at the site of vascular abnormalities in A-T patient (Habek et al., 2008). Indeed, one of the case studies showed the prior occurrence of WMH before any neuroimaging symptom of A-T in MRI (Chung et al., 1994). This provided an insight that myelin pathology might prior to the occurrence of other A-T hallmark symptoms such as cerebellar atrophy or telangiectasia.

6.3 Ataxia Telangiectasia Mutated (ATM)

6.3.1 ATM gene and protein structure

ATM gene is located on chromosome 11q23, which spans around 150kb with 66 exons (Mavrou et al., 2008). The transcription of ATM gene is highly conserved in vertebrates with 12 kb coding length (Meyn, 1999). ATM protein is a high molecular weight protein kinase (350 kDa) with 3056 residues, which belongs to the phosphoinositidyl 3-kinase-related protein kinase (PIKK) family. Similar to other DNA-dependent protein kinases (DNA-PK) and mammalian target of rapamycin (mTOR) in the PIKK family, ATM is also a serine/ threonine protein kinase (Kastan & Lim, 2000). ATM protein could be separated into two parts, a N-solenoid and a FATKIN. N-solenoid is composed of Spiral (a.a. 1-1160), an α -helical structure, and a Pincer (a.a. 1161-1890) (Figure 3) (Huret, 2021). On the other side, FATKIN is divided

into five regions, tetratricopeptide repeat domains TRD1 (a.a. 1903-2025), TRD2 (a.a. 2032 - 2190), and TRD3 (a.a. 2195-2476); HRD (HEAT-repeats domain) (a.a. 2484-2612); and a kinase domain (a.a. 2618-3056) (Figure 3). According to data in Table 1, one of the A-T patients with severe cerebral white matter manifestation showed a missense mutation on the site of HEAT repeats in the FAT domain. The FAT domain was reported to mediate the protein-protein or protein-DNA interaction as a scaffold region (Lau et al., 2016).



- **Protein kinase C phosphorylation sites:** 21,39,127,151,274,305,373,475,491,554,571,616,775,791,808,917,1037,1104,1179,1487,1558,1769,1770,1857,1880,1905,1990,2058,2134,2146,2194,2242,2264,2329,2434,2438,2513,2608,2611,2640,2685,2745,2754,2761
- ✦ **cAMP/cGMP dependent protein kinase (PKA) phosphorylation site:** 1923,2751
- ◆ **Casein kinase II phosphorylation site:** 127,200,274,373,403,470,515,571,629,644,646,655,710,767,837,865,891,934,1004,1048,1100,1118,1143,1179,1212,1242,1263,1350,1403,1589,1601,1609,1721,1748,1819,1891,1966,1988,1993,2000,2011,2123,2134,2142,2184,2218,2242,2333,2348,2359,2375,2408,2476,2573,2592,2812,2921,2947,299
- **Tyrosine kinase phosphorylation site:** KcqEllnY (116-123), KtqEkgY (296-303), RhgErtpY (447-454), KvsEtfY (1196-1203), KevEgtsY (2117-2124), KrsIEsvY (2160-2167), KksfEekY (2810-2817)
- **N-glycosylation sites:** 81,272,567,591,704,765,789,1230,1240,1356,1660,1719,1855,2994,3044
- ◆ **N-myristoylation sites:** 134,138,301,506,558,724,774,1016,1302,1456,1458,1672,1817,1925,1980,2020,2063,2342,2369,2678,2917,3019,3023,3029

Spiral (aa 1-1160)

TAN (aa 15-27)

Chromatin-association domain (aa 5-224)

NLS: Nuclear localization signal KRKK (aa 385-388)

Pincer (aa 1161-1890)

N-pillar (aa 1161-1430) **Bridge** (aa 1431-1600) **C-pillar** (aa 1601-1680)

Railing (aa 1681-1800) **Cap** (aa 1801-1890)

L: Leucine zipper (aa 1217-1238)

FATKIN (aa 1891-3056).

Tetratricopeptide repeat domains **TRD1** (aa 1903-2025), **TRD2** (aa 2032-2190), and **TRD3** (aa 2195-2476); **HEAT** (aa 2484-2612); **kinase domain** (aa 2618-3056)

FAT domain (aa 1903-2612)

PI: Phosphatidylinositol 3- and 4-kinases signature 1 (aa 2716-2730)

PI: Phosphatidylinositol 3- and 4-kinases signature 2 (aa 2855-2875)

FATC domain (aa 3024-3056)

LBE (aa 2791-2829) **Activation Loop** (aa 2888-2910)

S Serine 1981

ATM (11q22.3) – Gene and Protein

6.3.2 Splicing event on ATM gene

6.3.2.1 Alternative splicing

According to recent human genome database (GRCh38.p14) retrieved from Ensembl (release 113), there are 62 different ATM transcripts with unique alternative splicing (Harrison et al., 2024). Only 20 ATM transcripts are protein coding, while four of them were regarded as known transcripts in RefSeq database (O'Leary et al., 2016). Each protein coding transcript translates into protein isoforms with unique Ensembl protein identifier, while the size of ATM isoforms ranges from 112 to 3056 amino acids (Harrison et al., 2024). Among all ATM transcripts, ENST00000675843.1 (NM_000051.4) is the Matched Annotation from NCBI and EMBL-EBI project (MANE) selected canonical transcript. The encoded protein (ENSP00000501606.1) was mapped as Q13315 in Uniprot (2024).

Regarding the mouse genome database (GRCm39) retrieved from Ensembl (release 113), there are only 6 unique Atm transcripts (Harrison et al., 2024). Among the four protein coding transcripts, ENSMUST00000232179.2 is the canonical transcript, which was mapped to Uniprot (Q62388) with 3066 amino acids.

6.3.2.2 Pathogenic splicing mutations

The majority of the pathogenic mutations of A-T patients on ATM gene results in protein truncation (Gilad et al., 1996). Generally, A-T patients with frameshift mutation might result in truncated ATM protein expression. Indeed, missense variants in FAT region could also lead to a decline of ATM protein kinase function, which might result in different kinds of manifestation. However, the effect of pathogenic splicing effect was often neglected.

In the primary clinical research on 62 ATM mutations, an abnormally high portion of mutations (30/62) was found affecting ATM transcript splicing (Teraoka et al., 1999). Under another

large-scale survey of genetic mutation report on A-T patients conducted in 1997, half of the ATM coding exon (32/64) was observed to undergo exon skipping resulting by mRNA splicing (Concannon & Gatti, 1997). Within the 1013 pathogenic or likely pathogenic mutations unique to A-T disease in ClinVar database, there are also 144 intronic mutations (14.2%) (Landrum et al., 2014), which is leading to pathogenic mRNA splicing event. Indeed, effect of splicing event on long ATM gene was often neglected, including different ATM isoform caused by alternative splicing and pathogenic splicing event resulted by intronic variant.

6.3.3 ATM nucleus function

6.3.3.1 ATM protein kinase activity

ATM functions as protein kinase that can phosphorylate more than 700 proteins and 900 phosphorylation sites together with ATR (ATM and Rad3-related), another PIKK-like family member (Matsuoka et al., 2007). ATM protein level elevates in response to DSB through self-activation by autophosphorylation at Ser1981 residue (Bakkenist & Kastan, 2003). MRN complex (MRE11, RAD50, and NBS1) recruits the activated ATM, which phosphorylates substrates involved DSB repair subsequently (Nissenkorn & Ben-Zeev, 2015), pATM will then activate key checkpoint kinase Chk2 and the tumor suppressor p53. The kinase activity of ATM is important for DSB repair in cell nucleus as it stabilizes genome by coordinating the DNA-damage response and regulating cell-cycle checkpoints. For instance, downstream phosphorylation on p53 at Ser15 coordinates the cell cycle at G1-S checkpoint, phosphorylation on Chk2 at Thr68 controls G2/M checkpoint (Ambrose & Gatti, 2013). By controlling the checkpoints of cell cycle, ATM could halt proliferation in cells with irreparable genomic injury and prevent defective cell undergoing unregulated division and turn into

malignant cells. Recently, ATM was also reported crucial for unfolding 3D chromatin structure at DSB site for increasing accessibility of DNA repair machinery (Goodarzi et al., 2010).

6.3.3.2 ATM-mediated DSB repair mechanism

Among the 650 upregulated phosphorylation sites in a phosphoproteomics analysis on ATM immunoprecipitation, there are 170 (26%) phosphorylation sites on serine/threonine residues that are followed by a glutamine (S/T-Q motif) (Munk et al., 2017). Regions with closely packed S/T-Q motif were then termed S/T-Q cluster domains (SCDs), which already considered the structural hallmark of DNA-damage-response proteins in response to ATM/ATR phosphorylation (Traven & Heierhorst, 2005).

ATM is a major component of an indispensable DNA repair mechanism, the non-homologous end joining (NHEJ). NHEJ is initiated by recognition and binding of Ku70/Ku80 heterodimer onto the DSB site (Mari et al., 2006). It serves as a scaffold to recruit other NHEJ factors, such as X-ray cross complementing protein 4 (XRCC4), DNA Ligase IV, XRCC4-like factor (XLF), Aprataxin-and-PNK-like factor (APLF), and ATM (Figure 4) (Davis & Chen, 2013). ATM contains a leucine zipper which provides innate affinity toward DNA strand. Moreover, a recent structural study showed that the C-terminal domain of DNA-PKs forms a crown shape with N-terminal domain and result in a pincer-like structure that promotes its binding ability to DNA (Sibanda et al., 2010).

XRCC4 serves as the second scaffold for NHEJ factors recruitment, while most of the DNA end processing enzymes are recruited by this Ku-XRCC4 complex (Davis & Chen, 2013), including Artemis, PNKP, APLF, Polymerases μ and λ , Werner (WRN), and aprataxin. PNKP work as a kinase to add a phosphate to DSB 5' hydroxyl and serve as a phosphatase to remove 3' phosphate group (Bernstein et al., 2005). Apratxin could remove adenylate group attached to 5' phosphate end of DSB (Ahel et al., 2006). Artemis is responsible for cleaving the 5' end

overshoot and leave a blunt end for ligation, but it needs to be first activated by ATM or DNA-PK activation (Povirk et al., 2007). WRN is stimulated as 3' to 5' exonuclease via its interaction with Ku heterodimer and XRCC4 (Perry et al., 2006). APLF is also suggested to serve as a 3' to 5' exonuclease. With all the contribution of DNA end processing enzyme, 3' and 5' terminal would become blunt end before the ligation step of NHEJ.

Eventually, DSB ligation is mediated by DNA ligase IV. XRCC4 stabilises and stimulates the activity of DNA ligase IV by promoting adenylation on DNA ligase IV (Davis & Chen, 2013). NHEJ is important for its role on maintaining the genome stability, and it prevents the DNA damaged cell turning into cancerous cell .

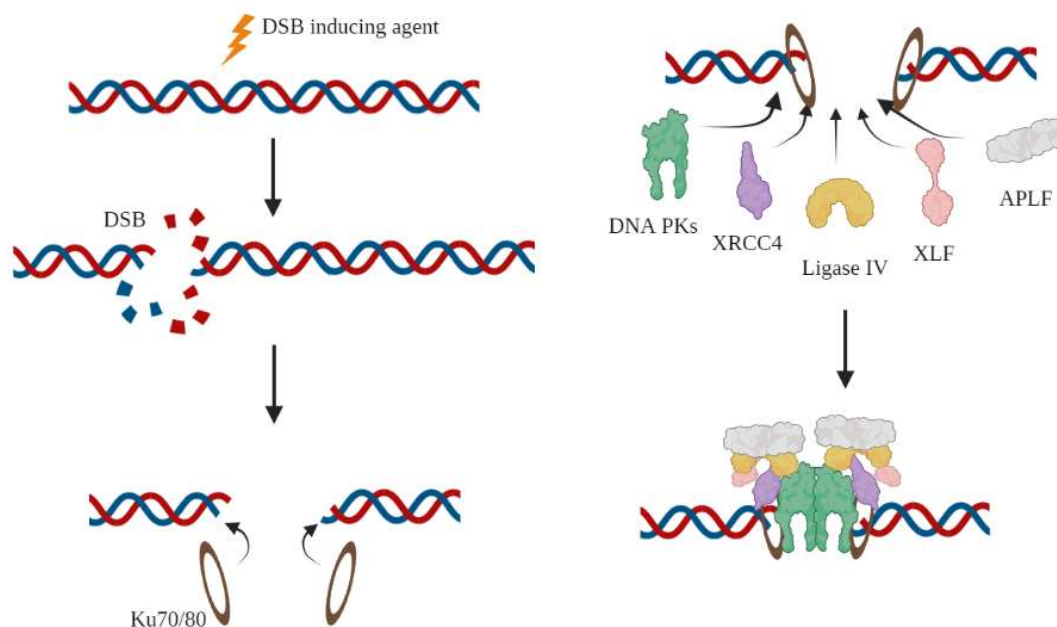


Figure 4. Ku 70/80 heterodimer recruits other core NHEJ factors, DNA-PKs, XRCC4, XLF, DNA Ligase IV, and APLF.

Created with BioRender.com.

6.3.4 ATM cytoplasmic function

As discussed above, the ATM protein is well known for its nuclear function in cell cycle and DSB repair, but there is emerging evidence proving that ATM has extra-nucleus function. According to the phosphoproteomics analysis on ATM immunoprecipitation upon mitomycin

C treatment, over 40% ATM substrates were found in nucleus while around 20% substrates were found in the cytoplasm (Munk et al., 2017). A significant portion of ATM was found in peroxisomes, which was potentially associated with oxidative metabolism (Watters et al., 1999). It was shown that reactive oxygen species (ROS) could activate cytoplasmic ATM (Alexander & Walker, 2010). Independent to p53 signaling pathway, activated ATM triggered the LKB1-AMPK signaling cascade and suppressed mTORC1 through TSC2 activation (Alexander & Walker, 2010). Cytoplasmic ATM was also found associated with vesicle trafficking in neuronal pre-synapse (Vail et al., 2016). Recent studies revealed that ATM plays an important role on regulating autophagy-lysosomal pathway (Barlow et al., 2000; Cheng et al., 2021; Cheng et al., 2018). Further investigation discovered the association between regulation of ATM and SLC2A4 translocation towards plasma membrane, while ATM deficiency would lead to abnormal lysosome upsurge (Cheng et al., 2021). Moreover, ATM was associated with ceramide synthesis in endoplasmic reticulum (Liao et al., 1999), metabolic pathways (Marzano et al., 2012), insulin signaling pathway (Yang et al., 2011) and a role as a homeostatic protein kinase (Shiloh & Ziv, 2013). As the full cytoplasmic functions of ATM remains obscure, it is possible that some of the A-T related phenotypes could be caused by the loss of ATM cytoplasmic function.

6.4 Sequencing diagnosis of rare disease

Recently, over 10000 rare genetic diseases are affecting millions of people on earth (Boycott et al., 2019). However, the difficulty of diagnosing rare genetic diseases almost always results in a diagnostic odyssey, which explains the prolonged journey for rare genetic disease patients towards accurate diagnosis. During the long journey, around 38 per cent of patients had to consult more than six clinicians, and 30 per cent of them needed to wait for more than five years towards diagnosis, but half of them could be misdiagnosed (Baynam et al., 2016). In Hong Kong, approximately 1.5 per cent of population suffer from rare genetic disease (Chiu et al., 2018).

In order to develop a better practice on genetic diagnosis, this study aimed to review the current diagnostic practice on genetic diseases in Hong Kong and investigate the current research advancement on sequencing technique for clinical application.

6.4.1 Current background on sequencing diagnosis

6.4.1.1 Current application on rare disease diagnosis in Hong Kong

According to Hong Kong Hospital Authority (HA) (2019), Genetic and Genome (G/G) service was provided in 11 hospitals, but their workflow wasn't clearly streamlined for diagnosis. If the patients were suspected to suffer from rare genetic disease, they have to apply for professional clinical assessment, which was mainly provided by Clinical Genetic Service in Department of Health (DH CGS). Specific diagnostic modalities would be applied to suspected patients for narrowing the scope of disease type. Then, the genetic test serves as the confirmational approach for final diagnosis. However, DH CGS service was transferred to the HA's Clinical Genetic Service Unit (CGSU) at Hong Kong Children's Hospital (HKCH) from July 1, 2023 (GovHK, 2022). Moreover, Hong Kong Genome Institute (HKGI) was established

in 2021 by Hong Kong Government (HKG) for launching the Hong Kong Genome Project (HKGP) (GovHK, 2024). HKGP is the first large-scale genome sequencing project initiated by HKG, which targets the patients and their family members with undiagnosed rare diseases and hereditary cancer (Legislative Council Secretariat, 2024). In a recent implementation of HKGP in laboratories of HKGI and Centre of PanorOmics Sciences (CPOS) in the University of Hong Kong, 240 genomes were sequenced with Illumina NovaSeq 6000 sequencer using S4 flow cells with 300 cycles, achieving 41x mean coverage and 162 Gb mean data yield per sample (Chu et al., 2023). To achieve the final target of HKGP, HKGI collaborated with HA, The Chinese University of Hong Kong and The University of Hong Kong to establish partnering centers at HKCH, Prince of Wales Hospital (PWH) and Queen Mary Hospital (QMH) respectively (Hospital Authority, 2024). Indeed, the participation of HKCH, PWH and QMH in HKGP reflects their capacity on routine diagnosis of rare genetic diseases. Apart from the above three hospitals, the G/G service provided in other hospitals might not sufficient to support daily clinical implementation (Steering Committee of Genomic Medicine, 2020).

According to previous guideline provided by DH CGS (2022), four modalities are provided in the cytogenetic services and another four modalities are provided in molecular services. Blood sample is either delivered with heparin in room temperature or with EDTA at 4°C. Type of genetic test is determined by the specific mutation pattern of the rare genetic disease. In cytogenetic services, large scale mutations on chromosome level are detected. For instance, karyotyping detects chromosomal copy variation, DEB fragility assay detects chromosome breakage, chromosome microarray and fluorescence *in situ* hybridization detect chromosomal micro-deletion or duplication. In contrast, molecular service distinguishes mutations on nucleotide level. For instance, multiplex ligation dependent probe amplification detects frameshift mutations, PCR fragment analysis detects trinucleotide repeat, Sanger sequencing

and next-generation sequencing detect single nucleotide polymorphism (SNP) mutation. 132 out of 172 genetic diseases listed on laboratory user guide of DH CGS (2022) is caused by SNP mutation, which can only be detected by sequencing. Recent research revealed that over 60 per cent of the rare genetic patients presented with a monogenic condition, which could only be confirmed with sequencing (Vaisitti et al., 2021). Therefore, sequencing plays a crucial role in the current diagnostic procedure.

6.4.1.2 Limitation on current clinical application

Difficulties in the diagnosis of rare genetic disease led to the averaged six-year diagnostic odysseys. Over 35 per cent of patients born with genetic disease cannot survive until their first birthday (Vaisitti et al., 2021). They barely received an accurate diagnosis throughout their lifespan, which might easily result in incorrect healthcare arrangement that worsen patient condition and waste unnecessary expenditure. According to DH CGS (Mok, 2022), four to six month turnaround time was expected for suspected patient to receive molecular test result in Hong Kong. The long turnaround time of genetic testing could be attributed to the lack of genetic specialties and non-systematic workflow for diagnosis. Long waiting period for only one test contributes to the long journey of diagnostic odyssey. Between tests, patients need to consult clinical geneticists for result interpretation and advice towards diagnosis. However, HA (2019) revealed their shortage of clinical geneticist in their report. It reflected the fact that patients of rare genetic disease also face difficulties in finding professional clinical geneticists. Sequencing undiagnosed rare genetic disease costs around USD\$7300 in non-urgent situations, and around USD\$17000 for critically ill infants. High cost of genomic sequencing is also forbidding patients towards diagnosis as many families might not have enough money. Moreover, the development of sequencing diagnosis in Hong Kong was also limited by the

non-effective existing funding mechanism in HA for implementing advanced genetic tests (Steering Committee of Genomic Medicine, 2020).

6.4.2 Genetic sequencing technique

Genetic sequencing is the direct and detailed method for A-T diagnosis. Moreover, direct genetic sequencing is the standard for confirming ATM gene variant in A-T patients. The whole exome sequencing is currently the clinical standard practice, which is used to screen the gene that is involved in the pathogenesis of some rare genetic diseases with a customized panel (Graziola et al., 2019). The site of mutation on ATM gene is a determinant factor for the level of A-T severity. Heterozygous mutations on different sites and splicing site mutation could do much less harm to the patient since they usually still have a detectable level of ATM protein. In contrast, homozygotes and truncating mutation usually cause the severe classic A-T phenotype with no residual ATM protein (Verhagen et al., 2012). The advantage of diagnosis by sequencing is the accuracy on identifying the late-onset A-T patient with a milder phenotype.

6.4.2.1 First generation sequencing

The major breakthrough of DNA sequencing came at 1977 when Frederick Sanger invented the “Sanger sequencing” (Heather & Chain, 2016). It terminates the DNA extension with a analog of deoxyribonucleotide (dNTP), called dideoxynucleotides (ddNTPs), which lacks the 3' hydroxyl group required for DNA extension. It is accurate within the range 300 to 1000 base pairs, which was a known accuracy standard for DNA sequencing.

6.4.2.2 Next generation sequencing (NGS)

NGS technology revolutionized genetic research for its innovative method of sequencing by synthesis and its faster turn around time compared to Sanger sequencing. Illumina-NGS is one of the second-generation sequencing methods that is based on technique known as “bridge amplification” (Slatko et al., 2018). Cleaved DNA strands (around 500bp) was ligated with proper adapter on each ends, followed by synthesizing short-read nucleic acids for massive parallel sequencing. It recently becomes the gold standard of genomic sequencing study due to its low error rate.

6.4.2.3 Third generation sequencing

The MinION sequencer is developed by Oxford Nanopore Technologies, one of the third-generation sequencing platform. The flow cells used in MinION sequencer are arrayed with nanopore sensor on-chip (2048 nanopore flowcell pores per each flow cell), which emits a specific electric signals on reading each specific nucleotide (A, T, C, G) throughout the sequencing . Firstly, double strand DNA was bound to a processive enzyme, for instance phi29 polymerase. Single DNA strand enters the nanopore when the complex encounters the nanopore, while the translocation rate through the pore is regulated by DNA polymerase synthesis and translocation. Current disruption occurred every time after a nucleotide passing through, which provides a characteristic electronic signal for each nucleotide. Theoretically, the nanopore is available again for sequencing a different DNA molecule everytime after the the sequenced DNA left (Slatko et al., 2018). The extended long reads at the kilobase scale favors mutational detection on specific site without whole genome sequencing.

6.4.3 Targeted sequencing development

There are three kinds of sequencing approaches with different sizes of scope for genomic investigation, including whole genome sequencing, whole exome sequencing and targeted sequencing.

Whole-genome sequencing covers the whole genome including all coding and non-coding regions, which maps the whole genomic of patient with around 3 billion base pair regional size.

Whole-exome sequencing only covers the coding regions in the human genome, which maps only 1-3% of human genome with around 60 million base pair regional size. Targeted sequencing only covers the target regions of interest. Variation of sequencing region depends on the panel size designed for each sequencing experiment, ranging from 1 hundred thousand to 10 million base pair.

Indeed, the cost per experiment and data size for analysis of targeted sequencing should make it the most optimal choice for lowering the difficulty of sequencing and applying to daily clinical diagnostic usage. Whole-genome sequencing cost USD\$1000-3000 to yield 60-350Gb data, whole exome sequencing cost \$500-2000 to yield 5-20Gb data, and targeted sequencing cost \$300 -1000 to yield around 100Mb-5Gb data (Incerti et al., 2022). Among three approaches, targeted sequencing cost least to generate minimal data size targeting the pathogenic mutation regions, so that hospital can perform sequencing easily without very high standard equipment.

6.4.4 Targeted sequencing with current sequencing technique

There are four major methods for targeting regions of interest for sequencing, amplicon-based, probe capturing, Cas9-targeted and adaptive sampling (AS) sequencing methods. The first

three methods were proved to be executable with next-generation sequencer, Illumina, and third-generation sequencer, Nanopore.

6.4.4.1 Amplicon-based targeted sequencing

Amplicon-based targeted sequencing is dependent on the PCR amplification (Zucca et al., 2016). Firstly, raw DNA sample was amplified with designed primer probes targeting regions of interest. Then, unwanted product of PCR is removed by size selection with beads or gel. After ligation to suitable barcode and adapter, libraries are ready for multiplexing and sequencing. The following paragraphs list two applicational research of amplicon-based targeted sequencing on Illumina and Nanopore correspondingly.

The first research aimed to develop four gene panels, including Parkinson disease, Intracerebral Hemorrhage Diseases (COL4) and Familial Hemiplegic Migraine (CACNA1A & ATP1A2) with amplicon-based Illumina targeted sequencing method (Zucca et al., 2016). They designed four gene panels with an average 250 base pair amplicon size using DesignStudio. Illumina Truseq Custom Amplicon sequencing assay kit was chosen in this study for downstream library preparation and the multiplexed libraries were sequenced with MiSeq 300 V2 flow cell. Result showed coverage of four panels ranging from 90.8% to 96.6%. Interestingly, the self-developed bioinformatic pipeline could averagely identify a greater number of variants in each panel. Averaged number of variants detected with Illumina pipeline for four panels ranged from 16.1 to 69.4, while the number detected with self-developed bioinformatic pipeline ranged from 30.3 to 89.2.

The second research aimed to develop genetic loci specific sequencing workflow with MinION for mutation identification in high-throughput way (Whitford et al., 2022). Ten amplicons' libraries were pooled into one R9.4 flow cell for sequencing on MinION. FastQ files basecalled by guppy were then demultiplexed and aligned with minimap2. Over 90% of total reads passed

quality qscore of seven and 94.32% of them were successfully aligned to the reference genome by minimap2. 29 out 30 variants were successfully called with nanopolish, while the unsuccessful variant calling was attributed by poor homopolymer detection with MinION.

6.4.4.2 Probe capturing targeted sequencing

Targeted capturing sequencing is dependent on probe hybridization and capturing (Lazaro-Guevara et al., 2021). Firstly, DNA samples were fragmented to desirable size and followed by adapter and barcode ligation. Then, customized biotinylated capturing nucleotide probe was incubated with the library overnight. Fragments with attachment of biotinylated bait were then retained by streptavidin-coated beads capturing. After that, the capturing libraries are ready for multiplexing and sequencing. The following paragraphs list two applicational research of probe capturing targeted sequencing on Illumina and Nanopore correspondingly.

The first research aimed to delineate variants in kidney disease-related genes between patients with diabetic kidney disease (DKD) and non-diabetic disease (NDKD) (Lazaro-Guevara et al., 2021). Customized gene panel composed of 345 kidney disease-related genes were designed with 972,494 base pair capturing size. Libraries were pooled before hybridization to targeted panel. Then, the target-captured libraries were sequenced by HiSeq2500. The base-called FastQ file were aligned to hg19 before variant calling with Sentieon DNaseq pipeline. Average read depth was 309X among all samples while 98.1% of bases were above 15X coverage. In total 1259 functional variants were detected and 63 of them were identified pathogenic or likely pathogenic. The result showed differential gene location distribution of diagnostic variants between DKD and NDKD, which might reflect different pathogenesis pathways among two types of patients.

The second research aimed to characterize the regulatory region of ABCB1 gene of acute myeloid leukemia (AML) and high-grade serous ovarian cancer (HGSC) with long read

sequencing (Williams et al., 2020). Customized probes were designed with Agilent SureDesign, including ABCB1 and upstream neighbouring gene with targeted size 500 kb. Hybridized libraries were pooled before sequencing with R9.4.1 flow cell in MinION sequencer. The base-called FastQ files were aligned to hg38 with LAST, an adaptive aligner previously trained with 10,000 reads. Over 95% reads were successfully mapped to hg38 genome with an average 1581 read length. Results from MinION sequencing also demonstrated better efficiency for identifying promotor translocation with lower coverage.

6.4.4.3 Cas9-targeted sequencing

Gene selection of Cas9-targeted sequencing depends on clustered regularly interspaced short palindromic repeats (CRISPR) /Cas9 cleavage on targeted regions (Thyme et al., 2016). Each target spot for sequencing requires a guided RNA (gRNA) with specificity to targeted genomic region. Specific cleavage on the complementary site of gRNA would be mediated by interaction between Cas9 protein and gRNA. Different modifications of CRISPR/Cas9-based targeted sequencing enable the detection of various types of mutation, including structural variants, short tandem repeats (STR), fusion genes and low frequency mutations (Malekshoar et al., 2023). CRISPR system-assisted dsDNA capture (CRISPR-Cap) was applied to enrich coding sequence regions of 20 genes from the human genome with Illumina (Lee et al., 2019). However, the result from CRISPR-Cap revealed a low on-target ratio (0.65%) and low average percentage of targeted region with 1x coverage (79.6%) under 100 ng initial genomic DNA input. Cas9-assisted targeting of chromosome segments (CATCH) aid sequencing of large gene fragments, which promotes detection of structural variants. The CATCH method employs Cas9 to specifically fragment genomic DNA in vitro, then separates the targeted genomic DNA with pulsed field gel electrophoresis (PFGE). CATCH was applied for Nanopore sequencing on a 200 kb target human genome region in a study, which included short tandem repeats of the

gene of interest (Gabrieli et al., 2018). The application of CATCH demonstrated an average 70x sequencing depth in targeted region and 237-fold enrichment. Moreover, fusion genes could be identified with FUSion Detection from Gene Enrichment (FUDGE) incorporate with specific CRISPR-Cas9 activity and Nanopore sequencing (Stangl et al., 2020). In another modification of Cas9 targeted sequencing with Nanopore (nCATS) on tumor samples, only 4.62% on-target reads were recorded with the multi-gRNA panel (Gilpatrick et al., 2020).

6.4.4.4 Adaptive sampling targeted sequencing

Adaptive sampling is unique for Nanopore sequencing since gene selection of AS is dependent on the computational power behind the real-time basecalling by using graphical processing unit (Payne et al., 2021). However, general NGS technology and even other third generation sequencing like PacBio were not capable of real time basecalling. The major advantage of target adaptive sampling long read sequence (TAS-LRS) is that no wet lab preparation is required for specific genomic regions of interest. In a recent study, 16Mb region was targeted with AS on cancer sample. The median depth of on-target region could achieve 21.9x which was ten times higher than the off-target region (Nakamura et al., 2024). However, this study only demonstrated high accuracy on genotyping SNPs. Attributed to low coverage, the efficiency of Indels calling was greatly reduced compared to the performance of high-coverage whole-genome short-read sequence (Nakamura et al., 2024). Indeed, AS with Nanopore sequencing successfully confirmed 55 clinically relevant copy number variants (CNVs) with a concordance rate of 100% in another clinical study (Greer et al., 2023). Moreover, this study extended for the detection of methylation status and phase with TAS-LRS, which enabled the clinical confirmation of a chromosome 15q duplication syndrome (Greer et al., 2023). Yet, there was not much applicational research with AS technique on Nanopore sequencing because of its novelty.

6.4.4.5 Comparison between different targeted sequencing methods

In general comparison, amplicon-based targeted sequencing is the easiest approach for targeted sequencing. Limited technique and resources are required as PCR amplification with DNA polymerase is the major step for this approach. However, targeting by amplification is considered more error-prone which leads to low fidelity result and its efficiency is reduced in sequencing long ROI and large scale of targets. Probe-capturing targeting method was developed for highly scalable sequencing with the capacity to examine up to millions of target sequences (Kuchinski et al., 2022). However, this approach requires tailor-made gene panel probe for capturing which is not cost-effectively customized. Generally, gene panels were the commercial products with preset target genes, which limited the flexibility of targeted sequencing application. The application of CRISPR-Cas9 cleavage to target sequencing enhanced the target specificity and fidelity of large ROI sequencing. Yet, the complexity of CRISPR-Cas9 wet lab procedure is not favorable for clinical application. AS has more advantages in targeting accuracy and the convenience on sequencing preparation as it does not require wet lab procedure for gene targeting. Moreover, due to the amplification-free nature of this approach, it is capable of sequencing methylation groups with Nanopore sequencer. The major limiting factor of AS is the requirement on advanced GPU for real time basecalling.

6.4.5 Feasibility towards clinical application in Hong Kong

Recently, there is no specific regulations for performing genetic and genomic testing in Hong Kong, but the performance of medical testing for clinical diagnosis is restricted for Medical Laboratory Technologists (MLTs) under the Supplementary Medical Professions Ordinance (Cap. 359) (SMPO) (Steering Committee of Genomic Medicine, 2020). Specific professionals

like biomedical scientists that were not under SMPO might also be capable of performing genetic and genomic tests.

According to the Hong Kong Laboratory Accreditation Scheme (HOKLAS), whole genetic testing procedure need to be accredited in accordance with ISO 15189: 2022 "Medical laboratories – Requirements for quality and competence" in all medical laboratories before the test could be applied to the public. As genetic testing was considered point-of-care testing in ISO 15189, detailed requirements for testing resources, technical processes and management system would be examined (International Organization for Standardization, 2022). Application of novel targeting sequencing technique in Hong Kong would also require accreditation of ISO 15189.

Targeted sequencing is suggested as an alternative to current diagnostic practice. It is because targeted sequencing has shortened turnaround time that prohibits diagnostic odysseys. Moreover, it is more cost-effective and saving unnecessary costs for hospitalizations, treatments and procedures. The design of panels also allows room of flexibility for targeting different variants of interest. Although it might be limited by the range of sequencing throughput, it could still be a powerful tool to effectively narrow down the scope of undiagnosed patients towards diagnosis.

7. Hypothesis

In this thesis, we hypothesize that ATM deficiency imposes an inhibitory effect on myelination of both cerebellar and cerebral regions of A-T patients, and the site of ATM mutation plays an important role in the myelin pathology.

First, the project aims to delineate effect of ATM deficiency on OPC cell cycle progression. Primary murine and cell line OPC cultures were employed for investigation on cell cycle proteins expression upon pharmacological Atm inhibition. Expression of cell cycle proteins representable for each cell cycle stage were further confirmed with immunoblotting studies of cell line culture.

Second, this project aims to delineate effect of ATM deficiency on CNS myelination in different brain regions and mutation sites. Effect of different ATM mutation sites on myelination were studied in cerebellum and cerebral cortex of A-T human and Atm knock-out mice. Immunohistology and immunoblotting studies were employed for investigation on expression pattern of ATM protein and pathology of myelin and OL under ATM deficiency. Transcriptional shift in two brain regions with loss-of-function ATM mutation were investigated for mutation-site-dependent change in gene expression. Effect of Atm-mutation-dependent pathology on OL and myelination was further confirmed with primary OL culture with Atm knock-out mice. Interactions between Atm and Mbp protein was investigated for potential effect of Atm on myelination through immunoprecipitation experiment.

Thirdly, the project aims to delineate transcriptional and translational differences of ATM mRNA and ATM protein in A-T patients with distinct mutations. The cost effectiveness in

genomic mutation profiling and exome sequencing between Nanopore-based and Illumina-based targeted sequencing were further compared for genetic diagnosis of A-T disease.

Finally, the project aims to investigate of the effect of WMH-specific genetic risk factors on endothelial cells and its potential effect cerebrovascular pathology of A-T. Mouse brain derived endothelial cell line, bEnd3 was employed for the immunocytochemistry and immunoblotting studies upon pharmacological induction and suppression of autophagy.

8. Methodology

8.1 Postmortem human cerebellar and cerebral tissues

The postmortem tissues were approved by Human Subjects Ethics Application Review board at the Hong Kong Polytechnic University (PolyU). Details of all cases are listed in Supplementary Table 2. Postmortem cerebellar and cerebral tissues from ten A-T cases were kindly provided by the NeuroBioBank of the National Institutes of Health at The University of Maryland with approvals from Tissue Access Committee at NeuroBioBank. All postmortem tissues isolated from left cerebellar cortex and cerebral prefrontal cortex were frozen without fixation and stored at -80°C. After being embedded in TissueTek O.C.T. medium, tissue samples were cryosectioned at 16 µm and mounted on glass slides. The slides mounted with tissue sample were kept at -80°C again until use.

8.2 Animal ethics

All experiments are done with compliance to The Government of the Hong Kong Special Administrative Region Department of Health's Animals (Control of Experiments) Ordinance Chapter 340. Mice were treated under the regulations for reference number: (19-252) in DH/HT&A/8/2/4 Pt.1. All mice were kept and cared at the Centralized Animal Facilities (CAF) at the PolyU in compliance with the Ordinance and The Code of Practice for Care and Use of Animals for Experimental Purposes of Hong Kong. All animal experiments and analysis were approved by the Animal Subjects Ethics Sub-Committee at The Hong Kong Polytechnic University.

8.3 Animal models

Colonies of B6;129S4-*Atm*^{tm1Bal/J} (Bal), B6.129S6-*Atm*^{tm1Awb/J} (Awb) and wildtype C57BL/6J mice (WT) were used in this study. Both transgenic mice were generated from embryonic stem cell transfection through electroporation. The transfected stem cells were then injected into C57BL/6 blastocysts for creating chimeric mice.

Bal mice was designed and created by Drs. Yang Xu and David Baltimore from Massachusetts Institute of Technology in 1996 (Xu & Baltimore, 1996). The targeted construct of Bal mice was designed by inserting PGK-neomycin cassette into exon responsible for encoding conserved sequence of ATM kinase while deleting the adjacent exon simultaneously (Figure 5) (Xu & Baltimore, 1996). The C57BL/6 blastocysts with transfected stem cells injection were then implanted into the uterus of Balb/c mice for breeding.

Awb mice was designed by the research team led by Professor Anthony Wynshaw-Boris from the Laboratory of Genetic Disease Research in 1996 (Barlow et al., 1996). The targeted construct of Awb mice was designed by inserting PGK-neomycin cassette into a 178 bp exon (located at *Atm* transcript 5705 - 5882) that was corresponding to the approximate location of at least ten protein truncation mutations found in A-T patients (located at *ATM* transcript 5178 - 5979) (Figure 5) (Barlow et al., 1996). The C57BL/6 blastocysts with transfected stem cells injection were then inbred with 129/SvEv mice or bred under mixed genetic background (129/SvEv mice crossing NIH Black Swiss mice).

In this study, both Bal and Awb mice strains were provided with ad libitum water and diet. Mice employed at different age cohort (1MO, 4MO) would be compared with different experiments, including behavioral test, immunostaining and gene expression experiments. For a better communication in this report, the names of heterozygous and homozygous Bal mice are abbreviated as Bal^{+/-} and Bal^{-/-}, while the names of heterozygous and homozygous Awb mice are abbreviated as Awb^{+/-} and Awb^{-/-}.

All animals were housed in individually ventilated cages (IVC) in a temperature and humidity-controlled animal facility at the CAF in the PolyU with a 12 hour light-dark rotation. Cages containing controlled diets were kept in IVC common housing 29 rooms. Genetic identities were confirmed 4 weeks of age and post-mortem by ear pinch genotyping.

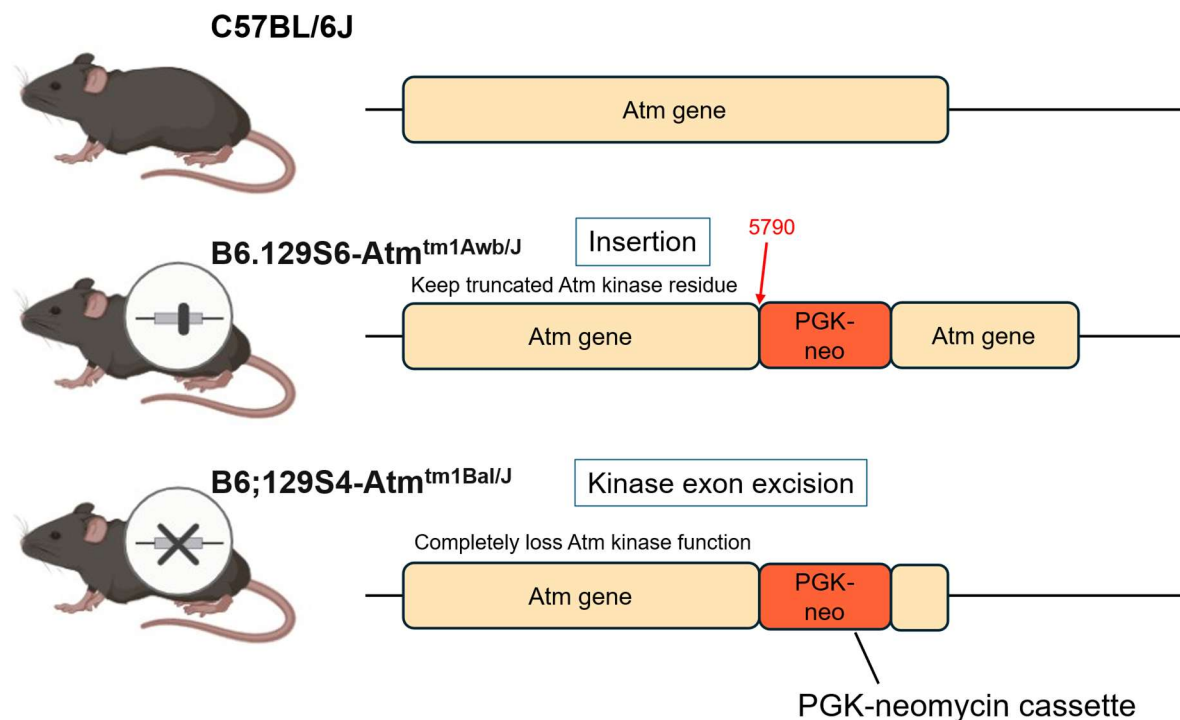


Figure 5. Genetic construct design of ATM knock out mice.

Created with BioRender.com.

8.4 Behaviour test

For better adaptation to the testing environment, mice were assimilated in the procedure room for at least 30 minutes before behaviour tests. Two types of Y-maze based behavioural tests are employed in our behavioural study, spatial and non-spatial working memory test. Spatial spontaneous alternation takes advantage of a mice's preference for exploring novel environment. Consecutive entries into each arm of Y-maze without repetition is measured in 8

minutes for the spatial memory testing. Non-spontaneous working memory test has two parts. First, mice were given 4 minutes to explore the Y-maze, while one arm of the maze is closed off. After 60 minutes of rest, mice were put back into the maze for another 4 minutes with all arms open. The percentage of spontaneous alternation is then measured with ANY-Maze for all three trials using the following formula:

$$\text{Spontaneous Alternation} = 100 \times \left(\frac{\text{Number of Alternations}}{\text{Number of Entries}} \right)$$

8.5 Brain and other tissue extraction and processing

Mice were irreversibly anesthetized by intraperitoneal injection with Avertin (1.25% 2,2,2-tribromoethanol) before transcardial perfusion with phosphate buffer solution (PBS) through peristaltic pump at 4.500 mL/min. Extracted brain was dissected between the left hemisphere, which was snap frozen in dry ice, and right hemisphere, which was fixed in 8mL of 4% paraformaldehyde for 24 h at 4°C. Fixed brain tissues were then dehydrated with 30% w/v sucrose/PBS for a minimum of 48 h, embedded into cassettes with optimal cutting temperature (OCT) in dry ice, and cryosectioned using Cryostar NX70 Cryostat at 10 µm sections on charged slides. Embedded tissues and unused sectioned slides were stored in -80°C while unused brain tissues were cryopreserved with 20% glycine/sucrose in -20°C.

8.6 Brain extraction for primary cell culture

Mouse pups at postnatal day 2 to day 7 were sacrificed by decapitation, while brain was extracted through skull dissection. Olfactory bulbs and cerebellum were then removed in a cold glucose-rich PBS buffer (33.3mM), while the remaining cerebrum was placed under a light microscope for detachment of meninges with fine forceps. After that, the cerebrum without

meninges was minced and homogenised in a eppendorf with fine scissors on ice, followed by trypsin-based enzymatic digestion (0.25%, 30 minutes, 37°C) with manual shaking every 10 minutes. After neutralization with DNase and complete medium, the cell suspension was filtered through a cell strainer with 40 µm pore size. The filtered suspension was then centrifuged (1500 rpm, 5 minutes), the centrifuged cell pellet was resuspended and transferred to a dish coated with BSL-1 (1:500, L1100, Vector Laboratories) for 15 minutes at room temperature in order to remove isolectin B4+ microglia. Supernatant was then collected without disturbing the attached cells, followed by another centrifugation (1500 rpm, 5 minutes). After resuspension, cell number was counted with a haematocytometer and the cell suspension was plated onto the poly-L-lysine (PLL) coated dish for proper attachment (30 minutes, 37°C), followed by topping-up with proper medium.

8.7 Primary oligodendrocyte cell culture

Cell plated on PLL coated dish was suspended with OPC growth medium that was composed of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Gibco), 1% fetal horse serum (FHS), 1% N2 supplement (bovine serum albumin [70 µg/mL], insulin [5 µg/mL], human transferrin [5 µg/mL], putrescine [1.6 µg/mL], progesterone [60 ng/mL], sodium selenite [5 ng/mL] and L-thyroxine [400 ng/mL], Sigma), 20 ng/mL platelet derived growth factor-AA (PDGF-AA, Sigma), and 1% insulin-transferrin-selenium (ITS, Gibco). OPCs were harvested after in vitro for 5 days and plated on PLL coated 12mm glass coverslip (50,000 cells each), followed by 7 days differentiation using growth medium with 34ng/mL triiodothyronine (T3) in the absence of growth factors, and reduced FHS concentration upon 0.1%. The purity of OPC culture differentiation is illustrated in Supplementary Figure 1.

8.8 Cell line culture

Oli-neu is an immortalized OPC cell line which was kindly provided by Dr Jacqueline Trotter (University of Mainz, Germany). Oli-neu cells recapitulate the morphology of OPC and remain the potential to differentiate into mature OL with MAG expression upon 1 mM db-cAMP stimulation (De Vries & Boullerne, 2010; Jung et al., 1995; Söhl et al., 2013). Oli-Neu cells were cultured in DMEM/F12 medium with 1% FHS, 1% N2 supplement and 1% penicillin-streptomycin (P/S) mixture at 37°C with 5% CO₂, while FHS concentration was reduced to 0.1% upon differentiation.

bEnd3 (CCL-2299, ATCC), an endothelial cell line derived from mouse brain, was employed for studying the potential effect of autophagy on cerebrovasculature (Montesano et al., 1990). bEnd3 cells were cultured in complete Dulbecco's Modified Eagle Medium (cDMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO₂. To study autophagy, 3-methyladenine (3-MA, #1292 Tocris, MN, USA) or rapamycin (Rapa, #3977 Tocris, MN, USA) were employed as the treatment for bEnd3 cells.

8.9 Chromogenic immunohistochemistry and imaging

Slides with mounted brain sections were defrosted from -80°C to room temperature before washing with PBS for 3 times. Converters manufactured for Leica Bond Rx were then placed on each slide after filling with PBS. Leica Bond Polymer Refine Detection was used as the staining consumables inserted with pre-made cocktails of targeted primary antibody. Staining protocols were optimized for heat induced epitope retrieval (HIER) pH, time necessary for antigen retrieval, antibody dilutions, and incubation period (Table 2). After staining, tissues were immersed into distilled water immediately after removing converter. Slides were then placed into a slide staining rack for dehydration and washed for 30 s with agitation in order

from 70% ethanol, 90% ethanol, 100% ethanol twice, and xylene twice. Tissues were mounted using DPX high grade xylene mountant after the last wash with xylene. Finally, slides were imaged with Leica Aperio CSO Scanner in 40x double magnification.

Primary antibody target	Concentration	Category number	Manufacturer	HIER method
PLP1	1:1000	CST 28702S (Clone E9V1N)	Cell Signalling Technology	Incubate with ER1 solution for 20 mins
Olig2	1:500	MABN50	Millipore	Incubate with ER1 solution for 20 mins
ASPA	1:500	ABN1698	Millipore	Incubate with ER1 solution for 20 mins
APC-CC1	1:750	MABC200 (Clone CC1)	Millipore	Incubate with ER1 solution for 20 mins
Nkx2.2	1:1000	ab191077	Abcam	Incubate with ER1 solution for 20 mins

Table 2. Primary antibody list for chromogenic immunohistochemistry

8.10 Fluorescent immunohistochemistry and immunocytochemistry

OLs cultured on 13mm coverslip (Marienfield) in 24 well cell culture plate was washed with filtered PBS three times before fixation. Frozen postmortem cryosectioned human cerebellar and cerebral tissues were defrozed in a chamber at room temperature for 20 minutes before fixation.

Afterwards, the specimens were fixed with paraformaldehyde (PFA, 4%) for 20 minutes before three times PBS washing. Then, they were permeabilized using 0.3% Triton X-100 and blocked with 5% normal donkey serum (NDS) in PBS for 30 min. Primary antibodies were applied for specimen incubation overnight at 4°C followed by three times PBS washes. Details of primary antibody were also listed in Table 3. Meanwhile, negative controls were proceed with plain

blocking solution without primary antibody. Fluorochrome conjugated secondary antibodies (Alexa Fluor 488, 555 or 647) were then applied for 1 hour specimen incubation at room temperature in darkness. After PBS washing, sepcimens were counterstained with 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 5 minutes. Finally, specimens were mounted with Hydromount (National Diagnostics).

Primary antibody target	Concentration	Category number	Manufacturer	Staining target
NeuN	1:500	MAB377C3 (clone A60, Cy3 Conjugate)	Millipore	Human tissue
Olig2	1:200	MABN50 (clone 211F1)	Millipore	Human tissue
pATM	1:400	CST 4526S (Ser1981) (10H11.E12)	Cell Signalling Technology	Human tissue
CCA2	1:250	ab181591 (EPR17351)	Abcam	Cell culture
CCD1	1:250	sc-450 (72-13G)	Santa Cruz	Cell culture
γ H2AX	1:250	ab2893	Abcam	Cell culture
MBP	1:500	SMI-99P	Covance	Cell culture
Olig2	1:500	MABN50A4 (clone 211F1.1)	Millipore	Cell culture
NG2	1:400	ab5320	Abcam	Cell culture
MAG	1:400	ab89780 [3C7]	Abcam	Cell culture
Olig2	1:500	MABN50 (clone 211F1)	Millipore	Cell culture
MAG	1:800	MAB1567A4 (clone 513)	Millipore	Cell culture
pATM	1:400	ab81292 (S1981) [EP1890Y]	Abcam	Cell culture
LC3B	1:200	CST 83506S (E5Q2K)	Cell Signalling Technology	Cell culture
TRIM47	1:200	ab72234	Abcam	Cell culture

Table 3. Primary antibody list for fluorescent immunohistochemistry and immunocytochemistry

The antibody for pATM (Ser1981) [4526S] is a well cited antibody with over 250 citations, which had demonstrated its specificity by multiple reports (Yalçın et al., 2024; Yasuda et al., 2024).

8.11 Fluorescent microscopy imaging

Specimens were visualised on Nikon Eclipse Ti2-E live-cell fluorescent imaging system equipped with scientific-grade monochrome CMOS camera (Nikon DS-Qi2), and visualised through a 20×/0.75 N.A Dry lens (CFI Plan Apo 20×/0.75 Dry λ) and 60×/1.40 N.A Oil lens (CFI Apo 60x Oil λ S). Squares with sides around 892 μ m and 298 μ m were imaged with 20x and 60x lens corresponsive in each experiment using multi-wavelength LED illumination system (CoolLED pE-4000) and appropriate filters.

8.12 Structural illumination microscopy imaging

Specimen preparation for structural illumination microscopy (SIM) imaging was slightly different from normal fluorescent microscopy. Cultured cells were plated on 1.5H 13mm coverslips (Marienfield) on 24 well cell culture plate. Fixation was identical to the procedure of normal fluorescent immunocytochemistry. After permeabilization with 0.3% Triton X-100 and blocking with 5% NDS, specimens were immunostained with 4-5 times concentrated primary antibody and fluorochrome conjugated secondary antibody stepwisely. Following the counterstain of DAPI, specimens were mount ProLong™ Diamond Antifade Mountant (Thermo Fisher).

Mounted specimens were visualised on Nikon N-SIM super-resolution microscopy with 100x/1.35 N.A lens (CFI SR HP Plan Apo 100×/1.35 Silicone Oil). SIM Images were acquired with 405nm, 488nm, 561nm and 640nm excitation lasers in 0.05 μ m Z-step under 3D-SIM modality. Slice reconstruction of SIM image was performed by NIS-Elements AR software under 3D-SIM modality (Illumination Modulation Contrast = 1.0, High Resolution Noise Suppression = 0.5 and Out of Focus Blur Suppression = 0.15). The three dimension SIM images

were further constructed from the slice reconstruction of SIM image by Imaris 9.5 Image Visualisation and Analysis Software.

8.13 Imaging analysis

For fluorescent immunohistochemistry of human tissues, five randomized images ($892\ \mu\text{m}^2$, 2424×2424 pixels) were acquired from cerebellar grey matter (spanning the molecular, Purkinje and granule cell layer), cerebellar white matter (spanning folia and deep regions), cerebral grey matter (prefrontal cortex) and cerebral white matter (prefrontal cortex).

For chromogenic immunohistochemistry of mouse tissues, whole sagittal mouse brain images acquired with Leica Aperio CSO Scanner were divided into different regions, including frontal cortex (FCx), motor cortex (MCx), anterior cingulate cortex (ACCx), retrosplenial cortex (RsCx), genu, corpus callosum body (CC body), hippocampus (Hippo), splenium, fornix, thalamus, anterior commissure (Ant Com), cerebellum (CB) grey matter and CB white matter with QuPath annotation before quantification (Figure 6).

For fluorescent immunocytochemistry of cell culture, five randomized images ($892\ \mu\text{m}^2$, 2424×2424 pixels) were acquired with at least 50 nuclei under each conditions for quantification. Image quantification was performed with QuPath (v 0.5.0). Individual cells were identified by nuclei detection of DAPI or haematoxylin optical density in QuPath. Nuclei detection parameters were set as follow, pixel size of $0.5\ \mu\text{m}$, background radius of $8\ \mu\text{m}$, median filter radius of $0\ \mu\text{m}$, sigma of $2\ \mu\text{m}$, minimum area of $10\ \mu\text{m}^2$, and maximum area of $400\ \mu\text{m}^2$, and cell expansion of $3\ \mu\text{m}$. Watershed and smooth boundaries were applied to the detection. The threshold of DAPI detection was set at 100 and haematoxylin threshold was set at 0.1. Positive cell density was calculated by immunoactive cell count per size of region.

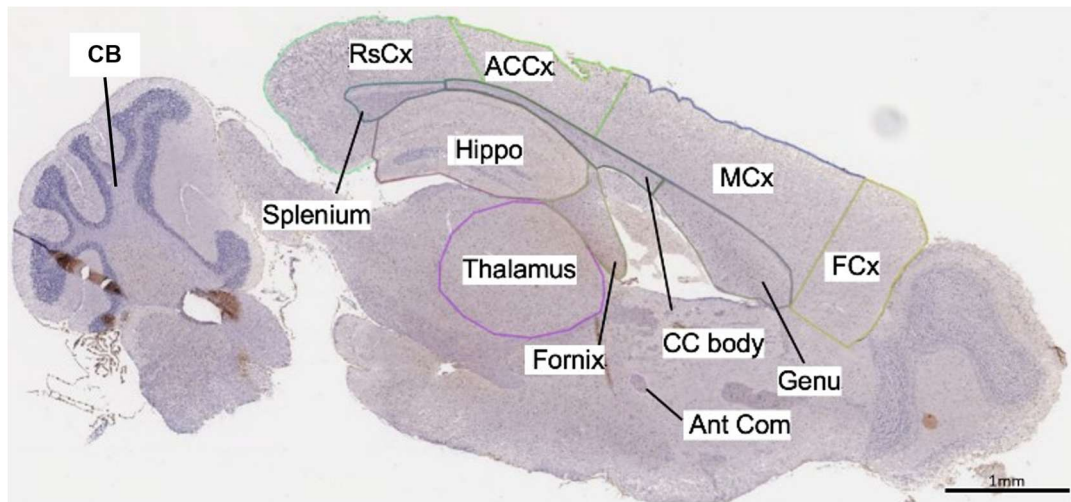


Figure 6. Annotation map for histopathological study on Atm deficient mice brain.

FCx: Frontal cortex, MCx: Motor cortex, ACCx: Anterior cingulate cortex, RsCx: Retrosplenial cortex, Genu, CC body: Corpus callosum body, Splenium, Hippo: Hippocampus, Fornix, Thalamus, Ant Com: Anterior commissure, CB: cerebellum.

8.14 Immunoprecipitation and western blotting

Separation of the left cortex from each mouse was followed by protein extraction using radioimmunoprecipitation assay (RIPA) buffer. The lysate was supplemented with protease inhibitors and phosphatase (Roche Diagnostic). Protein sample concentrations were quantified by bicinchoninic acid assay (Bio-Rad) and normalized to same quantity. Denaturation of protein sample was done by putting cap-sealed samples into dry heat block at 98°C with 5% beta-mercaptoethanol (β -ME, ThermoFisher) for 5 minutes. It was followed by electrophoresis on 8 – 12% SDS-polyacrylamide gels. After transfer to PVDF membranes with Trans-Blot Turbo System, non-specific binding was blocked with 5% non-fat milk followed by primary antibody incubation overnight at room temperature with rotation. Membrane was then washed with Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST) solution, followed by probing with horseradish peroxidase-linked secondary immunoglobulins (Cell signalling) before visualization with chemiluminescent substrates (SuperSignal™ West Dura, ThermoFisher) (Immobilon Forte, Millipore). The chemiluminescent signals were detected in

Bio-Rad ChemiDoc MP Integrated Imaging System, while the resulting band intensity were formatted and quantified using ImageJ software. In each set of experiment, GAPDH was commonly used as the protein loading control. For immunoprecipitation, proteins of interests were pulled down by Dynabeads™ Protein G (ThermoFisher Scientific) using specific antibody prior to western blotting with target antibody. The specificity of ATM antibody (ATM (ab78) [2C1]) used for the characterization of total ATM expression level of human brain tissue had already been demonstrated by a previous work, which showed differential expression of different ATM fragment peptides in the comparative analyses involving Atm-deficient versus wild-type mice, as well as A-T patients versus healthy controls (Li et al., 2011).

8.15 DNA and RNA preparation

To prepare human DNA and RNA specimens, fresh frozen human cerebellar and cerebral tissues were cryosectioned at 20 µm for ten times for each preparation and collected in 2 ml tubes. Followed by one time cold PBS washing, DNA specimens were incubated with 265 µl lysis buffer from QIAamp® Fast DNA Tissue Kit while RNA specimens were incubated 1 ml TRIzol reagent (Thermo Fisher). After applying three 3 mm stainless steel beads to screw cap 2ml tube (BioFil) with the tissue, both DNA and RNA specimens were homogenized by Precellys Evolution tissue homogenizer with Cryolys cooling system with 3 times 30-seconds-cycle of 5500 rpm spinning speed and 10 seconds pause in between cycles. Homogenised human DNA was then extracted with QIAamp® Fast DNA Tissue Kit protocol that ensured purified DNA content free of protein, nucleases or other impurities. For RNA specimen, 200 µl chloroform was applied to the homogenised TRIzol mixtures and vortexed for 30 seconds. After sitting the mixture at room temperature for 3 minutes, it was centrifuged with 13,000 rpm for 15 minutes at 4°C. Transparent aqueous phase on the top of the spinned mixture was

carefully transferred to a new sterile RNase-free Eppendorf. Equal volume of RNase-free absolute ethanol was applied to the transparent specimen. After by 10 seconds vortexing, the mixture was applied to the RNeasy spin column. The remaining procedures followed the menu of RNeasy mini kit (Qiagen) with DNase I on-column digestion.

To prepare mouse RNA specimen, frontal lobe of mouse brain was dissected at bregma 0 after defrost (Figure 7). Followed by the incubation of 1 ml TRIzol reagent (Thermo Fisher) with dissected frontal lobe and application of the mixture into screw cap 2ml tube (BioFil) with three 3 mm stainless steel beads, specimens were homogenized by Precellys Evolution tissue homogenizer with Cryolys cooling system with 3 times 30-seconds-cycle of 5500 rpm spinning speed and 10 seconds pause in between cycles. The homogenised TRIzol mixtures were mixed with 200 μ l chloroform and vortexed for 30 seconds. After sitting the mixture at room temperature for 3 minutes, it was centrifuged with 13,000 rpm for 15 minutes at 4°C. Transparent aqueous phase on the top of the spinned mixture was carefully transferred to a new sterile RNase-free Eppendorf. Equal volume of RNase-free absolute ethanol was applied to the transparent specimen. After by 10 seconds vortexing, the mixture was applied to the RNeasy spin column. The remaining procedures followed the menu of RNeasy mini kit (Qiagen) with DNase I on-column digestion.

To prepare RNA specimens from cell culture, four wells of cultured cells from 24-well plate was lysed with RLT buffer with 1% β -ME. The remaining procedures followed the menu of RNeasy mini kit (Qiagen) without DNase I on-column digestion as the RNA specimens from cell culture were prepared for quantitative PCR.

Before converting RNA sample to cDNA for quantitative PCR, contaminating genomic DNA was digested using DNase I (1 U/ μ L). Then, all cDNA samples were reversed transcribed using High-capacity RNA-to-cDNA kit (Applied Biosystem).

Qualification control and quantity measurement were done with Nanodrop ND2000 Spectrophotometer (ThermoFisher Scientific), Qubit2 Fluorometer (Invitrogen), Agilent High Sensitivity DNA Kit and Agilent RNA 6000 Nano Kit for 2100 Bioanalyzer system (Agilent).

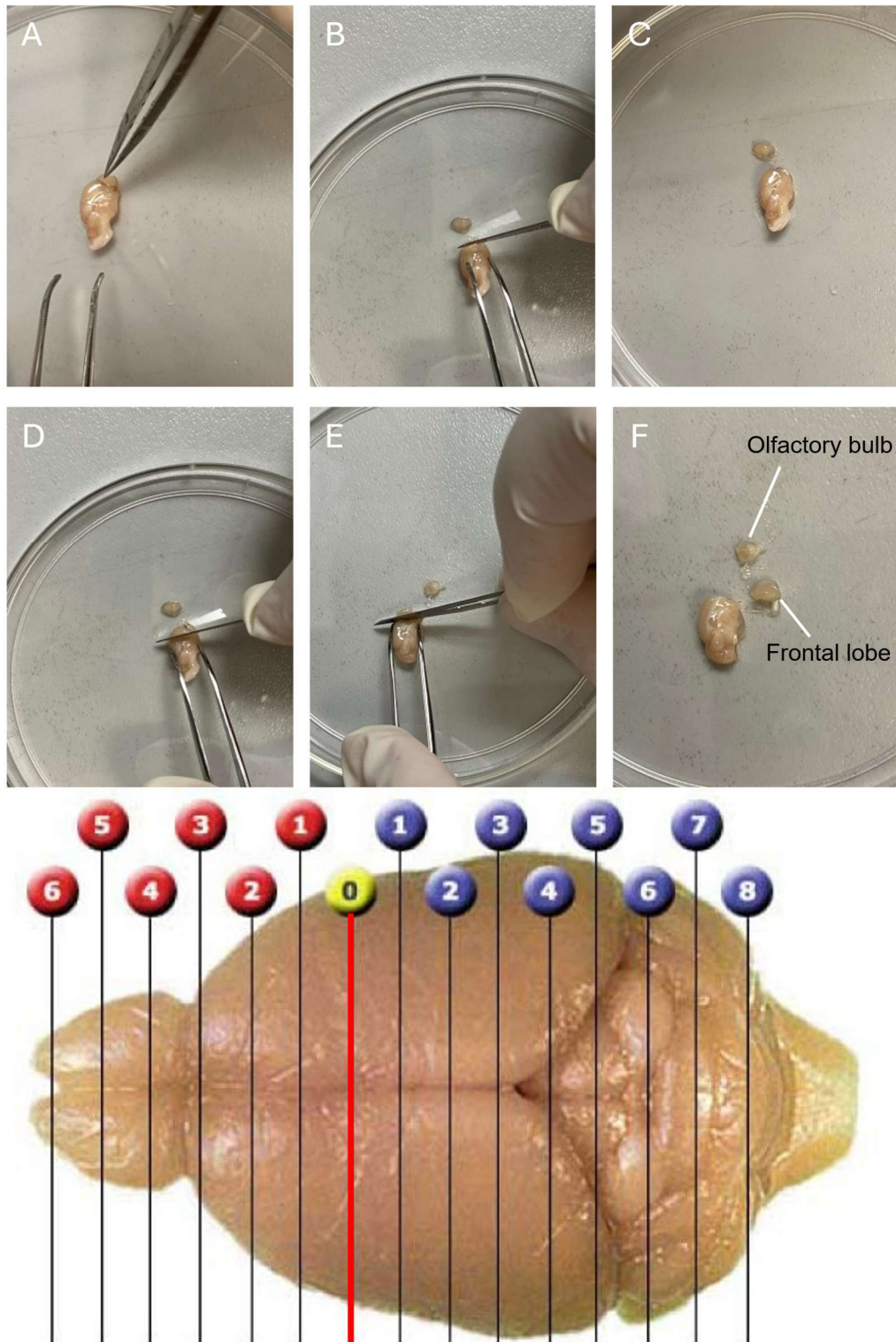


Figure 7. Stepwise procedure of frontal lobe dissection at bregma 0 for mouse RNA specimen preparation

8.16 Gene expression analysis

Gene expression analysis was performed using Fast SYBR® Green Master Mix (Applied Biosystem) in a Light Cycler 480 II (Roche) using the human or mouse specific primers optimized by PrimerBank (Spandidos et al., 2010). The quantitative PCR protocol was set as follow, initial denaturation at 95°C for 30 seconds, quantification PCR cycles 40 times (denaturing: 95°C for 5 seconds, primer annealing and extension with intensity single acquisition: 60°C for 30 seconds) and melting curve analysis (denaturing: 95°C for 5 seconds, final extension: 60°C for 1 minute, followed by gentle increase of temperature until 95°C with ramp rate 0.11°C per second and continuous intensity acquisition mode with 5 acquisitions per 1°C). All gene expression levels were calculated relative to housekeeping genes (18S, β -actin and GAPDH). Data analysis was performed using Microsoft excel based on the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001).

8.17 Illumina-based targeted DNA sequencing

Quantifications were done with Quant-iT™ PicoGreen™ dsDNA Assay Kit on a NanoDrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific). Using TruSight® Inherited Disease Sequencing Panel, the samples were then tagged, captured, amplified and processed for targeted genomic sequencing. The sample prepared with TruSight were normalized, indexed, and pooled into libraries with quality control using High Sensitivity NGS Analysis Kit (DNF-474, Advanced analytical) on Fragment Analyzer™. The pooled libraries were then sequenced using Illumina Mid-Output Kit with The aligned reads in the targeted genome were analyzed for SNPs,

insertions or deletions (Indels) based on Genome Analysis Toolkit (GATK, Broad Institute). NextSeq550.

8.18 Nanopore-based targeted DNA sequencing

DNA concentration of human DNA samples were quantified with Nanodrop ND2000 Spectrophotometer (ThermoFisher Scientific). Then, 3.5 µg DNA sample was extracted and diluted upto 130 µl with Tris-HCl (pH 8.0, 10mM). Aiming 1500bp target fragment size, diluted DNA specimens were fragmented with M220 ultrasonicator (Covaris) with following parameters, peak incident power of 50 W, duty factor of 2%, 200 cycle per burst, treatment time of 20 seconds and temperature at 20°C (Leung et al., 2022). Fragmented DNA specimens were then purified with 0.6x AMPure XP magnetic beads (Beckman Coulter Genomics). Purified DNA specimens were then repaired and end-prepared with NEBNext FFPE Repair Mix (M6630) and NEBNext End Repair/ dA-tailing Module (E7546). Followed by another purification step with 0.6x AMPure XP magnetic beads (Beckman Coulter Genomics), DNA specimens were ligated with Nanopore PCR adapter from PCR Expansion kit (EXP-PCA001) by NEB Blunt/TA Ligase Master Mix (M0367). DNA products from the ligation were further purified with 0.6x AMPure XP magnetic beads (Beckman Coulter Genomics). Then, purified DNA specimens were amplified with Primer mix from PCR Expansion kit (EXP-PCA001) by 2x Long AMP Taq (M0287). After another purification with 0.6x AMPure XP magnetic beads (Beckman Coulter Genomics), DNA specimens were blocked with Cot-1 DNA (ThermoFisher Scientific 15279-011) and Blocking oligo at 1 mM, sequence 5'-AGGTTAAACACCCAAGCAGACGCCGCAATATCAGCACCAACAGAAACAA 3', before probe capturing. Probe hybridization of targeted gene panel, ClearSeq Inherited Disease Panel (Agilent), was then performed with SureSelect TE Reagent Kits (G9605A), while DNA

specimens were hybridized with targeted gene panel at 65 °C for 16 to 24 hours. Hybridized DNA specimens with targeted probes were pulled down with Dynabeads MyOne Streptavidin T1 (ThermoFisher Scientific, 65601) in the aid of SureSelect TE Reagent Kits (G9605A). The captured DNA specimens were further amplified with Primer mix from PCR Expansion kit (EXP-PCA001) by 2x Long AMP Taq (M0287), followed by purification with 0.6x AMPure XP magnetic beads (Beckman Coulter Genomics). Then, DNA specimens were end-prepared with NEBNext End Repair/ dA-tailing Module (E7546), followed by purification with 0.6x AMPure XP magnetic beads (Beckman Coulter Genomics). End-prepared DNA specimens were ligated with adapter mix (AMX) from Nanopore Ligation Sequencing Kit (SQK-LSK109) by T4 ligase from NEBNext Quick Ligation Module (E6056). Purification with 0.6x AMPure XP magnetic beads (Beckman Coulter Genomics) was performed on ligated DNA with S Fragment buffer (SFB) from SQK-LSK109 as washing buffer and Elution Buffer (EB) from SQK-LSK109 as reagent for elution. Finally, 100 fmol DNA Library was loaded to R 9.4.1 flowcell on Mk1b device, which was sequenced with Minknow software and basecalled with computer with RTX 3090 GPU device.

8.19 PCR confirmation of genetic mutation

DNA extracted from human patients were amplified by Q5[®] High-Fidelity DNA Polymerase at identified A-T pathogenic mutation sites with specifically designed primers. PCR mixtures of each sample were denatured initially at 98°C for 30 seconds, followed by customised PCR cycles 35 times (denaturing: 98°C for 10 seconds, primer annealing: temperature specific to primers for 30 seconds, extension: 72°C for 30 seconds). Followed by the final extension at 72°C for 2 minutes, the amplified PCR products were collected for 0.7% agarose gel electrophoresis (70V, 50 minutes) with ladder. The gel was further imaged with Bio-Rad

GelDoc XR+ System. Sequence of designed primers and corresponding optimal binding temperature were listed in Supplementary Table 3.

8.20 Illumina-based targeted RNA sequencing

Quantification and qualification were performed with Agilent RNA 6000 Nano Kit on 2100 Bioanalyzer system (Agilent). After that, 100 ng human RNA extracted from cerebellar and cerebral tissue were fragmented with Elute, Prime, Fragment High Mix (EPH) from TruSight® RNA Pan-Cancer Panel library preparation kit (Illumina). Then, first and second strand cDNA stepwise synthesis was employed for fragmented RNA samples with First Strand Synthesis Act D Mix (FSA) and Second Strand Marking Master Mix (SMM) from TruSight® RNA Pan-Cancer Panel library preparation kit (Illumina) correspondingly. After 1.8x AMPure XP magnetic beads (Beckman Coulter Genomics) purification, the synthesized cDNA was adenylated at 3' ends with A-Tailing Mix (ATL) from TruSight® RNA Pan-Cancer Panel library preparation kit (Illumina) and then ligated to Illumina RNA index adapters respectively with Ligation Mix (LIG) from TruSight® RNA Pan-Cancer Panel library preparation kit (Illumina). Followed by 1x AMPure XP magnetic beads (Beckman Coulter Genomics) purification, indexed cDNA samples were amplified by PCR Master Mix (PMM) and PCR Primer Cocktail (PPC) from TruSight® RNA Pan-Cancer Panel library preparation kit (Illumina). After another step of 1x AMPure XP magnetic beads (Beckman Coulter Genomics) purification, amplified cDNA samples were quantified with Agilent DNA 1000 chips on 2100 Bioanalyzer system (Agilent). Size detected with each specimen was expected within the range of 250 to 300 bp. Then, 200 ng cDNA from each specimen was hybridized with RNA PanCancer Oligos (RPO) using Capture Target Buffer 3(CT3) from TruSight® RNA Pan-Cancer Panel library preparation kit (Illumina). The hybridized cDNA specimens with targeted

probes were pulled down with Dynabeads MyOne Streptavidin T1 (ThermoFisher Scientific, 65601) in the aid of Enrichment Elution Buffer 1 (EE1) and Elute Target Buffer 2 (ET2) from TruSight® RNA Pan-Cancer Panel library preparation kit (Illumina). After 2x AMPure XP magnetic beads (Beckman Coulter Genomics) purification, captured cDNA samples were further amplified by Enhanced PCR Mix (EPM) and PCR Primer Cocktail (PPC) from TruSight® RNA Pan-Cancer Panel library preparation kit (Illumina). Followed by another step of 1.8x AMPure XP magnetic beads (Beckman Coulter Genomics) purification, amplified cDNA samples were quantified with Agilent DNA 1000 chips on 2100 Bioanalyzer system (Agilent). There were eight specimens pulled into one 4 nM library prepared for one set of sequencing. The pooled library was diluted to 12 pM with 1% PhiX spike-in. Finally, the pooled libraries were sequenced with MiSeq Reagent Kit v3 (150 cycles) in MiSeq platform (Illumina).

8.21 Illumina-based transcriptomic sequencing

The human and mouse RNA samples (1000ng input) were captured by 1x RNA purification beads from Illumina Stranded mRNA Prep Ligation library preparation kit and were fragmented with Elute, Prime, Fragment 3HC Mix (EPH3) from Illumina Stranded mRNA Prep Ligation library preparation kit. Then, the first and second strand cDNA stepwise synthesis was employed for fragmented RNA samples with First Strand Synthesis Mix (FSA) and Second Strand Marking Master Mix (SMM) from Illumina Stranded mRNA Prep Ligation library preparation kit. After 1.8x AMPure XP magnetic beads (Beckman Coulter Genomics) purification, the synthesized cDNA was adenylated at 3' ends with A-Tailing Mix (ATL4) from Illumina Stranded mRNA Prep Ligation library preparation kit and ligated to Illumina RNA index anchors with Ligation Mix (LIG) from Illumina Stranded mRNA Prep Ligation library

preparation kit. Followed by 0.8x AMPure XP magnetic beads (Beckman Coulter Genomics) purification, cDNA samples were amplified and indexed respectively by Enhanced PCR Mix (EPM) and RNA indexes in IDT for Illumina RNA UD Indexes Set A from Illumina Stranded mRNA Prep Ligation library preparation kit. Followed by another step of 1x AMPure XP magnetic beads (Beckman Coulter Genomics) purification, amplified cDNA samples were quantified with Agilent DNA HS chips on 2100 Bioanalyzer system (Agilent). Finally, the 16 mouse cDNA specimens were pooled into one library and were sequenced on an NextSeq 1000/2000 p2 reagent (200 cycles) in NextSeq2000 (Illumina), while 32 human cDNA specimens were pooled into one library with 14 extra other samples and were sequenced on an NextSeq 1000/2000 p3 reagent (200 cycles) in NextSeq2000 platform (Illumina).

8.22 Bioinformatic analysis for sequencing results

For the analysis of Illumina-based targeted DNA sequencing, the raw reads were aligned against the human reference genome (hg19). There are 550 genes targets in the TruSight® Inherited Disease Sequencing Panel, which are known as the cause of rare inherited diseases. There are around 30,000 probes, covering 8801 exons and exon-intron boundaries of these genes, which include ATM and other DNA repair genes that cause progeria syndromes. The aligned reads were then analyzed for SNPs, insertions or deletions (Indels) using Genome Analysis Toolkit (GATK, Broad Institute). The annotated genetic variants were visualised and further annotated with variant details by the Illumina VariantStudio™ 3.0, including variant types, genotype (hetero/homozygosity), coordinates, exon/intron, protein and cDNA consequences, potential pathogenicity and variant details matching against multiple SNP databases including ClinVar (NCBI), COSMIC (Sanger Institute), dbSNPs (NCBI) and HGVS (Human Genome Organization).

For the analysis of Nanopore-based targeted DNA sequencing, raw fast5 files were real-time base-called using ONT basecaller Guppy (v 6.5.7) with SUP config model in Minknow (minimum q-score = 10). Meanwhile, processed fastq file were further trimmed out of adapter sequence and aligned against the human reference genome (hg19) with Guppy (v 6.5.7) in the same time. Aligned bam files were analysed for SNPs variant calling with Clair3 (v 1.0.0) and large Indels variant calling with Sniffles2 (v 2.2). The variants called by Clair3 and Sniffles2 were then annotated with Ensembl Variant Effect Predictor (VEP) with following attributes, variant types, zygosity, coordinates, exon/intron, protein and cDNA consequences in dbSNPs and HGVS, potential pathogenicity in ClinVar, SIFT score, PolyPhen score, gnomAD score, MaxEntScan score, CADD score and SpliceAI prediction score.

For the analysis of targeted RNA sequencing of human tissue with TruSight[®] RNA Pan-Cancer Panel, raw read files were aligned with hg19 (UCSC) by RNA-Seq Alignment software (v 1.0.0, BaseSpace) with STAR aligner (v 2.5.0). Aligned reads proceeded with differentially expressed genes (DEGs) discovery with DESeq2 (v 1.6.3) from BaseSpace.

For the analysis of transcriptomic sequencing of human tissue, raw read files were aligned with hg38 (1000 Genomes v4) by DRAGEN RNA (v 0.7.11, BaseSpace). The aligned reads proceeded with DEGs discovery with DRAGEN Differential Expression (v 4.3.7, BaseSpace).

For the analysis of targeted RNA sequencing of mouse tissue with Qiagen Mouse Cancer Transcriptome (RMM-003Z), raw read counts generated from STAR alignment pipeline were then normalized by geNorm with GeneGlobe Data Analysis Center (Qiagen).

For the analysis of RNA sequencing of mouse tissue with Illumina Stranded mRNA Prep Ligation, raw read files were aligned with GRCm39 (Ensembl) by Dragen-RNA application (v 4.3.4, BaseSpace). Aligned reads proceeded DEGs discovery with DESeq2 (v 1.42.1) with RStudio.

8.23 Statistical analysis

All statistical analysis were done on GraphPad Prism 9 (version 9.5.0., San Diego, US). Two-way analysis of variance (ANOVA) was performed with Tukey post-hoc multiple comparison test on all comparisons including two independent variables, which minimize Type I error due to differing sample. One-way ANOVA was performed on comparisons including one independent variable. Tukey post-hoc multiple comparison test and Fisher's LSD multiple comparison test were applied for one-way ANOVA tests dependent on size of comparison group (Tukey: more than three groups; LSD: only three groups). Dunnett's multiple comparisons test was applied in one-way ANOVA when there was only one control group for comparison. All data in graphs are expressed as mean $\pm$ standard error of mean (SEM) with minimum statistical significance at $p \leq 0.05$.

9. Results

Chapter 1. ATM protein expression upon pharmacological inhibition and DSB induction in oligodendrocyte

Summary

In this chapter, mixed glial primary culture and pure oligodendrocyte cell line culture were experimented with pharmacological ATM inhibition and DSB formation. Cell cycle proteins expression in OPC primary culture were highly suppressed by ATM inhibition, while selective suppression on S phase entry was caused by DSB formation. Meanwhile, DSB formation in mOL led to cell cycle re-entry in mOL with significant increase in G₁ phase protein expression. However, suppression of cell proliferation upon DSB formation was found in Oli-Neu culture.

Central hypothesis

ATM protein regulates cell cycle and repair DNA to maintain genome integrity (Shiloh & Ziv, 2013). Re-expression of cell cycle proteins in postmitotic neuron cell in both human A-T patients and ATM deficient mouse were associated with neuronal cell death (Kruman et al., 2004; Yang & Herrup, 2005). However, the role of ATM on OL cell cycle regulation remains obscure. As demonstrated in different genetic diseases with deficits in DNA repair, numerous DNA repair genes are uniquely important for OL and their myelination (Tse & Herrup, 2017). We therefore hypothesize that ATM functions are required for the regulation of the cell cycle progression in self-renewing OPC and post-mitotic mOL (Figure 8).

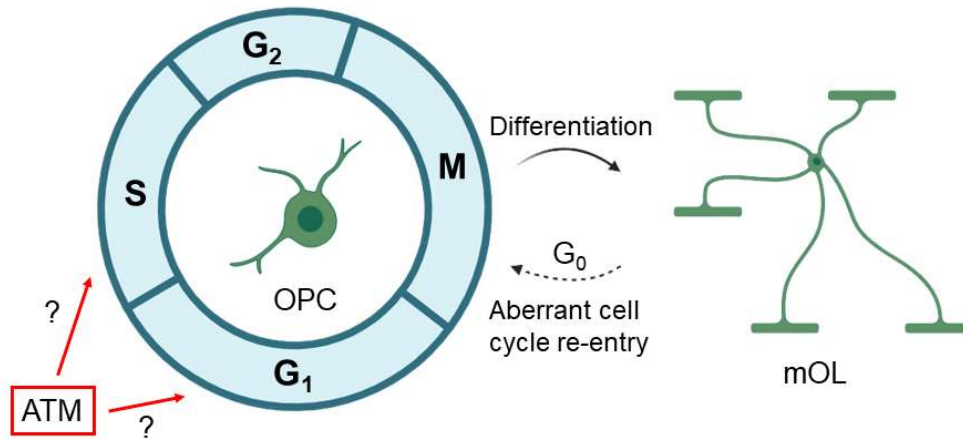


Figure 8. Central hypothesis 1: ATM functions are required for the regulation of the cell cycle progression in self-renewing OPC and post-mitotic mOL

To investigate, a selective topoisomerase II inhibitor, etoposide was applied to primary murine OL culture at different stages to induce DSB formation and ATM activation, and a selective ATM kinase inhibitor, KU-60019 was applied to inhibit ATM activity (Golding et al., 2009). Significant reduction of ATM activity on mOL soma and processes was found with KU-60019 application at 10 μ M in our preprint manuscript, which demonstrated over 50% inhibition of S-phase entry in both OPC and OL population without significant cell loss (Tse et al., 2023). Although KU-60019 is not a unique ATM inhibitor, it showed a highly specificity to ATM with IC₅₀ at 6.3nM, while it's specificity (IC₅₀) to DNA-PKcs and ATR are just 1.7 μ M and >10 μ M (Golding et al., 2009). In this chapter, expression of cell cycle protein, cyclin D1 and cyclin A2 represents cells in G1 and S phase correspondingly (Gong & Ferrell, 2010; Welsh et al., 2001).

Cell cycle protein expression in primary OPC cell upon Atm inhibition

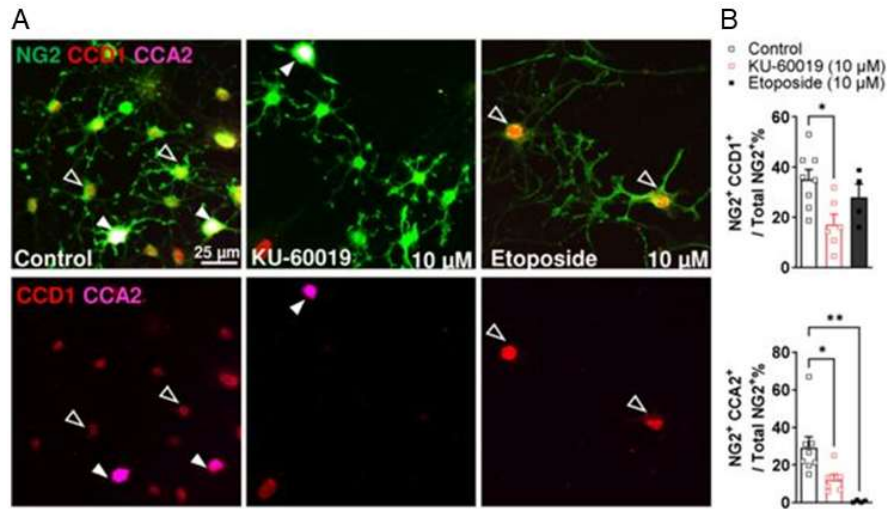


Figure 9. OPC cell cycle activity upon KU-60019 inhibition and etoposide treatment in primary OPC culture.

A) Representative immunocytochemistry image of primary OPC culture denoting NG2-positive OPC cell cycle activity with cyclin D1 (CCD1: red) and cyclin A2 (CCA2: magenta). B) Quantification of cyclin D1 positive OPC and cyclin A2 positive OPC over total OPC population in different treatment (One-way ANOVA with Dunnett's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

In primary OPC culture (5 DIV), KU-60019 (10 μ M) and etoposide (10 μ M) were applied for 24 hours correspondingly. Atm inhibition through KU-60019 limited cell cycle activity of OPC through suppression of cyclin D1 and cyclin A2 expression by more than half (Figure 9B). DSB formation through etoposide barely affected the G₁ phase entry of OPC, but the expression of cyclin A2 was virtually abolished (Figure 9B).

Cell cycle protein expression in primary mOL cell upon Atm inhibition

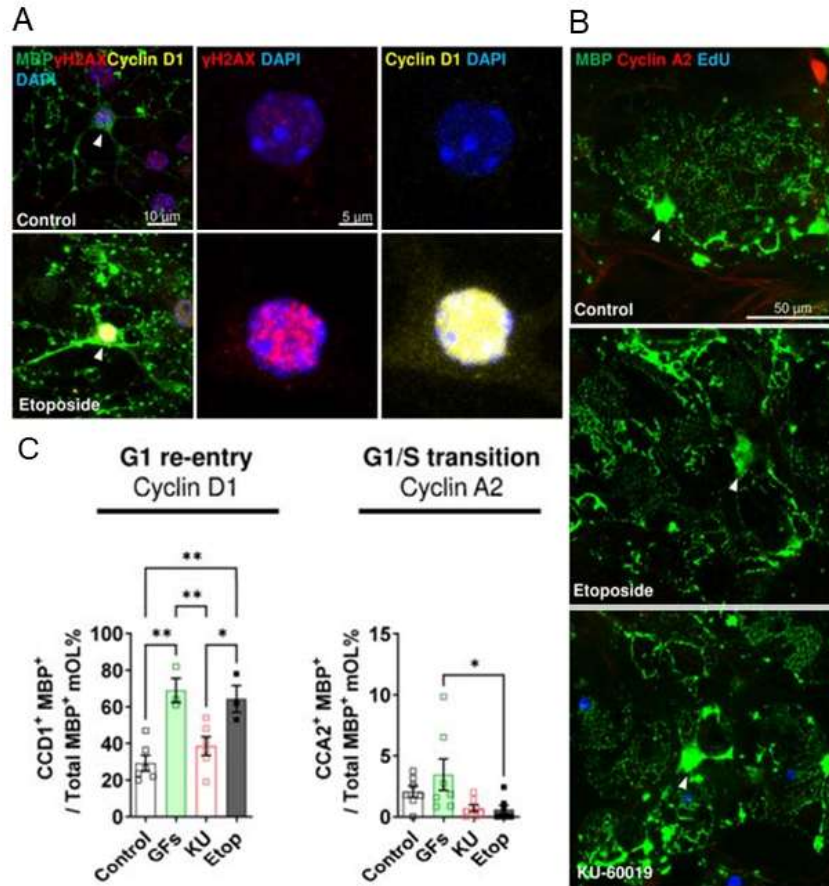


Figure 10. mOL cell cycle activity upon growth factors application KU-60019 inhibition and etoposide treatment in primary mOL culture.

A) Representative immunocytochemistry image of primary mOL culture denoting MBP-positive mOL cell cycle activity with cyclin D1 (CCD1: yellow). B) Representative immunocytochemistry image of primary mOL culture denoting MBP-positive mOL cell cycle activity with cyclin A2 (CCA2: red). C) Quantification of cyclin D1 positive mOL and cyclin A2 positive mOL over total mOL population in different treatment (One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

After differentiation with T3, primary mOL culture (14 DIV) was incubated with growth factors (PDGF-AA and bFGF, 10 ng/mL), KU-60019 (10 μM) and etoposide (10 μM) for 24

hours correspondingly. Significant abnormal cell cycle re-entry was found when growth factors and etoposide (GFs: 64%; Etop: 69%) were applied (Figure 10C). In contrast, KU-60019-mediated ATM inhibition on mOL had no effect on the expression of cyclin D1 or cyclin A2 (Figure 10C). Hence, ATM inhibition barely imposed effect on cell cycle regulation after maturation of OL. Neither growth factors nor etoposide significantly altered cyclin A2 expression in mOL (Figure 10C), which implied a relatively unaffected transition of mOL from G1 to S phase.

Cell cycle protein expression in OPC cell line upon Atm inhibition

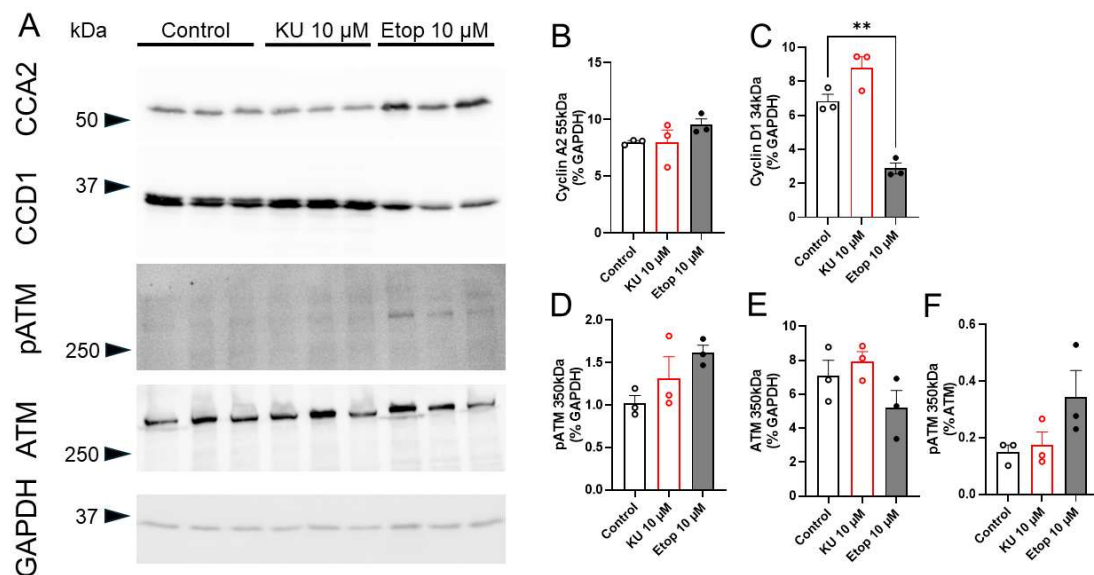


Figure 11. KU-60019-mediated and etoposide-mediated aberrant cell cycle protein expression in Oli-Neu cell culture.

A) Immunoblot images of cell cycle proteins (cyclin A2 and cyclin D1), pATM(S1981) and ATM(2C1) expression in Oli-Neu cell culture with different treatment (KU-60019: 10 μ M; etoposide: 10 μ M). Representative analysis of the B) cyclin A2, C) cyclin D1, D) pATM and E) ATM expression against GAPDH, and F) pATM expression against ATM are shown. (Ordinary one-way ANOVA with Dunnett's multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

To study the pharmacological effect of KU-60019 (10 μ M) and etoposide (10 μ M), a pure mural clonal oligodendrocyte culture, Oli-Neu was employed upon 24 hours incubation of each drug respectively.

There was no significant change in phospho-ATM^{S1981} and ATM expression after KU-60019 and etoposide treatment in Oli-Neu (Figure 11D, E). Despite the visual increase of pATM expression with etoposide treatment in immunoblot image (Figure 11A), the levels of pATM expression were levelled off after normalized to GAPDH and ATM expression (Figure 11D, F). Decrease of cyclin D1 expression (62.1%, $p=0.0021$) was found in etoposide-treated Oli-Neu (Figure 11C), which reflected the suppression of cell proliferation upon DSB formation in Oli-Neu. Hence, KU-60019 treatment has no significant effect on cyclin D1 and cyclin A2 expression in Oli-Neu (Figure 11B, C). In the immunoblotting image, etoposide treatment imposed a slight upregulation of cyclin A2 expression in Oli-Neu (Figure 11A), but the trend levelled off after normalization of GAPDH expression (Figure 11B).

Conclusion

	OPC	Postmitotic OL
ATM inhibition	Reduced cyclin D1 and A2 expression	Apoptosis
DNA damage	Reduced cyclin A2 expression	Increased cyclin D1 expression and apoptosis

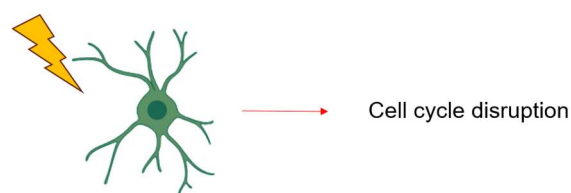


Figure 12. Conclusion for chapter 1: ATM functions are important for the regulation of the cell cycle progression in self-renewing OPC and post-mitotic mOL

Chapter 2. ATM regulation on oligodendrocyte differentiation

Summary

Apart from the classic symptoms of cerebellar ataxia and oculocutaneous telangiectasia, demyelination was found in cerebellum and spinal cord of A-T patients in histopathological study (Aguilar et al., 1968; De León et al., 1976). Cerebral WMH found in A-T patients in MRI study suggested potential demyelination in cerebral region (Chung et al., 1994). The causal relationship between ATM deficiency and neurological damage in A-T patients was well known with reproducible neuronal loss in cerebellum in experimental mouse A-T models (Kuljis et al., 1997). The pathogenesis of A-T myelin pathology and neurological damage in cerebral cortex remain largely unknown. Functions of ATM were associated with cell cycle regulation in OL, the only cell type producing myelin in CNS. However, OL cell cycle dysregulation associated with ATM deficiency might not be the direct cause of demyelination.

Central Hypothesis

We hypothesis that *ATM* mutations are not only affecting cerebellum but also cerebral cortex, and ATM deficiency directly imposes an inhibitory effect on myelination (Figure 12).

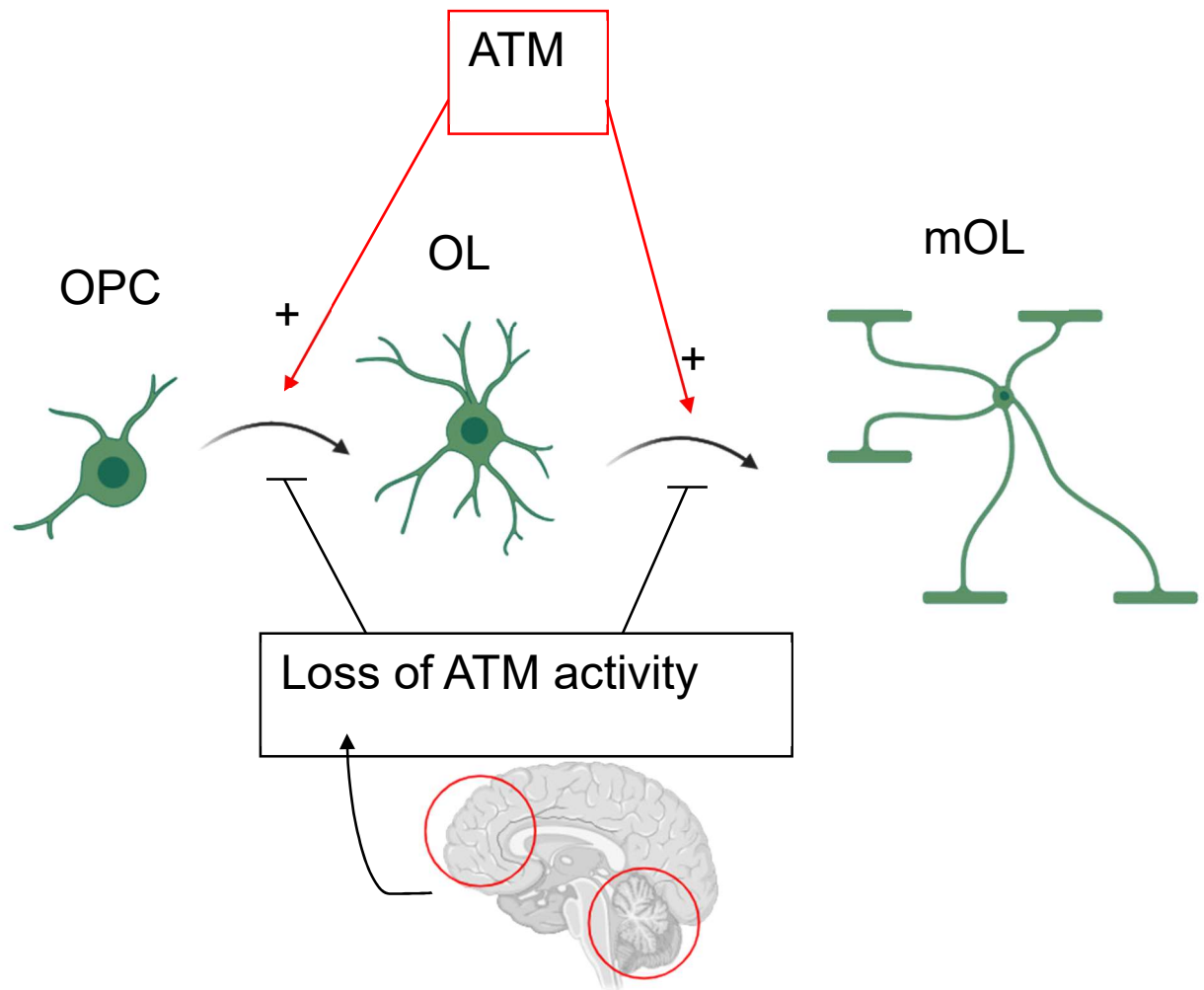


Figure 13. Central hypothesis 2: ATM functions are required for the regulation of differentiation progress from OPC to mOL, while loss of ATM activity led to inhibitory effect on OL differentiation in both cerebral and cerebellar region.

As pathology of ATM deficiency in A-T patients was primarily known as neuronal loss in cerebellum, OL pathology of A-T was often neglected. It was proven in the last chapter that ATM deficiency led to dysregulation of cell cycle in OL in vitro. We aimed to investigate OL pathology of A-T in vivo and delineate the region-specificity of OL pathology. To test the hypothesis, tissue specimens from the frontal cortex and cerebellum of A-T patients were examined histologically for neuronal and myelin degeneration. The goal of the comparison between frontal cortex and cerebellar cortex is to delineate A-T myelin pathology subject to regional specificity. To verify these changes in the A-T tissues at the biochemical level, and provide an analysis between cerebral cortex and cerebellum, the protein expression of these

tissues was examined by immunoblotting. Further, the mRNA extracted from these human tissues were sequenced for the global changes in the transcriptome.

Using two distinct *Atm* knock-out mice models, *Atm*^{tm1Awb/J} and *Atm*^{tm1Bal/J}, we further confirm human findings of whether *ATM* mutation has any effects on oligodendrocyte differentiation using a battery of behavioural test, histological and molecular analysis on myelin pathologies. Further, by using primary OL culture derived from ATM knock-out mice, as well as using KU-60019 to pharmacologically inhibit ATM kinase *in vitro*, we validated if ATM kinase activity has any implications on OL differentiation. Finally, based on a series of *in silico* analysis, we proposed that one of the key myelin proteins, MBP, is a potential substrate of ATM. To confirm, we conducted a co-immunoprecipitation using specific antibodies against MBP and ATM, and conducted a preliminary proteomic study of cell lysate pulled down by anti-ATM. These findings were supplemented by visualization using super-resolution study using N-SIM system.

Oligodendrocyte and myelin degeneration in human A-T patient

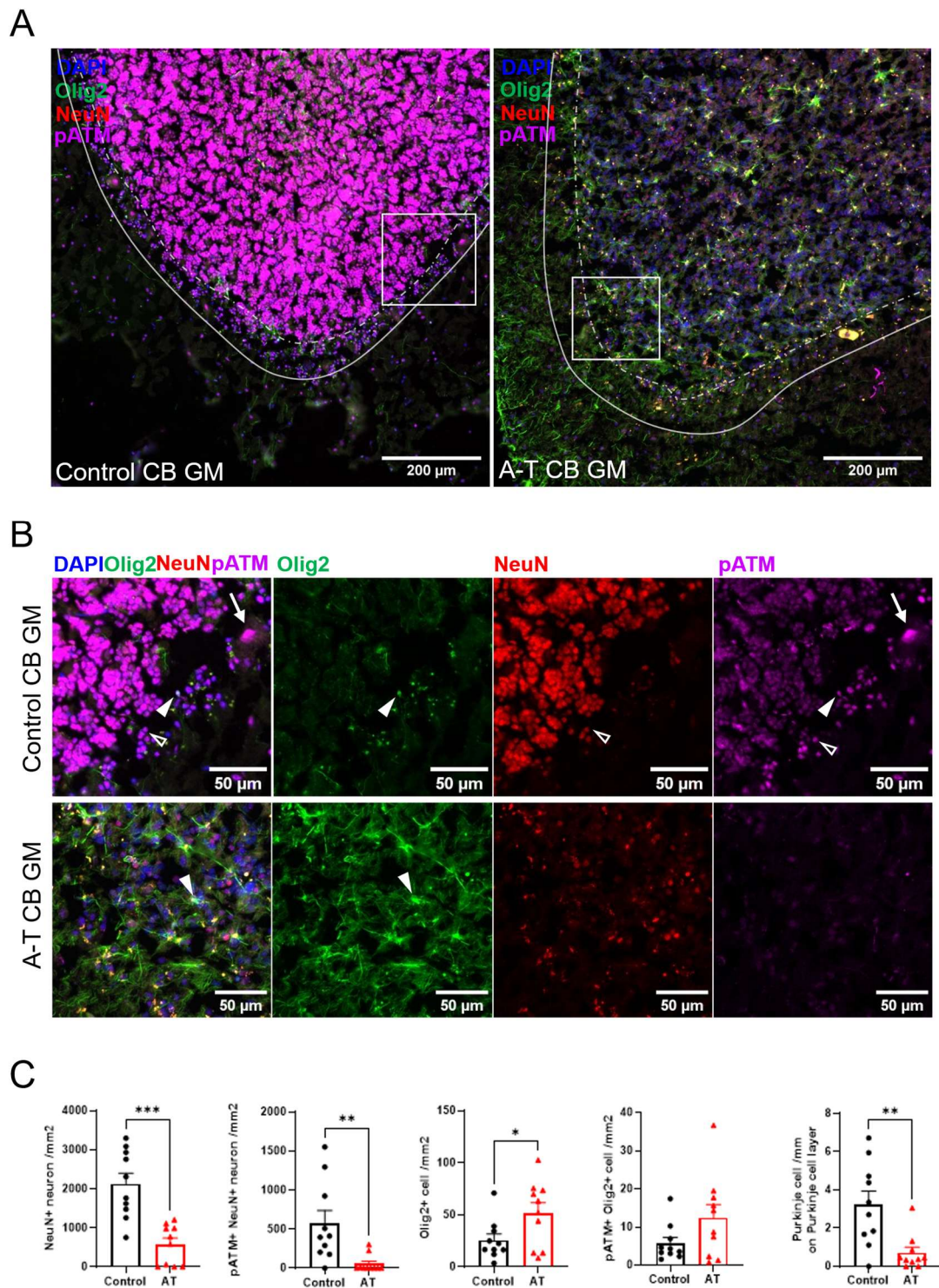


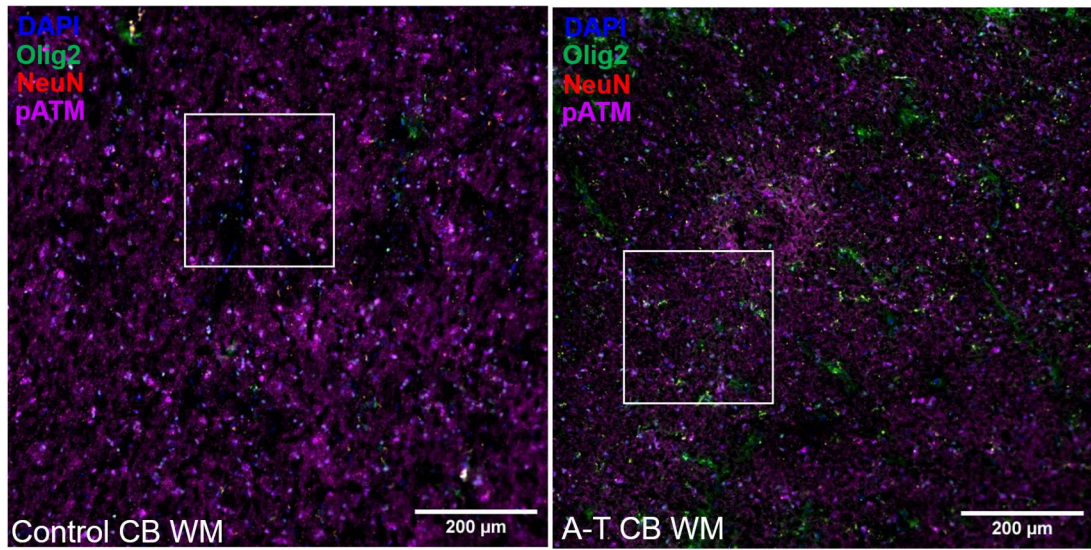
Figure 14. Immunohistology study of OLs, neurons and Purkinje cells in cerebellar grey matter of A-T patients.

A) Representative immunohistochemistry images indicating expression of phosphorylated ATM (pATM) in neuron (NeuN+) and OL (Olig2+) in cerebellar grey matter. Area within dotted line indicates granule cell layer of cerebellum; area between dotted line and solid line indicates Purkinje cell layer; area outside solid line indicates molecular layer. B) Magnified image panels from the selected region (white square) in figure A. Solid arrowhead indicates Olig2+ OL; hollow arrowhead indicates NeuN+ neuron; tailed arrowhead indicates Purkinje cell expressing pATM. C) Quantification of density of NeuN+ neuron, pATM+ NeuN+ neuron, Olig2+ OL and pATM+ Olig2+ OL. Quantification of number of pATM+

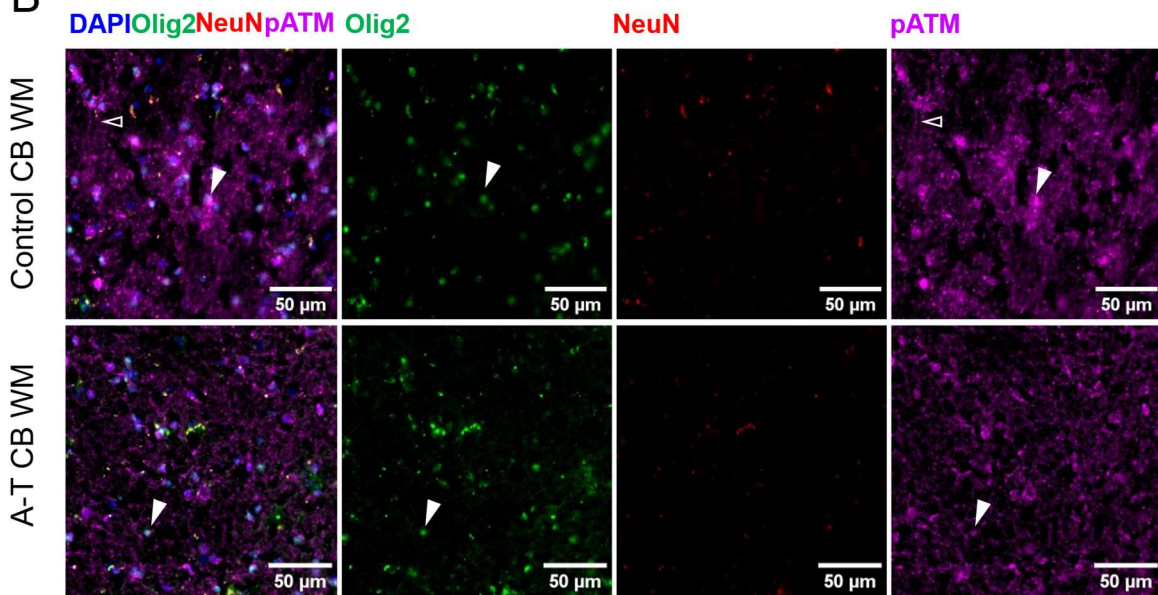
Purkinje cells against the distance of Purkinje cell layer. (Control: n=10, A-T subjects: n=10) (unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Fixed cerebellar tissues from A-T patients were probed with Olig2, NeuN and pATM to investigate neuronal and OL pathology with ATM S1981 autophosphorylation status. There was visually descended NeuN and pATM expression in granule cell layer indicated within dotted line (Figure 13A), while NeuN⁺ neuron and pATM⁺ NeuN⁺ neuron densities were significantly decreased by 73.1% ($p < 0.0001$) and 90.4% ($p = 0.0056$) in A-T subjects (Figure 13C). There were no NeuN expressions but persistent ATM reactivity in Purkinje cells demonstrated by previous immunohistochemistry studies (Deacon et al., 2024; Wolf et al., 1996). Based on the soma size, Purkinje cells (PCs) with pATM reactivity were identified in the Purkinje cell layer (Figure 13B). After normalization to the edge distance of Purkinje cell layer, PC number was decreased significantly by 78.2% ($p = 0.0032$) in A-T cerebellum (Figure 13C). Despite granule neurons and Purkinje cells, Olig2⁺ OLs were also found with pATM expression (Figure 13B). Cytoplasmic translocation of Olig2 expression was observed in A-T sample (Figure 13B). The Olig2 translocation indicates the conversion from OL progenitor to reactive astrocyte upon inflammatory response (Cassiani-Ingoni et al., 2006). Olig2⁺ cell density was increased by 131% ($p=0.0381$) in A-T samples (Figure 13C).

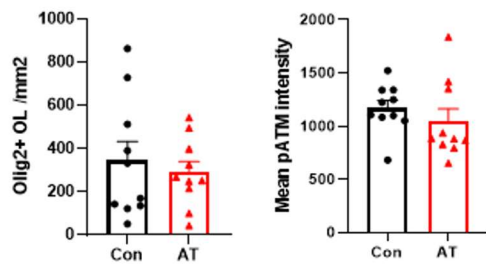
A



B



C



D

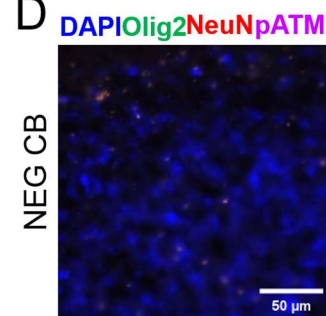


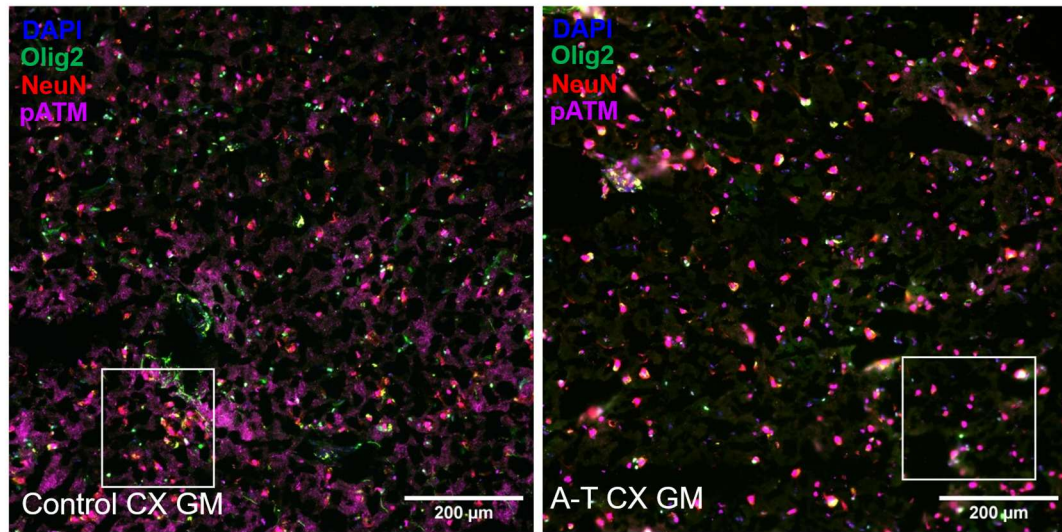
Figure 15. Immunohistology study of OLs in cerebellar white matter of A-T patients.

- A) Representative immunohistochemistry images indicating expression of pATM in OL (Olig2+) in cerebellar white matter.
 B) Magnified image panels from the selected region (white square) in figure A. Solid arrowhead indicates Olig2+ OL;

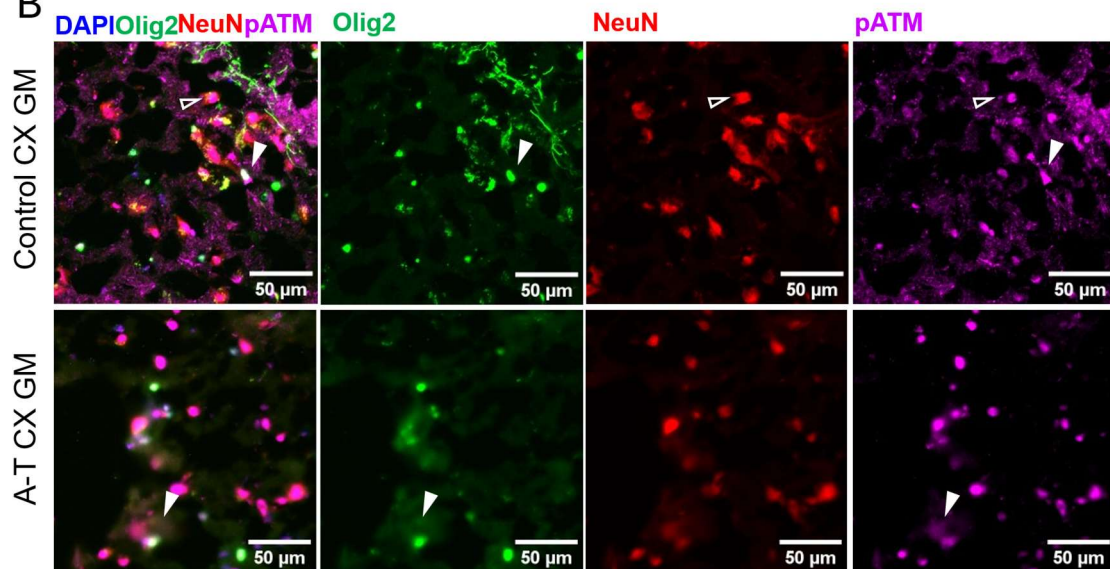
hollow arrowhead indicates thread-like structure probed with phosphorylated ATM. C) Quantification of Olig2⁺ OL density and mean pATM intensity. D) Representative immunohistochemistry images of negative control cerebellar tissue sample. (Control: n=10, A-T subjects: n=10) (unpaired t-test; * p < 0.05, ** p < 0.01, *** p < 0.001)

Within the cerebellar white matter region, there was no Olig2 translocation observed in A-T samples (Figure 14A, B). Co-expression of Olig2 and pATM was also seen in the cerebellar white matter region, while pATM expression was observed in cytoplasmic region (Figure 14B). Thread-like structure was probed with pATM expression in the cerebellar white matter (Figure 3B). Olig2⁺ OL density has no significant change in A-T samples (Figure 14C). Mean pATM intensity against white matter area has no significant change (Figure 14C).

A



B



C

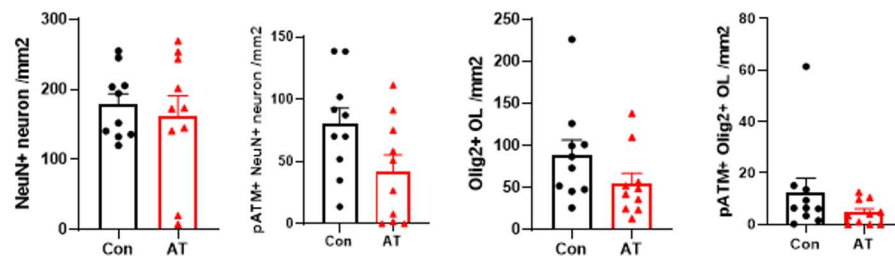


Figure 16. Immunohistology study of OLs, neurons in cerebral grey matter of A-T patients.

A) Representative immunohistochemistry images indicating expression of pATM in neuron (NeuN) and OL (Olig2+) in cerebral grey matter. B) Magnified image panels from the selected region (white square) in figure A. Solid arrowhead indicates Olig2+ OL; hollow arrowhead indicates NeuN+ neurons. C) Quantification of density of NeuN+ neuron, pATM+

NeuN⁺ neuron, Olig2⁺ OL and pATM⁺ Olig2⁺ OL. (Control: n=10, A-T subjects: n=10) (unpaired t-test; * p < 0.05, ** p < 0.01, *** p < 0.001)

To confirm the neuronal and OL pathology in cerebral region, the same immunohistology markers were probe with cerebral frontal cortex tissue from A-T subjects. Image from grey matter indicated co-expression pattern between NeuN⁺ neuron, Olig2⁺ OL and pATM⁺ respectively (Figure 15B). Density of NeuN⁺ neuron and pATM⁺ NeuN⁺ neuron remain unchanged according to statistical analysis (Figure 15C). The averaged densities of Olig2⁺ OL and Olig2⁺ pATM⁺ OL were slightly decreased in A-T grey matter, however the difference between control and A-T sample did not reach significance (Figure 15C).

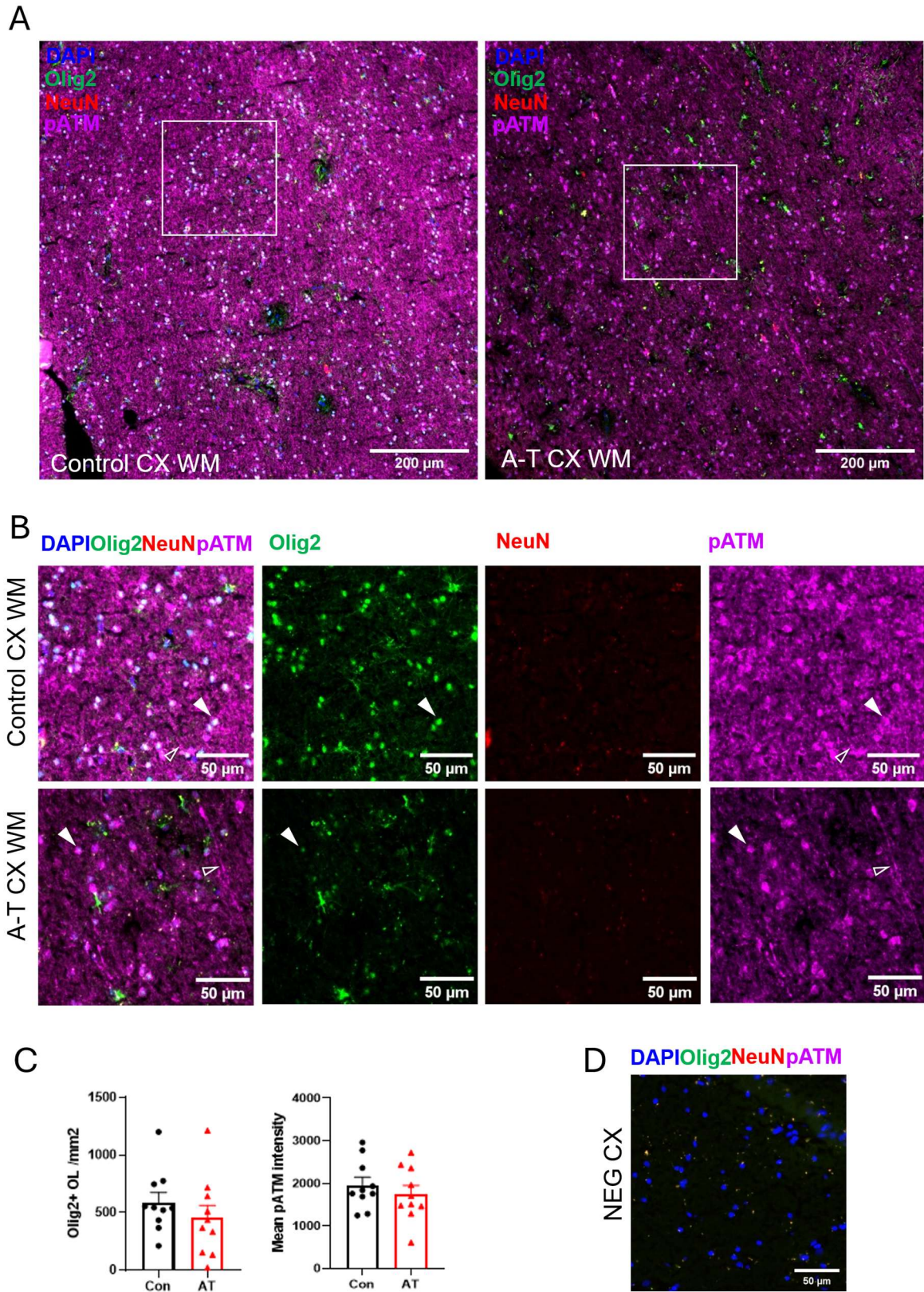


Figure 17. Immunohistology study of OLs in cerebral white matter of A-T patients.

A) Representative immunohistochemistry images indicating expression of pATM in OL (Olig2+) in cerebral white matter.

B) Magnified image panels from the selected region (white square) in figure A. Solid arrowhead indicates Olig2+ OL; hollow arrowhead indicates thread-like structure probed with phosphorylated ATM. C) Quantification of Olig2+ OL density

and mean pATM intensity. D) Representative immunohistochemistry images of negative control cerebellar tissue sample.
(Control: n=10, A-T subjects: n=10) (unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Compared to cerebral grey matter, similar co-expression between Olig2⁺ OL and pATM was found in cerebral white matter region (Figure 16B). In A-T cerebral white matter, the density of Olig2⁺ OL does not significantly alter. Meanwhile, mean pATM intensity has no significant change (Figure 16C).

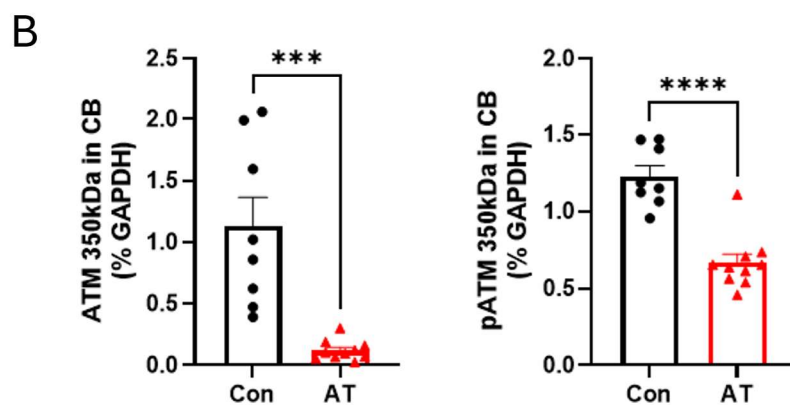
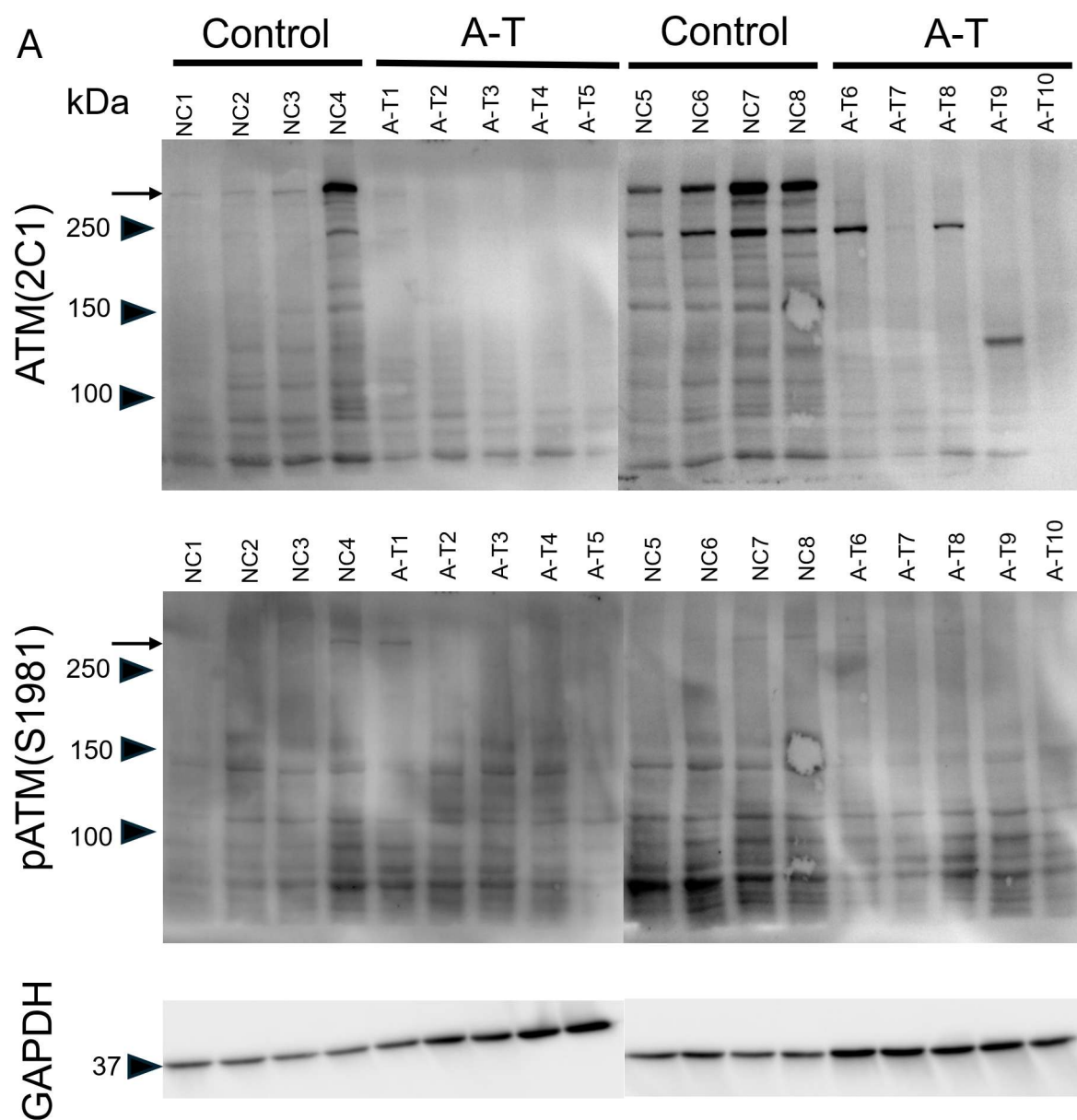


Figure 18. Immunoblotting result of ATM activity in human cerebellar samples.

A) Immunoblot images of ATM(2C1), pATM(S1981) and GAPDH expression in cerebellar tissue. Tailed arrow indicates detection with correct protein full length size. B) Analysis of cerebellar tissue on ATM and pATM expression against GAPDH. (Control: n=8, A-T subjects: n=10) (unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

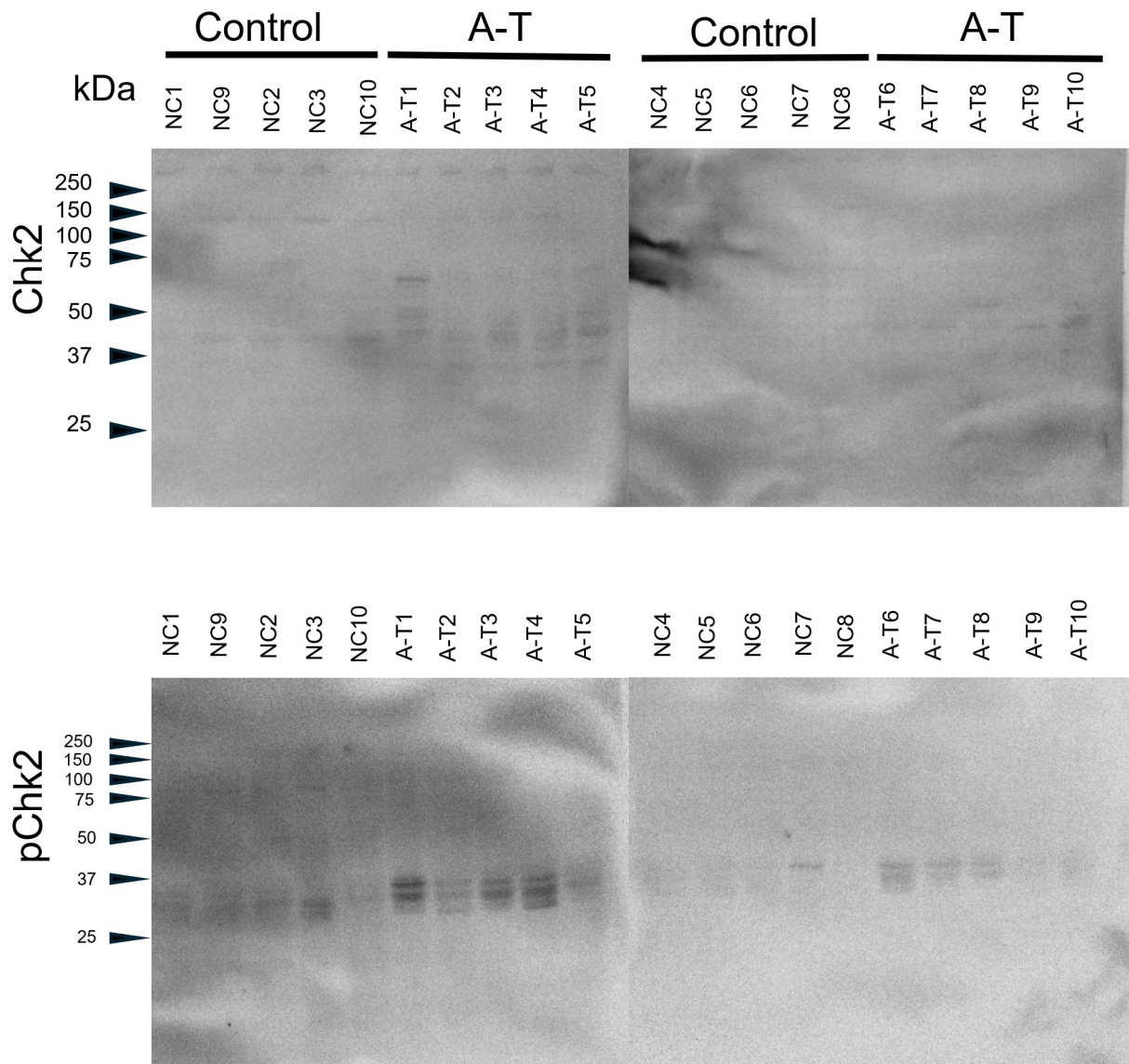


Figure 19. Immunoblotting result of Chk2 and pChk2 expression in human cerebellar tissue.

To verify the total ATM expression and its self-phosphorylation status at S1981 in cerebellar tissue, immunoblotting experiments were employed for comparison between control and A-T samples.

There were multiple bands on both ATM and pATM immunoblot images, which were attributed to different sizes of ATM protein due to truncation or splicing effect (Figure 17A). ATM (2C1) antibody demonstrated its ability to capture 40 peptide fragments from control mice in immunoprecipitation experiment (Li et al., 2011). There was significant reduction of full-length (350kDa) ATM expression and pATM expression in cerebellar samples to 89.3% ($p = 0.0002$) and 45.6% ($p < 0.0001$) respectively (Figure 17B). Immunoblot image of Chk2 and pChk2 in cerebellar tissue failed to demonstrate a quantifiable band at its full length (62 kDa) (Figure 18). Therefore, the images were not quantified for statistical analysis, due to large variations in the blotting result.

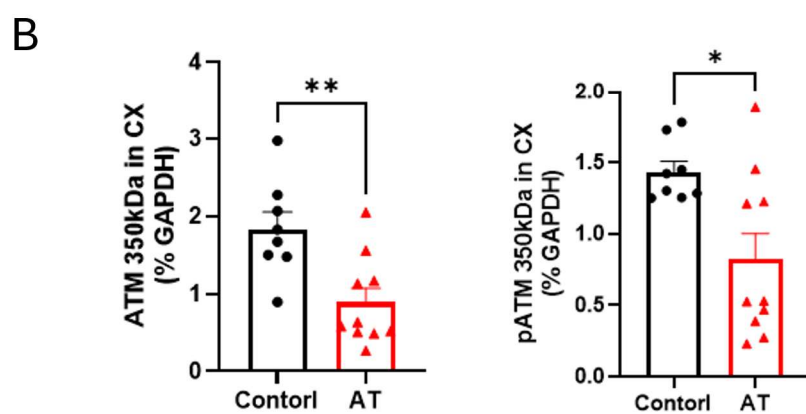
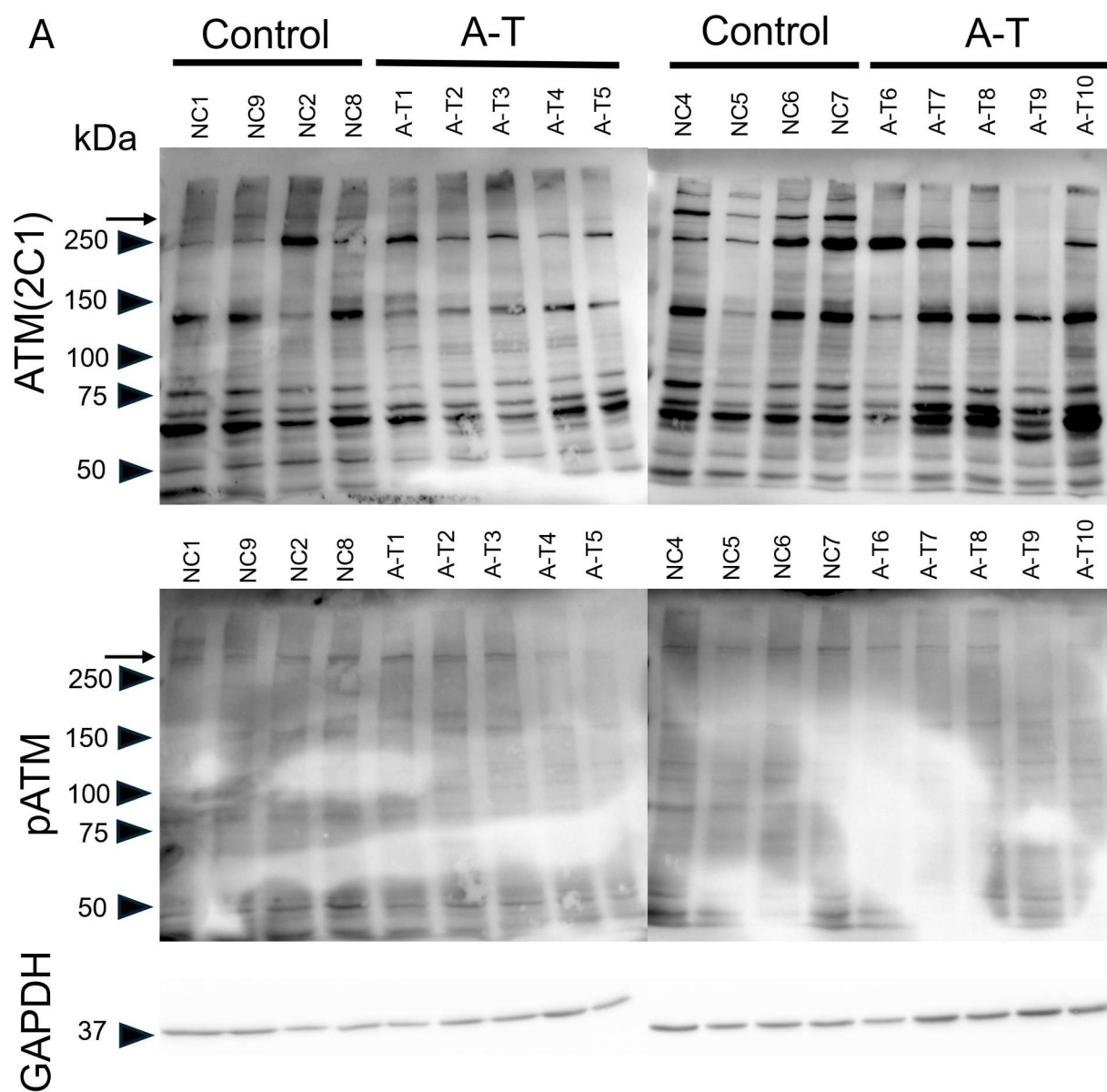


Figure 20. Immunoblotting result of ATM activity in human cerebral cortex samples.

A) Immunoblot images of ATM(2C1), pATM(S1981) and GAPDH expression in cerebral cortex tissue. Tailed arrow indicates detection with correct protein full length size. B) Analysis of cerebral cortex tissue on ATM and pATM expression against GAPDH. (Control: n=8, A-T subjects: n=10) (unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

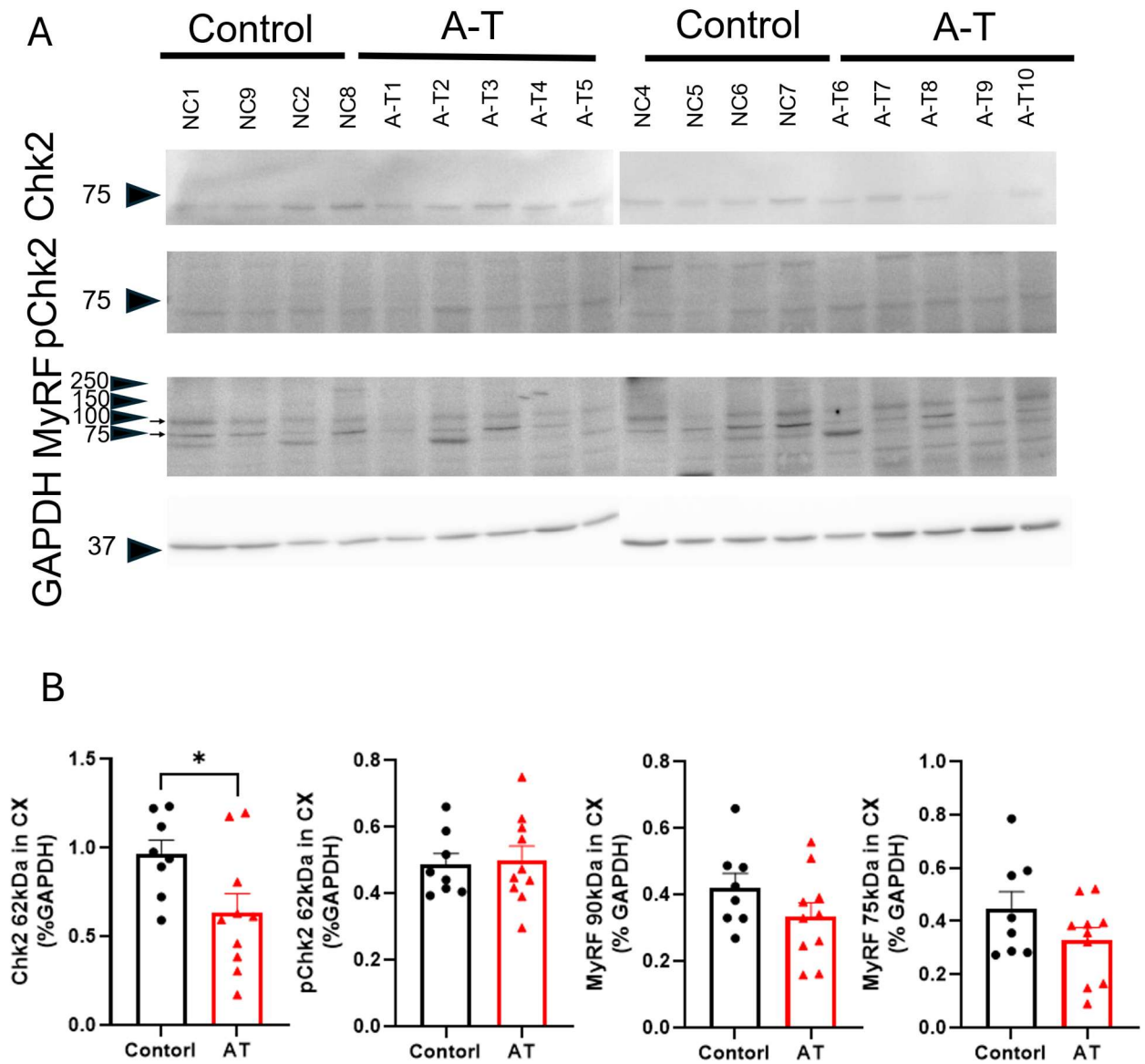


Figure 21. Immunoblotting result of Chk2 and pChk2 expression in human cerebral cortex samples.

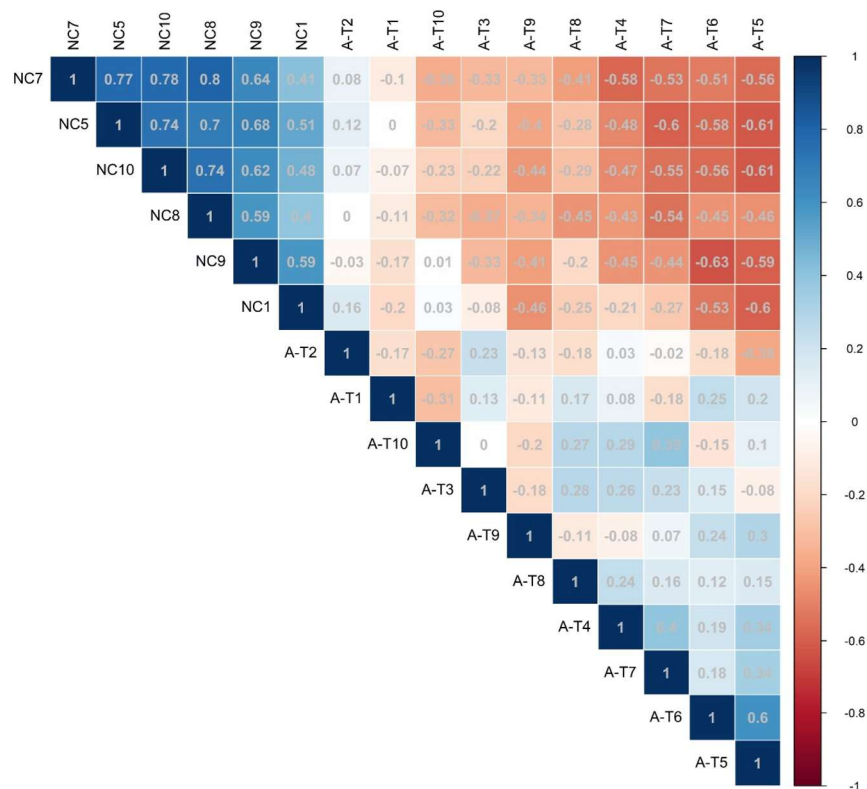
A) Immunoblot images of Chk2, pChk2, MyRF and GAPDH expression in cerebral cortex tissue. Tailed arrowhead indicates specific truncated MyRF protein size at 90 kDa and 75 kDa. B) Analysis of cerebral cortex tissue on Chk2, pChk2 and MyRF (90 kDa and 75 kDa) expression against GAPDH. (Control: n=8, A-T subjects: n=10) (unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Total ATM expression and its phosphorylation status on S1981 were also examined with cerebral cortex tissue. Similarly, full-length ATM expression and pATM expression decreased by 51.2% ($p = 0.004$) and 42.9% ($p = 0.0115$) correspondingly in A-T cerebral sample (Figure 19B). Otherwise, Chk2 expression decreased by 34.7% ($p = 0.034$) in A-T frontal cortex without significant change in phosphorylated chk2 (Thr68) expression (Figure 20B). Threonine 68 phosphorylation of chk2 was mediated by ATM upon DNA double strand break (Ward et al., 2001). Full length MyRF protein (~160kDa) is cleaved into N-fragment (~80-90 kDa) and C-fragment (~75 kDa) in mOL, and the cleaved N-fragment of MyRF would undergo nuclear translocation (Huang et al., 2018). Full length MyRF protein expression was not detectable in the immunoblot image (Figure 20A). Cleaved form of N-fragment (~90 kDa) and C-fragment (~75 kDa) of MyRF were shown in the immunoblot image (Figure 20A). However, both N-fragment and C-fragment had no differences in the A-T cerebral tissues (Figure 20B).

Targeted transcriptomic changes in A-T brain

The loss of ATM expression and auto-phosphorylation at S1981 in cerebellum and cerebral frontal cortex were confirmed with the immunoblotting experiment. To further examine the transcriptomic change in both region upon ATM deficiency, targeted RNA sequencing was employed (TruSight RNA Pan-Cancer Panel) for investigation of mRNA alteration in ATM related pathway, for instance, cell cycle regulation, DNA double strand break repair mechanism. While cerebellar pathology of ATM deficiency in A-T patients was primarily associated with neuronal loss, the pathology in cerebral cortex was often neglected. The targeted RNA sequencing aimed to delineate the potential pathology in A-T cerebral cortex and to empirically compare it with the cerebellar pathology in A-T.

A



B

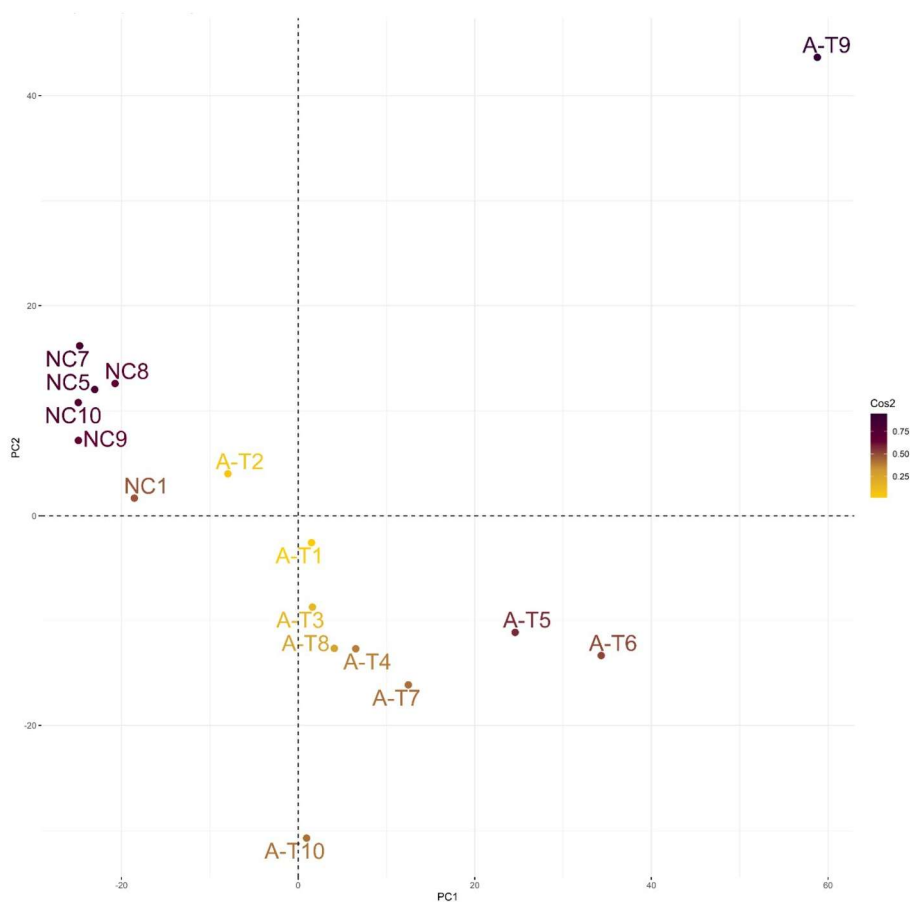


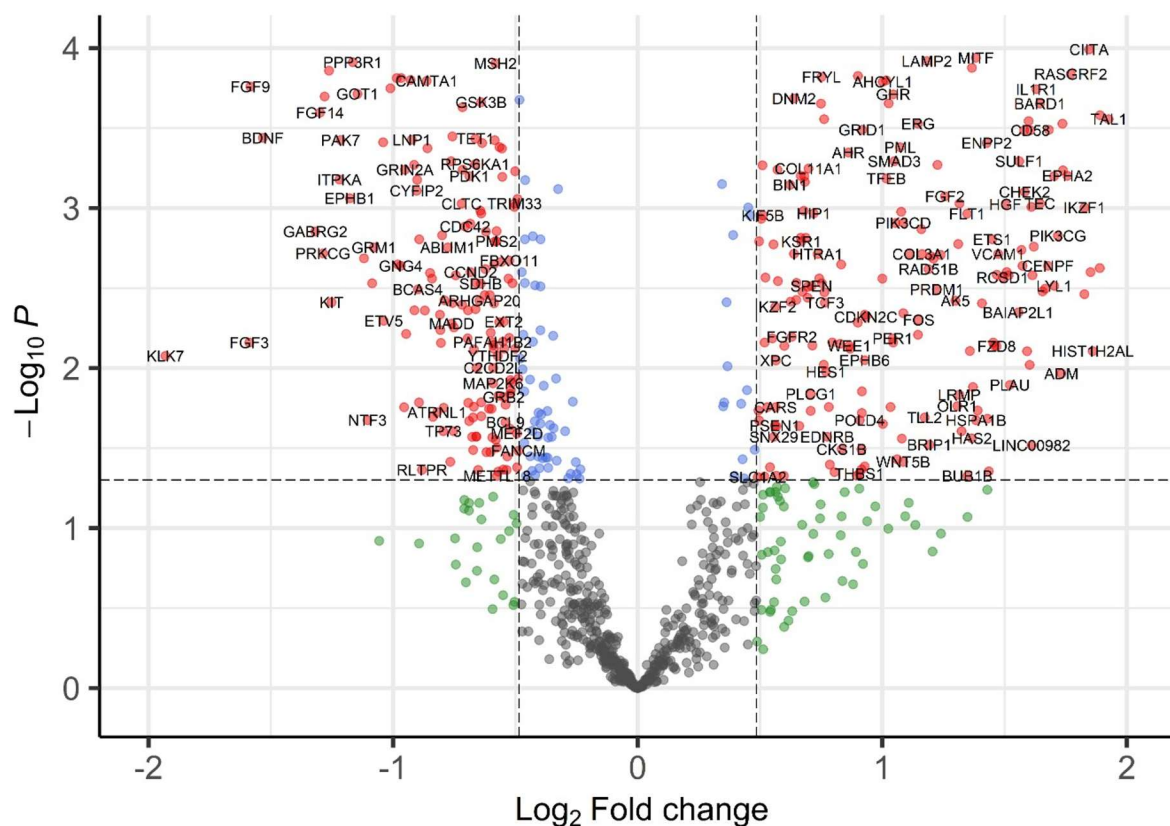
Figure 22. Quality assessment of the targeted RNA sequencing of A-T cerebellum.

(Control: n=6, A-T subjects: n=10) A) Pearson correlation matrix of each sample in the targeted RNA sequencing of A-T cerebellum. B) Principal component analysis (PCA) of each sample in the targeted RNA sequencing of A-T cerebellum.

The Pearson correlation coefficients implying linear correlation among two set of data were normalized between -1 to 1 in the correlation matrix (Figure 21A). Control cerebellum tissues expression patterns were closely correlated to each other in the correlation heatmap. The normalized correlation coefficient had at least 0.4 among control samples, while 52 over 60 comparisons between control and A-T cerebellum scored less than 0 (Figure 21A). According to the principal component analysis (PCA) plot, control cerebellum samples were well clustered in small area (Figure 21B). Otherwise, A-T cerebellum samples demonstrated larger differences among each other with less clustered distribution (Figure 21B).

Cerebellum Control VS AT RNA expression TruSight RNA Pan-Cancer Panel

EnhancedVolcano



total = 1412 variables

- not significant
- 0.4 fold change
- BH-adjusted p-value<0.05
- significant in fold change & BH-adjusted p-value

Figure 23. Volcano plot illustrating DEG from RNA sequencing analysis in cerebellar tissue between control and A-T samples.

(DEG indicated as red dots) (DEG definition: BH-adjusted p-value < 0.05; |fold change| > 0.4) (Control: n=6, A-T subjects: n=10)

Total 1412 genes were annotated with each sample by human reference genome (UCSC hg19). The volcano plot demonstrated fold change against Benjamini–Hochberg false discovery rate (BH-FDR) adjusted p-value of each annotated gene that was calculated by DESeq2 (V 1.6.3) in the comparison between control and A-T cerebellum. The criteria of differential expressed gene (DEG) were percentage of fold change over 0.4 and adjusted p-value less than 0.05. The best cut-off for volcano plot was expected at least around 2-fold change (Love et al., 2014; Stupnikov et al., 2021). However, attributed to the huge variance in A-T clinical samples and restriction of targeted sequencing power, we lift the standard of percentage of fold change to be considered as DEG, which was comparable to another bulk RNA sequencing study of human brain (Wan et al., 2020). There were 368 differentially up-regulated genes and 219 differentially down-regulated genes in A-T cerebellum sample (Figure 22).

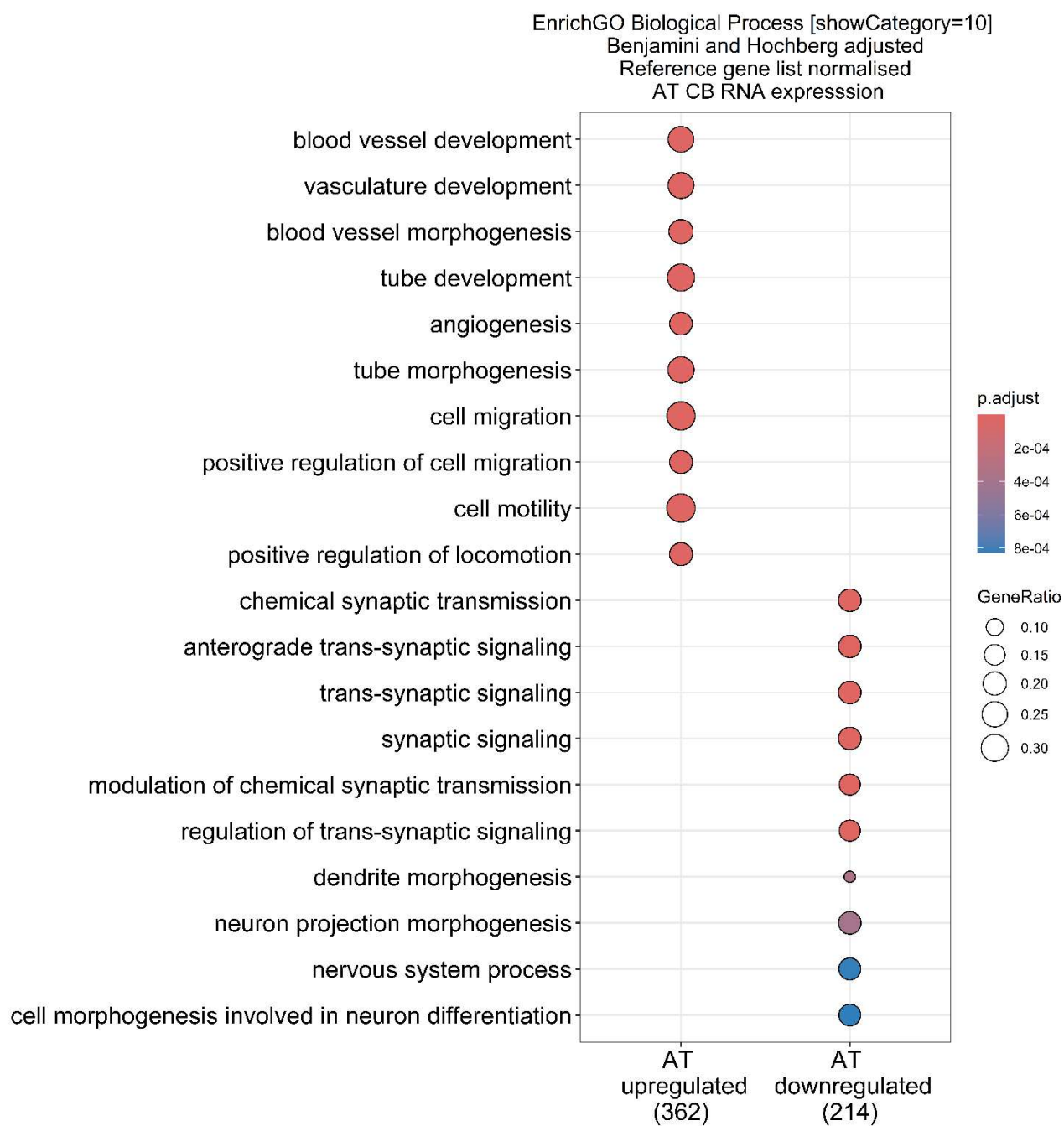


Figure 24. Dot plot for gene set enrichment of DEGs upregulated and downregulated in A-T cerebellar tissue with EnrichGO biological process pathway sets.

Significance of enrichment results were normalized with BH-FDR method. Top 10 significantly enriched pathways were selected for demonstration.

All DEGs of A-T cerebellar tissue were subjected to gene set enrichment analysis which used EnrichGO biological process gene sets to identify enriched pathways. The p-value of each enriched pathway was also adjusted with BH-FDR method. There were 170 and 52 pathways enriched respectively for up-regulated and down-regulated DEGs of A-T cerebellum. Among all enriched pathways, the ten most significant pathways in each enrichment were shown in Figure 23. Blood vessel development (GO:0001568; adjusted p-value= 3.16×10^{-9}), vasculature development (GO: 0001944; adjusted p-value= 3.16×10^{-9}) and blood vessel morphogenesis (GO: 0048514; adjusted p-value= 1.19×10^{-8}) were the most significant pathways enriched with up-regulated DEGs of A-T cerebellum RNA expression. Chemical synaptic transmission (GO: 0007268; adjusted p-value= 6.14×10^{-7}), anterograde trans-synaptic signaling (GO: 0098916; adjusted p-value= 6.14×10^{-7}) and trans-synaptic signaling (GO: 0099537; adjusted p-value= 6.14×10^{-7}) were the most significant pathways enriched with down-regulated DEGs of cerebellum comparison.

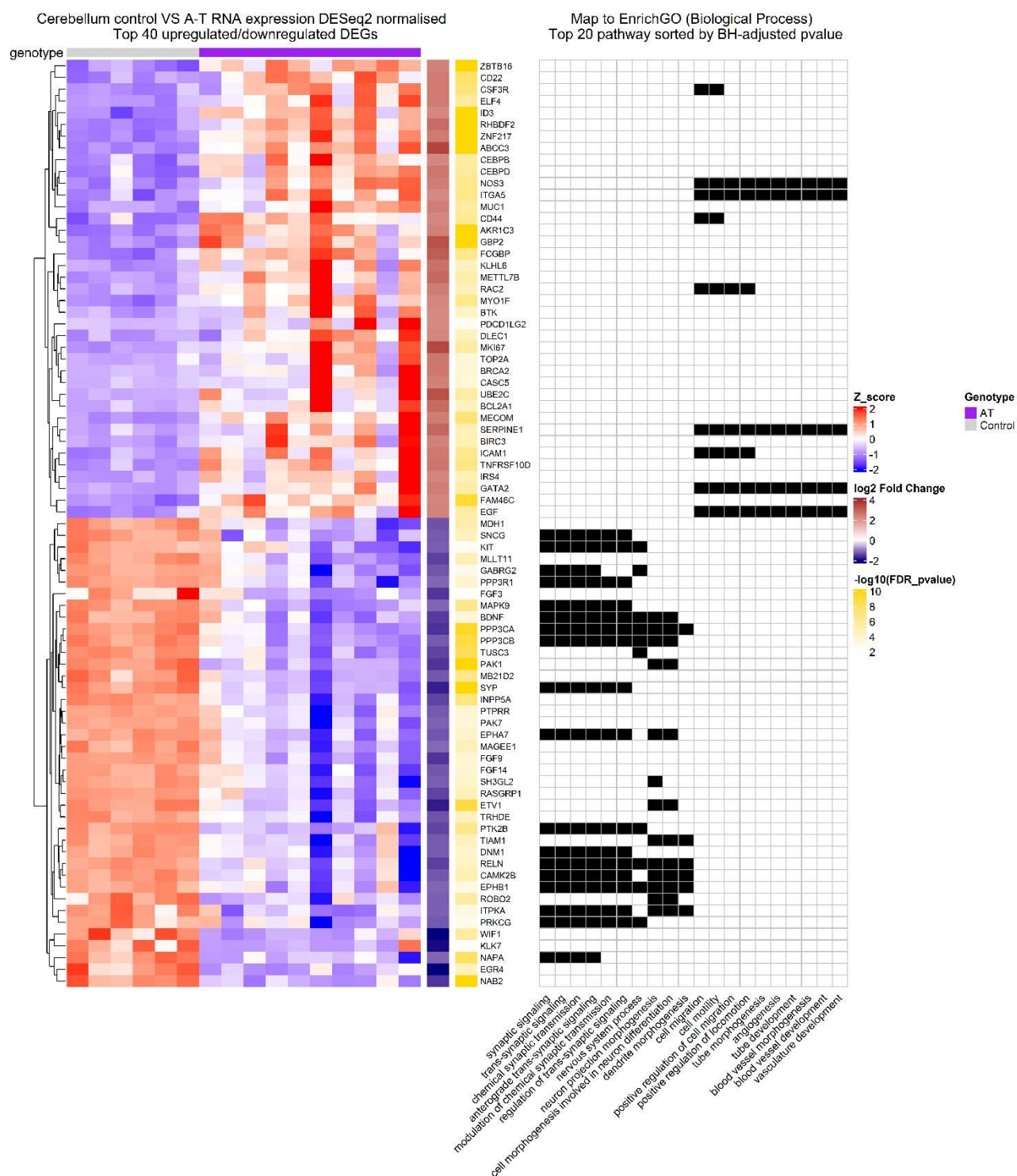


Figure 25. Heatmap illustrating adjusted read counts of top 40 upregulated and downregulated A-T cerebellum DEGs with Z-score normalization.

Normalized fold change and adjusted p-value of each DEG were leveled with color gradient aside the heatmap. Ten of the most significantly enriched pathways (EnrichGO: biological process) from each up-regulated and down-regulated DEG sets were matched with the DEGs illustrated in the heatmap.

The heatmap was plotted with A-T cerebellar DEGs of 40 highest fold change for both up- and down- regulation. Scaled fold change (FC) and adjusted p-value (p^{adj}) were aligned on the right side of the heatmap. All top 40 upregulated and downregulated genes were then mapped onto top 10 significantly enriched pathways from EnrichGO gene set enrichment analysis.

Among all upregulated DEGs, *CSF3R* ($\log_2$ FC = 2.53; p^{adj} = 4.24×10^{-9}), *NOS3* ($\log_2$ FC = 2.47; p^{adj} = 3.36×10^{-7}), *ITGA5* ($\log_2$ FC = 2.26; p^{adj} = 2.72×10^{-8}), *CD44* ($\log_2$ FC = 2.27; p^{adj} = 8.58×10^{-8}), *RAC2* ($\log_2$ FC = 2.48; p^{adj} = 2.53×10^{-5}), *SERPINE1* ($\log_2$ FC = 2.94; p^{adj} = 1.74×10^{-5}), *ICAM1* ($\log_2$ FC = 2.55; p^{adj} = 4.63×10^{-7}), *GATA2* ($\log_2$ FC = 2.50; p^{adj} = 3.63×10^{-6}) and *EGF* ($\log_2$ FC = 2.37; p^{adj} = 3.18×10^{-6}) were associated with the upregulation pathways. Among all downregulated DEGs, *SNCG* ($\log_2$ FC = -1.27; p^{adj} = 2.84×10^{-5}), *KIT* ($\log_2$ FC = -1.25; p^{adj} = 3.88×10^{-3}), *GABRG2* ($\log_2$ FC = -1.32; p^{adj} = 0.0014), *PPP3R1* ($\log_2$ FC = -1.17; p^{adj} = 1.22×10^{-4}), *MAPK9* ($\log_2$ FC = -1.18; p^{adj} = 3.56×10^{-7}), *BDNF* ($\log_2$ FC = -1.53; p^{adj} = 3.64×10^{-4}), *PPP3CA* ($\log_2$ FC = -1.62; p^{adj} = 2.72×10^{-10}), *PPP3CB* ($\log_2$ FC = -1.27; p^{adj} = 1.25×10^{-9}), *TUSC3* ($\log_2$ FC = -1.47; p^{adj} = 5.2×10^{-9}), *PAK1* ($\log_2$ FC = -1.6; p^{adj} = 1.82×10^{-14}), *SYP* ($\log_2$ FC = -1.78; p^{adj} = 9.57×10^{-11}), *EPHA7* ($\log_2$ FC = -1.3; p^{adj} = 9.97×10^{-5}), *SH3GL2* ($\log_2$ FC = -1.24; p^{adj} = 8.74×10^{-5}), *ETV1* ($\log_2$ FC = -1.8; p^{adj} = 1.49×10^{-9}), *PTK2B* ($\log_2$ FC = -1.45; p^{adj} = 3.6×10^{-7}), *TLAM1* ($\log_2$ FC = -1.28; p^{adj} = 3.28×10^{-5}), *DNMI* ($\log_2$ FC = -1.26; p^{adj} = 1.38×10^{-4}), *RELN* ($\log_2$ FC = -1.5; p^{adj} = 4.38×10^{-5}), *CAMK2B* ($\log_2$ FC = -1.29; p^{adj} = 8.76×10^{-6}), *EPHB1* ($\log_2$ FC = -1.18; p^{adj} = 8.67×10^{-4}), *ROBO2* ($\log_2$ FC = -1.23; p^{adj} = 2.15×10^{-6}), *ITPKA* ($\log_2$ FC = -1.22; p^{adj} = 6.63×10^{-4}), *PRKCG* ($\log_2$ FC = -1.29; p^{adj} = 1.92×10^{-3}) and *NAPA* ($\log_2$ FC = -1.18; p^{adj} = 1.26×10^{-8}) were associated with the downregulation pathways.

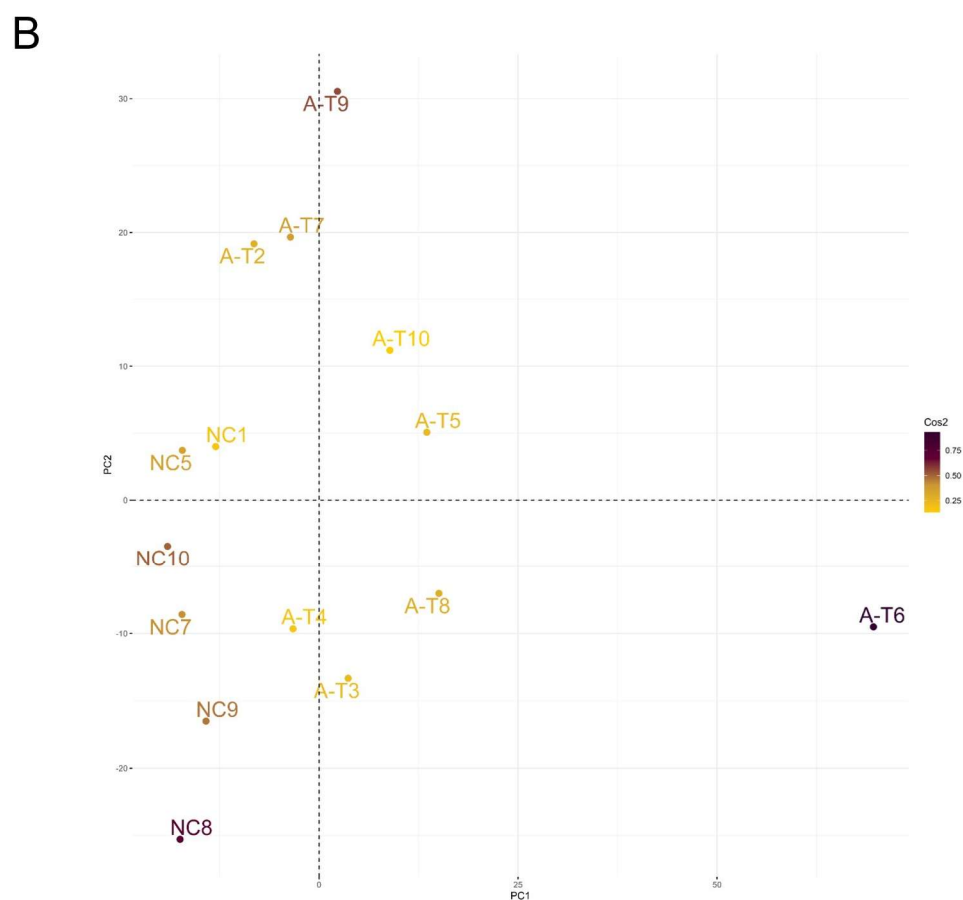
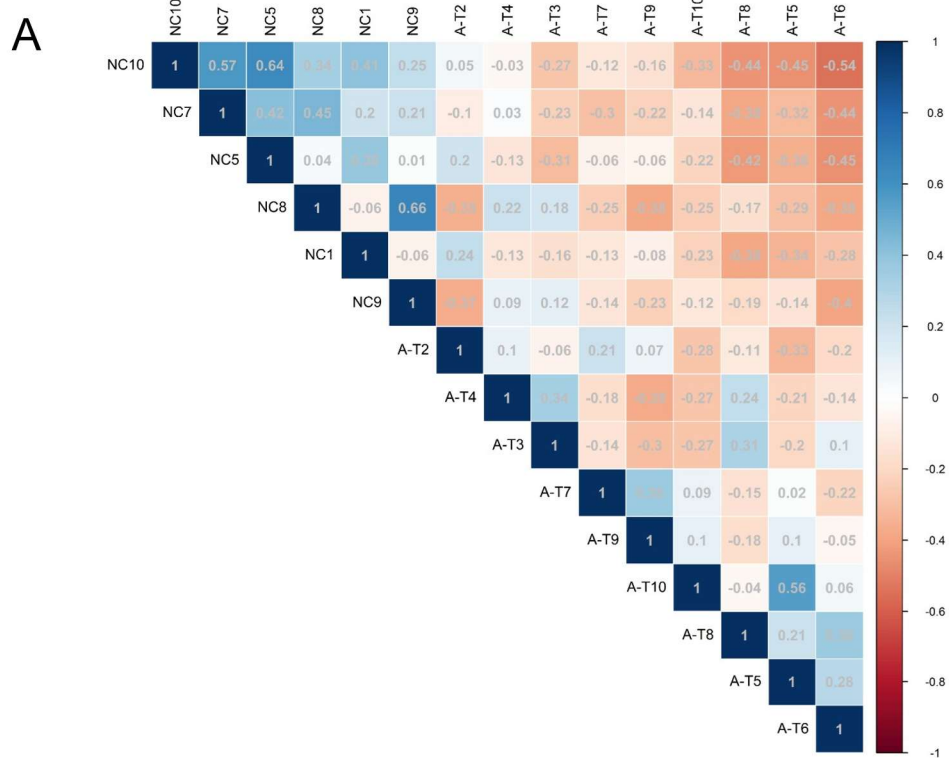


Figure 26. Quality assessment of the targeted RNA sequencing of A-T cerebral frontal cortex.

(Control: n=6, A-T subjects: n=9). A) Pearson correlation matrix of each sample in the targeted RNA sequencing of A-T cerebral frontal cortex. B) PCA of each sample in the targeted RNA sequencing of A-T cerebral frontal cortex.

The Pearson correlation coefficients were normalized between -1 to 1 in the correlation matrix. Compared with cerebellum tissue, control cerebral frontal cortex tissues RNA expressions were less closely correlated to each other in the correlation heatmap. Only 6 over 15 normalized correlation coefficients of internal comparison among control samples were more than 0.4 (Figure 25A). Difference between control and A-T samples remained huge in cerebral frontal cortex tissues, 52 over 60 comparisons between control and A-T cerebral frontal cortex tissues scored less than 0 (Figure 25A). According to the PCA plot, control cerebral frontal cortex samples were clustered in larger ellipse area compared with the PCA plot of cerebellum (Figure 25B). Yet, A-T cerebral frontal cortex samples retained huge differences among each other with wide-spread distribution (Figure 25B).

EnhancedVolcano

(DEG indicated as red dots) (DEG definition: BH-adjusted p-value < 0.05; |fold change| > 0.4) (Control: n=6, A-T subjects: n=9)

The volcano plot demonstrated fold change against BH-FDR adjusted p-value of each annotated gene that was calculated by DESeq2 (V 1.6.3) in the comparison between control and A-T cerebral frontal cortex. There were 169 differentially up-regulated genes and 81 differentially down-regulated genes in A-T cerebral frontal cortex sample (Figure 26).

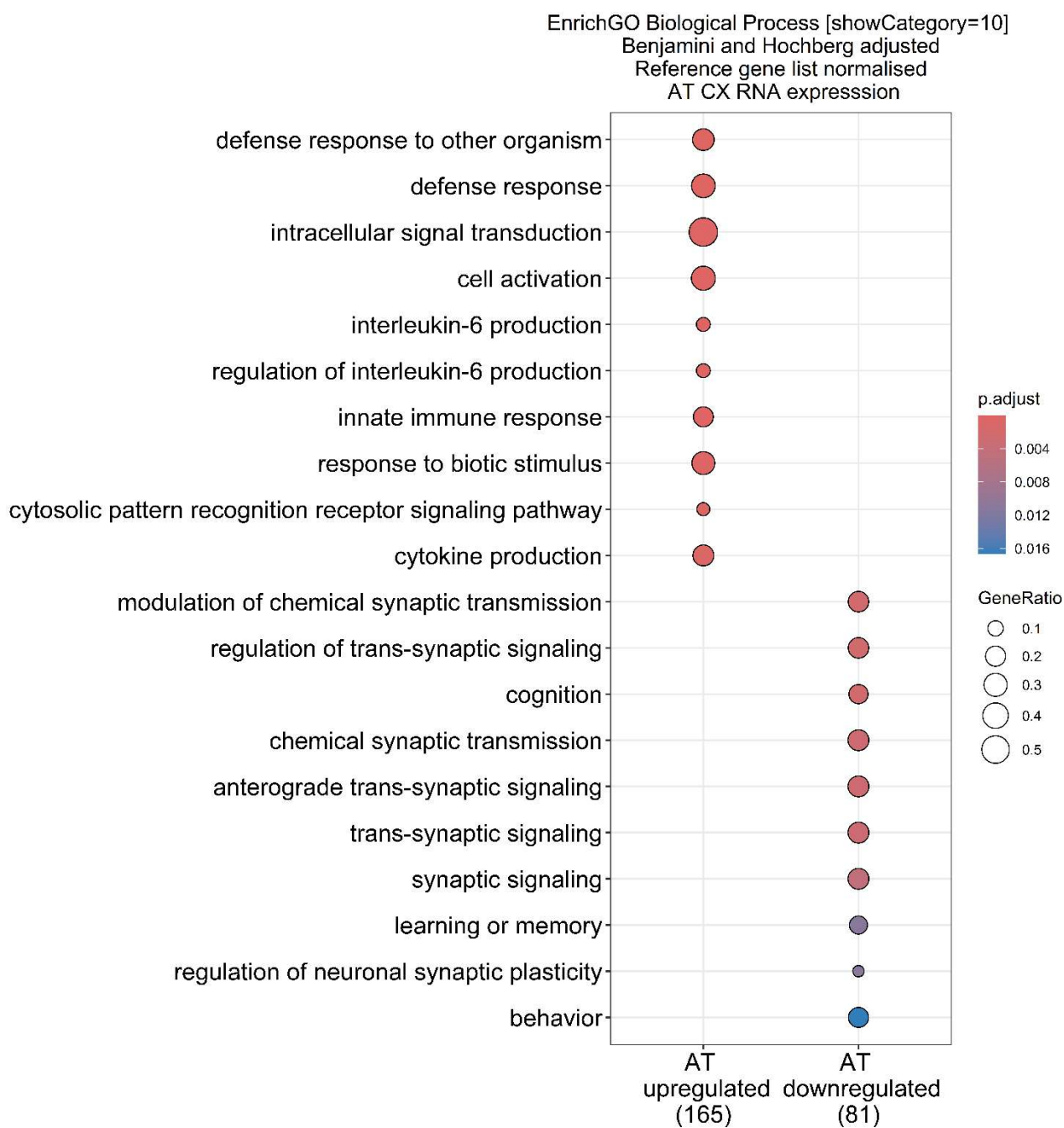


Figure 28. Dot plot for gene set enrichment of DEGs upregulated and downregulated in A-T cerebral frontal cortex tissue with EnrichGO biological process pathway sets.

Significance of enrichment results were normalized with BH-FDR method. Top 10 significantly enriched pathways were selected for demonstration.

There were 223 and 13 pathways enriched respectively for up-regulated and down-regulated DEGs of cerebral frontal cortex comparison.

Defense response to other organism (GO: 0098542; adjusted p-value = 7.82×10^{-6}), defense response (GO: 0006952; adjusted p-value = 1.02×10^{-5}) and intracellular signal transduction (GO: 0035556; adjusted p-value = 1.88×10^{-4}) were the most significant pathways enriched with up-regulated DEGs of A-T cerebral frontal cortex RNA expression. Modulation of chemical synaptic transmission (GO: 0050804; adjusted p-value = 0.00215), regulation of trans-synaptic signaling (GO: 0099177; adjusted p-value = 0.00215) and cognition (GO: 0050890; adjusted p-value = 0.00215) were the most significant pathways enriched with down-regulated DEGs of cerebral comparison (Figure 27).

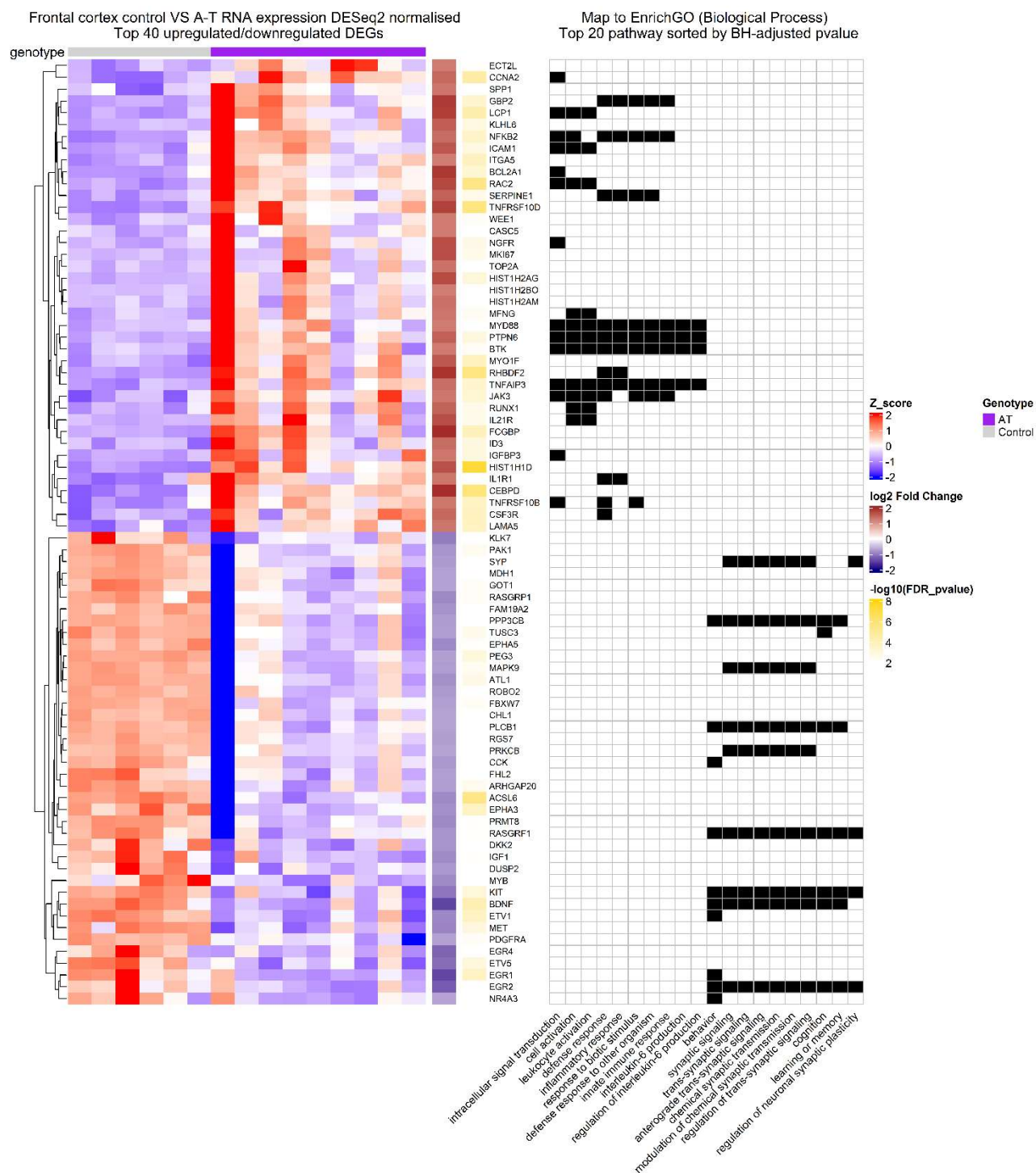


Figure 29. Heatmap illustrating adjusted read counts of top 40 upregulated and downregulated A-T cerebral frontal cortex DEGs with Z-score normalization.

Normalized fold change and adjusted p-value of each DEG were leveled with color gradient aside the heatmap. Ten of the most significantly enriched pathways (EnrichGO: biological process) from each up-regulated and down-regulated DEG sets were matched with the DEGs illustrated in the heatmap.

The heatmap was plotted with DEGs of 40 highest fold change for both up- and down-regulation in A-T cerebral frontal cortex (Figure 28).

Among all the upregulated DEGs listed in the heatmap, *CCNA2* ($\log_2$ FC = 1.45; $p^{\text{adj}} = 2.81 \times 10^{-5}$), *GBP2* ($\log_2$ FC = 1.87; $p^{\text{adj}} = 8.82 \times 10^{-6}$), *LCPI* ($\log_2$ FC = 1.84; $p^{\text{adj}} = 5.28 \times 10^{-7}$), *NFKB2* ($\log_2$ FC = 1.41; $p^{\text{adj}} = 2.51 \times 10^{-4}$), *ICAMI* ($\log_2$ FC = 1.62; $p^{\text{adj}} = 1.50 \times 10^{-3}$), *BCL2A1* ($\log_2$ FC = 1.89; $p^{\text{adj}} = 5.17 \times 10^{-4}$), *RAC2* ($\log_2$ FC = 1.74; $p^{\text{adj}} = 3.60 \times 10^{-6}$), *SERPINE1* ($\log_2$ FC = 1.51; $p^{\text{adj}} = 2.34 \times 10^{-3}$), *NGFR* ($\log_2$ FC = 1.71; $p^{\text{adj}} = 1.99 \times 10^{-3}$), *MFNG* ($\log_2$ FC = 1.33; $p^{\text{adj}} = 4.57 \times 10^{-3}$), *MYD88* ($\log_2$ FC = 1.27; $p^{\text{adj}} = 0.0104$), *PTPN6* ($\log_2$ FC = 1.53; $p^{\text{adj}} = 1.11 \times 10^{-3}$), *BTK* ($\log_2$ FC = 1.26; $p^{\text{adj}} = 5.63 \times 10^{-3}$), *RHBDF2* ($\log_2$ FC = 1.94; $p^{\text{adj}} = 2.25 \times 10^{-6}$), *TNFAIP3* ($\log_2$ FC = 1.39; $p^{\text{adj}} = 4.39 \times 10^{-4}$), *JAK3* ($\log_2$ FC = 1.30; $p^{\text{adj}} = 4.95 \times 10^{-4}$), *RUNXI* ($\log_2$ FC = 1.50; $p^{\text{adj}} = 2.39 \times 10^{-3}$), *IL2IR* ($\log_2$ FC = 1.70; $p^{\text{adj}} = 2.69 \times 10^{-3}$), *IGFBP3* ($\log_2$ FC = 1.31; $p^{\text{adj}} = 1.09 \times 10^{-3}$), *IL1RI* ($\log_2$ FC = 1.32; $p^{\text{adj}} = 8.17 \times 10^{-3}$), *TNFRSF10B* ($\log_2$ FC = 1.31; $p^{\text{adj}} = 1.91 \times 10^{-4}$), and *CSF3R* ($\log_2$ FC = 1.44; $p^{\text{adj}} = 1.41 \times 10^{-4}$) were associated with the upregulated pathways.

Among all the downregulated DEGs listed in the heatmap, *SYP* ($\log_2$ FC = -0.76; $p^{\text{adj}} = 0.0196$), *PPP3CB* ($\log_2$ FC = -0.75; $p^{\text{adj}} = 0.0123$), *TUSC3* ($\log_2$ FC = -0.74; $p^{\text{adj}} = 2.77 \times 10^{-3}$), *MAPK9* ($\log_2$ FC = -0.74; $p^{\text{adj}} = 4.15 \times 10^{-3}$), *PLCBI* ($\log_2$ FC = -0.69; $p^{\text{adj}} = 0.02$), *PRKCB* ($\log_2$ FC = -0.69; $p^{\text{adj}} = 0.0368$), *CCK* ($\log_2$ FC = -0.78; $p^{\text{adj}} = 0.0229$), *RASGRF1* ($\log_2$ FC = -0.81; $p^{\text{adj}} = 7.01 \times 10^{-3}$), *KIT* ($\log_2$ FC = -1.05; $p^{\text{adj}} = 7.73 \times 10^{-4}$), *BDNF* ($\log_2$ FC = -1.45; $p^{\text{adj}} = 5.91 \times 10^{-5}$), *ETVI* ($\log_2$ FC = -0.91; $p^{\text{adj}} = 1.91 \times 10^{-4}$), *EGR1* ($\log_2$ FC = -1.44; $p^{\text{adj}} = 2.04 \times 10^{-4}$), *EGR2* ($\log_2$ FC = -1.27; $p^{\text{adj}} = 0.0136$), *NR4A3* ($\log_2$ FC = -0.70; $p^{\text{adj}} = 0.0425$) were associated with the downregulated pathways.

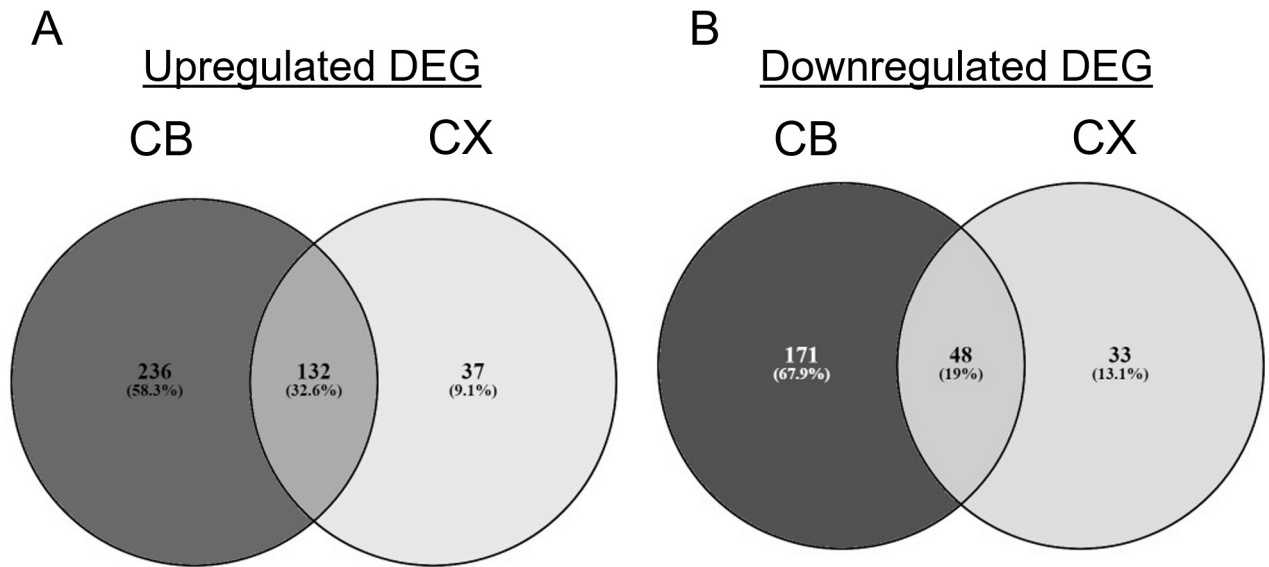


Figure 30. Venn diagram illustrating similarity between up-regulated and down-regulated DEG sets for cerebellum and cerebral frontal cortex comparison.

A) Venn diagram with up-regulated DEG sets. B) Venn diagram with down-regulated DEG sets.

Gene sets of upregulate and downregulate DEGs were collected from cerebellar and cerebral frontal cortex comparison. To investigate the region-specific effect of ATM deficiency in each brain region, venn diagram was plotted against the similarity of upregulated and downregulated DEG gene sets from the comparison of two brain regions (Figure 29). There were 236 and 37 upregulated DEGs unique to cerebellum and cerebral comparison respectively, while 132 common upregulated DEGs were found between two sets of gene. There were 171 and 33 downregulated DEGs unique to cerebellum and cerebral comparison respectively, while 48 common upregulated DEGs were found between two sets of genes. To extend the investigation, each group in upregulated and downregulated DEGs was subjected to gene set enrichment analysis with EnrichGO biological process gene sets.

Cluster	ID	Description	Count	pvalue	p.adjust
cortex only	GO:0140888	interferon-mediated signaling pathway	5	0.000102	0.129715
cortex only	GO:0060337	type I interferon-mediated signaling pathway	4	0.000251	0.129715
cortex only	GO:0071357	cellular response to type I interferon	4	0.000251	0.129715
cortex only	GO:0034340	response to type I interferon	4	0.000355	0.137603
cortex only	GO:0006334	nucleosome assembly	4	0.000849	0.26311
cortex only	GO:0065004	protein-DNA complex assembly	5	0.001308	0.337638
cortex only	GO:0034728	nucleosome organization	4	0.003568	0.721735
cortex only	GO:0046580	negative regulation of Ras protein signal transduction	3	0.004115	0.721735
cortex only	GO:0007266	Rho protein signal transduction	4	0.004193	0.721735
cortex only	GO:0051058	negative regulation of small GTPase mediated signal transduction	3	0.005243	0.812113
cerebellum only	GO:0001568	blood vessel development	61	6.45E-07	0.001677
cerebellum only	GO:0001944	vasculature development	61	2.72E-06	0.003474
cerebellum only	GO:0035239	tube morphogenesis	66	4.01E-06	0.003474
cerebellum only	GO:0048514	blood vessel morphogenesis	51	7.21E-06	0.004686
cerebellum only	GO:0035295	tube development	71	1.22E-05	0.00632
cerebellum only	GO:0016477	cell migration	79	5.16E-05	0.020449
cerebellum only	GO:0048870	cell motility	81	5.51E-05	0.020449
cerebellum only	GO:0030198	extracellular matrix organization	20	8.55E-05	0.022096
cerebellum only	GO:0043062	extracellular structure organization	20	8.55E-05	0.022096
cerebellum only	GO:0045229	external encapsulating structure organization	20	8.55E-05	0.022096
common upregulated	GO:0098542	defense response to other organism	34	1.1E-07	0.000251
common upregulated	GO:0006952	defense response	44	2.16E-07	0.000251
common upregulated	GO:0032635	interleukin-6 production	13	4.12E-07	0.000251
common upregulated	GO:0032675	regulation of interleukin-6 production	13	4.12E-07	0.000251
common upregulated	GO:0002753	cytosolic pattern recognition receptor signaling pathway	12	2.12E-06	0.001034
common upregulated	GO:0035872	nucleotide-binding domain, leucine rich repeat containing receptor signaling pathway	8	3.27E-06	0.001132
common upregulated	GO:0070423	nucleotide-binding oligomerization domain containing signaling pathway	8	3.27E-06	0.001132
common upregulated	GO:0042060	wound healing	24	3.71E-06	0.001132
common upregulated	GO:0001775	cell activation	45	8.75E-06	0.00205
common upregulated	GO:0009607	response to biotic stimulus	40	9.24E-06	0.00205

Table 4. Summarized table demonstrating top 10 pathways significantly enriched from uniquely and commonly upregulated DEGs of cerebellum and cerebral frontal cortex.

(EnrichGO: biological process) (p.adjust: BH-adjusted p-value)

Interferon related pathways enriched for the upregulated DEGs unique to cerebral cortex have no significance due to small DEG size. Vasculature related pathways were significantly enriched for upregulated DEGs unique to cerebellum, such as blood vessel development ($p = 0.001677$), vasculature development ($p = 0.003474$) and tube morphogenesis ($p = 0.003474$). Two pathways related to defense response were significantly enriched with the common upregulated DEGs (GO:0098542, $p = 0.000251$; GO:0006952, $p = 0.000251$).

Cluster	ID	Description	Count	pvalue	p.adjust
cortex only	GO:0071417	cellular response to organonitrogen compound	12	0.000389	0.138487
cortex only	GO:0034762	regulation of transmembrane transport	8	0.000495	0.138487
cortex only	GO:1901699	cellular response to nitrogen compound	12	0.000531	0.138487
cortex only	GO:1901700	response to oxygen-containing compound	19	0.000563	0.138487
cortex only	GO:1901701	cellular response to oxygen-containing compound	16	0.000835	0.138487
cortex only	GO:0055007	cardiac muscle cell differentiation	5	0.001194	0.138487
cortex only	GO:0050804	modulation of chemical synaptic transmission	8	0.001208	0.138487
cortex only	GO:0099177	regulation of trans-synaptic signaling	8	0.001208	0.138487
cortex only	GO:0010817	regulation of hormone levels	7	0.001299	0.138487
cortex only	GO:0055085	transmembrane transport	9	0.00137	0.138487
cerebellum only	GO:0007268	chemical synaptic transmission	30	3.08E-06	0.003099
cerebellum only	GO:0098916	anterograde trans-synaptic signaling	30	3.08E-06	0.003099
cerebellum only	GO:0099537	trans-synaptic signaling	30	3.91E-06	0.003099
cerebellum only	GO:0048813	dendrite morphogenesis	13	6.5E-06	0.003861
cerebellum only	GO:0099536	synaptic signaling	30	9.74E-06	0.004627
cerebellum only	GO:0050804	modulation of chemical synaptic transmission	25	2.5E-05	0.008481
cerebellum only	GO:0099177	regulation of trans-synaptic signaling	25	2.5E-05	0.008481
cerebellum only	GO:0016358	dendrite development	16	4.06E-05	0.01205
cerebellum only	GO:0099504	synaptic vesicle cycle	12	6.72E-05	0.017745
cerebellum only	GO:0060997	dendritic spine morphogenesis	8	0.000134	0.031714
common downregulated	GO:0048168	regulation of neuronal synaptic plasticity	4	0.001446	0.706192
common downregulated	GO:0050890	cognition	8	0.002022	0.706192
common downregulated	GO:0048167	regulation of synaptic plasticity	6	0.002902	0.706192
common downregulated	GO:0007613	memory	5	0.002904	0.706192
common downregulated	GO:0007409	axonogenesis	10	0.003459	0.706192
common downregulated	GO:0048667	cell morphogenesis involved in neuron differentiation	11	0.004158	0.706192
common downregulated	GO:0010821	regulation of mitochondrion organization	5	0.004881	0.706192
common downregulated	GO:0043547	positive regulation of GTPase activity	5	0.004881	0.706192
common downregulated	GO:0050804	modulation of chemical synaptic transmission	9	0.005272	0.706192
common downregulated	GO:0099177	regulation of trans-synaptic signaling	9	0.005272	0.706192

Table 5. Summarized table demonstrating top 10 pathways significantly enriched from uniquely and commonly downregulated DEGs of cerebellum and cerebral frontal cortex.

(EnrichGO: biological process) (p.adjust: BH-adjusted p-value)

There was no significant pathway enriched from downregulated DEGs unique to cerebral cortex and the common downregulated DEGs. However, synaptic signaling related pathways were significantly enriched with downregulated DEGs unique to cerebellum, for example, chemical synaptic transmission ($p = 0.003099$), anterograde trans-synaptic signaling ($p = 0.003099$) and trans-synaptic signaling ($p = 0.003099$).

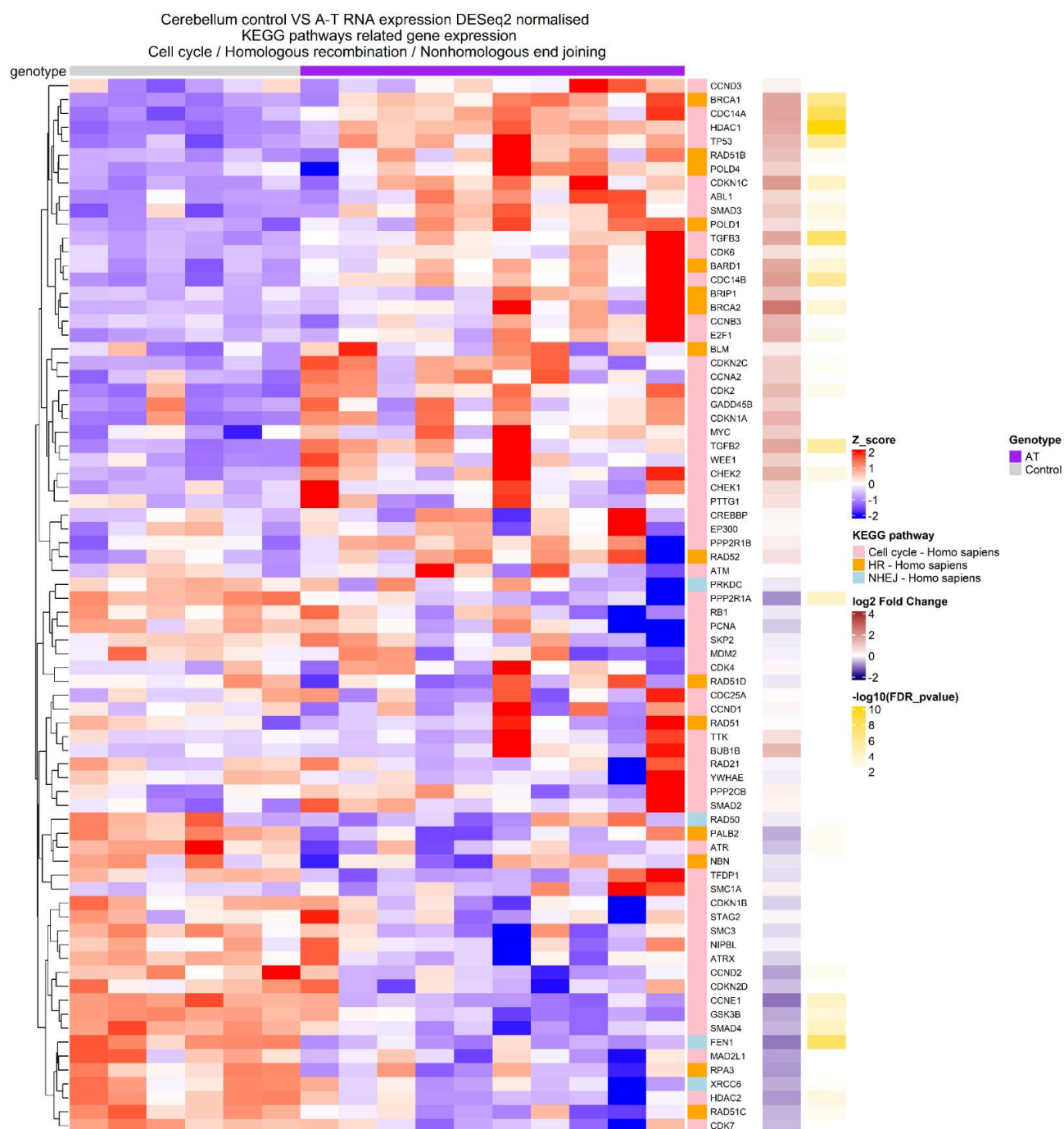


Figure 31. Heatmap illustrating A-T cerebellum DEGs involved in cell cycle (hsa04110), homologous recombination (hsa03440) and non-homologous end joining (hsa03450) KEGG pathways with Z-score normalization.

Normalized fold change and adjusted p-value of each gene were leveled with color gradient aside the heatmap.

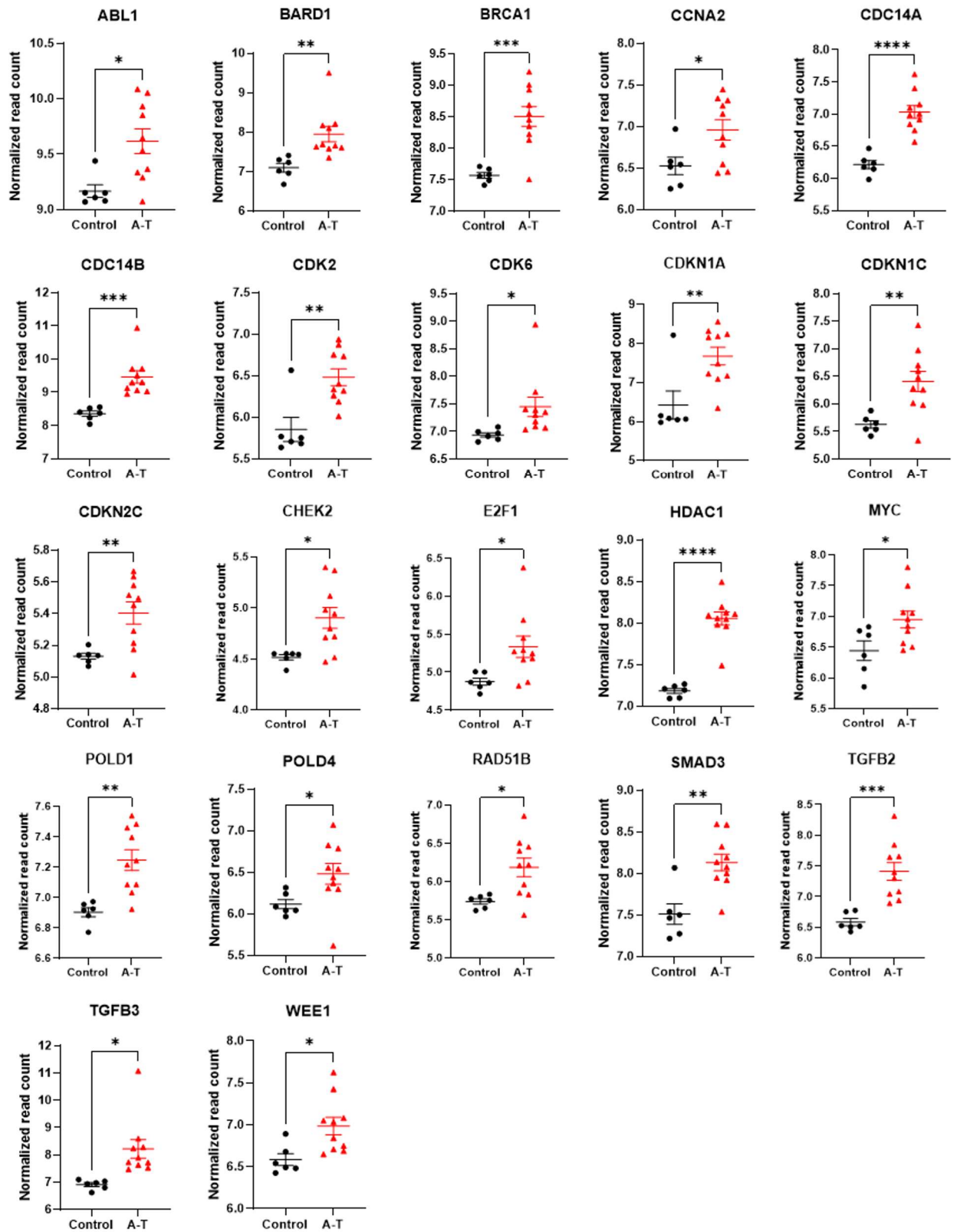


Figure 32. Column comparison illustrating upregulated DEGs with significant increased normalized read counts in A-T cerebellum related to KEGG pathways.

(unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

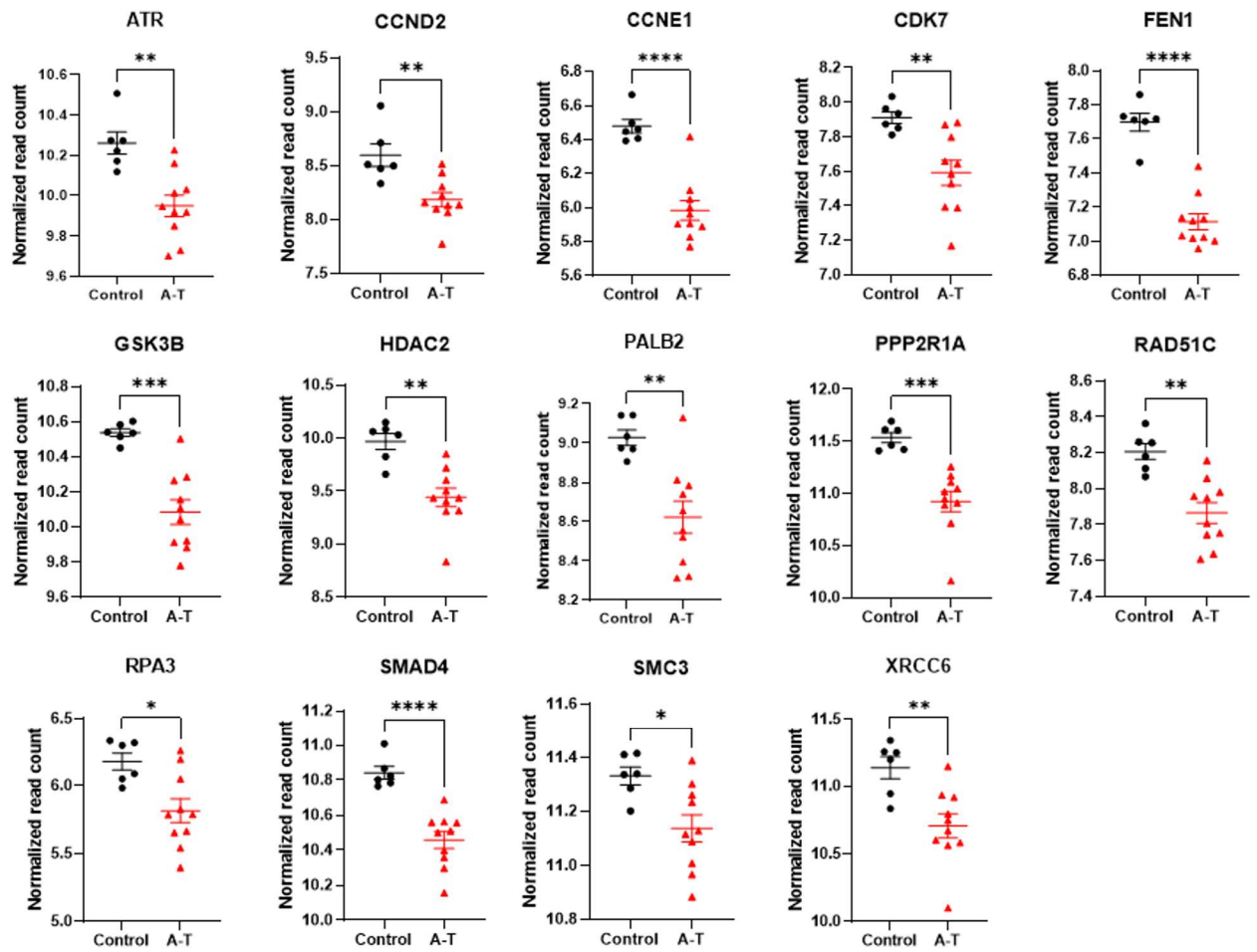


Figure 33. Column comparison illustrating downregulated DEGs with significant decreased normalized read counts in A-T cerebellum related to KEGG pathways.

(unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

To investigate the functional consequence of ATM deficiency in cell cycle and DSB repair related pathways, A-T cerebellum gene expression differences were identified in three KEGG pathways (cell cycle: hsa04110, homologous recombination: hsa03450, non-homologous end joining: hsa03440).

Cerebellum DEGs related to the KEGG pathways were illustrated with heatmap in figure 30. Among all plotted DEGs, 22 upregulated DEGs and 14 downregulated DEGs revealed significant difference in unpaired t-test with normalized read count.

Among all DEGs with t-test significance, there were 17 upregulated DEGs related to cell cycle pathway (Figure 31), including *ABLI*(log₂ FC = 0.75; p^{adj} = 2.94×10⁻³), *CCNA2*(log₂ FC = 0.92; p^{adj} = 0.014), *CDC14A*(log₂ FC = 1.75; p^{adj} = 4.94×10⁻⁹), *CDC14B*(log₂ FC = 1.89; p^{adj} = 9.13×10⁻⁷), *CDK2*(log₂ FC = 1.31; p^{adj} = 1.68×10⁻³), *CDK6*(log₂ FC = 0.65; p^{adj} = 2.93×10⁻³), *CDKN1A*(log₂ FC = 1.43; p^{adj} = 0.021), *CDKN1C*(log₂ FC = 1.93; p^{adj} = 5.98×10⁻⁵), *CDKN2C*(log₂ FC = 0.93; p^{adj} = 4.79×10⁻³), *CHEK2*(log₂ FC = 1.58; p^{adj} = 7.91×10⁻⁴), *E2F1*(log₂ FC = 1.57; p^{adj} = 1.82×10⁻³), *HDAC1*(log₂ FC = 1.59; p^{adj} = 5.68×10⁻¹⁴), *MYC*(log₂ FC = 1.0; p^{adj} = 0.022), *SMAD3*(log₂ FC = 1.05; p^{adj} = 5.09×10⁻⁴), *TGFB2*(log₂ FC = 1.68; p^{adj} = 2.66×10⁻⁶), *TGFB3*(log₂ FC = 1.67; p^{adj} = 1.30×10⁻⁸), and *WEE1*(log₂ FC = 0.86; p^{adj} = 7.25×10⁻³). The remaining 5 upregulated DEGs were related to homologous recombination pathway, including *BARD1*(log₂ FC = 1.64; p^{adj} = 2.22×10⁻⁴), *BRC1*(log₂ FC = 1.67; p^{adj} = 3.03×10⁻⁷), *POLD1*(log₂ FC = 0.69; p^{adj} = 1.53×10⁻³), *POLD4*(log₂ FC = 0.91; p^{adj} = 0.021), and *RAD51B*(log₂ FC = 1.19; p^{adj} = 2.41×10⁻³).

There were 9 downregulated DEGs with t-test significance associated with cell cycle pathway (Figure 32), including *ATR*(log₂ FC = -0.46; p^{adj} = 2.93×10⁻³), *CCND2*(log₂ FC = -0.68; p^{adj} = 2.51×10⁻³), *CCNE1*(log₂ FC = -1.07; p^{adj} = 4.91×10⁻⁵), *CDK7*(log₂ FC = -0.52; p^{adj} = 0.014), *GSK3B*(log₂ FC = -0.64; p^{adj} = 2.18×10⁻⁴), *HDAC2*(log₂ FC = -0.76; p^{adj} = 2.09×10⁻⁴), *PPP2R1A*(log₂ FC = -0.84; p^{adj} = 7.01×10⁻⁵), *SMAD4*(log₂ FC = -0.56; p^{adj} = 3.07×10⁻⁵), and *SMC3*(log₂ FC = -0.27; p^{adj} = 0.046). Three of the downregulated DEGs were related to homologous recombination pathway, including *PALB2*(log₂ FC = -0.61; p^{adj} = 3.50×10⁻³), *RAD51C* (log₂ FC = -0.56; p^{adj} = 2.16×10⁻³), and *RPA3* (log₂ FC = -0.77; p^{adj} = 0.038). The remaining two downregulated DEGs were related to non-homologous end joining pathway, including *FEN1* (log₂ FC = -1.05; p^{adj} = 1.16×10⁻⁸) and *XRCC6* (log₂ FC = -0.60; p^{adj} = 6.01×10⁻³).

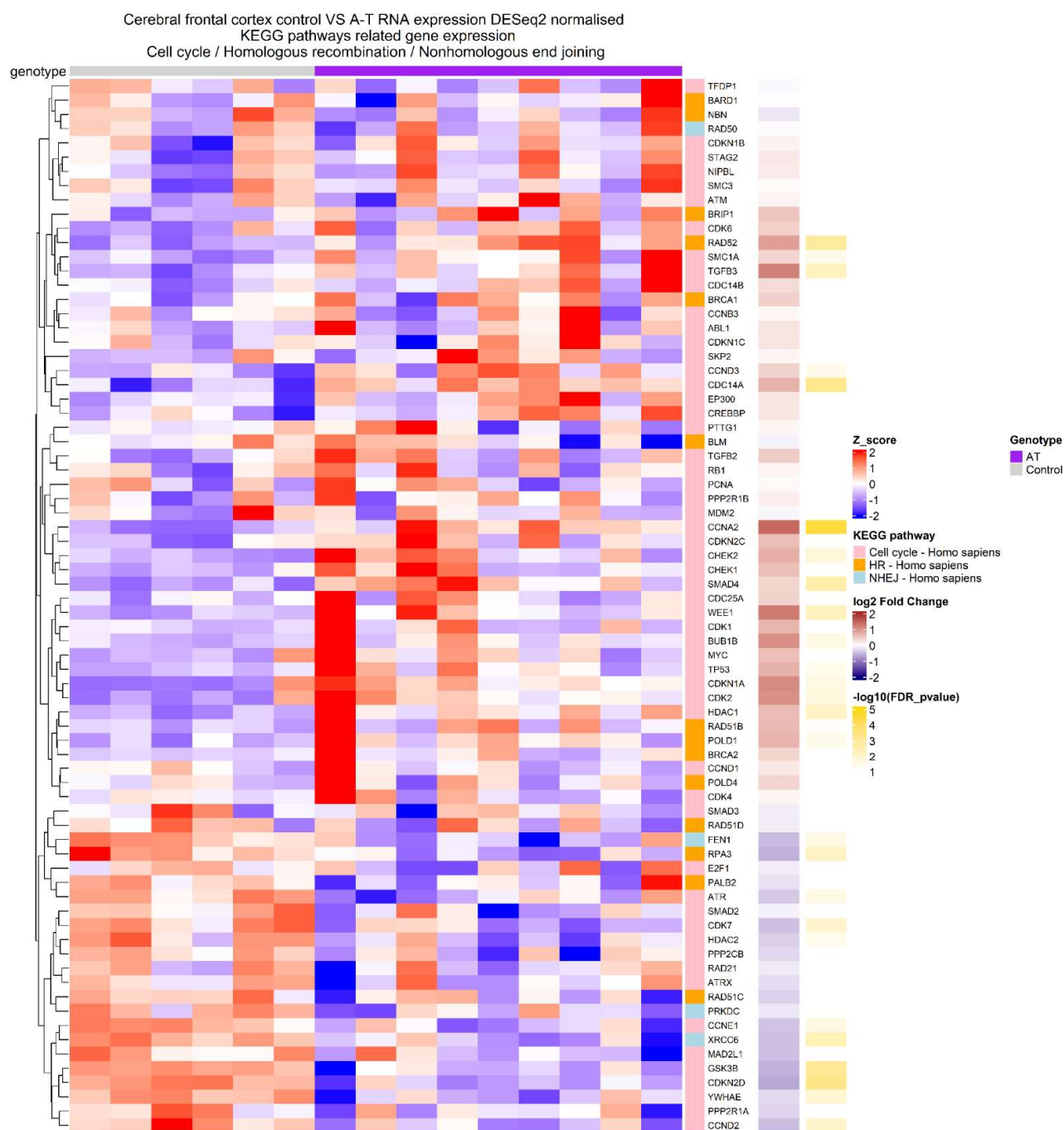


Figure 34. Heatmap illustrating A-T cerebral frontal cortex expression of gene involved in cell cycle (hsa04110), homologous recombination (hsa03440) and non-homologous end joining (hsa03450) KEGG pathways with Z-score normalization.

Normalized fold change and adjusted p-value of each gene were levelled with colour gradient aside the heatmap.

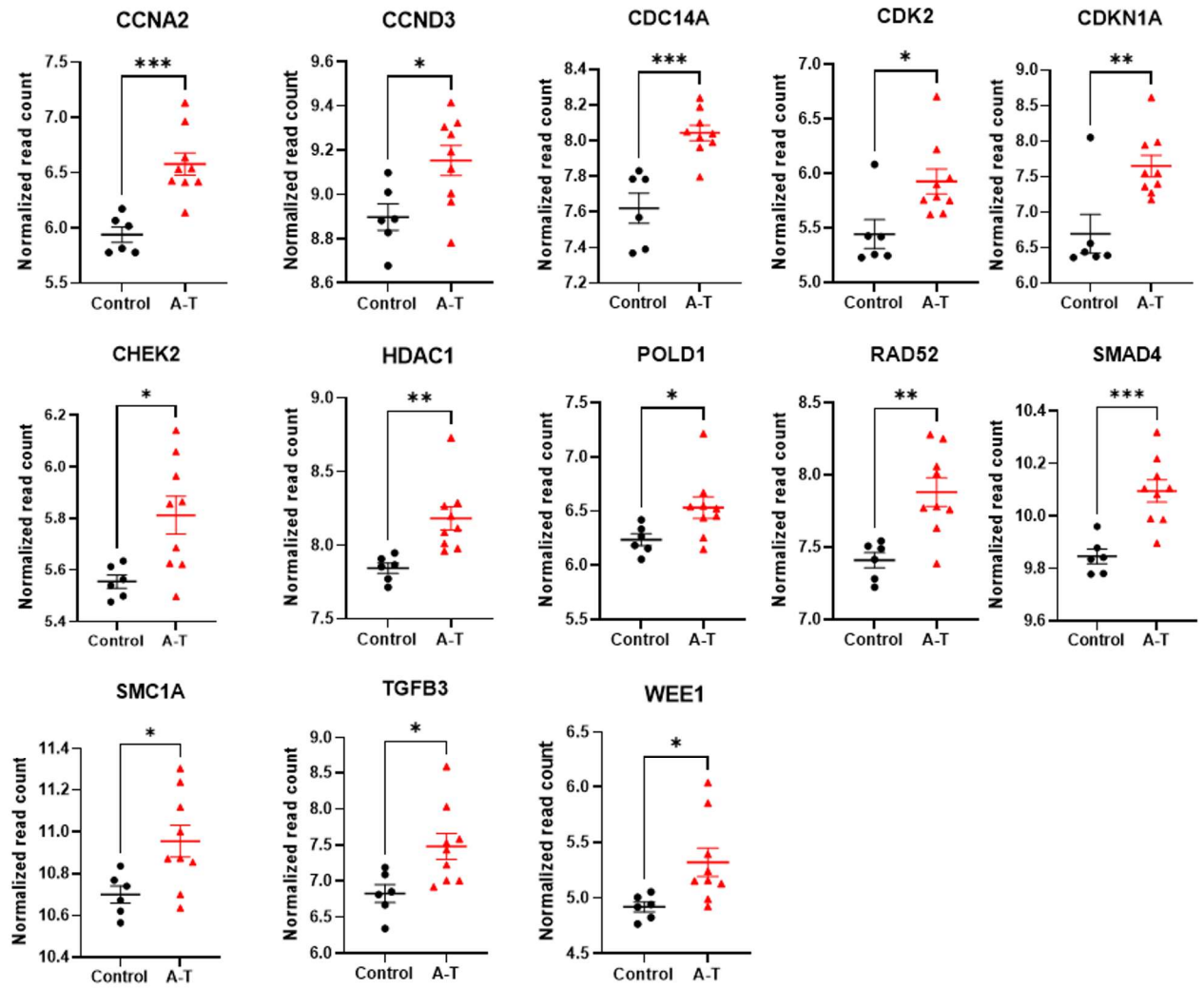


Figure 35. Column comparison illustrating upregulated DEGs with significant increased normalized read counts in A-T cerebral frontal cortex related to KEGG pathways.

(unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

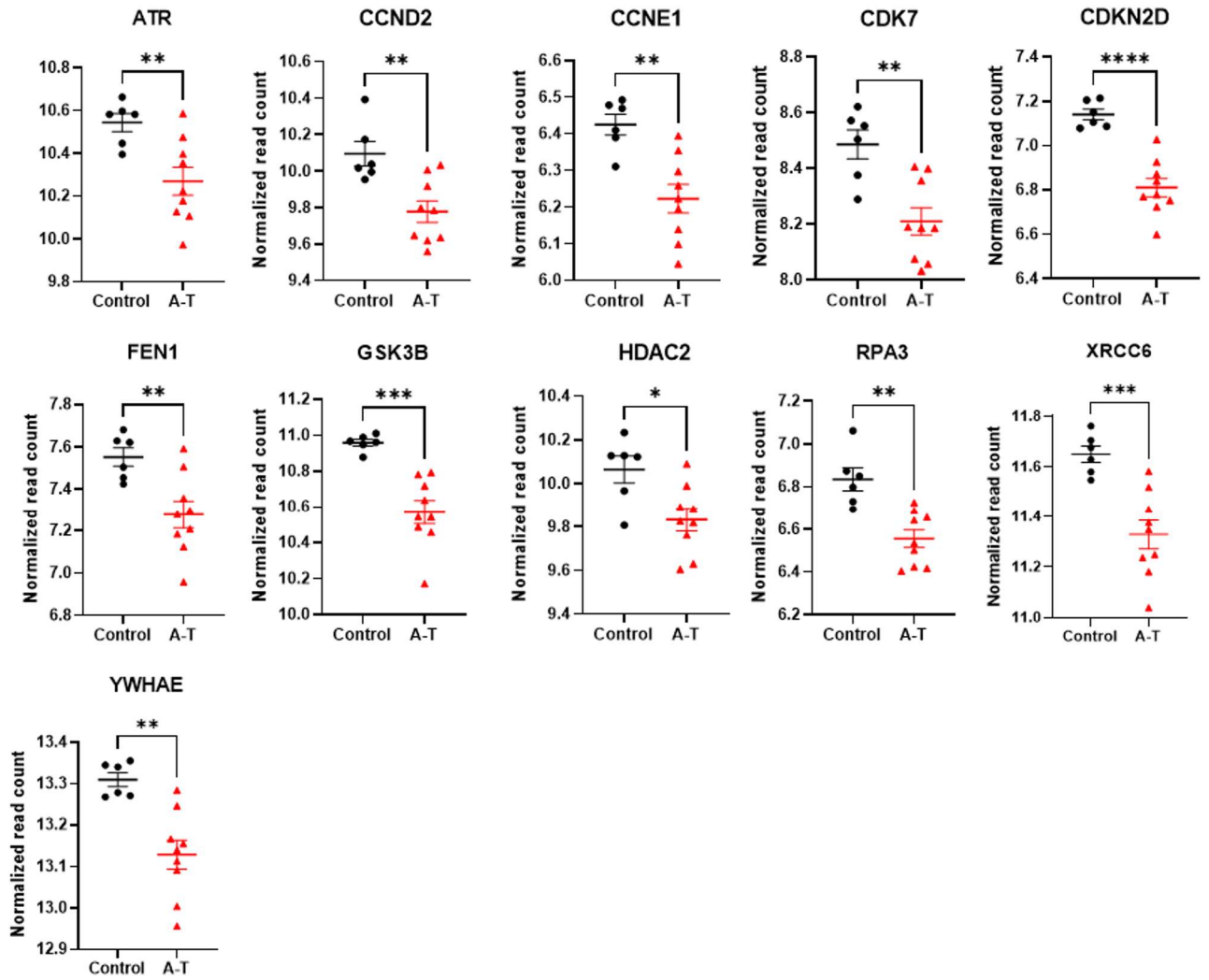


Figure 36. Column comparison illustrating downregulated DEGs with significant decreased normalized read counts in A-T cerebral frontal cortex related to KEGG pathways.

(unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Cerebral frontal cortex DEGs related to the KEGG pathways were illustrated with heatmap in Figure 34. Among all plotted DEGs, 13 upregulated DEGs and 11 downregulated DEGs revealed significant difference in unpaired t-test with normalized read count.

There were 11 upregulated DEGs with t-test significance associated with cell cycle pathway (Figure 34), including *CCNA2* ($\log_2$ FC = 1.45; $p^{\text{adj}} = 2.81 \times 10^{-5}$), *CCND3* ($\log_2$ FC = 0.43; $p^{\text{adj}} = 0.040$), *CDC14A* ($\log_2$ FC = 0.73; $p^{\text{adj}} = 5.65 \times 10^{-4}$), *CDK2* ($\log_2$ FC = 1.06; $p^{\text{adj}} = 0.0237$), *CDKN1A* ($\log_2$ FC = 1.11; $p^{\text{adj}} = 5.26 \times 10^{-3}$), *CHEK2* ($\log_2$ FC = 0.75; $p^{\text{adj}} = 0.0184$), *HDAC1* ($\log_2$ FC = 0.75; $p^{\text{adj}} = 0.0184$), *HDAC2* ($\log_2$ FC = 0.75; $p^{\text{adj}} = 0.0184$), *RPA3* ($\log_2$ FC = 0.75; $p^{\text{adj}} = 0.0184$), *XRCC6* ($\log_2$ FC = 0.75; $p^{\text{adj}} = 0.0184$), and *YWHAE* ($\log_2$ FC = 0.75; $p^{\text{adj}} = 0.0184$).

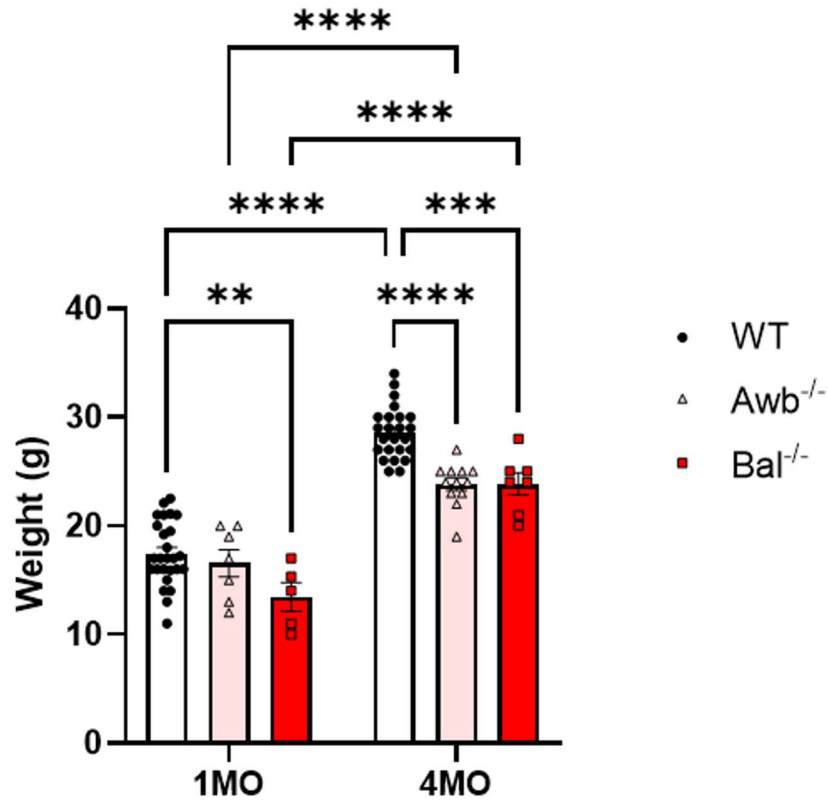
FC = 0.63; $p^{\text{adj}} = 7.82 \times 10^{-3}$), *SMAD4*(log₂ FC = 0.41; $p^{\text{adj}} = 2.46 \times 10^{-3}$), *SMC1A*(log₂ FC = 0.42; $p^{\text{adj}} = 0.0404$), *TGFB3*(log₂ FC = 1.20; $p^{\text{adj}} = 5.60 \times 10^{-3}$), and *WEE1*(log₂ FC = 1.26; $p^{\text{adj}} = 5.68 \times 10^{-3}$). Other two upregulated DEGs were related to homologous recombination pathway, including *POLD1*(log₂ FC = 0.71; $p^{\text{adj}} = 0.0397$), and *RAD52*(log₂ FC = 0.92; $p^{\text{adj}} = 1.30 \times 10^{-3}$). There were 8 downregulated DEGs with t-test significance associated with cell cycle pathway (Figure 35) , including *ATR*(log₂ FC = -0.41; $p^{\text{adj}} = 0.0313$), *CCND2*(log₂ FC = -0.49; $p^{\text{adj}} = 0.0119$), *CCNE1*(log₂ FC = -0.45; $p^{\text{adj}} = 0.0299$), *CDK7*(log₂ FC = -0.46; $p^{\text{adj}} = 0.0117$), *CDKN2D*(log₂ FC = -0.66; $p^{\text{adj}} = 5.17 \times 10^{-4}$), *GSK3B*(log₂ FC = -0.57; $p^{\text{adj}} = 4.10 \times 10^{-4}$), *HDAC2*(log₂ FC = -0.36; $p^{\text{adj}} = 0.0107$), *YWHAE*(log₂ FC = -0.27; $p^{\text{adj}} = 0.0137$) Only *RPA3*(log₂ FC = -0.58; $p^{\text{adj}} = 7.56 \times 10^{-3}$) was related to homologous recombination pathway among downregulated DEGs. Meanwhile, the other two DEGs were related to non-homologous end joining pathway, including *FEN1*(log₂ FC = -0.49; $p^{\text{adj}} = 0.0296$), and *XRCC6*(log₂ FC = -0.47; $p^{\text{adj}} = 3.42 \times 10^{-3}$).

Cognitive decline in ATMKO mice

Transcriptomic shifts were found in both cerebellum and cerebral frontal cortex of A-T samples. Sequencing results also revealed regional difference of transcriptional effect under ATM deficiency between cerebellum and cerebral frontal cortex. Meanwhile, disrupted cell cycle regulation and DNA repair mechanism were found in both cerebellum and cerebral frontal cortex of A-T samples. To further the investigation on ATM deficiency effect on myelin and oligodendrocyte pathology, two strains of ATMKO mice (*Atm*^{tm1Bal/J} and *Atm*^{tm1Awb/J}) were employed for the pathological study through behavioral test, histological investigation, transcriptional sequencing and primary oligodendrocyte culture.

A

Age-dependent weight



B

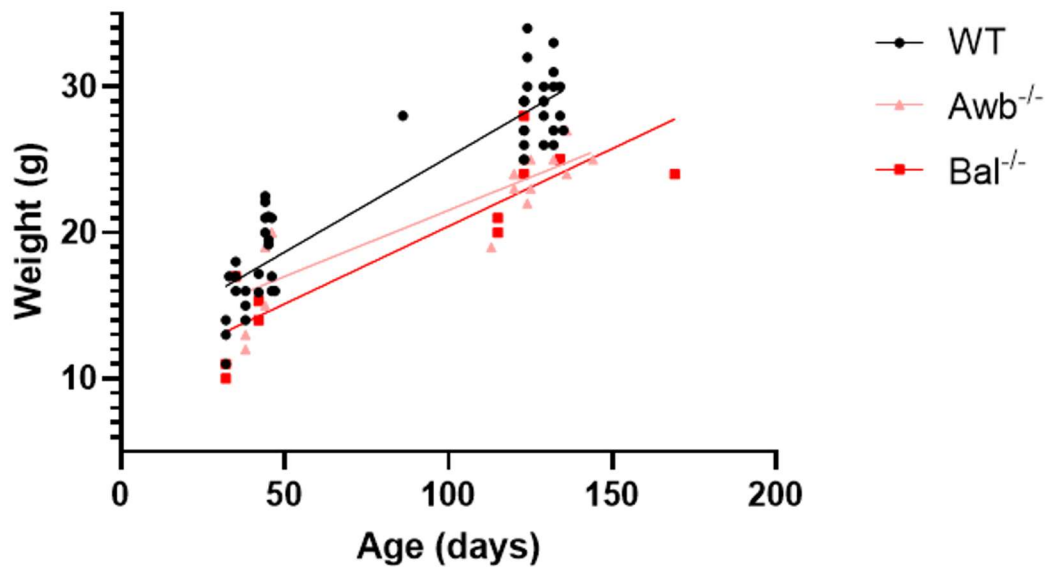


Figure 37. Weight loss in ATM deficient mice.

A) Grouped comparison of weight in 1MO and 4MO mice cohort. (1MO, WT: n=26, Awb^{-/-}: n=7, Bal^{-/-}: n=5; 4MO, WT: n=24, Awb^{-/-}: n=11, Bal^{-/-}: n=7). B) Correlation between age and weight of ATM deficient mice. (Two-way ANOVA with Tukey's multiple comparisons test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Weight loss was the primary phenotypic change found in ATM KO mice. The body mass of $Bal^{-/-}$ mice was reduced by 22.8% at 1MO ($p = 0.0274$) (Figure 36A). Meanwhile, the body mass of $Bal^{-/-}$ and $Awb^{-/-}$ mice were reduced by 16.5% and 16.6% respectively at 4MO ($p < 0.0001$) (Figure 36A). Declined weight gaining in $Awb^{-/-}$ mice was revealed during its development from 1MO to 4MO, while $Bal^{-/-}$ mice were generally lighter than wild type mice (Figure 36B).

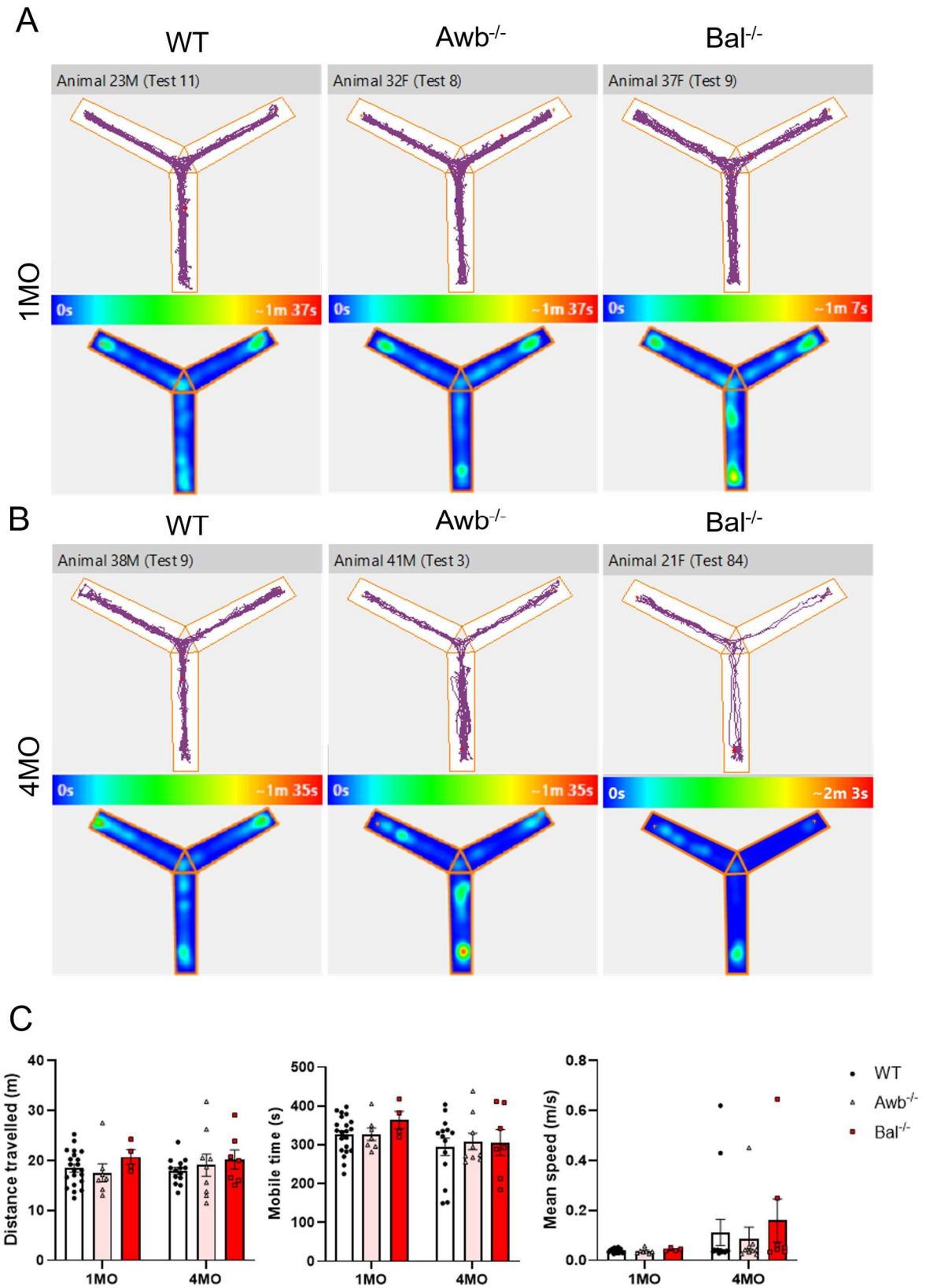


Figure 38. Behavioural test on ATM deficient mice with Y maze.

A) Y maze path record and corresponding heatmap of ATM deficient mice at 1MO. B) Y maze path record and corresponding heatmap of ATM deficient mice at 4MO. C) Grouped comparison on distance travelled, mobile time and mean speed of ATM deficient mice in Y maze at 1 and 4 MO. (Two-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

To examine the cognitive function of ATM KO mice, Y maze was employed to delineate their instant spatial memory performance and reference memory performance. The path record and corresponding heatmap of Y maze demonstrated the time spending preference of mice on each arm of Y maze (Figure 37A, B). There was no significant difference in total distance travelled and mobile time of mice in different genotype and age group (Figure 37C). The mean speed of mice at 4MO group was higher than 1MO group ($p = 0.0357$), but there was no genotypic difference among each age group (Figure 37C).



Figure 39. Cognitive performance of ATM deficient mice with age dependent analysis.

A) Analysis of spatial working memory performance with spontaneous alteration percentage for 1MO and 4MO ATM deficient mice. B) Analysis of spatial reference memory performance with time spent on correct arm in stage 3 of Y maze for 1MO and 4MO ATM deficient mice. (Two-way ANOVA with Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Spatial memory performance was calculated with spontaneous alteration percentages that attributed to the mice tendency of exploring a novel arm (Kraeuter et al., 2019). There was no significant difference of ATM KO mice in spatial memory performance at 1MO and 4MO (Figure 38A). Reference memory performance was measured by time spent of mice on blocked arm at stage 3 of Y maze behavioral test. Reference memory of $Bal^{-/-}$ mice was decreased by 51.8% ($p = 0.0268$) at 4MO (Figure 38B), but not in $Awb^{-/-}$ animals.

Oligodendrocyte and myelin pathology in ATMKO mice

Cognitive performance decline was found in $Bal^{-/-}$ mice at 4MO but the underlying pathology was not clear. As neuronal loss was only found in A-T cerebellum but not cerebral cortex, we examined myelin and oligodendrocyte pathology under ATM deficiency as white matter plasticity also contributed to cognitive development in the mice (Bells et al., 2019).

We divided sagittal mice brain slide into different regions for quantification of immunohistochemistry study, while frontal cortex (FCx), genu, hippocampus (Hippo) and cerebellum (CB) were the primary targets of this investigation (Figure 6).

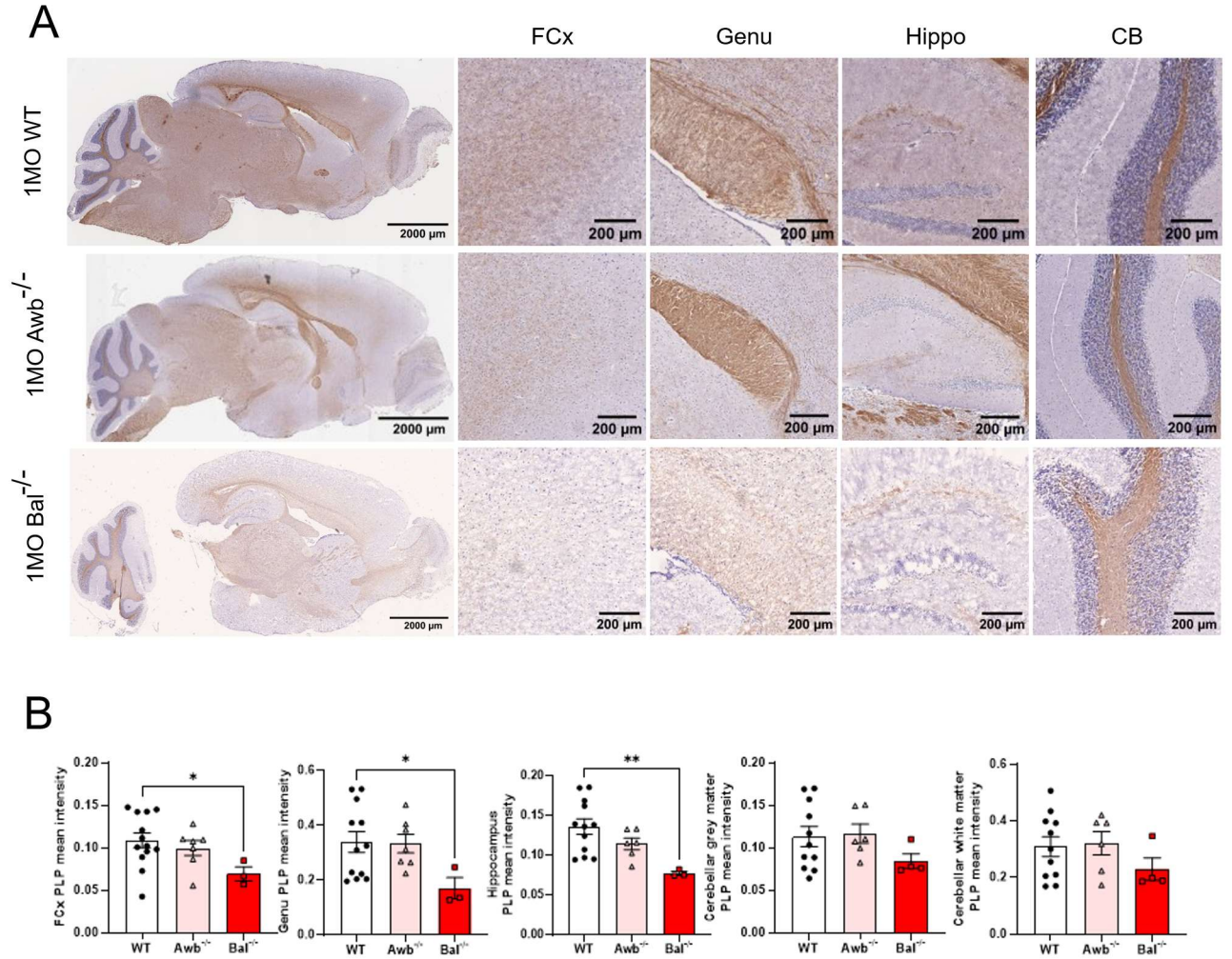


Figure 40. Immunohistology study of myelin pathology in ATM deficient mice at 1MO.

A) Representative BOND H-DAB images of PLP staining in whole brain slide and zoomed regions of Awb^{-/-} and Bal^{-/-} mice at 1MO. B) Quantification of PLP mean density in FCx, Genu, Hippo, CB grey matter and CB white matter. (WT: n=13, Awb^{-/-}: n=7, Bal^{-/-}: n=4) (One-way ANOVA with Fisher's LSD multiple comparison test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

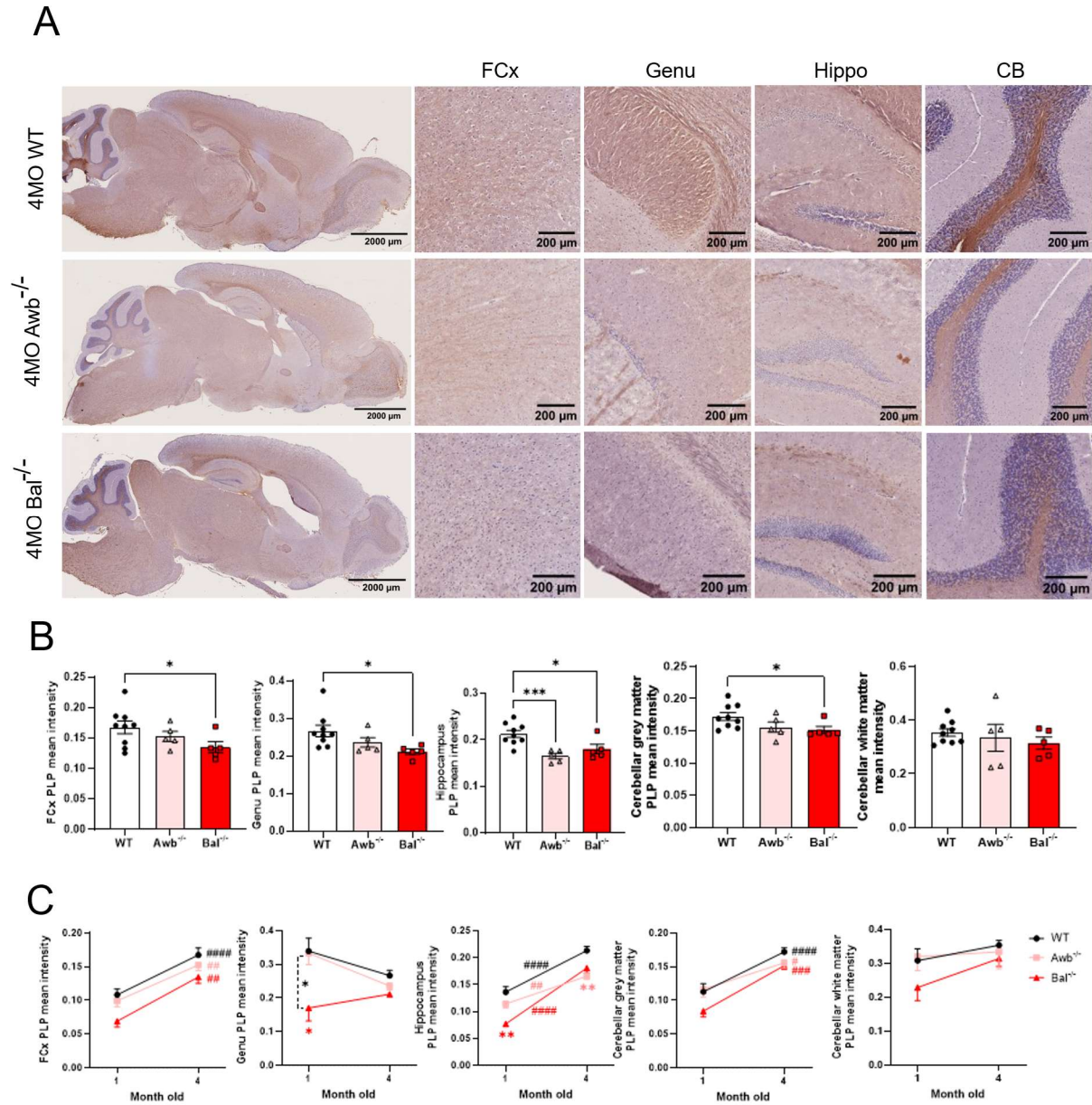


Figure 41. Immunohistology study of myelin pathology in ATM deficient mice at 4MO.

A) Representative BOND H-DAB images of PLP staining in whole brain slide and zoomed regions of $Awb^{-/-}$ and $Bal^{-/-}$ mice at 4MO. B) Quantification of PLP mean density in FCx, Genu, Hippo, CB grey matter and CB white matter. (WT: n=9, $Awb^{-/-}$: n=5, $Bal^{-/-}$: n=5). (One-way ANOVA with Fisher's LSD multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). C) Age-dependent change of PLP mean intensity in each region of $Awb^{-/-}$ and $Bal^{-/-}$ mice. (Two-way ANOVA with Tukey's multiple comparison test; p-value for row factor, i.e. mice genotype: *, hyphen-line * indicates p-value from comparison between $Awb^{-/-}$ and $Bal^{-/-}$ mice group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; p-value for column factor, i.e. mice age: #, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$).

Myelin pathology in ATM deficient mice was examined with proteolipid protein (PLP) staining. Representative image revealed an overall reduction in DAB staining intensity in 1MO Bal^{-/-} mice (Figure 39A). There were significant declines of PLP mean intensities in FCx, (36.1%, $p = 0.039$), genu (49.9%, $p = 0.0344$), and Hippo (43.4%, $p = 0.0044$) of 1MO Bal^{-/-} mice, but not the CB region (Figure 39B).

The contrast in PLP staining between gray and white matter regions of the brain was less pronounced in the 4MO cohort (Figure 40A). Substantial reductions in mean PLP intensities were found in FCx (21.0%, $p = 0.0384$), genu (21.0%, $p = 0.0148$), Hippo (15.0%, $p = 0.0134$), and CB grey matter (11.6%, $p = 0.0427$) of 4MO Bal^{-/-} mice (Figure 40B). Meanwhile, a significant decreased mean PLP intensity was also found in Hippo region of 4MO Awb^{-/-} mice (22.5%, $p = 0.0009$) (Figure 40B). There was no significant change in PLP intensity in Awb^{-/-} mice except Hippo region in 4MO cohort.

There were significant increasing trends of PLP intensity from 1MO to 4MO cohort in FCx, Hippo, CB grey matter regions among all genotypes (Figure 40C). Comparing to the corresponding WT mice region, the significant difference of PLP intensity was only found in 1MO Bal^{-/-} mice genu, 1MO Bal^{-/-} mice hippocampus and 4MO Awb^{-/-} mice hippocampus under the two-way ANNOVA test (Figure 40C).

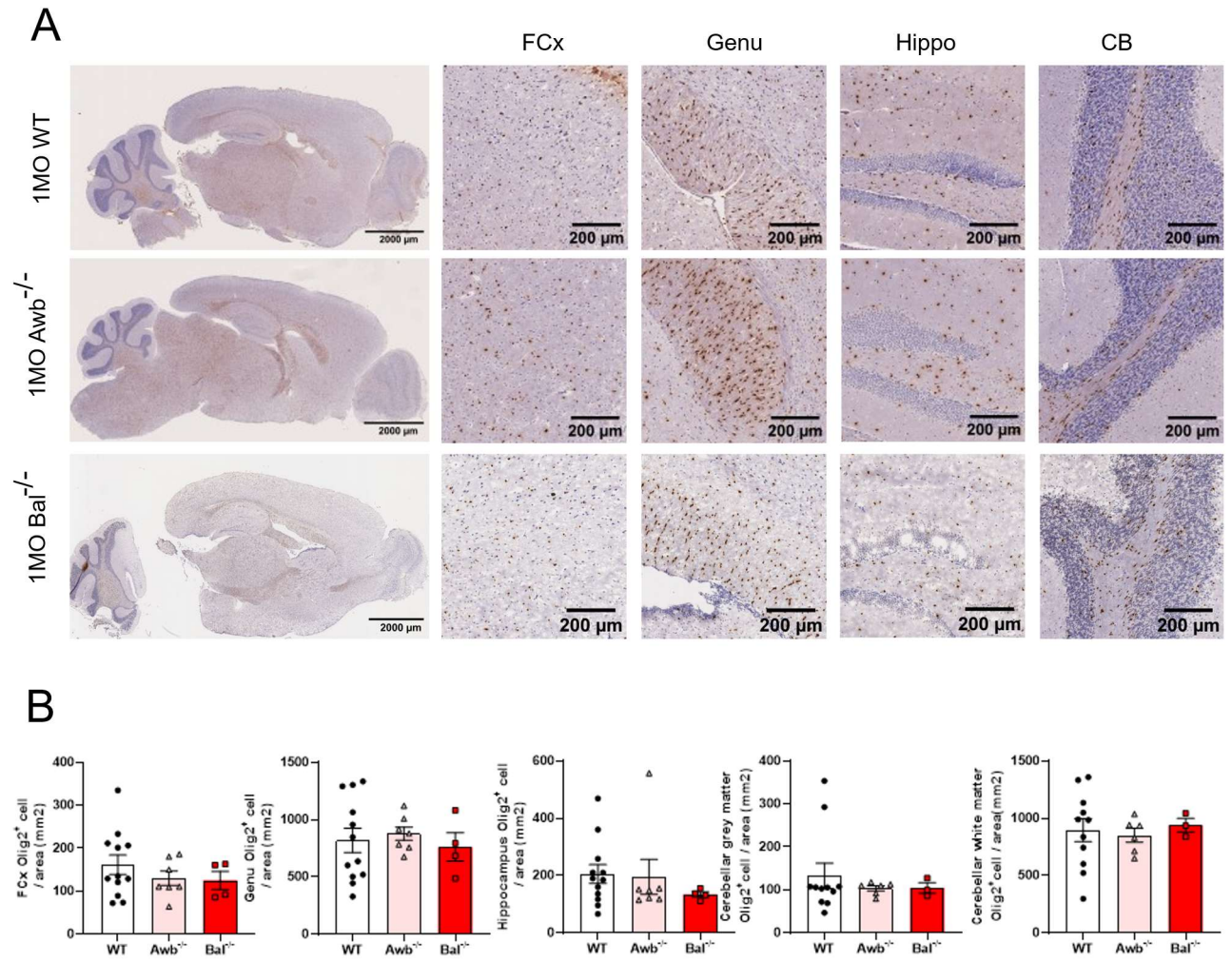


Figure 42. Immunohistology study of mass OL distribution in ATM deficient mice at 1MO.

A) Representative BOND H-DAB images Olig2 staining in whole brain slide and zoomed regions of Awb^{-/-} and Bal^{-/-} mice at 1MO. B) Quantification of Olig2⁺ cell density in FCx, Genu, Hippo, CB grey matter and CB white matter. (WT: n=11, Awb^{-/-}: n=7, Bal^{-/-}: n=4) (One-way ANOVA with Fisher's LSD multiple comparison test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

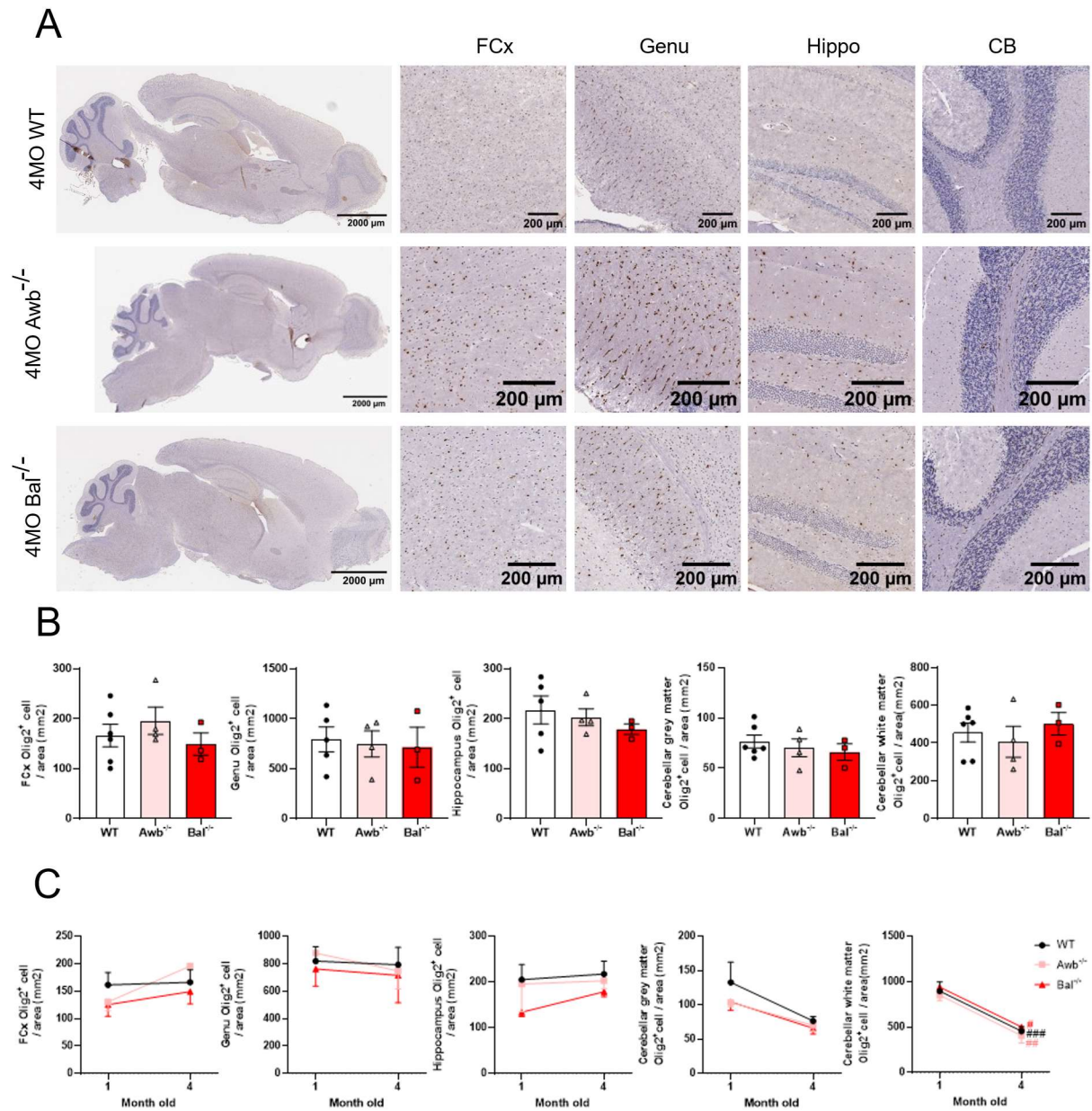


Figure 43. Immunohistology study of mass OL distribution in ATM deficient mice at 4MO.

A) Representative BOND H-DAB images of Olig2 staining in whole brain slide and zoomed regions of *Awb^{-/-}* and *Bal^{-/-}* mice at 4MO. B) Quantification of Olig2⁺ cell density in FCx, Genu, Hippo, CB grey matter and CB white matter. (WT: n=5, *Awb^{-/-}*: n=4, *Bal^{-/-}*: n=3) (One-way ANOVA with Fisher's LSD multiple comparison test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001). C) Age-dependent change of Olig2⁺ cell intensity in each region of *Awb^{-/-}* and *Bal^{-/-}* mice. (Two-way ANOVA with Tukey's multiple comparison test; p-value for row factor, i.e. mice genotype: *, hyphen-line * indicates p-value from comparison between *Awb^{-/-}* and *Bal^{-/-}* mice group, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; p-value for column factor, i.e. mice age: #, # p < 0.05, ## p < 0.01, ### p < 0.001, #### p < 0.0001).

Gross OLs population was examined in ATM deficient mice with Olig2 staining. There was no observable change in density of Olig2⁺ OL in the four regions in 1MO cohort of both Awb^{-/-} and Bal^{-/-} mice (Figure 41B). No significant difference in density of Olig2⁺ OL was found in 4MO ATM deficient mice cohort neither (Figure 42B). Significant decreasing trend of Olig2⁺ OL density from 1MO to 4MO was found in the white matter CB region among all mice genotype (Figure 42C). Otherwise, Olig2⁺ OL density remained consistent in FCx, Genu and Hippo region across different age and genotype cohorts (Figure 42C).

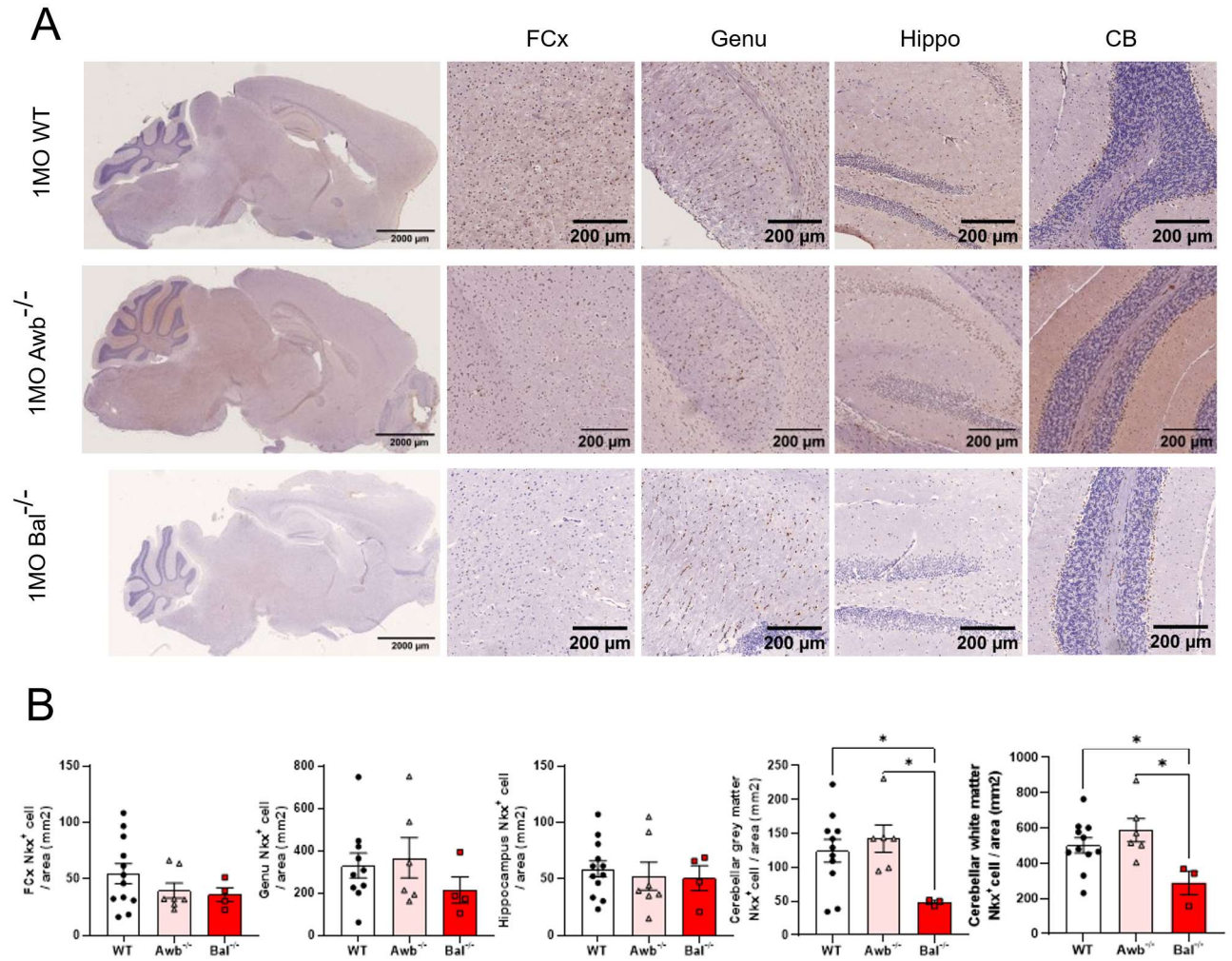


Figure 44. Immunohistology study of OPC distribution in ATM deficient mice at 1MO.

A) Representative BOND H-DAB images Nkx2.2 staining in whole brain slide and zoomed regions of *Awb*^{-/-} and *Bal*^{-/-} mice at 1MO. B) Quantification of Nkx2.2+ cell density in FCx, Genu, Hippo, CB grey matter and CB white matter. (WT: n=11, *Awb*^{-/-}: n=7, *Bal*^{-/-}: n=4) (One-way ANOVA with Fisher's LSD multiple comparison test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

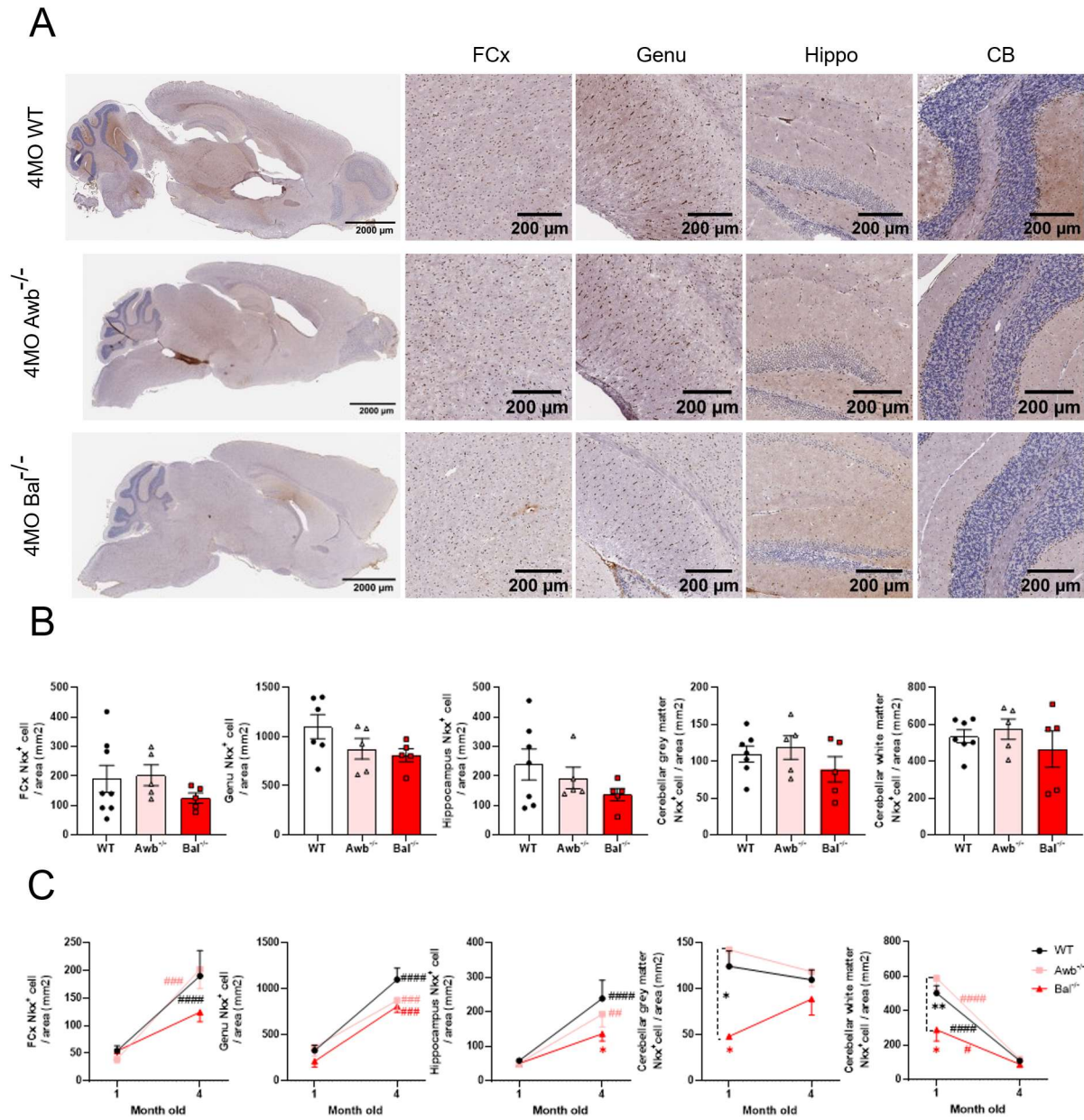


Figure 45. Immunohistology study of OPC distribution in ATM deficient mice at 4MO.

A) Representative BOND H-DAB images of Nkx2.2 staining in whole brain slide and zoomed regions of Awb^{-/-} and Bal^{-/-} mice at 4MO. B) Quantification of Nkx2.2+ cell density in FCx, Genu, Hippo, CB grey matter and CB white matter. (WT: n=5, Awb^{-/-}: n=4, Bal^{-/-}: n=3). (One-way ANOVA with Fisher's LSD multiple comparison test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001). C) Age-dependent change of Nkx2.2+ cell intensity in each region of Awb^{-/-} and Bal^{-/-} mice. (Two-way ANOVA with Tukey's multiple comparison test; p-value for row factor, i.e. mice genotype: *, hyphen-line * indicates p-value from comparison between Awb^{-/-} and Bal^{-/-} mice group, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; p-value for column factor, i.e. mice age: # p < 0.05, ## p < 0.01, ### p < 0.001, #### p < 0.0001).

Population of OPC was investigated with Nkx2.2 staining on ATM deficient mice. There was no notable change in Nkx2.2⁺ population in FCx, genu and Hippo at 1MO (Figure 43B). Yet, Nkx2.2⁺ cell density in grey and white matter of CB in Bal^{-/-} mice decreased by 61.2% (p = 0.0329) and 42.4% (p = 0.0329) correspondingly at 1MO (Figure 43B). Meanwhile, significant variation of Nkx2.2⁺ cell density was also found in the same regions between two strands of Atm deficient mice (grey matter: 66.1% decline in Bal^{-/-} mice, p = 0.0171; white matter: 50.8% decline in Bal^{-/-} mice, p = 0.0329). However, significant variations among genotype were not found in any of the four regions at 4MO cohort (Figure 44B).

Nkx2.2⁺ cell density was significantly increased from 1MO to 4MO cohort in genu and Hippo regions among all genotypes (Figure 44C). Significant increase from 1MO to 4MO cohort was also found in FCx of WT and Awb^{-/-} mice, but not in Bal^{-/-} mice (Figure 44C). However, Nkx2.2⁺ cell density significantly decreased in CB white matter region among all genotypes (Figure 44C).

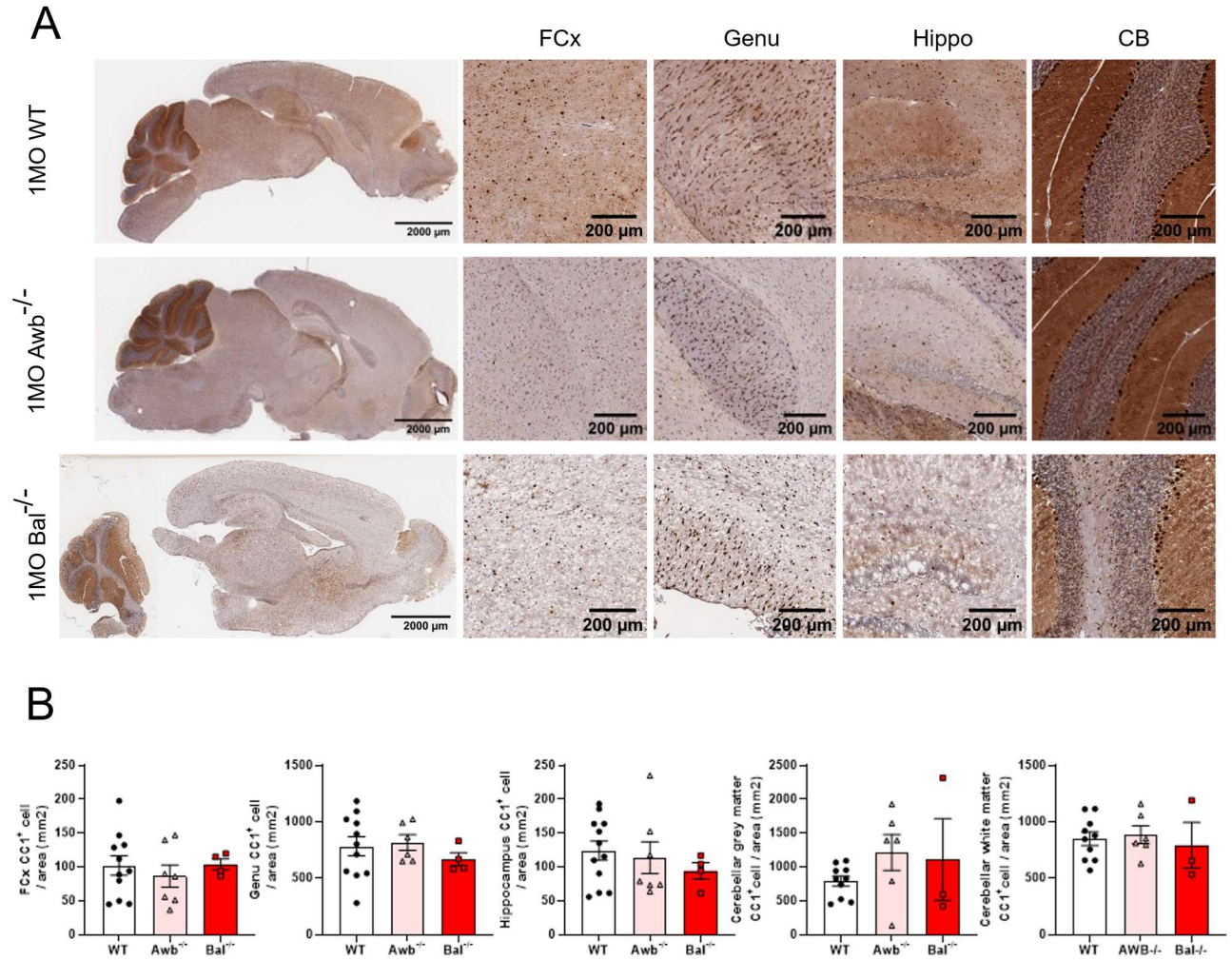


Figure 46. Immunohistology study of matured OL distribution in ATM deficient mice at 1MO.

A) Representative BOND H-DAB images CC1 staining in whole brain slide and zoomed regions of $Awb^{-/-}$ and $Bal^{-/-}$ mice at 1MO. B) Quantification of CC1+ cell density in FCx, Genu, Hippo, CB grey matter and CB white matter. (WT: n=11, $Awb^{-/-}$: n=7, $Bal^{-/-}$: n=4) (One-way ANOVA with Fisher's LSD multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

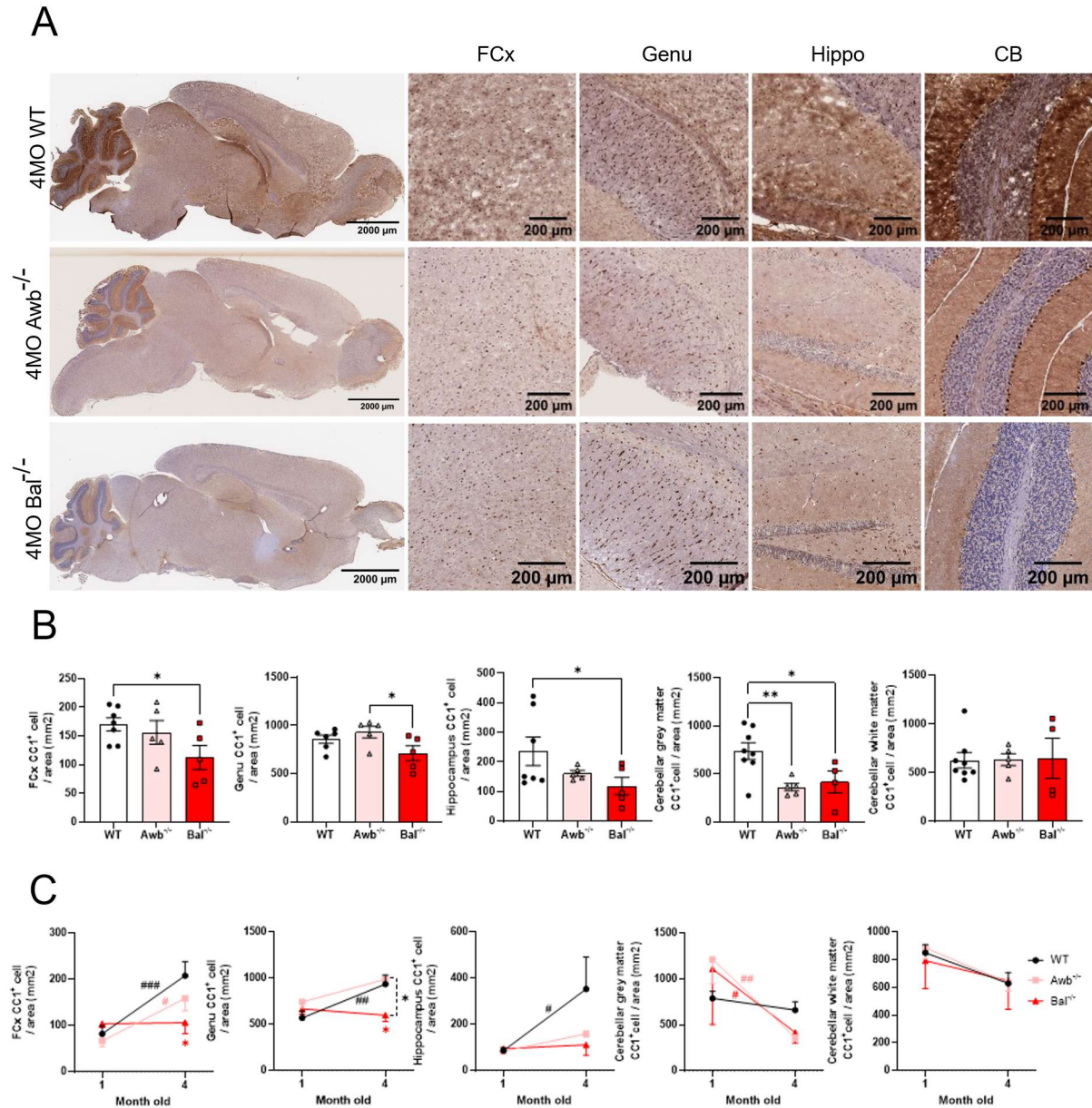


Figure 47. Immunohistology study of matured OL distribution in ATM deficient mice at 4MO.

A) Representative BOND H-DAB images of CC1 staining in whole brain slide and zoomed regions of $Awb^{-/-}$ and $Bal^{-/-}$ mice at 4MO. B) Quantification of CC1+ cell density in FCx, Genu, Hippo, CB grey matter and CB white matter. (WT: n=8, $Awb^{-/-}$: n=5, $Bal^{-/-}$: n=4). (One-way ANOVA with Fisher's LSD multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). C) Age-dependent change of CC1+ cell intensity in each region of $Awb^{-/-}$ and $Bal^{-/-}$ mice. (Two-way ANOVA with Tukey's multiple comparison test; p-value for row factor, i.e. mice genotype: *, hyphen-line * indicates p-value from comparison between $Awb^{-/-}$ and $Bal^{-/-}$ mice group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; p-value for column factor, i.e. mice age: #, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$).

Mature OLs population was detected with CC1 staining in this study. There was no significant difference among ATM deficient mice in the four regions at 1MO (Figure 45A, B). However, there was significant decrease of CC1⁺ cell density in FCx (33.5%, $p = 0.0294$) and Hippo (50.2%, $p = 0.0445$) region of Bal^{-/-} mice at 4MO (Figure 46B). Substantial declines were identified in CB grey matter in Awb^{-/-} (50.8%, $p = 0.0069$) and Bal^{-/-} (43.6%, $p = 0.0242$) mice at 4MO (Figure 46B).

Significant increasing trend of CC1⁺ cell density was shown from 1MO to 4MO in FCx of WT and Awb^{-/-} mice, but the trend levelled off in Bal^{-/-} mice (Figure 46C). Meanwhile, CC1⁺ cell density significantly increased in Hippo region from 1MO to 4MO WT mice, but the progression was flattened out for Awb^{-/-} and Bal^{-/-} mice (Figure 46C). Moreover, Awb^{-/-} and Bal^{-/-} mice demonstrated a significant decreasing trend of CC1⁺ cell density in cerebellar grey matter region at 4MO, but it remained consistent in WT mice at 4MO (Figure 46C).

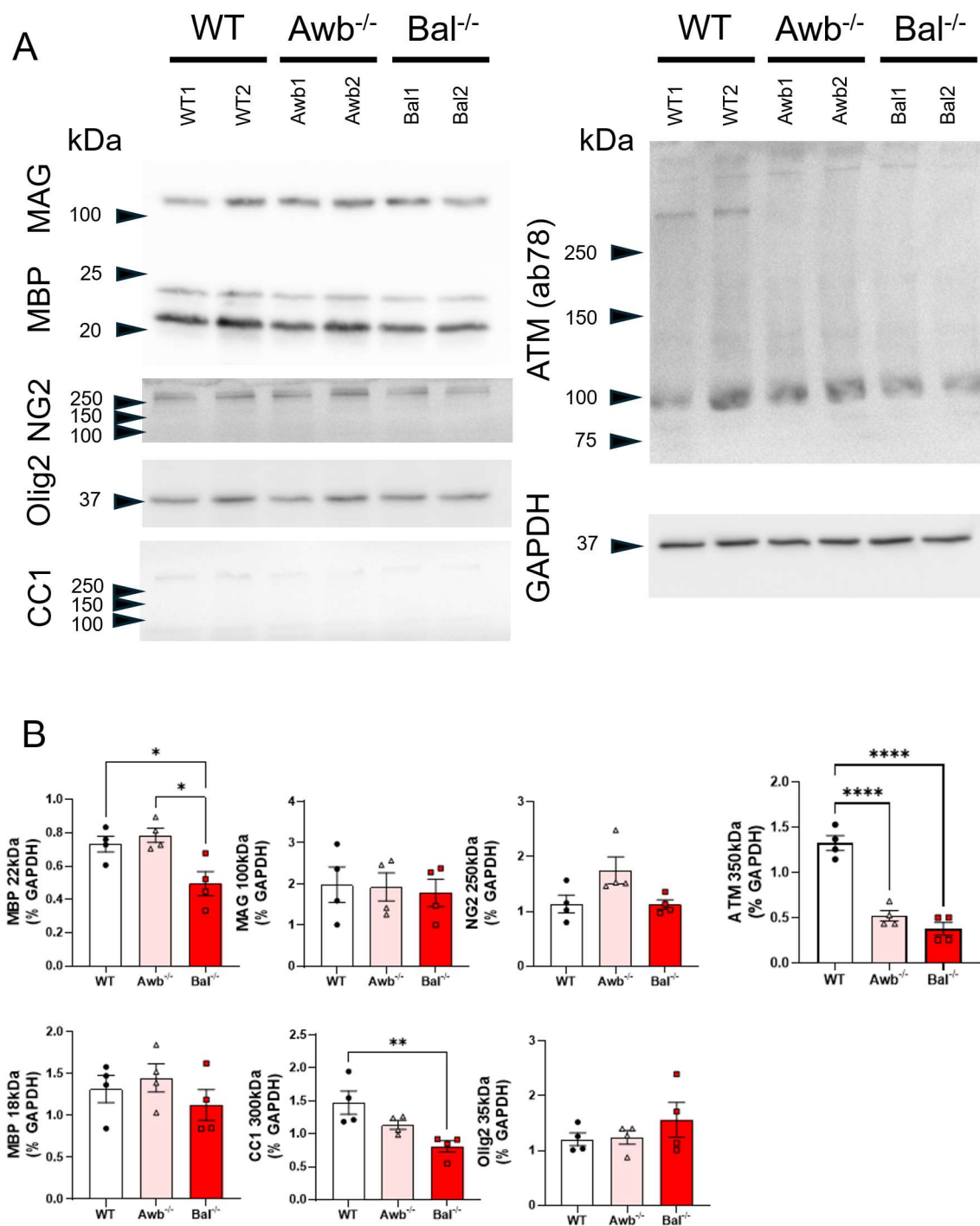


Figure 48 Immunoblotting study on cerebral cortex of 4MO ATM deficient mice.

A) Representative immunoblotting images of myelin, OL related protein and ATM protein expression in *Atm* deficient mice.
 B) Quantification of MBP (22kDa and 18kDa), MAG (100kDa), CC1 (300kDa), NG2 (250kDa), Olig2 (35kDa) and ATM (350kDa) protein expression against GAPDH expression. (One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Significant ATM protein loss was found in cerebral cortex of both Awb^{-/-} (60.7%, $p < 0.0001$) and Bal^{-/-} (71.4%, $p < 0.0001$) mice (Figure 48D). There were no observable band of full-length ATM on the immunoblotting image of both ATM deficient mice (Figure 48B).

To study the myelin related protein expression in ATM deficient mice, MAG and MBP were employed for the immunoblotting study (Figure 48A). No significant differences were found between wild type and ATM deficient mice in MAG expression and MBP expression at 18kDa (Figure 48C). Yet, MBP expression at 22kDa was significantly reduced in Bal^{-/-} mice comparing to wild type mice (32.4%, $p = 0.0356$) and even Awb^{-/-} mice (36.9%, $p = 0.0126$) (Figure 48C).

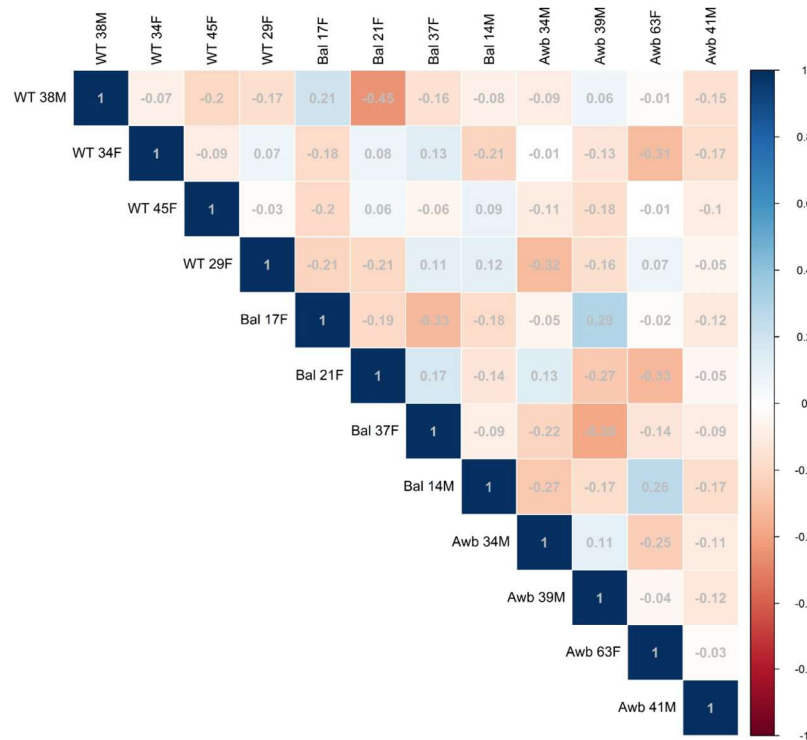
Corresponding to mass OL, OPC and mOL expression, Olig2, NG2 and CC1 were employed for studying the OL lineage (Figure 48A). There were no significant differences in Olig2 and NG2 expression among all mice genotype (Figure 48C). However, CC1 expression was significantly reduced in Bal^{-/-} mice compared to wild type mice (45%, $p = 0.0093$)

Transcriptomic change of ATMKO mice

Loss of myelination and mature OL population was found in both cerebral cortex and cerebellum of Bal^{-/-} mice in histological study. To further investigate transcriptomic change under ATM deficient in cerebral region, targeted RNA sequencing (TruSight RNA Pan-Cancer Panel) and transcriptomic RNA sequencing (Illumina Stranded mRNA Prep) were performed for both Awb^{-/-} and Bal^{-/-} mice at 4MO. Despite ATM related cell cycle and DNA repair mechanism, we aim to delineate the potential mechanisms leading to myelin and OL pathology in ATM deficient mice.

Targeted RNA sequencing (TruSight RNA Pan-Cancer Panel)

A



B

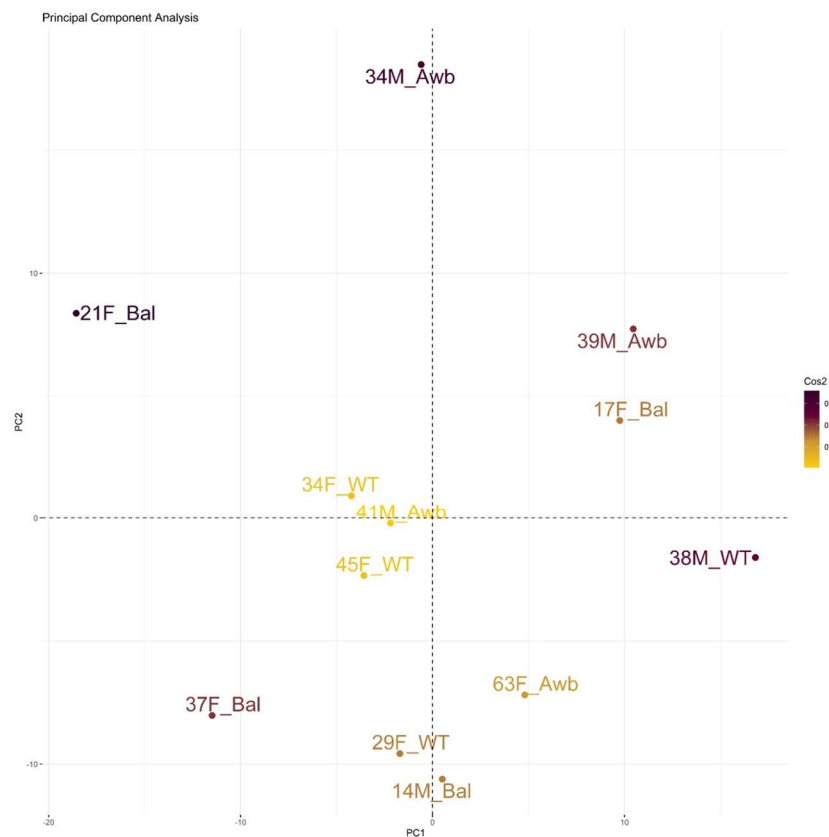


Figure 49. Quality assessment of the targeted RNA sequencing of cerebral frontal lobe of *Atm* deficient mice at 4MO.

(Control: n=4, Awb^{-/-}: n=4, Bal^{-/-}: n=4). A) Pearson correlation matrix of each sample in the targeted RNA sequencing. B) PCA displaying all 12 samples along PC1 and PC2.

Raw read counts generated from STAR alignment pipeline were then normalized by geNorm with GeneGlobe Data Analysis Center (Qiagen). Pearson's correlation coefficient was calculated with the normalized read count of each sample reflecting the linear correlations against other sample. The coefficients were then scaled from one to negative one in the heatmap (Figure 49A), reflecting the relative degree of correlation. Internal comparison among WT sample ranged from 0.07 to -0.2 (Figure 49A). Compared to the control sample in human targeted sequencing (Figure 22A, Figure 26A), this experiment demonstrated a less correlation within control group and a less difference between control group and target groups. Principal components analysis (PCA) plot demonstrated the variation among each sample with their greatest (PC1) and second greatest (PC2) variation within the data set, while clustering of samples reflected the proximity among samples. According to the PCA plot in Figure 49B, three groups of samples (WT, Awb^{-/-} and Bal^{-/-}) were not distanced with each other, which reflected the potential variation and proximity of three groups of samples.

The geNorm method identified the most stably expressed genes across whole panel for normalization. However, the average reference genes' molecular tags method did not reveal genes with highly significant expression (adjusted p-value < 0.05). Therefore, we did not imply p-value adjustment for DEGs discovery.

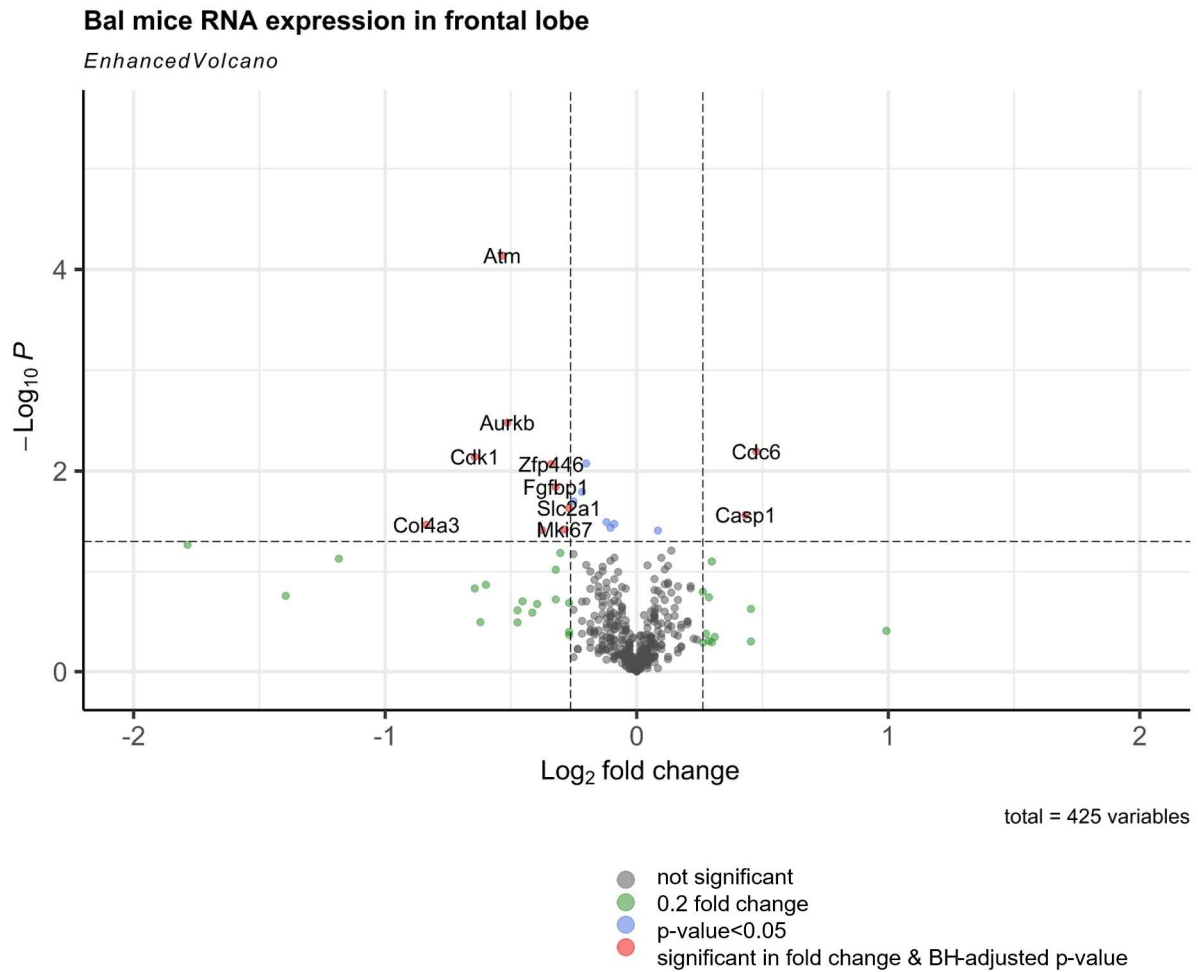


Figure 50. Volcano plot illustrating DEGs from RNA sequencing analysis in cerebral frontal lobe tissue between Bal^{-/-} and WT mice.

(DEGs indicated as red dots) (DEG definition: p-value < 0.05; |fold change| > 0.2)

Fold change and significance were calculated on each targeted gene and plotted against each group of samples, which formed volcano plot in every comparison. A total 425 genes were annotated after the alignment of targeted RNA sequencing. There were three DEGs with upregulation in the comparison between Bal and WT mice, including *Cdc6* ($\log_2$ fold change = 0.475, $p = 6.41 \times 10^{-3}$), *Casp1* ($\log_2$ fold change = 0.433, $p = 2.72 \times 10^{-2}$) and *Tinf2* ($\log_2$ fold change = 0.084, $p = 3.89 \times 10^{-2}$). There were 15 DEGs with downregulation, while *Atm* ($\log_2$ fold change = -0.535, $p = 7.3 \times 10^{-5}$), *Aurkb* ($\log_2$ fold change = -0.515, $p = 3.3 \times 10^{-3}$) and *Cdk1* ($\log_2$ fold change = -0.644, $p = 7.23 \times 10^{-3}$) were the most significant DEGs with downregulation.

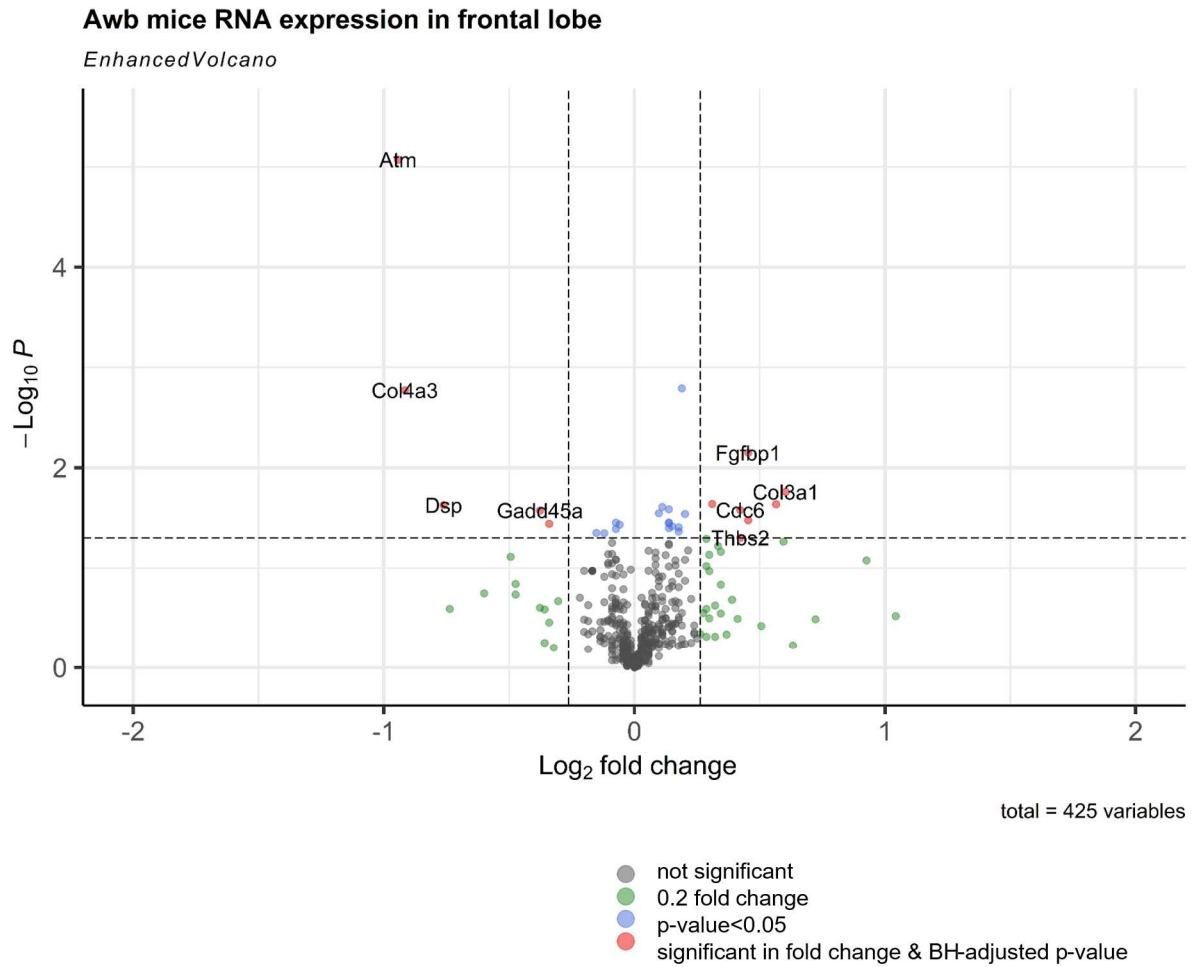


Figure 51. Volcano plot illustrating DEGs from RNA sequencing analysis in cerebral frontal lobe tissue between Awb^{-/-} and WT mice.

(DEGs indicated as red dots) (DEG definition: p-value < 0.05; |fold change| > 0.2)

There were 18 DEGs with upregulation and 10 DEGs with downregulation in the comparison between Awb and WT mice (Figure 51). *Pck2* ($\log_2$ fold change = 0.189, $p = 1.61 \times 10^{-3}$), *Fgfbp1* ($\log_2$ fold change = 0.454, $p = 7.1 \times 10^{-3}$) and *Col3a1* ($\log_2$ fold change = 0.604, $p = 1.74 \times 10^{-2}$) were the most significant DEGs with upregulation. *Atm* ($\log_2$ fold change = -0.943, $p = 8.48 \times 10^{-6}$), *Col4a3* ($\log_2$ fold change = -0.916, $p = 1.69 \times 10^{-3}$) and *Dsp* ($\log_2$ fold change = -0.761, $p = 2.35 \times 10^{-2}$) were the most significant DEGs with downregulation.

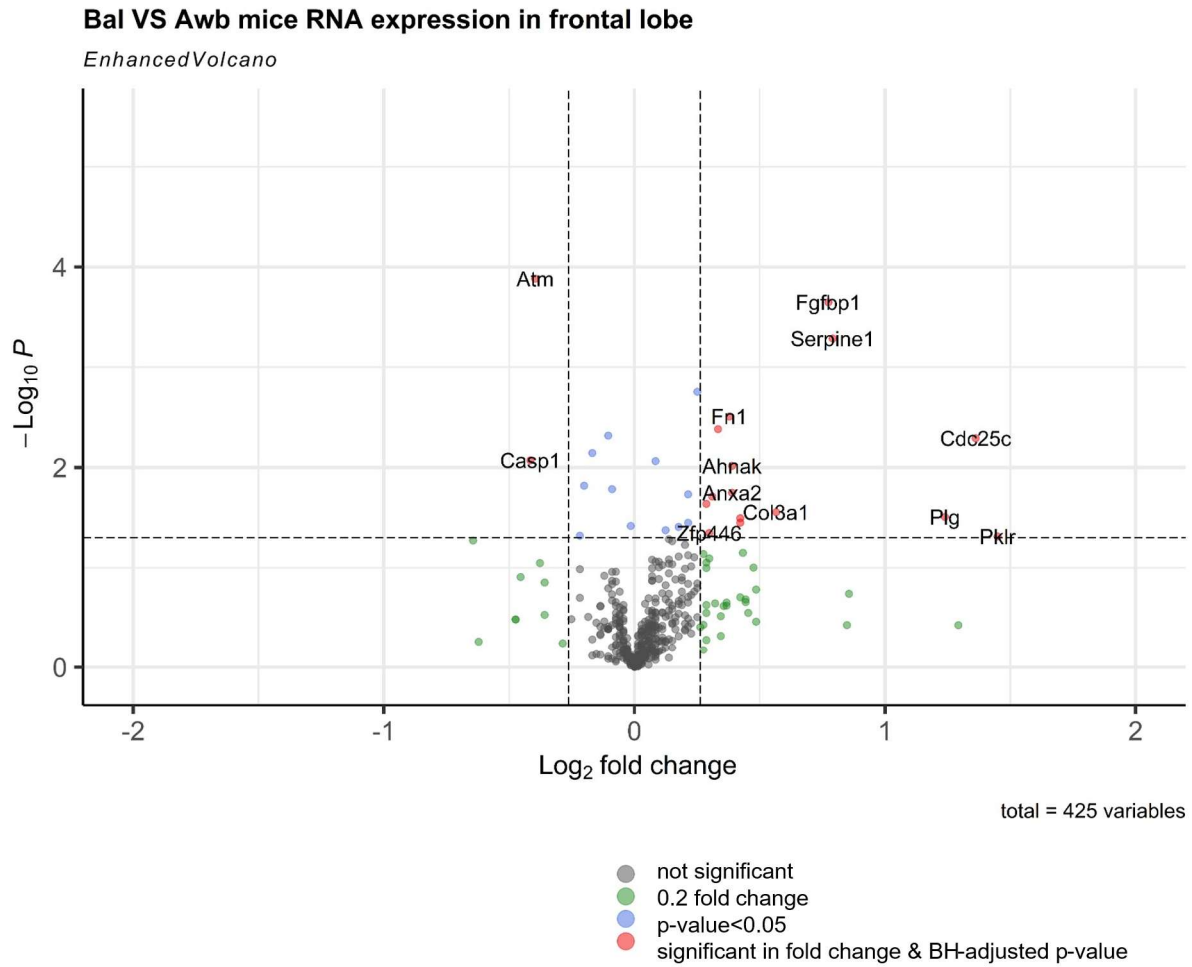


Figure 52. Volcano plot illustrating DEGs from RNA sequencing analysis in cerebral frontal lobe tissue between Bal^{-/-} and Awb^{-/-} mice.

(DEGs indicated as red dots) (DEG definition: p-value < 0.05; |fold change| > 0.2)

There were 21 DEGs with upregulation and 8 DEGs with downregulation in the comparison between Bal and Awb mice (Figure 52). *Fgf1p1* ($\log_2$ fold change = 0.774, $p = 2.25 \times 10^{-4}$), *Serpine1* ($\log_2$ fold change = 0.791, $p = 5.16 \times 10^{-4}$) and *Pms1* ($\log_2$ fold change = 0.251, $p = 1.76 \times 10^{-3}$) were the most significant DEGs with upregulation. *Atm* ($\log_2$ fold change = -0.394, $p = 1.31 \times 10^{-4}$), *E2f3* ($\log_2$ fold change = -0.105, $p = 4.81 \times 10^{-3}$) and *Ccne1* ($\log_2$ fold change = -0.168, $p = 7.17 \times 10^{-3}$) were the most significant DEGs with downregulation.

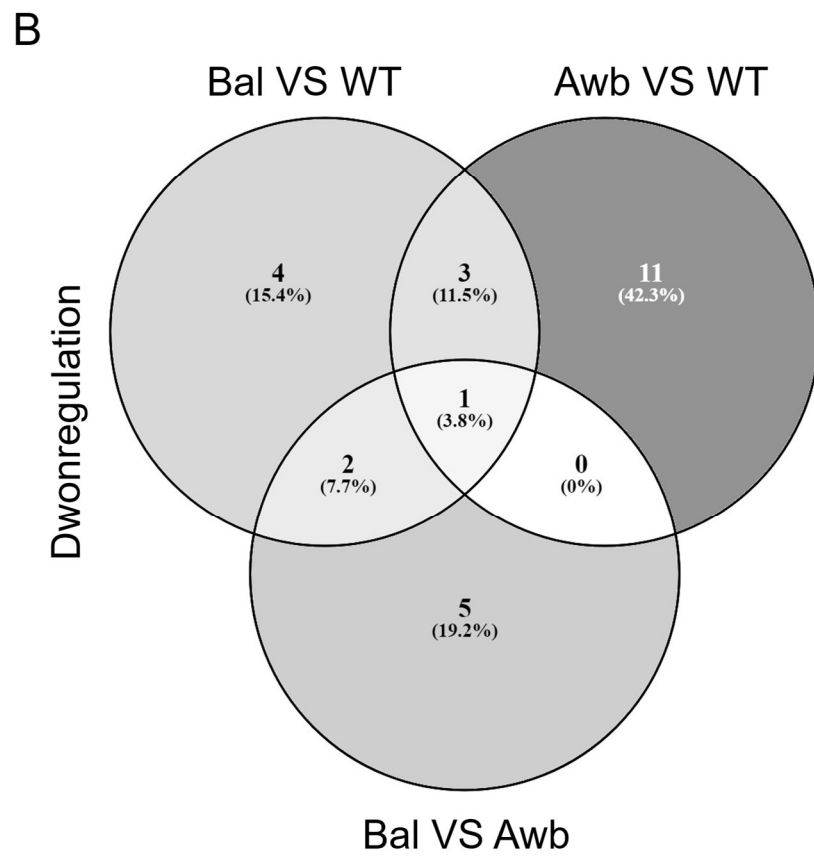
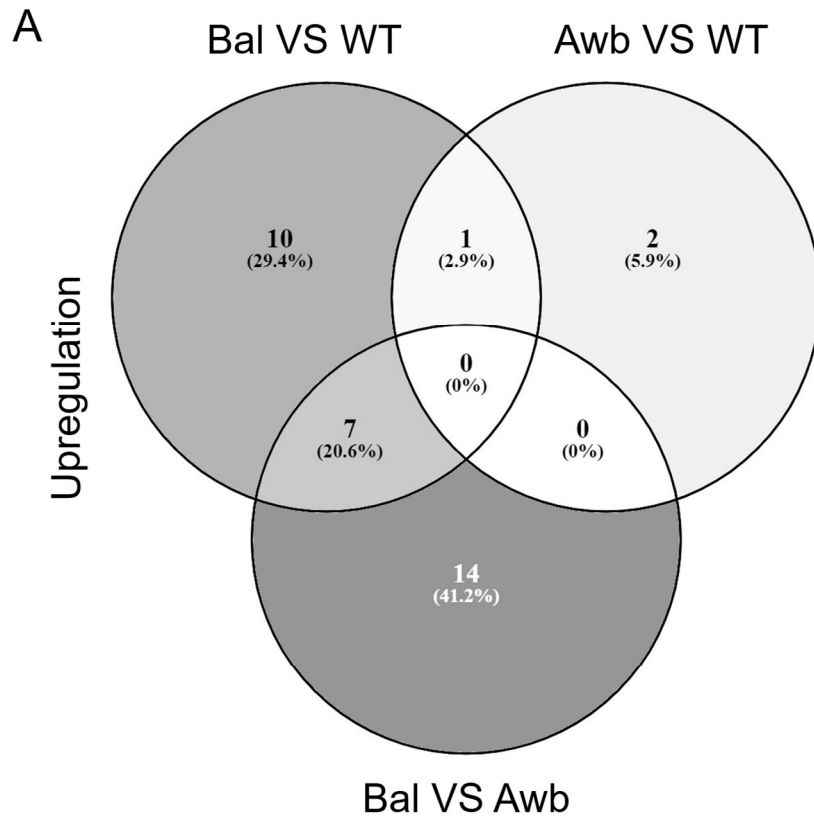
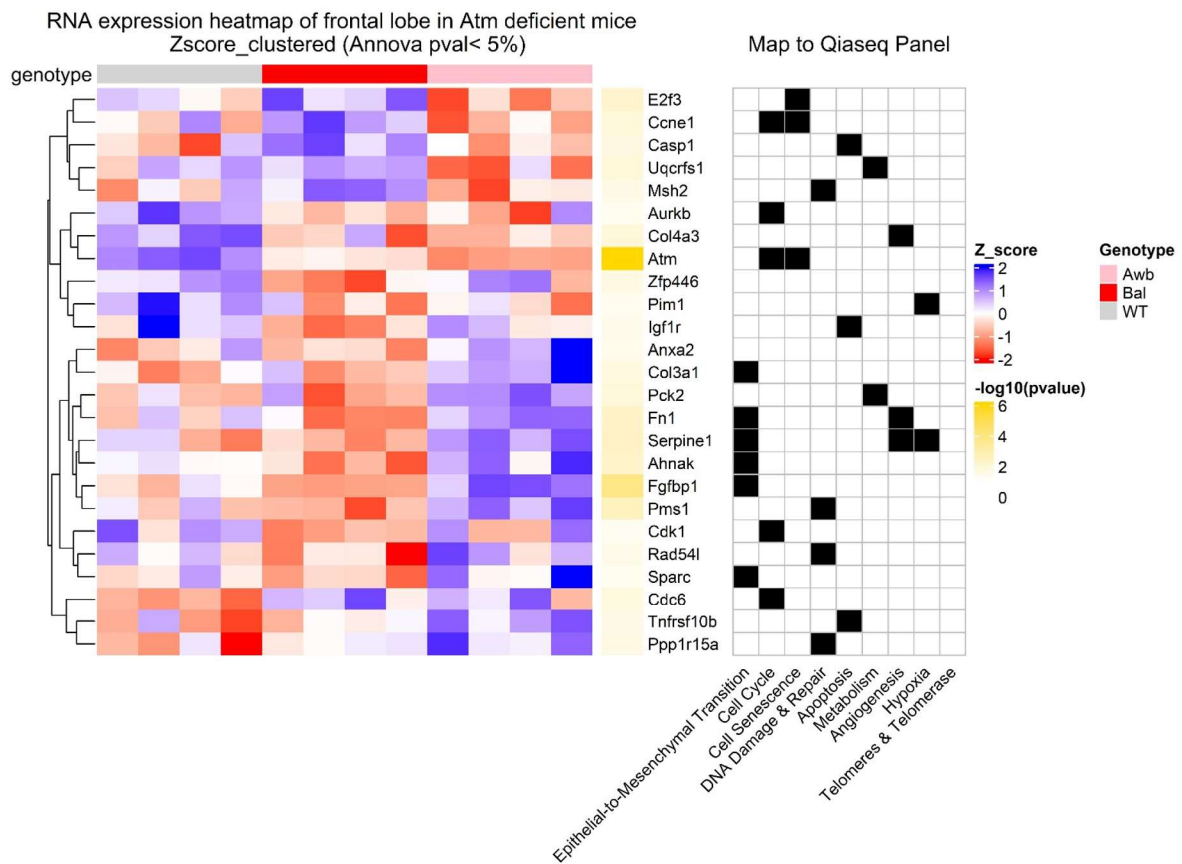


Figure 53. Venn diagram illustrating similarity between upregulated and downregulated DEG sets in three mice groups of frontal lobe comparison of targeted RNA sequencing.

A) Venn diagram with upregulated DEG sets. B) Venn diagram with downregulated DEG sets.

A



B

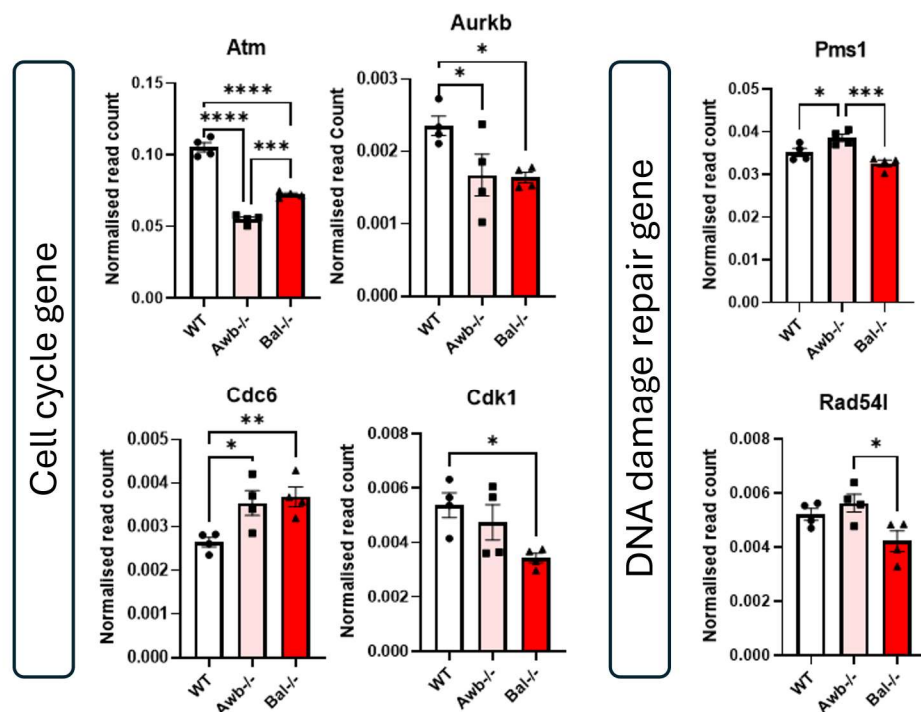


Figure 54. Mapping DEGs from targeted RNA sequencing to Qiaseq panel.

A) Heatmap illustrating DEGs with ANNOVA normalization among three groups of samples that were scaled with Z score from two to negative two, which mapped to Qiaseq panel. B) Representative normalised gene comparisons from cell cycle

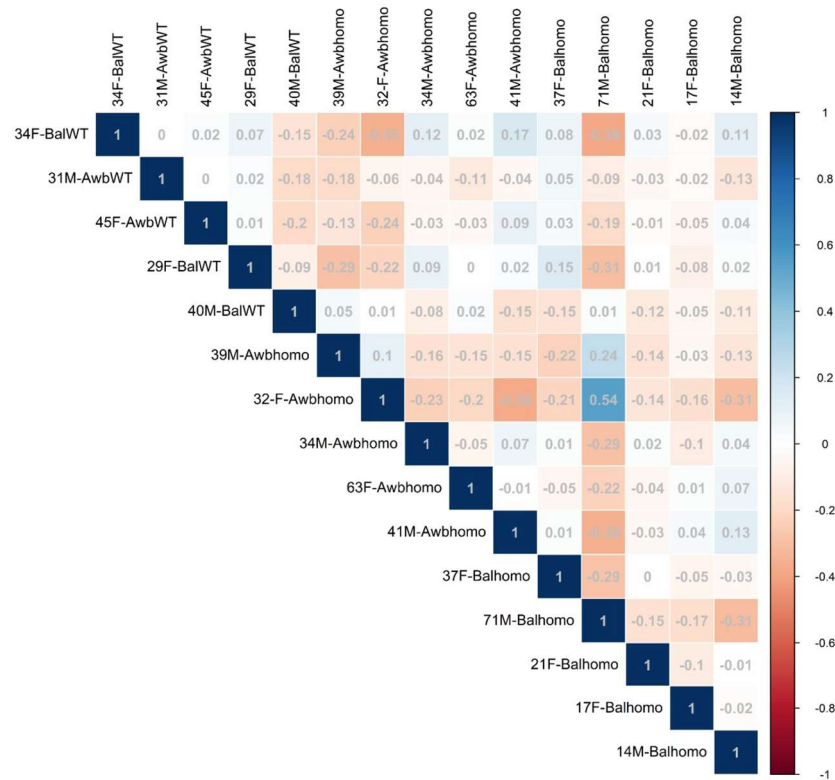
and DNA damage repair category. (One-way ANOVA with Fisher's LSD multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

There was no common DEG with upregulation in the three comparisons (Bal Vs WT, Awb Vs WT and Bal Vs Awb), but there was one common DEG (*Cdc6*) in the comparisons of each type of ATM deficient mice verses WT mice (Figure 53). Meanwhile, *Atm* was the common DEG with downregulation in the three comparisons, and there were three more common DEGs (*Col4a3*, *Pim1* and *Polb*) in the comparisons of each type of ATM deficient mice verses WT mice.

To further investigate the annotated gene with significance over three groups comparison, the aligned read count of three group samples was normalized one-way ANNOVA with Benjamini-Hochberg (BH) adjustment. After that, the significant genes (p -value < 0.05) were normalized with Z-score to plot on heatmap and mapped to their corresponding function under the Qiaseq panel. There were five significant genes related to cell cycle function and four significant genes related to DNA damage repair function (Figure 54A). *Atm* and *Aurkb* demonstrated a general reduced expression in the ATM deficient mice (*Atm*: Awb mice 47.6% reduction [$p < 0.0001$], Bal mice 31.4% reduction [$p < 0.0001$]; *Aurkb*: Awb mice 28.9% reduction [$p = 0.0314$], Bal mice 29.6% reduction [$p = 0.0253$]), while *Cdc6* expression was elevated in both Awb and Bal mice under the cell cycle function category (Awb mice 30.3% increase [$p = 0.0183$], Bal mice 36.8% reduction [$p = 0.0088$]) (Figure 54B).

Transcriptomic RNA sequencing (Illumina Stranded mRNA Prep)

A



B

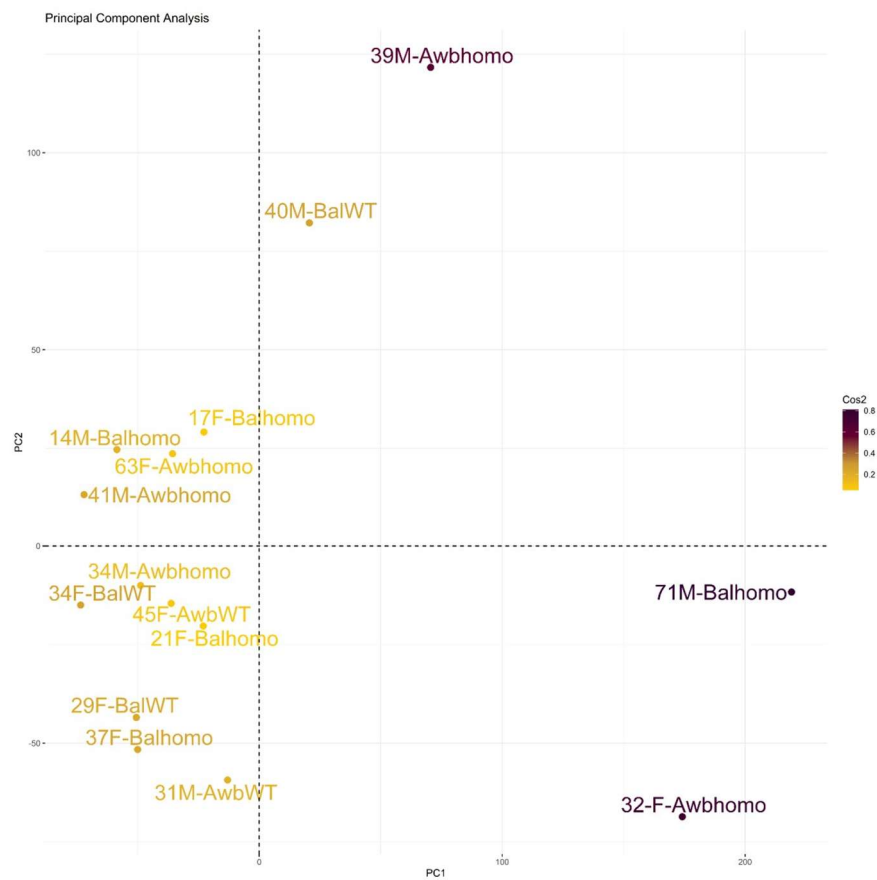


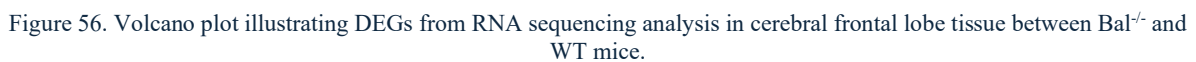
Figure 55. Quality assessment of the stranded RNA sequencing of cerebral frontal lobe of ATM deficient mice at 4MO.

(Control: n=5, Awb^{-/-}: n=5, Bal^{-/-}: n=5). A) Pearson correlation matrix of each sample in the targeted RNA sequencing. B) PCA displaying all 15 samples along PC1 and PC2.

Raw read counts generated from Dragen RNA pipeline were then normalized with DESeq2 (v 1.42.1). Pearson's correlation coefficient was calculated with the normalized read count of each sample reflecting the linear correlations against other sample. The coefficients were then scaled from one to negative one in the heatmap (Figure 55A), reflecting the relative degree of correlation. Internal comparison among WT sample ranged from 0.07 to -0.2, while one of the WT samples (40M-BalWT) revealed negative scaled correlation with other WT (Figure 55A). Compared to the control sample in human targeted sequencing (Figure 22A, Figure 26A), this experiment demonstrated a less correlation within control group and a less difference between control group and target groups. According to PCA plot in Figure 53B, the WT sample (40M-BalWT) with negative scaled correlation also demonstrated greater distance in the variation compared with other WT samples.

Due to the close proximity between samples in different group, we failed to obtain enough DEGs with adjusted p-value for pathway enrichment. In this study, we utilised the raw p-value for volcano plot and downstream pathway enrichment in order to simulate the potential effect of Atm deficiency on mice frontal lobe function.

Enhanced Volcano



Fold change and significance were calculated on each gene by DESeq2 and plotted against each group of samples, which formed volcano plot in every comparison. The whole transcriptome was mapped against the mouse reference genome GRCm39 with a total of 19760 genes annotated. In the comparison between Bal^{-/-} and WT mice, there were 270 genes reaching significance with p-value less than 0.05 (Figure 56). There were 123 genes upregulated with

fold change percentage more than 20%, while 74 genes were downregulated with fold change percentage over 20% (Figure 56).

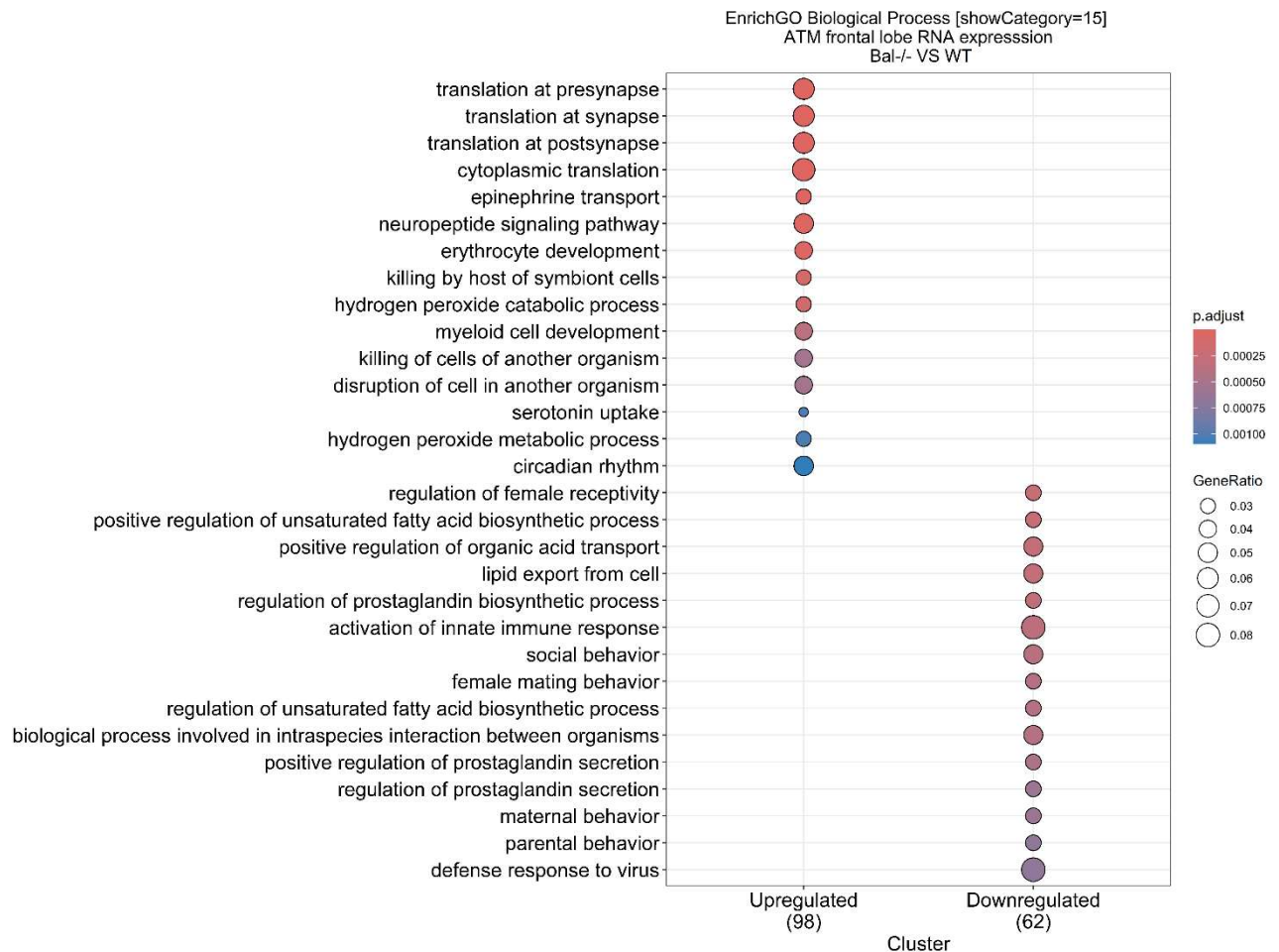


Figure 57. Dot plot for gene set enrichment of DEGs upregulated and downregulated in Bal^{-/-} mice frontal lobe tissue with EnrichGO biological process pathway sets compared with WT mice.

Significance of enrichment results were normalized with BH-FDR method. Top 10 significantly enriched pathways were selected for demonstration.

Synapse related pathways were highlighted in the enrichment of upregulated DEGs (Figure 55), which the top three enriched pathways were translation at presynapse ($p^{\text{adj}} = 1.23 \times 10^{-8}$), translation at synapse ($p^{\text{adj}} = 1.43 \times 10^{-8}$) and translation at postsynapse ($p^{\text{adj}} = 1.43 \times 10^{-8}$). Meanwhile, regulation of female receptivity ($p^{\text{adj}} = 2.46 \times 10^{-4}$), positive regulation of unsaturated fatty acid biosynthetic process ($p^{\text{adj}} = 2.46 \times 10^{-4}$) and positive regulation of organic

acid transport ($p^{\text{adj}} = 2.81 \times 10^{-4}$) were the most significant pathways enriched with downregulated DEGs.

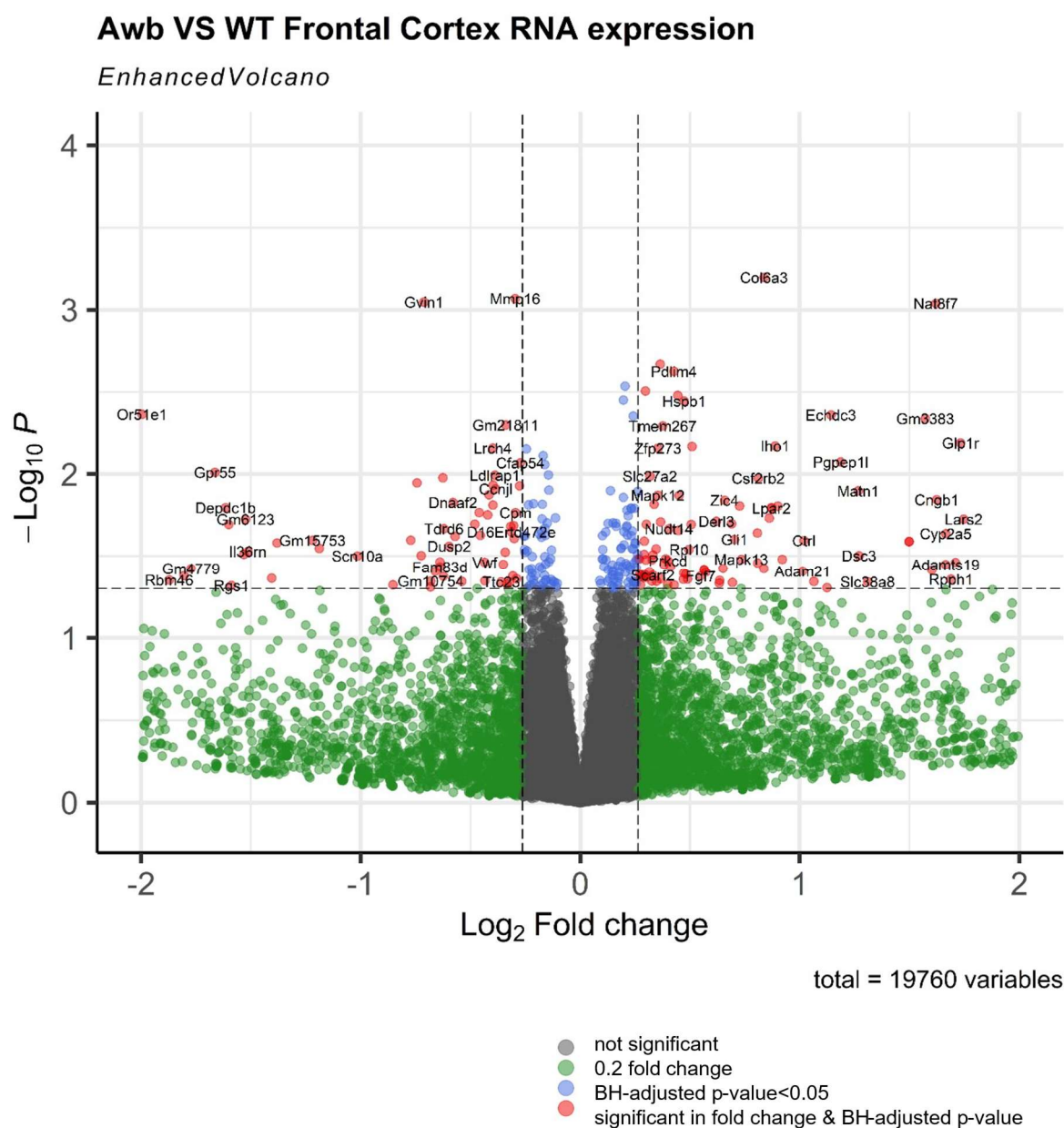


Figure 58. Volcano plot illustrating DEGs from RNA sequencing analysis in cerebral frontal lobe tissue between Awb^{-/-} and WT mice.

(DEGs indicated as red dots) (DEG definition: p-value < 0.05; |fold change| > 0.2)

In the comparison between Awb^{-/-} and WT mice, there were 313 genes with significance less than 0.05 (Figure 58). Considered having fold change over 0.2 as the threshold of DEG in this experiment, there were 111 upregulated DEGs and 77 downregulated DEGs (Figure 58).

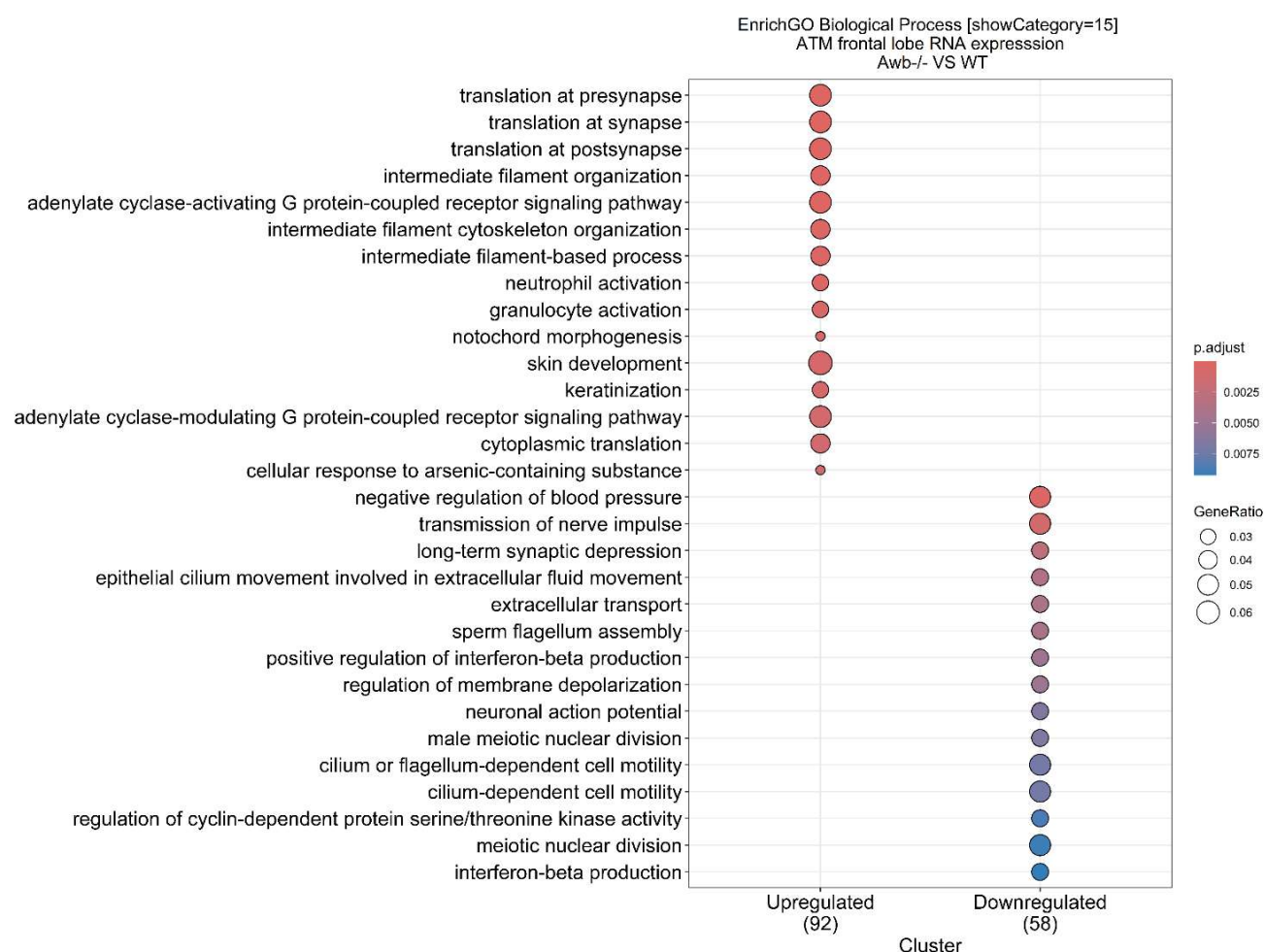


Figure 59. Dot plot for gene set enrichment of DEGs upregulated and downregulated in Awb^{-/-} mice frontal lobe tissue with EnrichGO biological process pathway sets compared with WT mice.

Significance of enrichment results were normalized with BH-FDR method. Top 10 significantly enriched pathways were selected for demonstration.

Alike the comparison of Bal^{-/-} and WT mice, synapse related pathways were also significantly enriched with the upregulated DEGs in the comparison of Awb^{-/-} and WT mice, including translation at presynapse ($p^{\text{adj}} = 4.05 \times 10^{-7}$), translation at synapse ($p^{\text{adj}} = 4.51 \times 10^{-7}$) and translation at postsynapse ($p^{\text{adj}} = 4.51 \times 10^{-7}$). Meanwhile, negative regulation of blood pressure ($p^{\text{adj}} = 3.36 \times 10^{-4}$), transmission of nerve impulse ($p^{\text{adj}} = 1.18 \times 10^{-3}$) and long-term synaptic depression ($p^{\text{adj}} = 2.80 \times 10^{-3}$) were the most significant pathways enriched with downregulated DEGs.

Bal VS Awb Frontal Cortex RNA expression

EnhancedVolcano

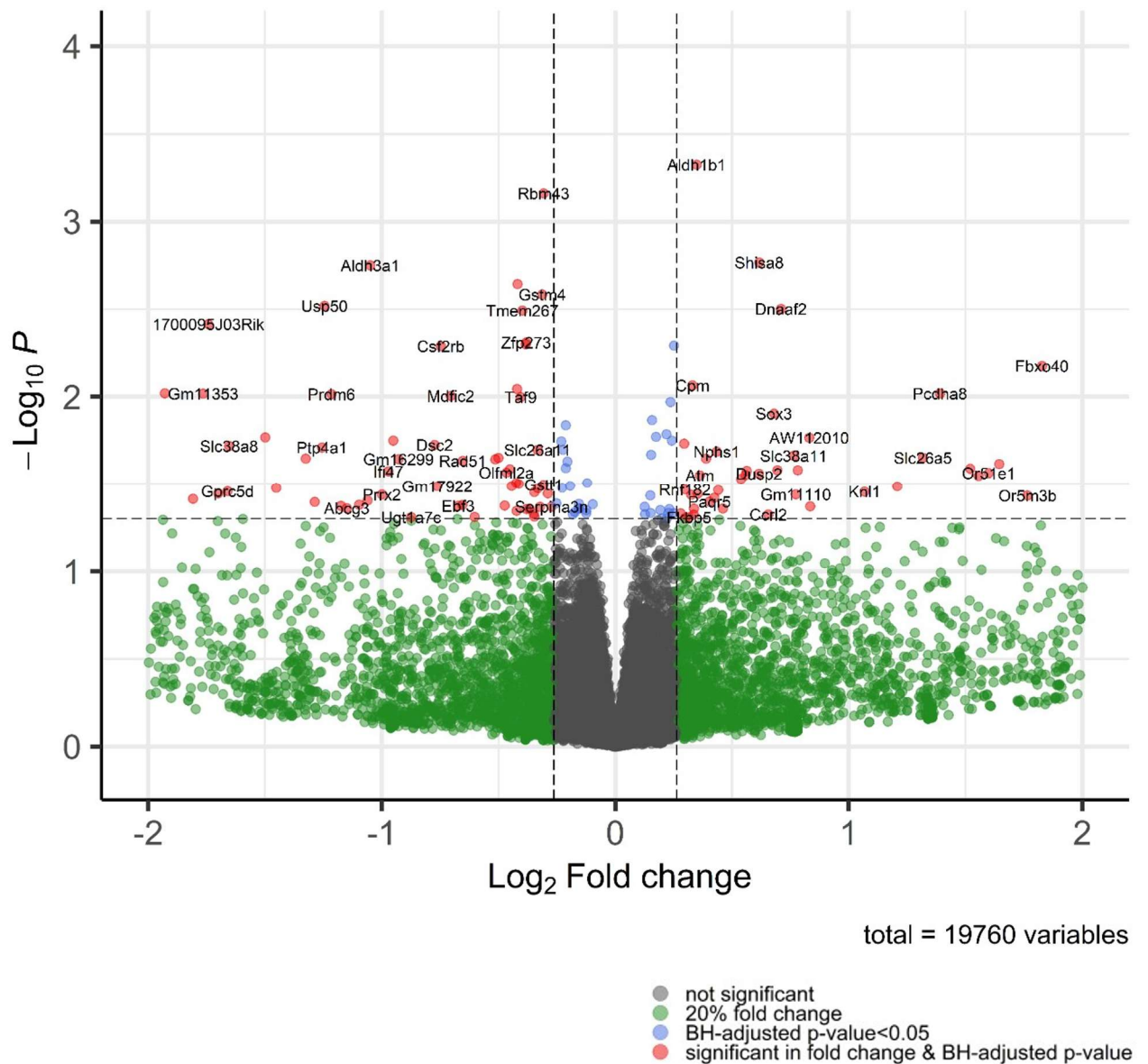


Figure 60. Volcano plot illustrating DEGs from RNA sequencing analysis in cerebral frontal lobe tissue between Bal^{-/-} and Awb^{-/-} mice.

(DEGs indicated as red dots) (DEG definition: p-value < 0.05; |fold change| > 20%)

In the comparison between Bal^{-/-} and Awb^{-/-} mice, there were 169 genes with significance less than 0.05 (Figure 60), while there were 60 upregulated DEGs and 70 downregulated DEGs (Figure 60).

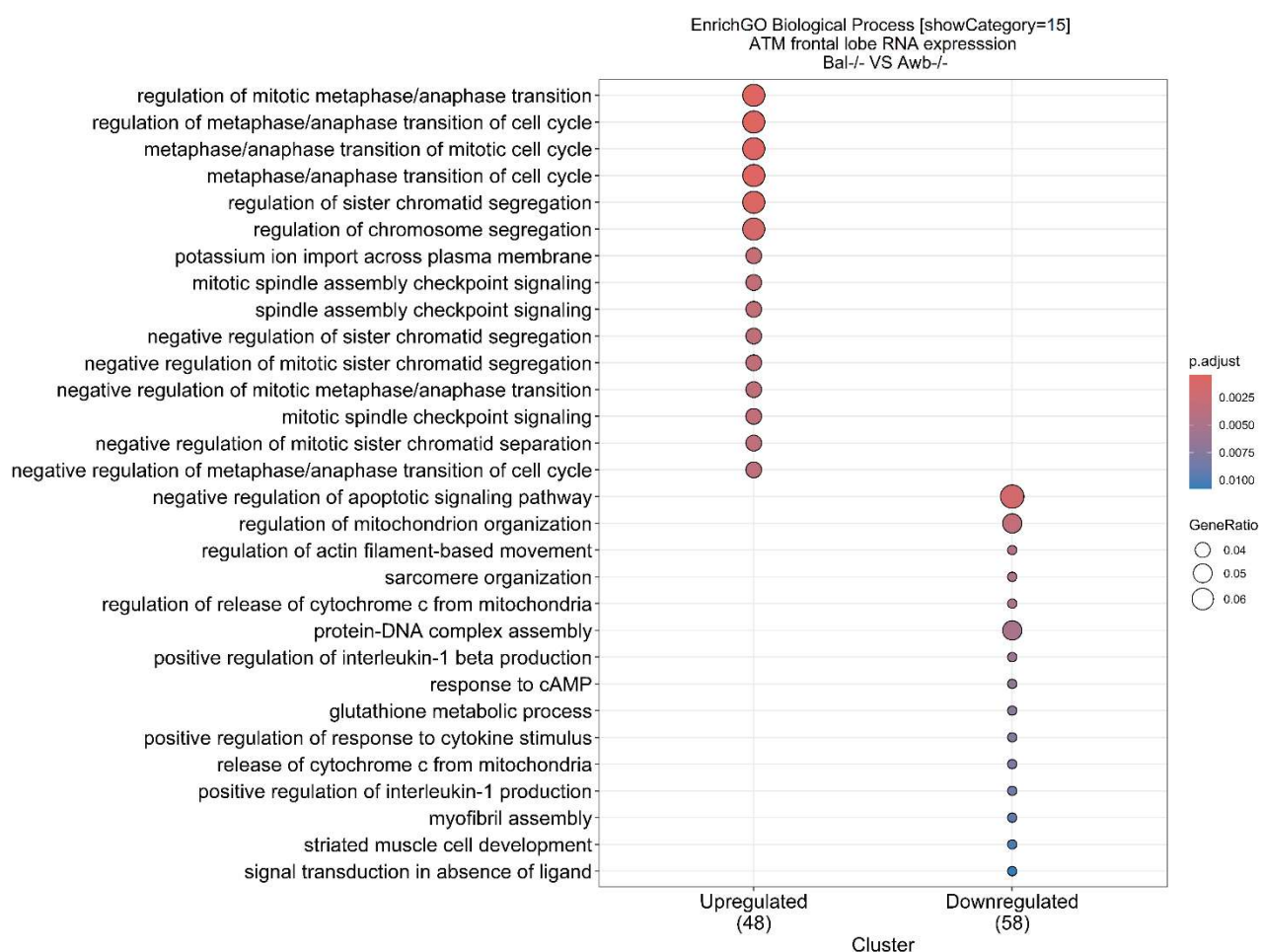


Figure 61. Dot plot for gene set enrichment of DEGs upregulated and downregulated in Bal^{-/-} mice frontal lobe tissue with EnrichGO biological process pathway sets compared with Awb^{-/-} mice.

Significance of enrichment results were normalized with BH-FDR method. Top 10 significantly enriched pathways were selected for demonstration.

Regulation of mitotic metaphase/anaphase transition ($p^{\text{adj}} = 4.57 \times 10^{-4}$), regulation of metaphase/anaphase transition of cell cycle ($p^{\text{adj}} = 5.03 \times 10^{-4}$) and metaphase/anaphase transition of mitotic cell cycle ($p^{\text{adj}} = 5.35 \times 10^{-4}$) were the most significant pathways enriched with the upregulated DEGs in comparison between Bal^{-/-} and Awb^{-/-} mice. Meanwhile, negative regulation of apoptotic signaling pathway ($p^{\text{adj}} = 1.68 \times 10^{-3}$), regulation of mitochondrion organization ($p^{\text{adj}} = 3.31 \times 10^{-3}$) and regulation of actin filament-based movement ($p^{\text{adj}} = 4.38 \times 10^{-3}$) were the most significant pathways enriched with the downregulated DEGs.

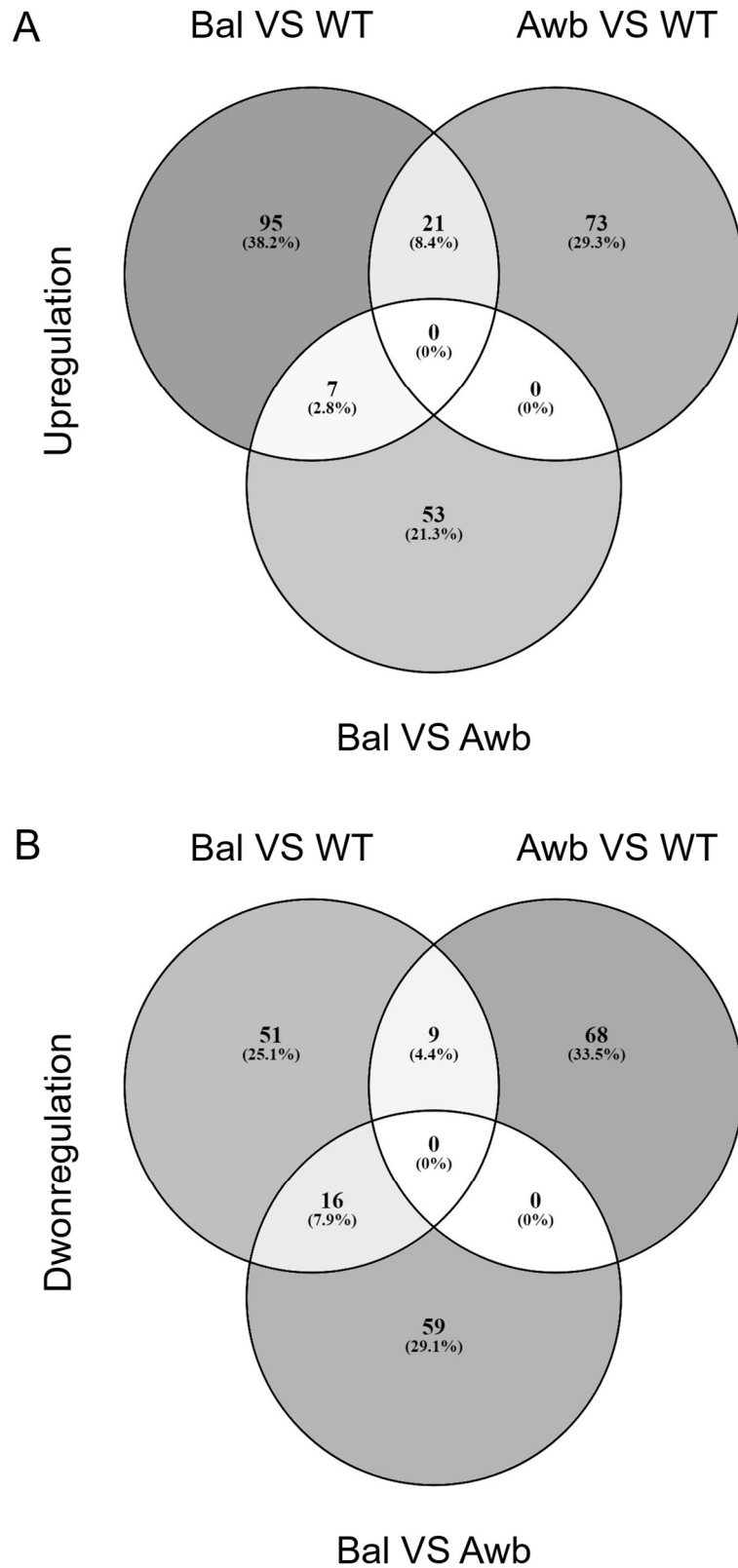


Figure 62. Venn diagram illustrating similarity between upregulated and downregulated DEG sets in three mice groups of frontal lobe comparison.

A) Venn diagram with upregulated DEG sets. B) Venn diagram with downregulated DEG sets.

genotype

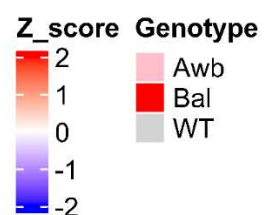
Z_score Genotype

2
1
0
-1
-2

Awb
Bal
WT

175

genotype



and Awb^{-/-}) that were scaled with Z score from two to negative two.

To have a better understanding of the specific *Atm* mutation effect on mice frontal lobe, venn charts were plotted against the upregulated and downregulated DEGs in each comparison. The DEGs unique to $Bal^{-/-}$ and $Awb^{-/-}$ mice are crucial for us to delineate the differential effect between total *Atm* knockout and *Atm* knockout with residual ATM kinase function on frontal lobe of the mouse brain. There were 95 DEGs uniquely upregulated in the $Bal^{-/-}$ WT mice comparison, 73 DEGs uniquely upregulated in the $Awb^{-/-}$ WT mice comparison and 53 DEGs uniquely upregulated in the $Bal^{-/-}$ $Awb^{-/-}$ mice comparison (Figure 62). There were 51 DEGs uniquely downregulated in the $Bal^{-/-}$ WT mice comparison, 68 DEGs uniquely downregulated in the $Awb^{-/-}$ WT mice comparison and 59 DEGs uniquely downregulated in the $Bal^{-/-}$ $Awb^{-/-}$ mice comparison (Figure 62). The heatmaps revealed the list of DEGs which were unique in each comparison (Figure 63, Figure 64).

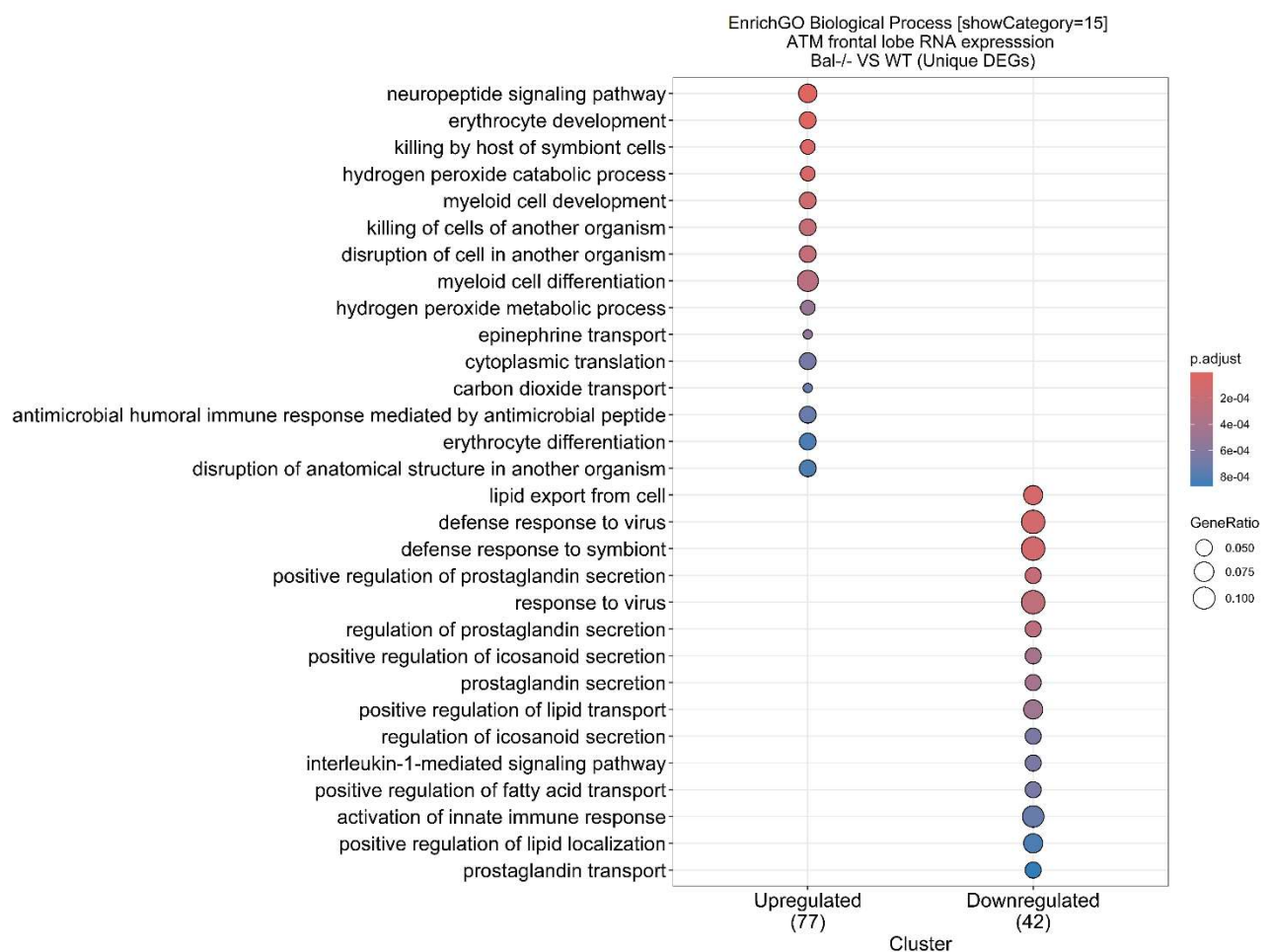


Figure 65. Dot plot for gene set enrichment of unique DEGs upregulated and downregulated in Bal^{-/-} mice frontal lobe tissue with EnrichGO biological process pathway sets compared with WT mice.

Significance of enrichment results were normalized with BH-FDR method. Top 10 significantly enriched pathways were selected for demonstration.

Neuropeptide signaling pathway ($p^{\text{adj}} = 4.84 \times 10^{-6}$), erythrocyte development ($p^{\text{adj}} = 9.02 \times 10^{-6}$) and killing by host of symbiont cells ($p^{\text{adj}} = 5.08 \times 10^{-5}$) were the most significant pathways enriched with the uniquely upregulated DEGs in the comparison of Bal^{-/-} and WT mice. Meanwhile, lipid export from cell ($p^{\text{adj}} = 8.77 \times 10^{-5}$), defense response to virus ($p^{\text{adj}} = 1.10 \times 10^{-4}$) and defense response to symbiont ($p^{\text{adj}} = 1.12 \times 10^{-4}$) were the most significant pathways enriched with the uniquely downregulated DEGs in the comparison of Bal^{-/-} and WT mice.

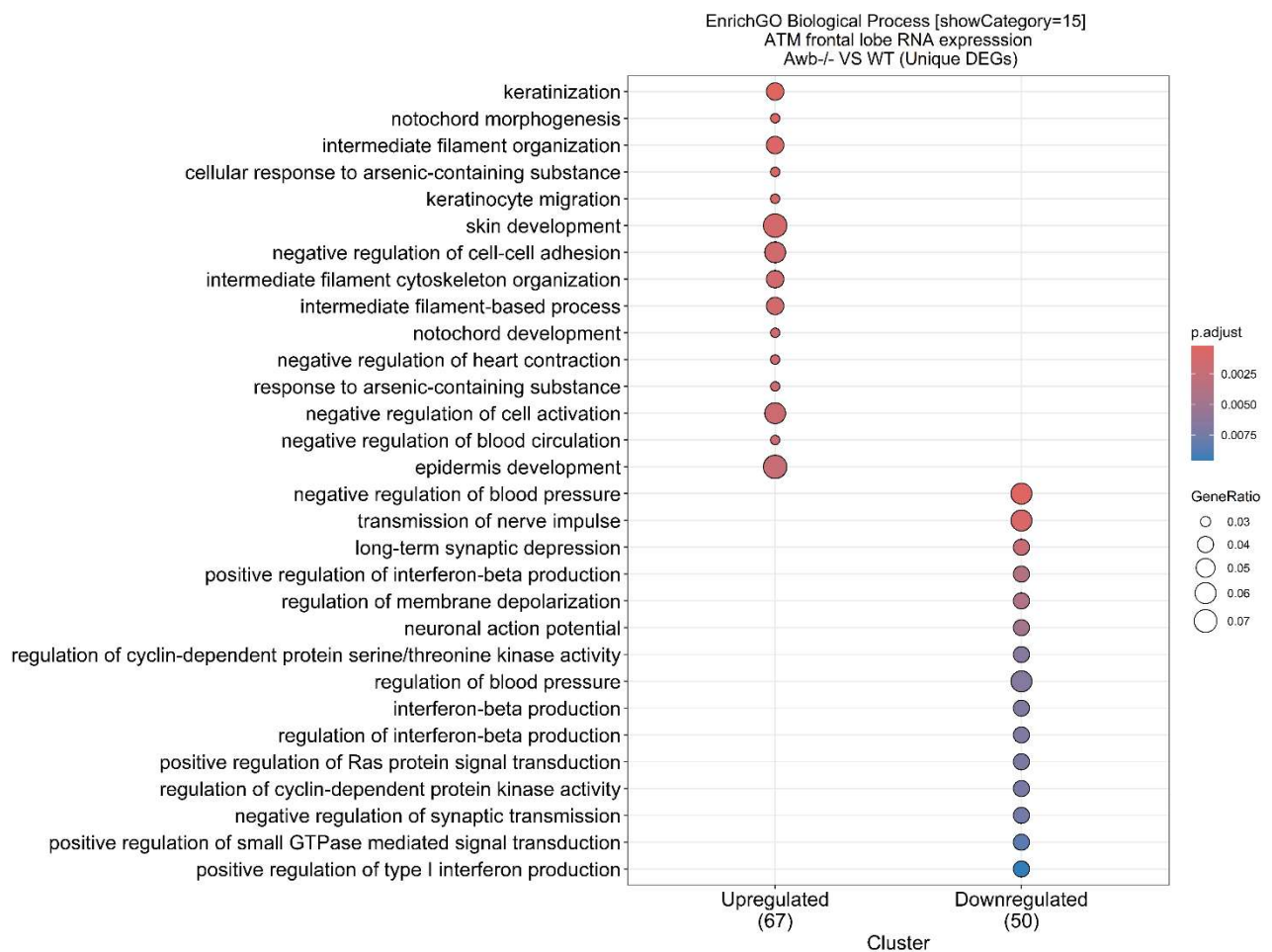


Figure 66. Dot plot for gene set enrichment of unique DEGs upregulated and downregulated in Awb^{-/-} mice frontal lobe tissue with EnrichGO biological process pathway sets compared with WT mice.

Significance of enrichment results were normalized with BH-FDR method. Top 10 significantly enriched pathways were selected for demonstration.

Keratinization ($p^{\text{adj}} = 3.71 \times 10^{-4}$), notochord morphogenesis ($p^{\text{adj}} = 4.06 \times 10^{-4}$) and intermediate filament organization ($p^{\text{adj}} = 7.42 \times 10^{-4}$) were the most significant pathways enriched with the uniquely upregulated DEGs in the comparison of *Awb*^{-/-} and WT mice. Meanwhile, negative regulation of blood pressure ($p^{\text{adj}} = 2.16 \times 10^{-4}$), transmission of nerve impulse ($p^{\text{adj}} = 7.64 \times 10^{-4}$) and long-term synaptic depression ($p^{\text{adj}} = 2.09 \times 10^{-3}$) were the most significant pathways enriched with the uniquely downregulated DEGs in the comparison of *Awb*^{-/-} and WT mice.

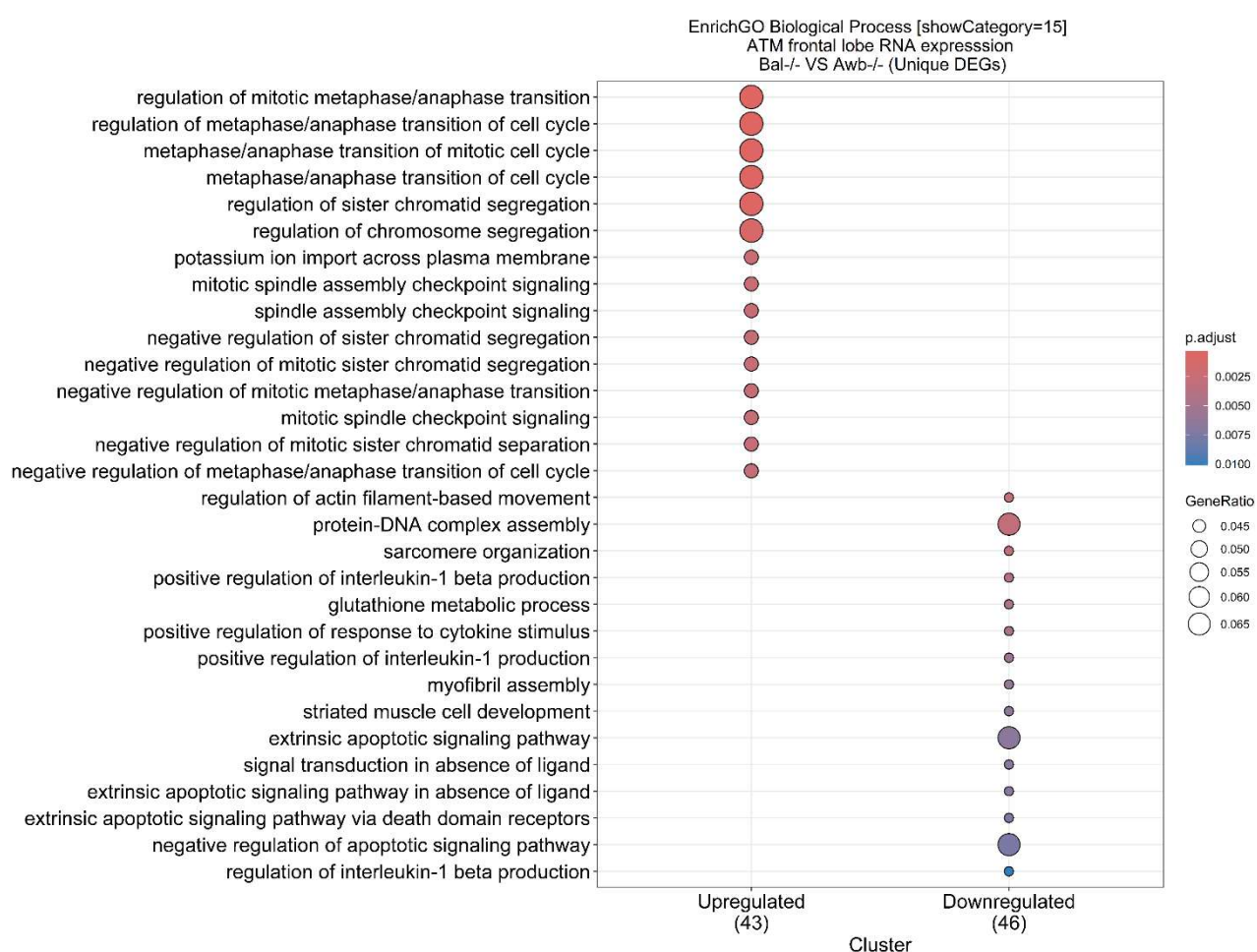


Figure 67. Dot plot for gene set enrichment of unique DEGs upregulated and downregulated in *Bal*^{-/-} mice frontal lobe tissue with EnrichGO biological process pathway sets compared with *Awb*^{-/-} mice.

Significance of enrichment results were normalized with BH-FDR method. Top 10 significantly enriched pathways were selected for demonstration.

Cell cycle related pathways were highlighted in the enrichment of unique upregulated DEGs of comparison between $Bal^{-/-}$ and $Awb^{-/-}$ mice, including regulation of mitotic metaphase/anaphase transition ($p^{adj} = 3.30 \times 10^{-4}$), regulation of metaphase/anaphase transition of cell cycle ($p^{adj} = 3.63 \times 10^{-4}$) and metaphase/anaphase transition of mitotic cell cycle ($p^{adj} = 3.86 \times 10^{-4}$). Meanwhile, regulation of actin filament-based movement ($p^{adj} = 2.78 \times 10^{-3}$), protein-DNA complex assembly ($p^{adj} = 2.78 \times 10^{-3}$) and sarcomere organization ($p^{adj} = 2.89 \times 10^{-3}$) were the most significant pathways enriched with the uniquely downregulated DEGs in the comparison of $Bal^{-/-}$ and $Awb^{-/-}$ mice.

Defective oligodendrocyte differentiation in ATM deficient primary OL culture

Despite the similarity in the pathways enriched by upregulated DEGs in two ATM deficient mice models, the frontal lobe RNA expression in the two models demonstrated distinct pathways associated with downregulated DEGs. Myelin pathology could be associated with the lipid transportation pathway enriched by unique downregulated DEGs in the comparison of $Bal^{-/-}$ and WT mice. However, the causal relationship between ATM deficiency and myelin pathology cannot be confirmed with the RNA sequencing experiment. To extend the investigation, primary glial culture was employed for the direct examination of mutational effect on myelination of OL.

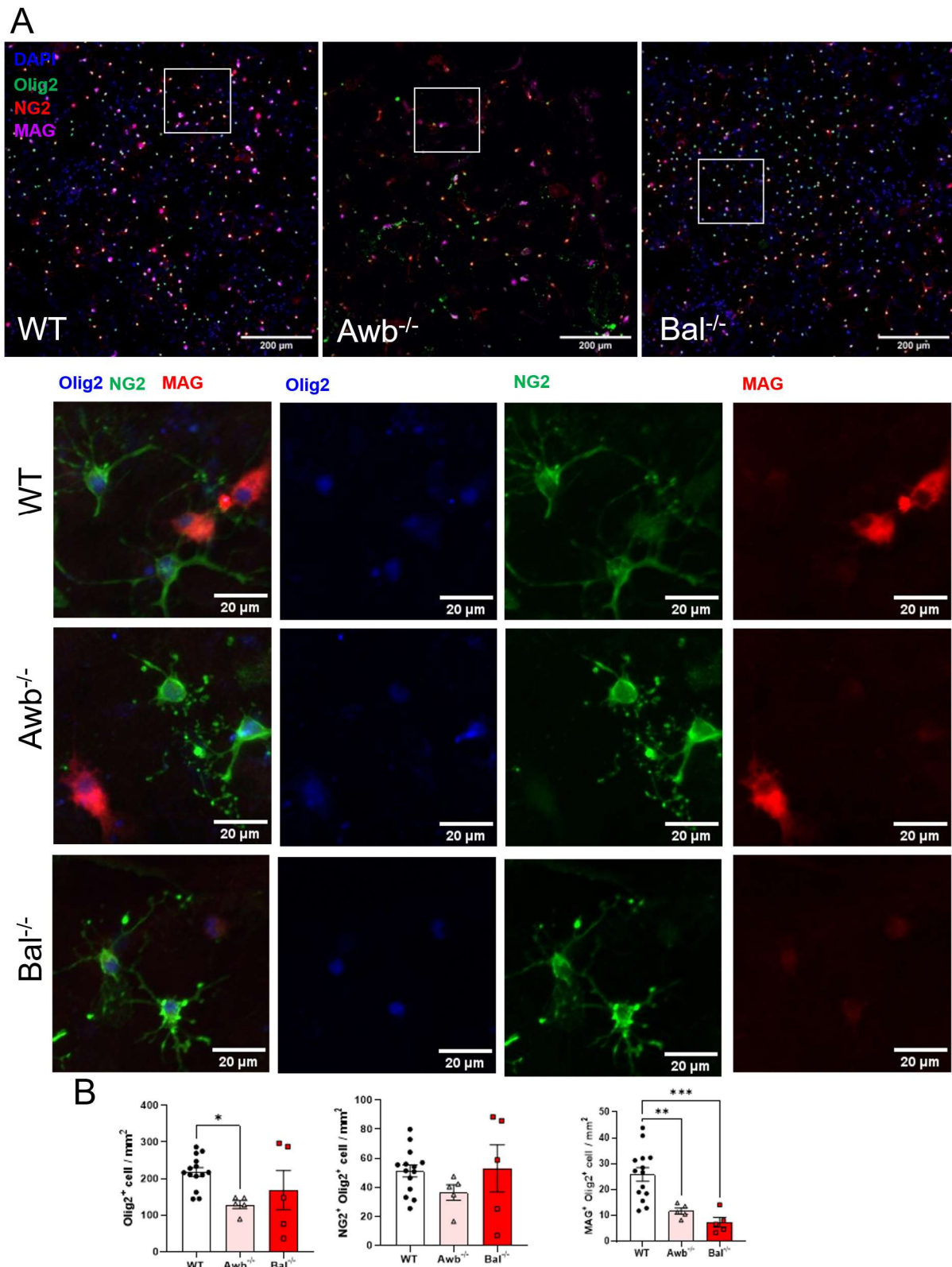


Figure 68. OL lineage of primary murine glial culture upon different *Atm* mutation effect.

A) Representative immunocytochemistry image of primary mOL culture of *Atm* deficient mice with different *Atm* mutation denoting OL lineage with Olig2(green), Ng2(red) and Mag(magenta). B) Quantification of Olig2+ OL, NG2+ OPC and MAG+ mOL cell density. (One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Primary mOL culture was induced by growth factors withdrawal (PDGF-AA and IGF 10 ng/mL) for twelve days DIV. The Olig2⁺ OL density was decreased by 40.8% (p = 0.0356) in Awb^{-/-} mice culture (Figure 68B). Yet, NG2⁺ OL cell density showed no difference among three groups of primary culture, which implied minimal effect of ATM deficiency on OPC populations development in primary culture. Noteworthy, MAG⁺ OL density was significantly reduced in both Awb^{-/-} mice and Bal^{-/-} mice primary culture by 54.7% (p = 0.0079) and 71.6% (p = 0.0007) respectively (Figure 68B).

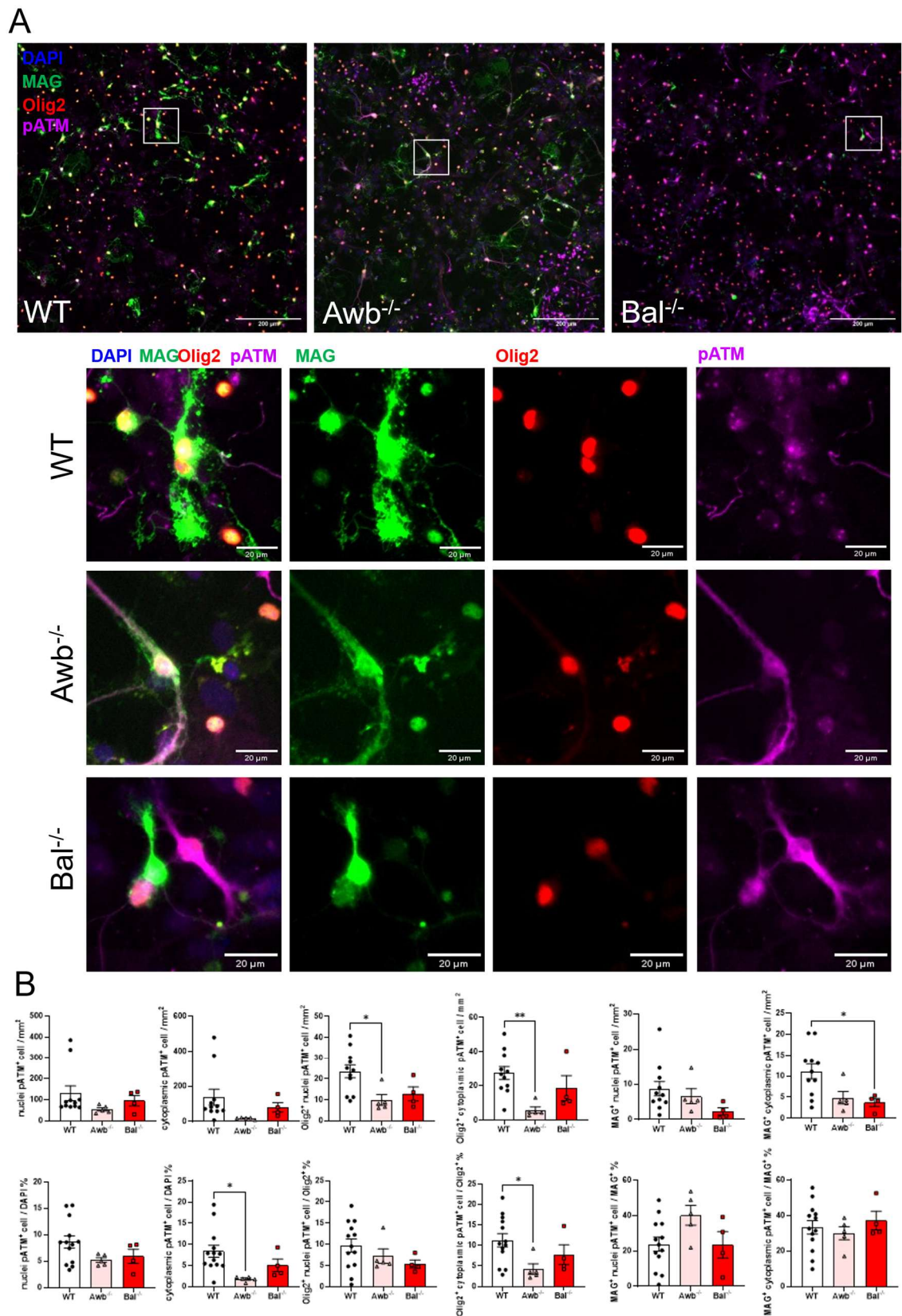


Figure 69. Expression of pATM in OL lineage of primary murine glial culture upon different *Atm* mutation effect.

A) Representative immunocytochemistry image of primary mOL culture of ATM deficient mice with different *Atm* mutation denoting OL lineage with Mag(green), Olig2(red) and pATM(magenta). B) Quantification of cell density population

percentage of nuclei and cytoplasmic pATM expression, and their co-expression with Olig2+ OL and MAG+ mOL. (One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

ATM function varies in nucleus and cytoplasm, DSB repair mechanism and cell cycle regulation were well known as nucleus function of ATM. To further examine the pattern of ATM expression in OL lineage, primary culture samples were probed with phosphorylated ATM marker, OL marker (Olig2) and mOL marker (MAG).

Representative image showing pATM expression in nucleus and cytoplasm of Mag⁺ mOL is presented in Figure 69A. There was slight reduction in the mean of nuclei pATM expressing cell density and population of Awb^{-/-} mice and Bal^{-/-} mice primary culture without significance. Slight reduction was also found in cytoplasmic pATM expressing cell density and population of Bal^{-/-} mice primary culture without significance. Population of cytoplasmic pATM expressing cell in Awb^{-/-} mice primary culture was significantly reduced by 78.5% ($p = 0.0214$). Density of nuclei pATM expressing OL was significantly decreased by 57.8% ($p = 0.0267$). Moreover, density and population of cytoplasmic pATM expressing OL was notably decreased by 78.5% ($p = 0.0081$) and 62.1% ($p = 0.0442$). Density and population of nuclei pATM expressing mOL had no significant change in both Awb^{-/-} mice and Bal^{-/-} mice primary culture. Indeed, density of cytoplasmic pATM expressing mOL was significantly reduced by 65.9% ($p = 0.0482$).

Potential interaction between ATM and myelin related protein

The cytoplasmic expression of pATM was found in Mag⁺ mOL in primary culture. To further examine the potential function of ATM on myelination, MBP and pATM colocalization was observed under Nikon structural illumination microscopy (N-SIM) with 100x lens.

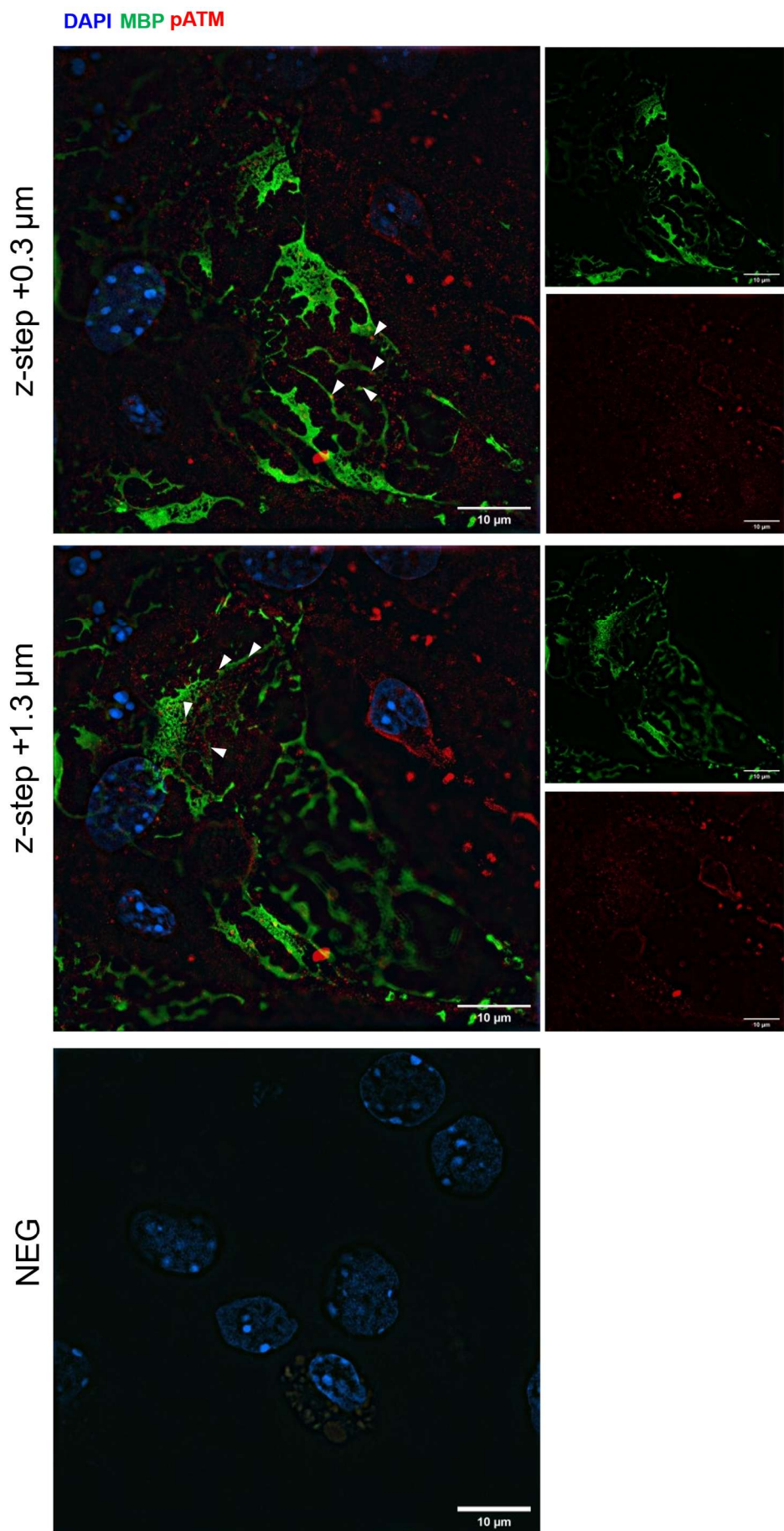


Figure 70. Nikon structural illumination microscopy (N-SIM) reconstructed image (100x) of myelinating OL.

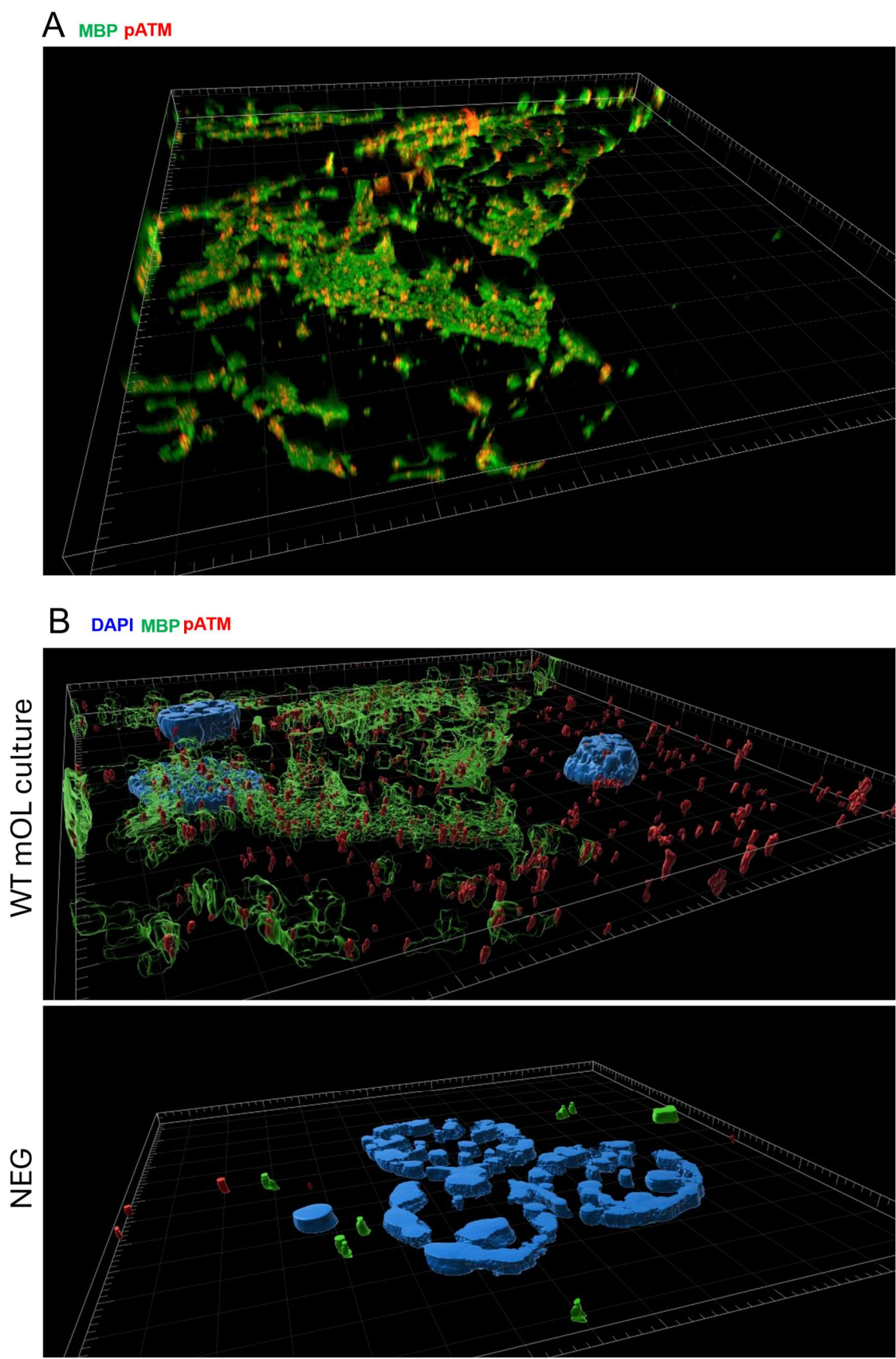


Figure 71. 3D construction of N-SIM image with Imaris 9.5.

A) Colocalization between pATM and MBP exclusively in the MBP membrane of mOL. B) Artificially constructed 3D volume of DAPI (blue), MBP (green) and pATM (red) of N-SIM image.

Image was acquired with three-dimensional structured illumination microscopy (3DSIM) illumination and capturing model, while the z step size was set at 0.1 μm . The acquired image was then reconstructed with local N-SIM reconstruction software in 3DSIM model (Illumination modulation contrast: 1.0, High resolution noise suppression: 0.5, Out of focus blur suppression: 0.15) and achieved reconstruction score eight.

The images shown in figure 68 were two z-plate cross-sections from one reconstructed N-SIM capture. White arrows indicated the in-focus colocalization between MBP and pATM, which was demonstrated at different z-plates (Figure 70).

Moreover, the reconstructed image was demonstrated as a 3D image with the aid of Imaris 9.5 software. Figure 71A demonstrated abundant colocalization regions between pATM and MBP exclusively in the MBP sheath of mOL. Artificial 3D volume created by Imaris also demonstrated actual contact between MBP and pATM in 3D view (Figure 71B).

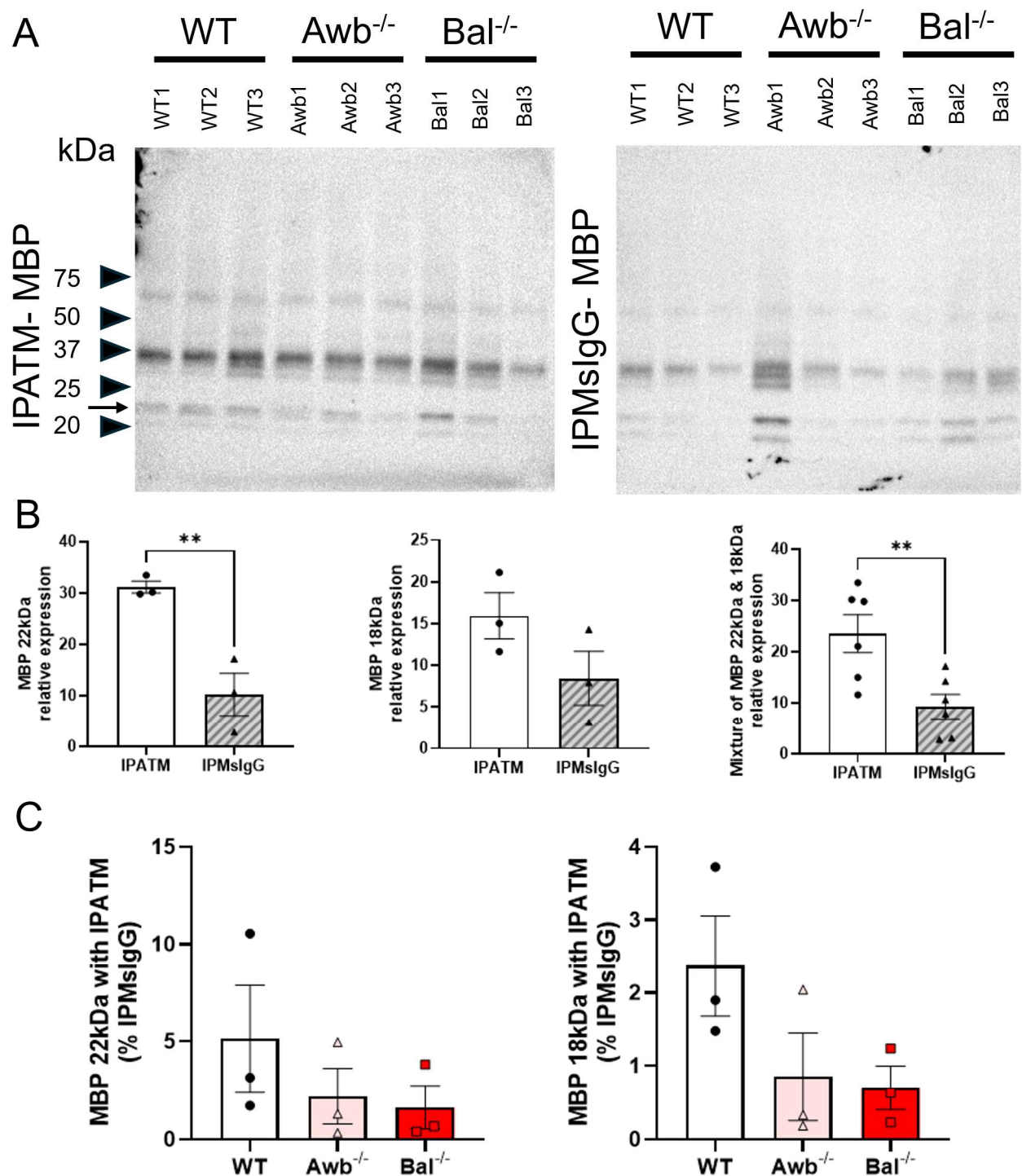


Figure 72. Co-immunoprecipitation experiment on interaction between MBP and ATM.

A) Representative immunoblotting images of control and ATM deficient mice brain lysate immunoprecipitated with ATM and MslgG respectively and probed with MBP. (Arrowhead indicating target MBP size at 22kDa) B) Quantification on MBP detection difference in WT mice brain lysate immunoprecipitated with ATM and MslgG. (unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) C) Quantification of MBP detection of ATM deficient mice brain lysate immunoprecipitated with ATM, while the relative expression level was normalized with corresponding MBP detection in immunoprecipitation with MslgG. (One-way ANOVA with Tukey's multiple comparisons test)

Co-immunoprecipitation experiment was conducted on brain lysate of WT and ATM deficient mice to examine the potential interactions between ATM and MBP. Representative immunoblotting image revealed clear bands on MBP target size (22kDa) in WT lysate immunoprecipitated with ATM (IP- ATM), but only faint bands could be observed in lysate immunoprecipitated with non-specific mouse IgG control (MsIgG, or IP-MsIgG) under same exposure time (Figure 72A). There was significant difference in the relative expression of MBP at 22 kDa between IP- ATM and IP-MsIgG (67.2% lower, $p = 0.0083$), and suggestive of a valid immunoprecipitation (Figure 72B). The normalized MBP expression in IP- ATM (over IP-MsIgG) was remarkably lower in both ATM deficient mice, but such difference did not reach statistical significance (Figure 72C).

Conclusion

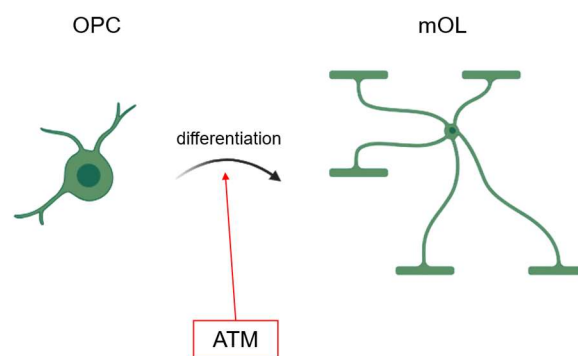


Figure 73. Conclusion for chapter 2: ATM directly affect OL differentiation

Chapter 3. Variant-specific and region-specific effect of ATM mutation on brain transcriptomes

Summary

The clinical manifestation of A-T is determined by the specific *ATM* mutation and the remaining kinase activity in the patient. This spectrum symptom spans from the classic form, which is characterized by severe, early-onset, and rapidly progressing neurodegeneration, to an atypical variant form, which is characterized milder neurological symptoms and a lower risk of systemic complications (Moeini Shad et al., 2021).

Central hypothesis

To study the site-directed mutational effect on *ATM* gene expression and protein expression, a correlation between clinical presentation, mutation sites, and the transcriptome in the cerebral cortex and cerebellar cortex of each patient were compared. In this chapter, we hypothesized that different mutation sites on *ATM* gene led to different splicing events before *ATM* mRNA translation, hence resulted in different ATM isoforms that attributed to the severity difference between A-T patients (Figure 74).

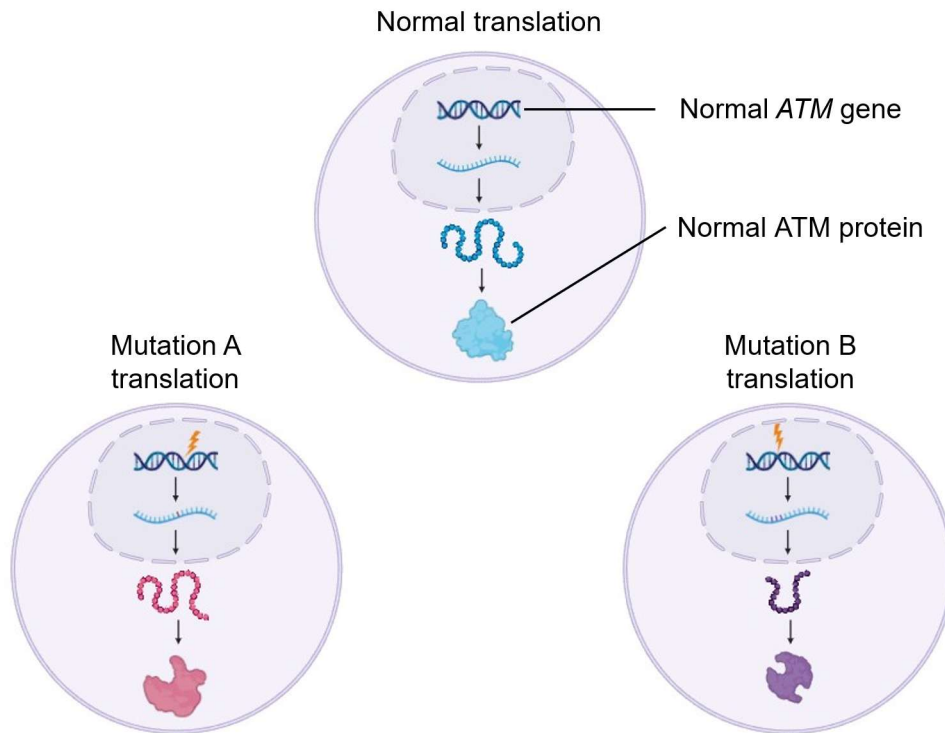


Figure 74. Central hypothesis 3: Different mutation sites on ATM gene result in different levels of damage to ATM protein translation.

Different severity levels of pathological findings were revealed from 10 A-T patients, including neuronal and OL pathology. Therefore, we hypothesized that the variation was attributed to the site of *ATM* mutations. Different forms of malfunctioning ATM protein resulted from differential *ATM* mutations could lead to different levels of loss of ATM function.

Detection of A-T pathogenic mutations by Illumina- and Nanopore-base targeted DNA sequencing

Illumina-based targeted DNA sequencing was performed with TruSight® Inherited Disease Sequencing Panel on NextSeq550, which detected 16 pathogenic mutations classified by ClinVar database (Table 4). According to the results detected from ten A-T patient samples by Illumina method, there were nine frameshift mutations, four missense mutations and three stop-gaining mutations. All pathogenic mutations in *ATM* gene were classified as heterogeneous in this sequencing experiment. However, only six out of ten A-T samples were identified with two heterogeneous pathogenic mutations. The remaining four A-T samples were identified with one heterogeneous pathogenic mutation.

To evaluate the effectiveness of long read sequencing for pinning down rare mutation, Nanopore targeted sequencing was employed for the detection of *ATM* gene pathogenic mutations. Due to the compatibility of panel, we performed the Nanopore targeted sequencing with another inherited disease panel covering only coding region of genome, named ClearSeq Inherited Disease Panel, while *ATM* gene was also included in the new targeted panel.

There were 19 mutations detected on *ATM* gene, which were classified by ClinVar database as pathogenic (Table 4). Nanopore targeted sequencing demonstrated 100% concordance rate at *ATM* gene loci comparing to Illumina targeted sequencing for all 10 A-T samples. Moreover, there was a large insertion (313 bp insertion) detected in A-T8 sample, which was considered highly possible to be a pathogenic mutation. All the 16 pathogenic mutations detected with Illumina targeted sequencing were also detected with Nanopore targeted sequencing. In addition, three pathogenic mutations located in splicing acceptor or donor region were only detected with targeted Nanopore sequencing. Therefore, Nanopore targeted sequencing was able to provide two pathogenic mutations for each A-T patient.

ID	Gene	Chr	Coordinate	Consequence	HGVSc	HGVSp	dbSNP ID	Exon	Intron	ClinVar Significance	Detection
A-T1	ATM	11	108202604	splice_acceptor_variant	NM_000051.4:c.7630-2A>G	NP_000042.3:p.Leu2544Splice	rs587779866	-	51/62	Pathogenic/Likely pathogenic	ONT
A-T1	ATM	11	108235838	stop_gained	NM_000051.4:c.8880G>A	NP_000042.3:p.Trp2960Ter	rs1060501650	62/63	-	Pathogenic	ONT & ILL
A-T2	ATM	11	108190736	frameshift_variant	NM_000051.4:c.6404_6405insTT	NP_000042.3:p.Arg2136Ter	rs587782554	44/63	-	Pathogenic/Likely pathogenic	ONT & ILL
A-T2	ATM	11	108205719	frameshift_variant	NM_000051.4:c.8036_8051del	NP_000042.3:p.Asn2679SerfsTer9	rs587780640	55/63	-	Pathogenic	ONT & ILL
A-T3	ATM	11	108117731	frameshift_variant	NM_000051.4:c.944del	NP_000042.3:p.Leu315TyrfsTer5	rs1565375035	8/63	-	Pathogenic	ONT & ILL
A-T3	ATM	11	108121752	frameshift_variant	NM_000051.4:c.1564_1565del	NP_000042.3:p.Glu522IlefsTer43	rs587779817	10/63	-	Pathogenic	ONT & ILL
A-T4	ATM	11	108143264	frameshift_variant	NM_000051.4:c.3085dup	NP_000042.3:p.Thr1029AsnfsTer19	rs876658502	21/63	-	Pathogenic	ONT & ILL
A-T4	ATM	11	108200960	stop_gained	NM_000051.4:c.7327C>T	NP_000042.3:p.Arg2443Ter	rs121434220	50/63	-	Pathogenic/Likely pathogenic	ONT & ILL
A-T5	ATM	11	108115594	stop_gained	NM_000051.4:c.742C>T	NP_000042.3:p.Arg248Ter	rs730881336	7/63	-	Pathogenic/Likely pathogenic	ONT & ILL
A-T5	ATM	11	108186638	missense_variant,splice_region_variant	NM_000051.4:c.6095G>A	NP_000042.3:p.Arg2032Lys	rs139770721	41/63	-	Pathogenic/Likely pathogenic	ONT & ILL
A-T6	ATM	11	108121479	frameshift_variant	NM_000051.4:c.1290_1291del	NP_000042.3:p.Cys430Ter	rs587781598	10/63	-	Pathogenic/Likely pathogenic	ONT & ILL
A-T6	ATM	11	108186638	missense_variant,splice_region_variant	NM_000051.4:c.6095G>A	NP_000042.3:p.Arg2032Lys	rs139770721	41/63	-	Pathogenic/Likely pathogenic	ONT & ILL
A-T7	ATM	11	108203575	missense_variant	NM_000051.4:c.7875T>G	NP_000042.3:p.Asp2625Glu	rs1196903858	53/63	-	Pathogenic	ONT & ILL
A-T7	ATM	11	108203576	missense_variant	NM_000051.4:c.7876G>C	NP_000042.3:p.Ala2626Pro	rs267606669	53/63	-	Pathogenic	ONT & ILL
A-T8	ATM	11	108128238	frameshift_variant	NM_000051.4:c.2284_2285del	NP_000042.3:p.Leu762ValfsTer2	rs587781658	15/63	-	Pathogenic/Likely pathogenic	ONT & ILL
A-T8	ATM	11	108201021	feature_elongation,coding_sequence_variant	NM_000051.4:c.7388delNinsN[313]	NP_000042.3:p.Leu2463fs	-	50/63	-	-	ONT
A-T9	ATM	11	108141874	splice_donor_variant	NM_000051.4:c.2921+1G>T	NP_000042.3:p.Ser974Splice	rs587781558	-	19/62	Pathogenic/Likely pathogenic	ONT
A-T9	ATM	11	108153564	frameshift_variant	NM_000051.4:c.3712_3716del	NP_000042.3:p.Leu1238LysfsTer6	rs786201675	25/63	-	Pathogenic/Likely pathogenic	ONT & ILL
A-T10	ATM	11	108190736	frameshift_variant	NM_000051.4:c.6404_6405insTT	NP_000042.3:p.Arg2136Ter	rs587782554	44/63	-	Pathogenic/Likely pathogenic	ONT & ILL
A-T10	ATM	11	108224608	splice_donor_variant	NM_000051.4:c.8786+1G>A	NP_000042.3:p.Arg2929Splice	rs17174393	-	60/62	Pathogenic/Likely pathogenic	ONT

Table 6. List of DNA mutations in ATM with pathological effect detected by Illumina and Nanopore

In comparison between targeted sequencing using Illumina and Nanopore, power of Illumina targeted sequencing was limited to the exon region, while Nanopore targeted sequencing could extend the search to adjacent intronic regions that was highly responsible for mRNA splicing event. Moreover, the large gene insertion was only detected with Nanopore targeted sequencing due to its nature of long read sequencing.

As Illumina targeted sequencing was limited to its fragment length and the association with sequencing panel, intronic region of *ATM* gene and large insertion might not be effectively detected.

PCR confirmation of ATM mutations

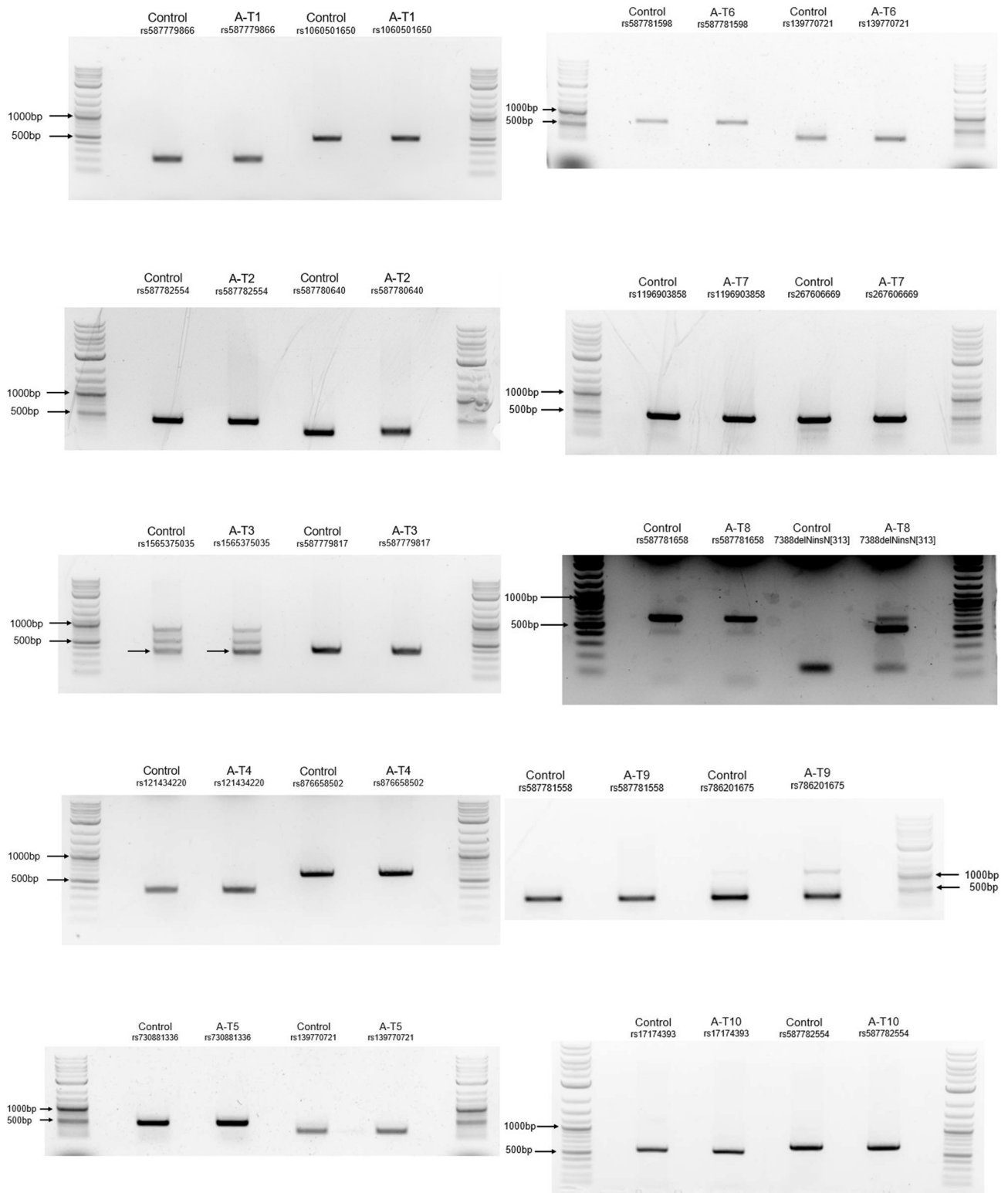


Figure 75. PCR confirmation of ATM mutation for 10 A-T samples

To verify the authenticity of Nanopore targeted sequencing result, PCR experiment was performed on A-T8 sample with primers targeting regions containing pathogenic mutation (Figure 75). Two pairs of primers were designed targeting exon 15 and 50 respectively for rs587781658 and 7388delNinsN[313] in A-T8 sample. For the primer pair targeting rs587781658, the expected product size was 629, so the result reflected a successful probe on the targeted region in both control and A-T8 sample. For the primer targeting 7388delNinsN[313], targeted product size in normal genome was 149bp. The result revealed the successful targeting of the region of interest in control sample. The expected band size of A-T8 was 462bp including the large insertion detected by Nanopore targeted sequencing. The result from Nanopore targeted sequencing was validated with the PCR experiment on the large insertion detection on A-T8 sample. Other A-T samples were also designed with specific primers on the pathogenic mutation site for further verification of authenticity. Result in figure 80 revealed the successfulness of specific primers of targeting the mutation sites. The amplicons were sent out for sanger sequencing, but the results were not finalised due to technical difficulties at the vendor.

ATM mutation site verification by Illumina- and Nanopore-base targeted DNA sequencing

The two pathological mutations detected with nanopore targeted DNA sequencing of each sample were visualized with Integrative Genomics Viewer (IGV). The effect of mutation on transcription was further investigated with transcriptomic sequencing of frontal cortex and cerebellum of A-T samples.

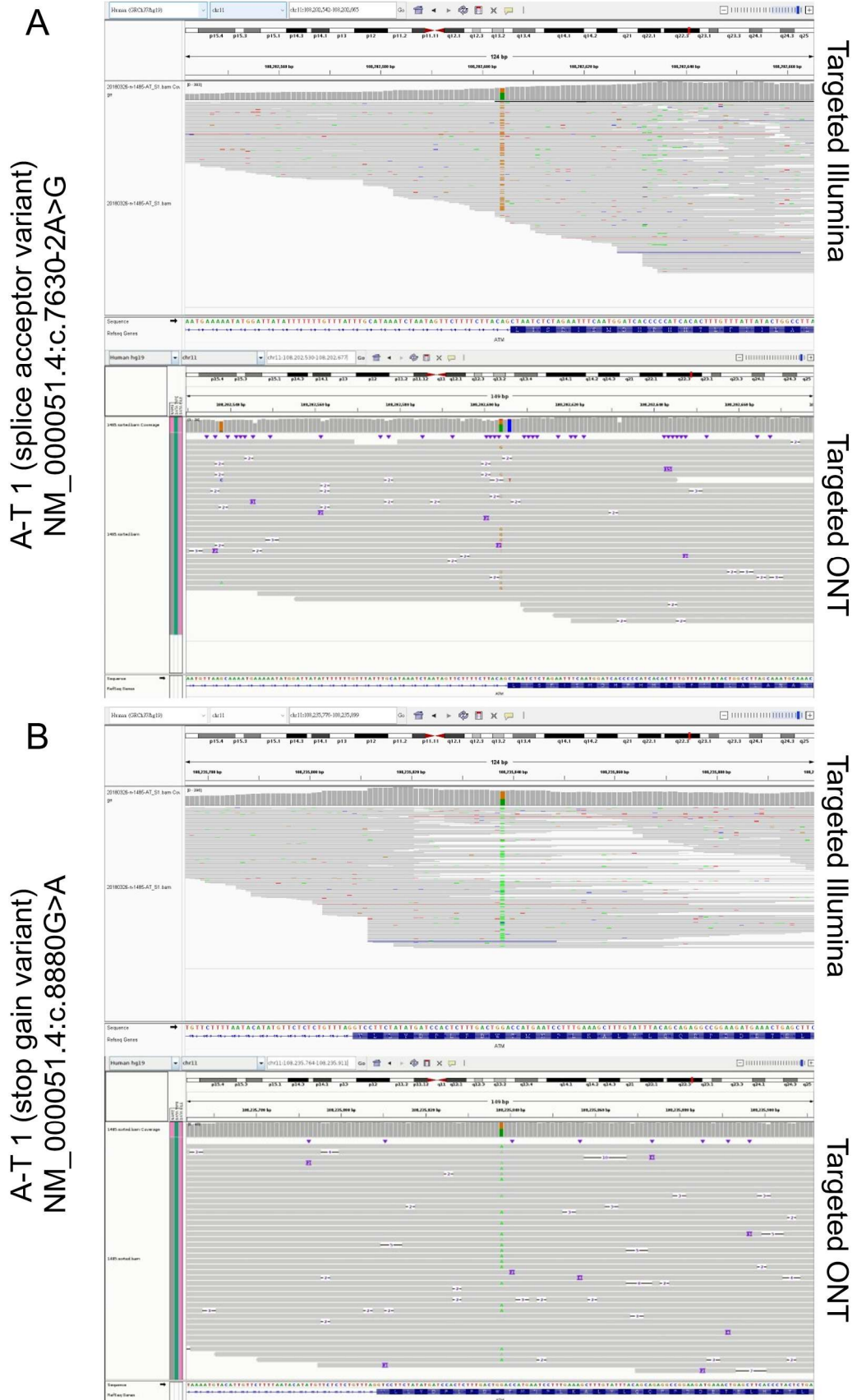


Figure 76. Gene coverage on pathogenic mutation site of A-T 1 detected with Illumina and Nanopore targeted DNA sequencing.

A) Pathogenic mutation NM_000051.4:c.7630-2A>G detected with Illumina and Nanopore on igv image. B) Pathogenic mutation NM_000051.4:c.8880G>A detected with Illumina and Nanopore on igv image.

Splice acceptor variant (NM_000051.4:c.7630-2A>G) and stop gain variant (NM_000051.4:c.8880G>A) mutation were found in the aligned read file of A-T 1 sample in IGV (Figure 73). According to Figure 76A, splice acceptor variant could be observed from the igv capture in targeted Illumina sequencing result, but the observation was not reflected in the variant calling result of targeted Illumina sequencing. Moreover, the base coverage percentage of targeted Illumina sequencing was gradually reduced in the edge region of *ATM* exon (Figure 76). However, the targeted Nanopore sequencing demonstrated a relative stable base coverage across the boundary between *ATM* exon and intron.

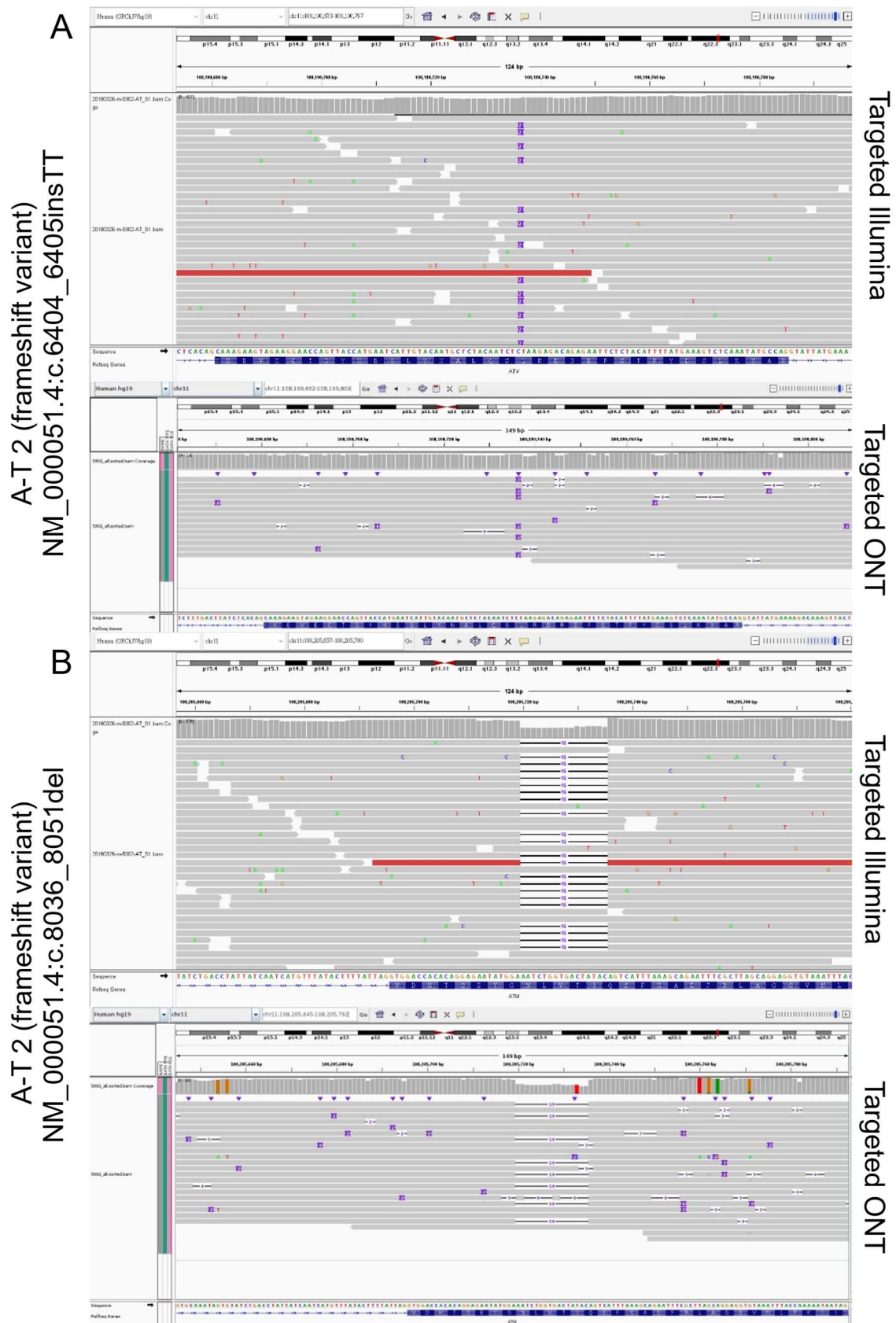


Figure 77. Gene coverage on pathogenic mutation site of A-T 2 detected with Illumina and Nanopore targeted DNA sequencing

A) Pathogenic mutation NM_000051.4:c.6404_6405insTT detected with Illumina and Nanopore on igv image. B)
Pathogenic mutation NM_000051.4:c.8036_8051del detected with Illumina and Nanopore on igv image.

Two frameshift mutations (NM_000051.4:c.6404_6405insTT, NM_000051.4:c.8036_8051del) were found in the aligned read file of A-T 2 sample in IGV (Figure 77).

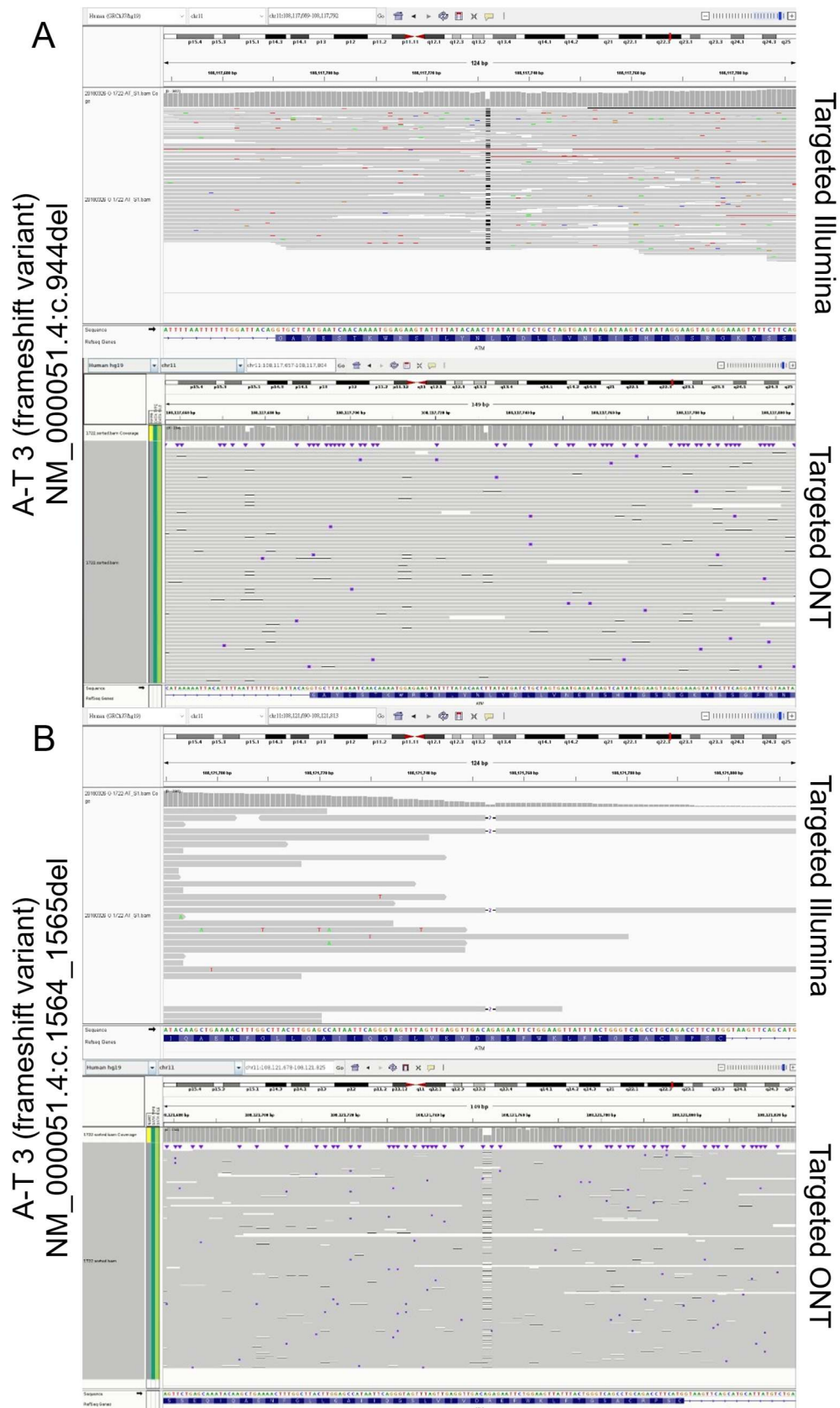


Figure 78. Gene coverage on pathogenic mutation site of A-T 3 detected with Illumina and Nanopore targeted DNA sequencing

A) Pathogenic mutation NM_000051.4:c.944del detected with Illumina and Nanopore on igv image. B) Pathogenic mutation NM_000051.4:c.1564_1565del detected with Illumina and Nanopore on igv image.

Two frameshift mutations (NM_000051.4:c.944del, NM_000051.4:c.1564_1565del) were found in the aligned read file of A-T 3 sample in IGV (Figure 78).

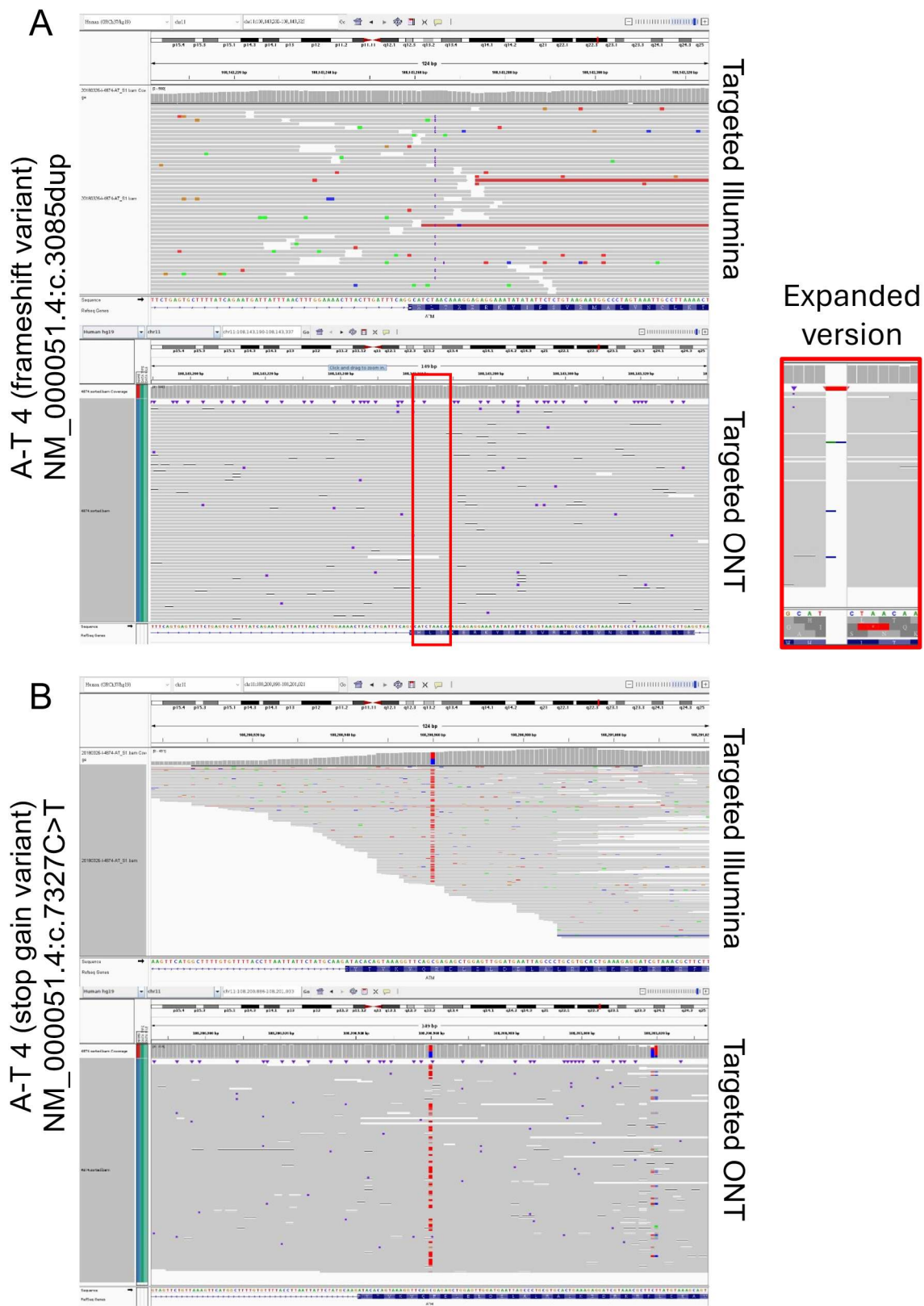


Figure 79. Gene coverage on pathogenic mutation site of A-T 4 detected with Illumina and Nanopore targeted DNA sequencing

A) Pathogenic mutation NM_000051.4:c.3085dup detected with Illumina and Nanopore on igv image. B) Pathogenic mutation NM_000051.4:c.7327C>T detected with Illumina and Nanopore on igv image.

Frameshift variant (NM_000051.4:c.3085dup) and stop gain variant (NM_000051.4:c.7327C>T) were found in the aligned read file of A-T 4 sample in IGV (Figure 79).

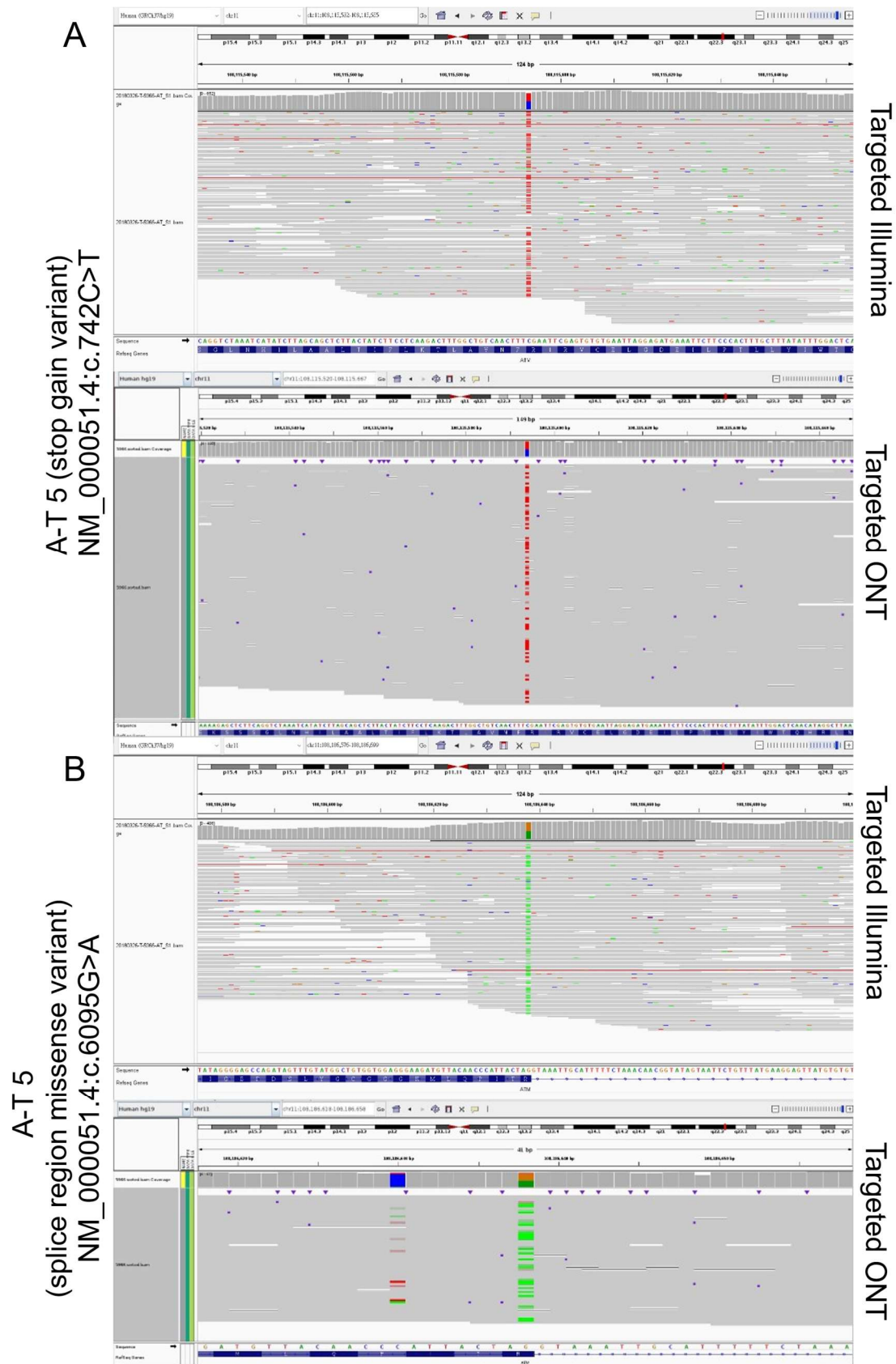


Figure 80. Gene coverage on pathogenic mutation site of A-T 5 detected with Illumina and Nanopore targeted DNA sequencing

A) Pathogenic mutation NM_000051.4:c.742C>T detected with Illumina and Nanopore on igv image. B) Pathogenic mutation NM_000051.4:c.6095G>A detected with Illumina and Nanopore on igv image.

Stop gain variant (NM_000051.4:c.742C>T) and splice region missense variant (NM_000051.4:c.6095G>A) were found in the aligned read file of A-T 5 sample in IGV (Figure 80).

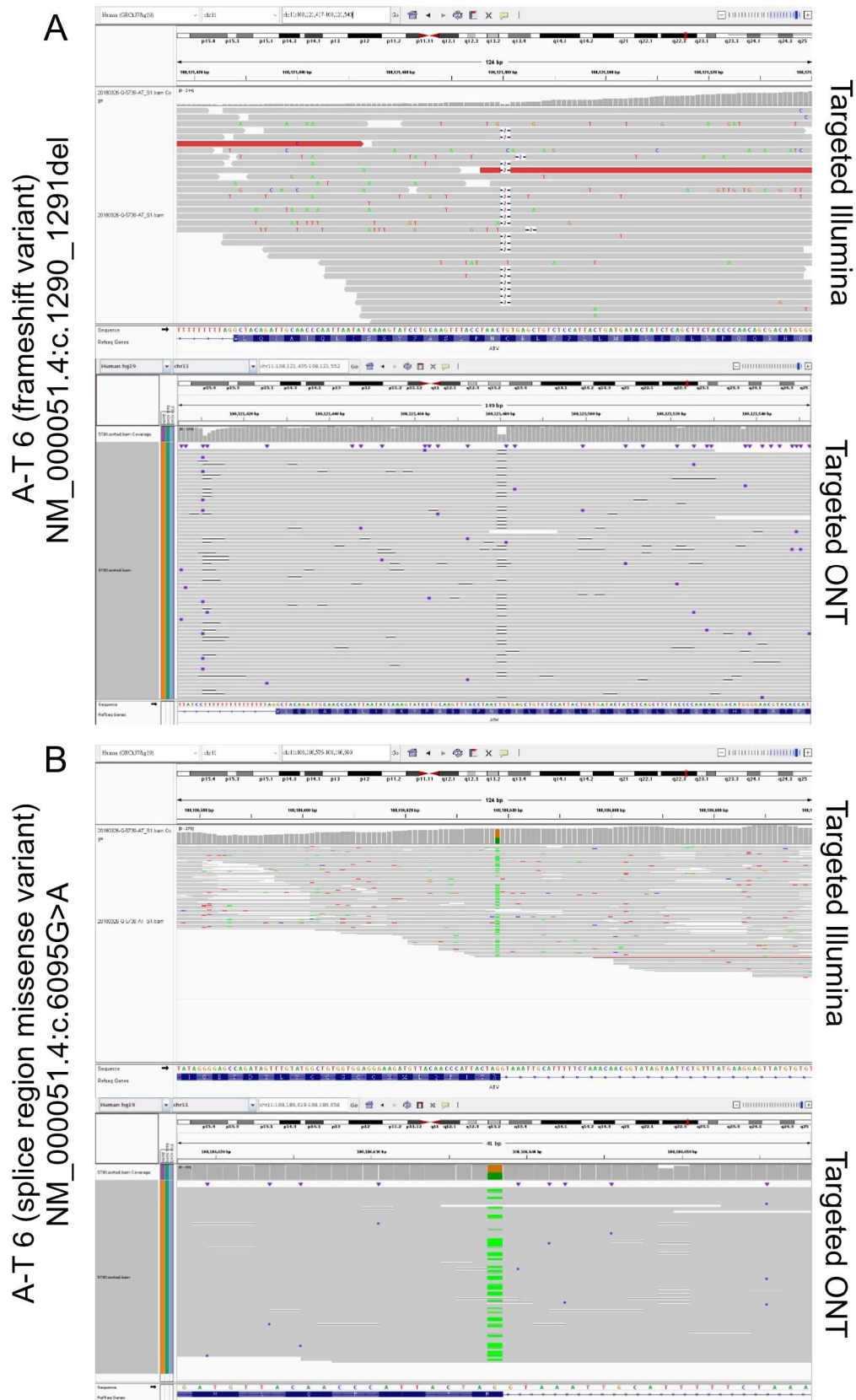


Figure 81. Gene coverage on pathogenic mutation site of A-T 6 detected with Illumina and Nanopore targeted DNA sequencing

A) Pathogenic mutation NM_000051.4:c.1290_1291del detected with Illumina and Nanopore on igv image. B) Pathogenic mutation NM_000051.4:c.6095G>A detected with Illumina and Nanopore on igv image.

Frameshift variant (NM_000051.4:c.1290_1291del) and splice region missense variant (NM_000051.4:c.6095G>A) were found in the aligned read file of A-T 6 sample in IGV (Figure 81).

A-T 7 (missense variant)
 NM_000051.4:c.7875T>G
 NM_000051.4:c.7876G>C



Figure 82. Gene coverage on pathogenic mutation site of A-T 7 detected with Illumina and Nanopore targeted DNA sequencing

Pathogenic mutations NM_000051.4:c.7875T>G and NM_000051.4:c.7876G>C detected with Illumina and Nanopore on igv image.

Two missense variants (NM_000051.4:c.7875T>G, NM_000051.4:c.7876G>C) were found adjacent to each other in the aligned read file of A-T 7 sample in IGV (Figure 82).

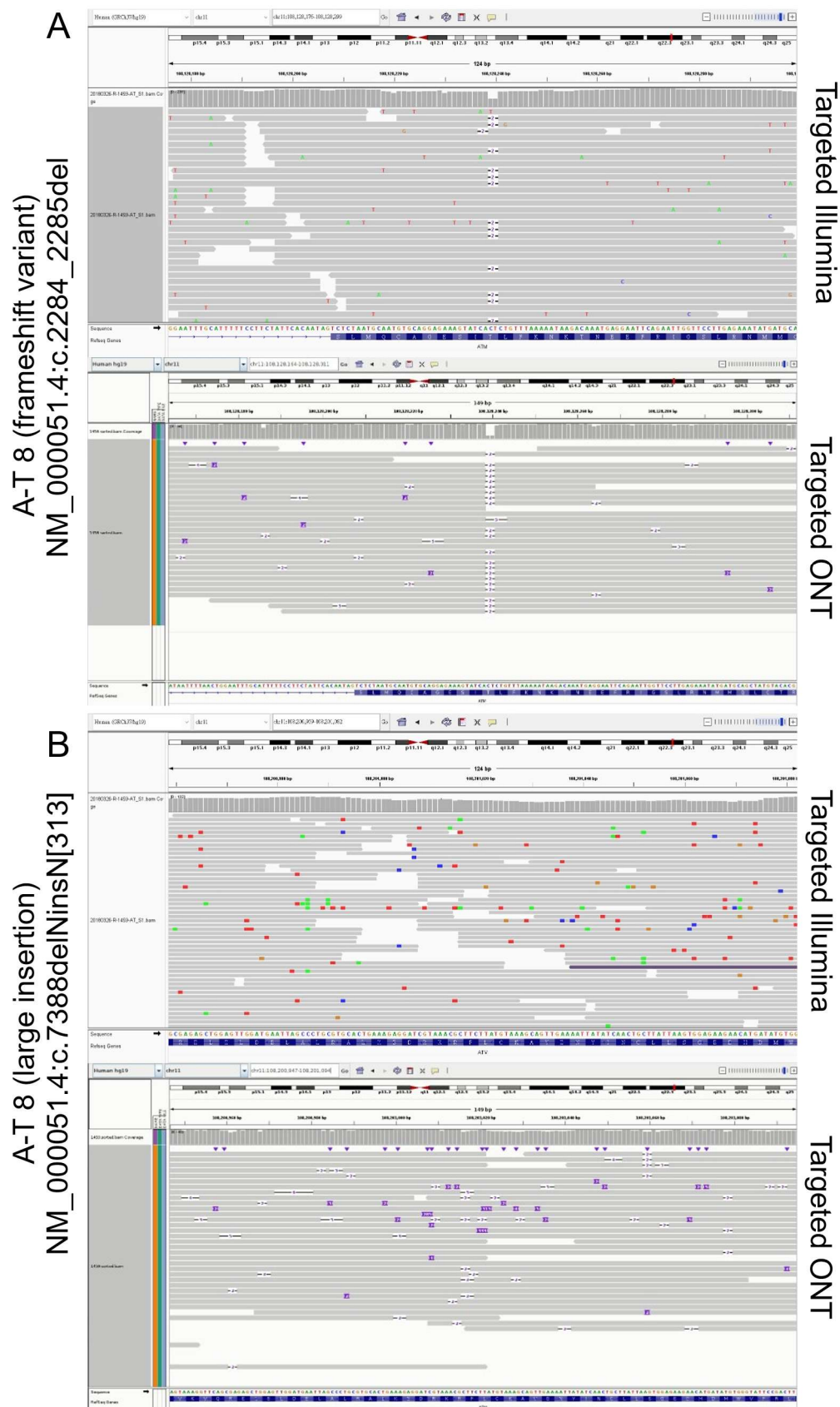


Figure 83. Gene coverage on pathogenic mutation site of A-T 8 detected with Illumina and Nanopore targeted DNA sequencing

A) Pathogenic mutation NM_000051.4:c.2284_2285del detected with Illumina and Nanopore on igv image. B) Pathogenic mutation NM_000051.4:c.7388delNinsN[313] detected with Illumina and Nanopore on igv image.

Frameshift variant (NM_000051.4:c.2284_2285del) and large insertion (NM_000051.4:c.7388delNinsN[313]) were found in the aligned read file of A-T 8 sample in IGV (Figure 83).

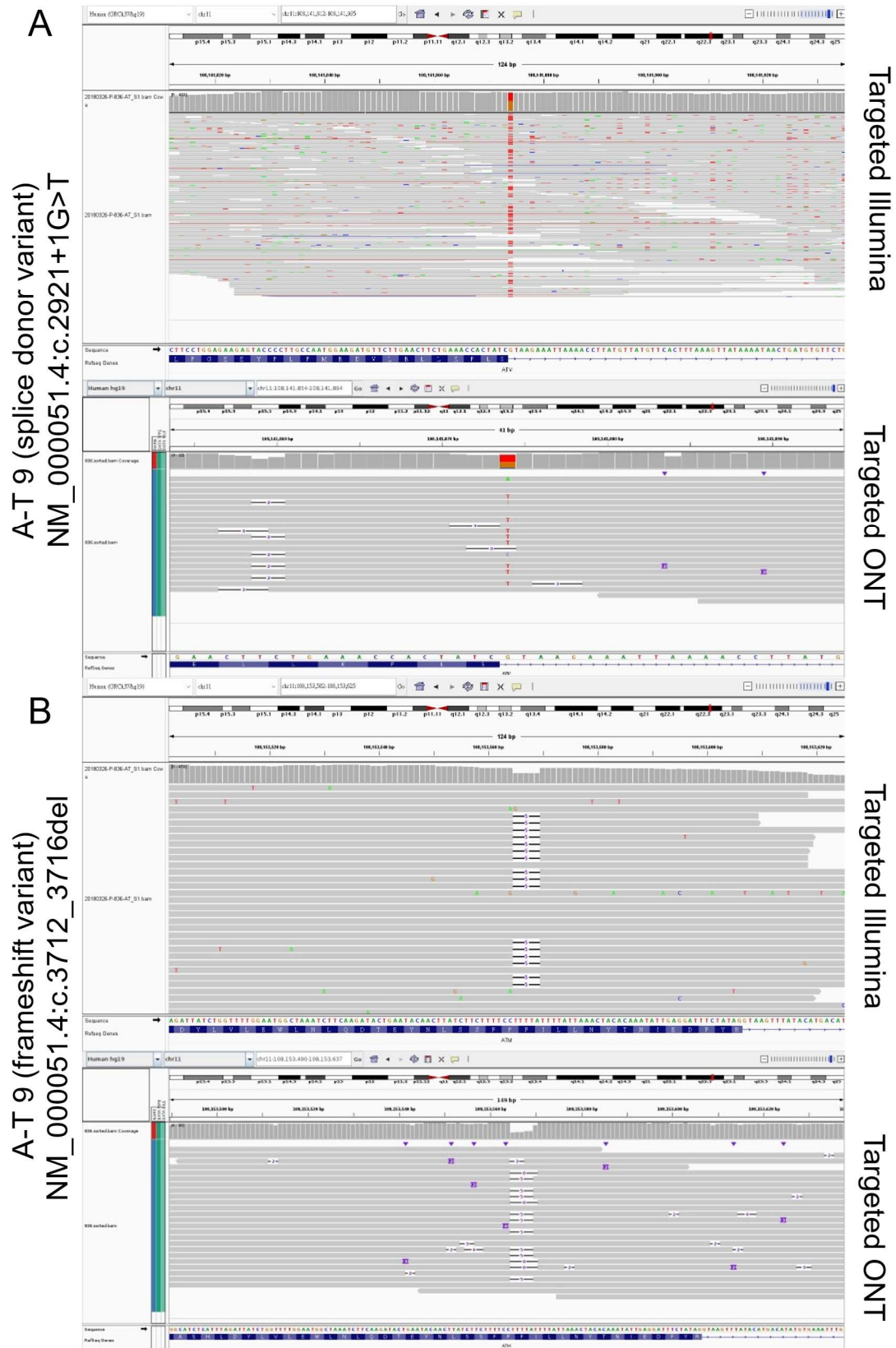


Figure 84. Gene coverage on pathogenic mutation site of A-T 9 detected with Illumina and Nanopore targeted DNA sequencing

A) Pathogenic mutation NM_000051.4:c.2921+1G>T detected with Illumina and Nanopore on igv image. B) Pathogenic mutation NM_000051.4:c.3712_3716del detected with Illumina and Nanopore on igv image.

Splice donor variant (NM_000051.4:c.2921+1G>T) and frameshift variant (NM_000051.4:c.3712_3716del) were found in the aligned read file of A-T 9 sample in IGV (Figure 84).

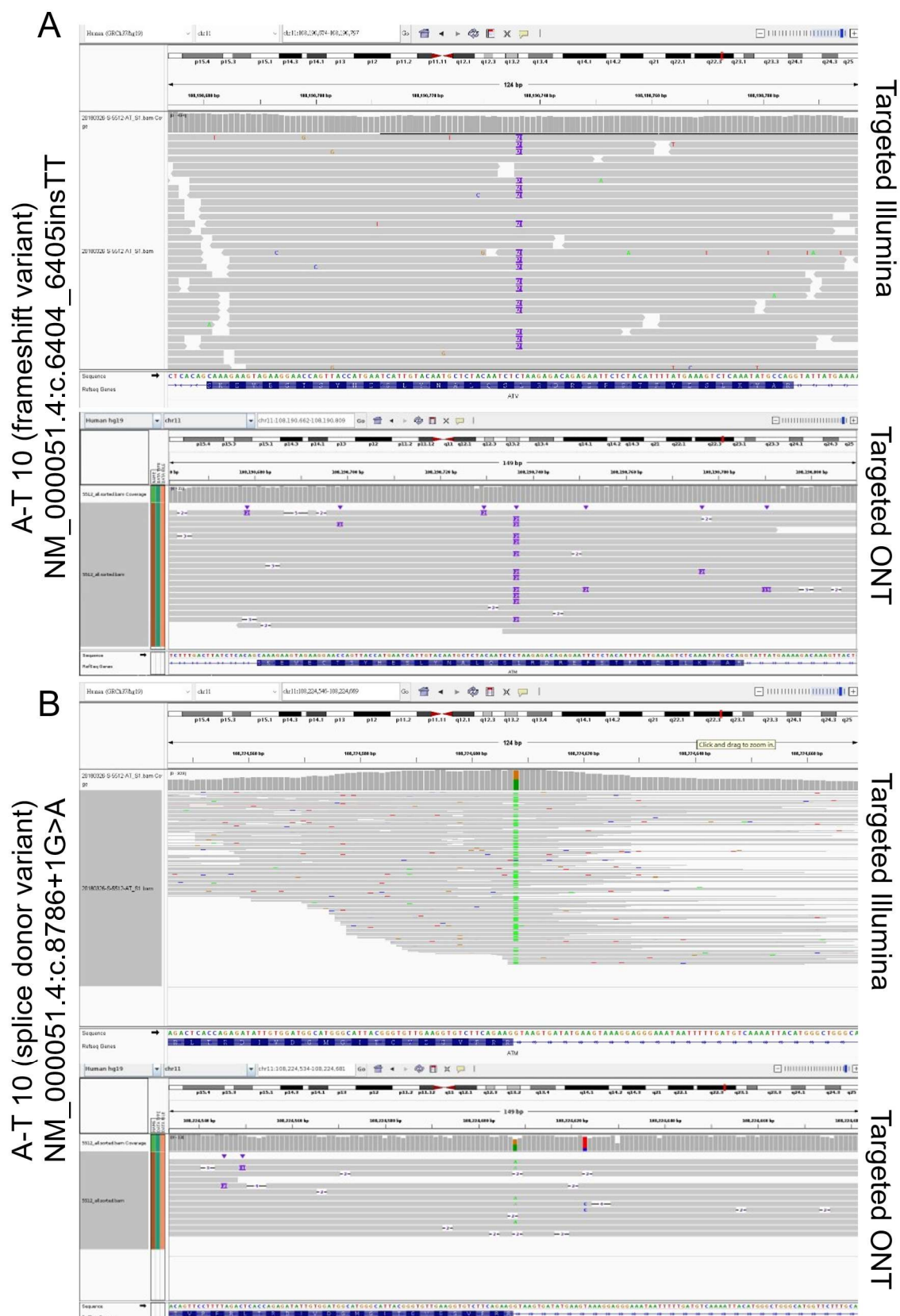


Figure 85. Gene coverage on pathogenic mutation site of A-T 10 detected with Illumina and Nanopore targeted DNA sequencing.

A) Pathogenic mutation NM_000051.4:c.6404_6405insTT detected with Illumina and Nanopore on igv image. B) Pathogenic mutation NM_000051.4:c.8786+1G>A detected with Illumina and Nanopore on igv image.

Frameshift variant (NM_000051.4:c.6404_6405insTT) and splice donor variant (NM_000051.4:c.8786+1G>A) were found in the aligned read file of A-T 10 sample in IGV (Figure 85).

Predicted damage of ATM mutation on transcription and protein translation

The pathogenicity of ATM variants in table 4 was identified as pathogenic or likely pathogenic in ClinVar database. However, the neuropathological findings of each A-T patients varied in their medical reports (Table 7).

	Key Clinical Manifestation	Sex	Brain Weight (g)	Age	Cerebellar Change	Cerebral Change	White Matter Change	Cerebrovascular Change
A-T1	Progressive dyspnoea and cough. Evaluation was felt to be consistent with chronic, persistent aspiration. Significant for... ataxia, telangiectasia and recurrent aspiration.	Female	980	24	MARKED CEREBELLAR ATROPHY CONSISTENT WITH ATAXIA-TELANGIECTASIA. Sections of the cerebellum reveal a marked loss of Purkinje and granule cells with associated Bergmann gliosis. The overlying molecular layer is thinner than normal.	A large area of predominantly cortical necrosis with an associated macrophage-rich infiltrate and associated reactive vascular proliferation involving the left occipital cortex. A similar but smaller wedge-shaped lesion is noted in the right occipital cortex. The adjacent cortex contains a focal area of neuronal loss and gliosis.	The underlying white matter contains scattered blood vessels surrounded by pigment-laden macrophages; these are most prominent in the right parietal white matter.	ORGANIZING INFARCTS, RIGHT AND LEFT OCCIPITAL LOBES.
A-T2	Male with ataxia telangiectasia and immune suppression... He has interstitial lung disease with chronic aspiration and has a PEG tube. Motor weakness(+), Ataxia(+). Telangiectasias...most prominent in the posterior frontal and parietal...white matter.	Male	1572	27	The cerebellar cortex shows granular neuron loss as well as less severe Purkinje neuron loss with Bergmann gliosis.	Acute infarct with mild haemorrhagic conversion, left posterior cerebral artery territory (8 cm). Focal acute ischemia with mild haemorrhagic conversion in the left posterior cerebral artery territory. Midbrain...focal ischemic changes, with acute inflammation	Scattered telangiectasias, most prominent in the posterior frontal and parietal, parasagittal white matter and measuring up to 0.5 cm in greatest dimension. Acute cytotoxic edema...consistent with acute...global ischemia.	TRANSIENT GLOBAL ISCHEMIA. ACUTE INFARCT WITH MILD HEMORRHAGIC CONVERSION, LEFT POSTERIOR CEREBRAL ARTERY TERRITORY. FOCAL ISCHEMIC CHANGES, WITH ACUTE INFLAMMATION, MIDBRAIN.
A-T3	Subject was a 17 year old boy with the diagnosis of Ataxia-Telangiectasia (A-T), also known as Louis-Bar syndrome. He had difficulty sleeping...frequent episodes of tachypnea...frequent respiratory infections, including pneumonias...He had severe ataxia, using a wheel chair for mobility.	Male	1400	17	There is severe cerebellar degeneration with sub complete loss of Purkinje cells and thinning and degeneration of the granular layer. Comparatively mild Bergmann's gliosis, and a hypercellular molecular layer.	Brain with degenerative and infectious features consistent with the clinical diagnosis of Ataxia-Telangiectasia (Louis-Bar disease). Meningoencephalitis, predominantly posterior fossa involvement...	Numerous lymphocytic infiltrates in the leptomeninges of the frontal cortex and especially the cerebellum... Numerous perivascular lymphocytic cuffs and intraparenchymal microglial and lymphocytic nodules...	Focal proteinaceous deposits in the subarachnoid space. No massive brain swelling nor herniation...inflammatory infiltrates...most likely the neurologic correlate of a generalized infection.
A-T4	Admitted for placement of a gastrostomy tube... He had a GJ tube in place that was not functioning...He was discharged home... He had malnutrition...Ataxia Telangiectasia Syndrome...Recurrent aspiration pneumonia...Anemia...Hx Pleural Effusion...	Male	1380	26	The cerebellum is globally atrophic. Severe cerebellar cortical atrophy with thinning of the folia and depletion of neurons in the Purkinje and granular layers.	The cortical ribbon is of normal thickness; the hippocampal formation is neither atrophic nor sclerotic, and the deep gray matter structures are well preserved.	No focal lesions...some infiltration in the cerebellar leptomeninges...focal lymphoplasmacytic infiltrate around vascular structures of the cerebral white matter leptomeninges...pigmented and phagocytic macrophages around the vessels.	No vascular anomalies, malformations or aneurysms...Neuropathologically, the disease belongs to the group of spinocerebellar degenerations.
A-T5	He had a gastrostomy, and occasional episodes of nausea, vomiting, abdominal pain and dehydration. He had scoliosis and small pleural effusions... At the end of his life he was hospice care. Final Neuropathologic Diagnosis: ... consistent with ataxia telangiectasia.	Male	1180	36	Atrophy or hypoplasia of the cerebellar folia, granule cell layer, and dentate nucleus with scattered microglial nodules... ..consistent with ataxia telangiectasia	Enlarged nuclei with irregular borders...in corpus callosum and thalamus... No evidence of ischemia or infection.	White matter perivascular spaces are mildly enlarged. No significant astrogliosis, demyelination, rarefaction, or vacuole change.	The parenchymal vasculature shows no significant atherosclerotic disease or arteriolosclerosis. No microorganisms are detected...The leptomeninges...demonstrate fibrosis.

A-T6	Diagnosis of Ataxia Telangiectasia. B-cell lymphoma diagnosis in 2013, underwent chemotherapy, died three-four months after diagnosis. Final Neuropathologic Diagnosis: 1. Cerebellar atrophy and degeneration, 2. Acute hypoxic-ischemic encephalopathy.	Male	1370	25	Nearby universal cortical atrophy and degeneration in the cerebellum. Folia are narrow, severe neuronal loss (Purkinje and granular) ...spongiosis of the Purkinje cell layer, Bergmann's gliosis. Rare Purkinje cells remain with degenerative features.	Acute hypoxic-ischemic encephalopathy...eosinophilic neurons in frontal and cingulate cortex, hippocampus (CA1), some thalamic neurons. Occipital cortex has more severe hypoxic damage (pink neurons, hydropic changes, vacuolation).	The deep gray and white matter structures are anatomically intact with no focal lesions. No mention of demyelination or significant white matter changes beyond normal architecture.	Leptomeninges are thick, scattered thick fibrotic arteries in leptomeninges and brain parenchyma. No major vascular anomalies or atherosclerosis in the circle of Willis. Significant vasculature changes relating to hypoxia/ischemia...some thick fibrotic arteries.
A-T7	(Deceased) had a long-standing diagnosis of [Ataxia Telangiectasia] with gradual deterioration...He was wheelchair bound...He had wasting of the muscles...He had long standing nystagmus...Severe headaches with sudden onset...	Male	1565	35	Moderate, diffuse atrophy of the cerebellar hemispheres...1) CLINICAL HISTORY OF ATAXIA TELANGIECTASIA. CEREBELLAR CORTICAL ATROPHY (SEVERE).	Neuropathology...Haemorrhagic necrosis in the right parietal occipital region. Necrotizing vasculitis, often with thrombus formation, and fungal hyphae invading blood vessel walls...(aspergillus species).	Scattered nodular collections of ectatic blood vessels...surrounding yellow discoloration and vasogenic edema.	Necrotizing vasculitis...especially involving the haemorrhagic focus...morphologically consistent with aspergillus species...fibrin thrombi. Marked cerebral edema, right parietal-occipital haemorrhagic necrosis, right transtentorial herniation.
A-T8	Ataxia Telangiectasia diagnosed ~7 y/o. Profoundly impaired...progressive ataxia, intention tremors... Possible 'Dandy-Walker' cyst...no definite cyst found at autopsy. Co-diagnosis (Fragile X) with >200 CGG repeats. Developed large B-cell lymphoma (2001).	Female	1153	19	Marked cerebellar cortical atrophy. Both vermis and lateral hemispheres are affected by this process. Significant decrease in Purkinje cells (with Bergmann gliosis) and in granule cells. Molecular layer also thinner than normal. Dentate nucleus...relatively spared.	Sections of the cerebral hemispheres...essentially unremarkable. Mild subependymal gliosis. No pathognomonic findings in Fragile X...cannot confirm or refute based on anatomic data.	Rare astrocytes with enlarged, hyperchromatic nuclei...in the deep white matter. Relatively spared.	Focus of petechial haemorrhages...in the left basis pontis (likely terminal coagulopathy). No evidence of a true vascular malformation...Telangiectatic changes not specifically localized. No aneurysms or significant vascular anomalies.
A-T9	28-year-old...history of ataxia telangiectasia since age 4...wheelchair-bound. Severe headaches...some subarachnoid hemorrhage in posterior fossa...Angio negative. MRI shows 'multiple punctate foci' of T2 signal...possible ADEM vs. multiple telangiectasias.	Female	1410	28	Cerebellar cortical atrophy, marked. Atrophic cerebellar hemispheres...cerebellar tonsils show evidence of tonsillar herniation with medullary compression. Serial parasagittal sections show marked atrophy of the cerebellar folia.	Diffuse confluent white matter disease...multiple punctate foci of high T2 signal which enhance after contrast...No evidence of aneurysm or AVM...No lymphomatosis.Global ischemic changes with diffuse cerebral edema...focal venous infarction...bilateral tonsillar herniation.	Multiple telangiectasis...in cerebral, cerebellar, brainstem, and spinal cord white matter. Capillary telangiectasia-like vascular malformations with chronic fibrillary gliosis, Rosenthal fibres, and remote haemorrhage. Both vasogenic and cytotoxic edema.	Multiple, recently thrombosed telangiectasis with vasogenic edema...etiology undetermined. Blood vessels of the circle of Willis...no atherosclerosis, vascular anomalies, malformations, or aneurysms. Staphylococcus epidermidis (coagulase negative) cultured from blood.
A-T10	History of recurrent GI bleeding from variceal bleeds... Chronic liver disease, iron deficiency anemia...fear of malabsorption. Ataxia telangiectasia with progressive weakness...had diabetes, pancreatic insufficiency, requiring enzyme therapy.	Female	N.D.	26	Profound cerebellar cortical atrophy with thinning of the folia, loss of Purkinje and granular neurons. Minor Bergmann's gliosis...some Purkinje cells misplaced into the molecular layer and appear deformed...also involvement of the vermis, lateral hemispheres.	Inferior olive is thin, atrophic, with significant neuronal loss, neuronal degeneration, gliosis, and vacuolation. Substantia nigra...rare degenerating neurons with Lewy-like eosinophilic inclusions. Hippocampal dentate gyrus minimally thinned, scattered pyknotic neurons; some thalamic neurons appear degrading... mild gliosis.	Meningeal and cortical vessels congested...occasional fibrin strands in lumina. White matter arteries show thick walls, occasional pigmented macrophages in perivascular spaces. Scattered parenchymal, perivascular, vascular mineralization in the globus pallidus.	Cerebellar atrophy, vascular thickening, and other neurodegenerative features...consistent with clinical diagnosis of Ataxia-Telangiectasia. Congestion of vessels, white blood cells, fibrin strands (terminal condition)...coupled with occasionally thickened arterial walls and mineralization.

Table 7. Summary table of A-T patients neurological severity based on their medical report

Indeed, all A-T patients showed cerebral manifestation in their corresponding medical reports. Myelination of A-T patients was not the primary pathological investigation target in the medical reports, but some cases revealed telangiectasias or infiltration near the cerebral white matter.

To extend the investigation of each pathological mutation effects detected with targeted DNA sequencing, the *ATM* variants in Table 3 were further annotated with Variant Effect Predictor (VEP) for their specific deleterious effect on *ATM* gene (Table 8).

GnomAD EAF represents frequency of effective allele in the Genome Aggregation Database. EAF of all detectable *ATM* pathogenic variants are less than 1.1×10^{-5} , showing relatively low frequency of the detected mutation in the population. GERP++ RS represents the rejected substitutions score which is defined as the position-specific estimation on the constrained elements in multiple alignments by quantifying substitution deficits. GERP++ RS range from -12.3 to 6.17. The genome site was considered highly constrained when $RS > 4.4$. Only three genomic sites with pathogenic mutation were highly constrained, including NM_000051.4:c.8880, NM_000051.4:c.6095 and NM_000051.4:c.742 (Table 8).

SIFT, PolyPhen and AlphaMissense predict pathogenicity of missense variants. SIFT predicts whether an amino acid substitution affects protein function, which is considered deleterious when it scores below 0.05 (ranging from 0 to 1). PolyPhen predicts the possible impact of an amino acid substitution on the structure and function of the corresponding protein, which is considered having damaging effect when it scores higher than 0.5 (ranging from 0 to 1). AlphaMissense predicts probability of the missense variant being pathogenic, which is considered pathogenic when it scores higher than 0.564 (ranging from 0 to 1). Only NM_000051.4:c.7876G>C was classified as pathogenic among all three indicators (Table 8). NM_000051.4:c.6095G>A showed deleterious effect in SIFT and PolyPhen score, but not in AlphaMissense (Table 8).

Sample ID	HGVSc	Consequence	ClinVar latest	gnomAD EAF	GERP++ RS	SIFT	PolyPhen	AlphaMissense pathogenicity	CADD PHRED	BayesDel addAF score	BayesDel noAF score	ClinPred	fathmm-MKL coding rankscore	fathmm-XF coding rankscore	PrimateAI score
A-T1	NM_000051.4:c.7630-2A>G	Splice acceptor variant	Pathogenic/Likely pathogenic	-	-	-	-	-	33	-	-	-	-	-	-
A-T1	NM_000051.4:c.8880G>A	Stop gained variant	Pathogenic	-	5.71	-	-	-	45	0.622	0.656	-	0.946	0.406	-
A-T2	NM_000051.4:c.6404_6405insTT	Frameshift variant	Pathogenic/Likely pathogenic	1.10E-05	-	-	-	-	34	-	-	-	-	-	-
A-T2	NM_000051.4:c.8036_8051del	Frameshift variant	Pathogenic	2.05E-06	-	-	-	-	36	-	-	-	-	-	-
A-T3	NM_000051.4:c.944del	Frameshift variant	Pathogenic	1.37E-06	-	-	-	-	24.7	-	-	-	-	-	-
A-T3	NM_000051.4:c.1564_1565del	Frameshift variant	Pathogenic	5.47E-05	-	-	-	-	31	-	-	-	-	-	-
A-T4	NM_000051.4:c.3085dup	Frameshift variant	Pathogenic	1.37E-06	-	-	-	-	34	-	-	-	-	-	-
A-T4	NM_000051.4:c.7327C>T	Stop gained variant	Pathogenic/Likely pathogenic	4.11E-06	-0.194	-	-	-	37	0.555	0.559	-	0.625	0.453	-
A-T5	NM_000051.4:c.6095G>A	Missense variant, Splice region variant	Pathogenic/Likely pathogenic	8.23E-06	5.33	deleterious (0)	Possibly damaging (0.591)	0.508	33	0.050	0.014	0.774	0.948	0.930	0.671
A-T5	NM_000051.4:c.742C>T	Stop gained variant	Pathogenic/Likely pathogenic	2.05E-06	4.6	-	-	-	36	0.477	0.617	-	0.622	0.180	-
A-T6	NM_000051.4:c.6095G>A	Missense variant, Splice region variant	Pathogenic/Likely pathogenic	8.23E-06	5.33	deleterious (0)	Possibly damaging (0.591)	0.508	33	0.050	0.014	0.774	0.948	0.930	0.671
A-T6	NM_000051.4:c.1290_1291del	Frameshift variant	Pathogenic/Likely pathogenic	1.03E-05	-	-	-	-	23.1	-	-	-	-	-	-
A-T7	NM_000051.4:c.7875T>G	Missense variant	Pathogenic	5.48E-06	3.84	tolerated (0.19)	Benign (0.061)	0.164	19.29	0.070	-0.137	0.462	0.623	0.509	0.568
A-T7	NM_000051.4:c.7876G>C	Missense variant	Pathogenic	5.48E-06	4.15	deleterious (0)	Probably damaging (0.971)	0.987	26.1	0.350	0.265	0.997	0.826	0.769	0.597
A-T8	NM_000051.4:c.2284_2285del	Frameshift variant	Pathogenic/Likely pathogenic	1.85E-05	-	-	-	-	33	-	-	-	-	-	-
A-T8	NM_000051.4:c.7388delNinsN[313]	Feature elongation, Coding sequence variant	null	-	-	-	-	-	-	-	-	-	-	-	-
A-T9	NM_000051.4:c.3712_3716del	Frameshift variant	Pathogenic/Likely pathogenic	7.95E-06	-	-	-	-	34	-	-	-	-	-	-
A-T9	NM_000051.4:c.2921+1G>T	Splice donor variant	Pathogenic/Likely pathogenic	2.74E-06	-	-	-	-	33	-	-	-	-	-	-
A-T10	NM_000051.4:c.6404_6405insTT	Frameshift variant	Pathogenic/Likely pathogenic	1.10E-05	-	-	-	-	34	-	-	-	-	-	-
A-T10	NM_000051.4:c.8786+1G>A	Splice donor variant	Pathogenic/Likely pathogenic	3.23E-05	-	-	-	-	34	-	-	-	-	-	-

Table 8. Pathogenicity prediction score table of the DNA variants called with targeted DNA sequencing

gnomAD EAF: The Genome Aggregation Database effect allele frequency, GERP++ RS: GERP rejected substitutions score, AlphaMissense pathogenicity: AlphaMissense pathogenicity score, CADD_PHRED: Combined Annotation-Dependent Depletion phred-like score, BayesDel addAF score: BayesDel deleteriousness meta-score incorporated with allele frequency data, BayesDel noAF score: BayesDel deleteriousness meta-score without allele frequency data, ClinPred: ClinPred pathogenicity score, fathmm-MKL coding rankscore: Functional Analysis Through Hidden Markov Models- machine learning approach functional consequence rank score for coding sequence, fathmm-XF coding rankscore: Functional Analysis Through Hidden Markov Models with eXtended Features functional consequence rank score for coding sequence, PrimateAI score: PrimateAI pathogenicity score (Red: pathogenicity score reaching significant effect)

CADD_PHRED represents an integrated quantitative phred-like score of Combined Annotation Dependent Depletion, which predicts deleteriousness effect of different mutations with cutoff score at 20 (ranging from 1 to 99) (Niroula & Vihinen, 2019). The CADD scores of nearly all ATM mutations were higher than 20, except NM_000051.4:c.7875T>G (Table 8). BayesDel is deleteriousness meta-score of different variants, while BayesDel addAF score represents the score with allele frequency normalisation and BayesDel noAF score represents the score without allele frequency normalisation. The universal cutoff of BayesDel score is 0.0693 with allele frequency normalisation and -0.0570 without allele frequency normalisation. NM_000051.4:c.8880G>A, NM_000051.4:c.7327C>T, NM_000051.4:c.6095G>A and NM_000051.4:c.7876G>C are pathogenic in both BayesDel addAF and noAF score (Table 8). NM_000051.4:c.6095G>A is only ranked as pathogenic in BayesDel noAF score and NM_000051.4:c.7875T>G is only ranked as pathogenic in BayesDel addAF score (Table 8). ClinPred represents a disease-relevant score of nonsynonymous variants with cutoff at 0.5 (ranging from 0 to 1). NM_000051.4:c.6095G>A and NM_000051.4:c.7876G>C are ranked as pathogenic in ClinPred score (Table 8). Fathmm-MKL and fathmm-XF coding rankscore are two generation functional consequence rank score of coding sequence with cutoff at 0.5 (ranging from 0 to 1), which work under Functional Analysis Through Hidden Markov Models (Shihab et al., 2015). NM_000051.4:c.6095G>A, NM_000051.4:c.7876G>C and NM_000051.4:c.2284_2285del are ranked as deleterious in both Fathmm rank score (Table 8). PrimateAI predicts the pathogenicity of missense variants in primate species using deep learning algorithms, which is considered pathogenic when it scores higher than 0.803 (ranging from 0 to 1). However, none of the *ATM* mutations detected in this study achieved the score of pathogenicity under PrimateAI (Table 8).

Sample ID	HGVSc	Consequence	ClinVar latest	MaxEntScan_ref	MaxEntScan_alt	MaxEntScan_diff	SpliceAI_DS_AG	SpliceAI_DS_AL	SpliceAI_DS_DG	SpliceAI_DS_DL	SpliceAI_DP_AG	SpliceAI_DP_AL	SpliceAI_DP_DG	SpliceAI_DP_DL
A-T1	NM_000051.4:c.7630-2A>G	Splice acceptor variant	Pathogenic/Likely pathogenic	6.962	-0.993	7.955	0.32	0.91	0	0	13	2	13	2
A-T1	NM_000051.4:c.8880G>A	Stop gained variant	Pathogenic	-	-	-	0.04	0	0	0	34	-29	-4	-17
A-T2	NM_000051.4:c.6404_6405insTT	Frameshift variant	Pathogenic/Likely pathogenic	-	-	-	-	-	-	-	-	-	-	-
A-T2	NM_000051.4:c.8036_8051del	Frameshift variant	Pathogenic	-	-	-	-	-	-	-	-	-	-	-
A-T3	NM_000051.4:c.944del	Frameshift variant	Pathogenic	-	-	-	0	0	0	0	-41	16	23	49
A-T3	NM_000051.4:c.1564_1565del	Frameshift variant	Pathogenic	-	-	-	0	0	0	0	4	2	46	50
A-T4	NM_000051.4:c.3085dup	Frameshift variant	Pathogenic	-	-	-	0	0.02	0	0	-20	-6	27	42
A-T4	NM_000051.4:c.7327C>T	Stop gained variant	Pathogenic/Likely pathogenic	-	-	-	0	0	0	0	-32	-11	-2	48
A-T5	NM_000051.4:c.6095G>A	Missense variant, Splice region variant	Pathogenic/Likely pathogenic	6.621	1.478	5.143	0	0	0.02	0.54	0	-19	25	0
A-T5	NM_000051.4:c.742C>T	Stop gained variant	Pathogenic/Likely pathogenic	-	-	-	0	0	0	0	-38	-18	-33	8
A-T6	NM_000051.4:c.6095G>A	Missense variant, Splice region variant	Pathogenic/Likely pathogenic	6.621	1.478	5.143	0	0	0.02	0.54	0	-19	25	0
A-T6	NM_000051.4:c.1290_1291del	Frameshift variant	Pathogenic/Likely pathogenic	-	-	-	0	0	0	0	-45	0	7	0
A-T7	NM_000051.4:c.7875T>G	Missense variant	Pathogenic	-	-	-	0	0	0.04	0	-5	17	-5	-27
A-T7	NM_000051.4:c.7876G>C	Missense variant	Pathogenic	-	-	-	0	0	0	0	16	25	-13	-28
A-T8	NM_000051.4:c.2284_2285del	Frameshift variant	Pathogenic/Likely pathogenic	-	-	-	0.06	0.03	0	0	-12	-31	-37	-7
A-T8	NM_000051.4:c.7388delNinsN[313]	Feature elongation, Coding sequence variant	null	-	-	-	-	-	-	-	-	-	-	-
A-T9	NM_000051.4:c.3712_3716del	Frameshift variant	Pathogenic/Likely pathogenic	-	-	-	-	-	-	-	-	-	-	-
A-T9	NM_000051.4:c.2921+1G>T	Splice donor variant	Pathogenic/Likely pathogenic	6.287	-2.216	8.504	0	0	0	0.91	20	-49	19	-1
A-T10	NM_000051.4:c.6404_6405insTT	Frameshift variant	Pathogenic/Likely pathogenic	-	-	-	-	-	-	-	-	-	-	-
A-T10	NM_000051.4:c.8786+1G>A	Splice donor variant	Pathogenic/Likely pathogenic	10.998	2.816	8.182	0	0	0.03	1	13	-13	13	-1

Table 9. Alternative splicing score table of the DNA variants called with targeted DNA sequencing

MaxEntScan_ref: the predicted fitness of the reference motif as a donor or an acceptor splice site based on maximum entropy model, MaxEntScan_alt: the predicted fitness of the alternative motif as a donor or an acceptor splice site based on maximum entropy model, MaxEntScan_dif: the difference between the predicted fitness of alternative motif and reference motif as a donor or an acceptor splice site based on maximum entropy model, SpliceAI DS AG: Delta score for acceptor gain predicted by SpliceAI on splicing effect, SpliceAI DS AL: Delta score for acceptor loss predicted by SpliceAI on splicing effect, SpliceAI DS DG: Delta score for donor gain predicted by SpliceAI on splicing effect, SpliceAI DS DL: Delta score for donor loss predicted by SpliceAI on splicing effect, SpliceAI DP AG: Delta position for acceptor gain predicted by SpliceAI on splicing effect, SpliceAI DP AL: Delta position for acceptor loss predicted by SpliceAI on splicing effect, SpliceAI DP DG: Delta position for donor gain predicted by SpliceAI on splicing effect, SpliceAI DP DL: Delta position for donor loss predicted by SpliceAI on splicing effect. (Red: alternative splicing score reaching significant effect)

MaxEntScan predicts the fitness of the sequence motif including dependencies between nonadjacent and adjacent positions (Yeo & Burge, 2004). The mutated splice site is considered severely losing its function when the MaxEntScan_alt score is less than 6.5, and the loss is considered significant when the MaxEntScan_dif is more than 1.15 (Eng et al., 2004). NM_000051.4:c.7630-2A>G, NM_000051.4:c.6095G>A, NM_000051.4:c.2921+1G>T and NM_000051.4:c.8786+1G>A revealed significant loss of splicing function on their corresponding site based on the MaxEntScan prediction (Table 9). SpliceAI predicts splice junctions from an arbitrary pre-mRNA transcript sequence based on deep neural network machine learning (Jaganathan et al., 2019). Delta score of SpliceAI represents the probability of the variant being splice-altering in the corresponding delta position. The recommended cutoff of delta score is higher than 0.5, while 0.8 is the standard with high precision (ranging from 0 to 1). NM_000051.4:c.7630-2A>G, NM_000051.4:c.2921+1G>T and NM_000051.4:c.8786+1G>A revealed high precision splice-altering effect on their corresponding splice site based on SpliceAI estimation (Table 9). Moreover, one of the missense variant, NM_000051.4:c.6095G>A, also showed its ability contributing to the splice donor loss (Table 9).

ATM transcript expression variation upon mutation

To extend the investigation of ATM gene mutation detected in DNA sequencing, transcriptomic RNA sequencing result was further analysed with DRAGEN RNA pipeline for transcript-level differential expression using DRAGEN Differential Expression (v 4.2.4). The result demonstrated expression level of each post-splicing transcript affected by ATM gene pathogenic mutations.

Transcript ID	Base Mean	log ₂ Fold Change	p-value	p ^{adj}	status	control mean	comparison mean	gene	Transcript start	Transcript end	Transcript length
ENST00000278616.8	68.857	-0.871	0.540	0.720	OK	89.024	48.690	ATM	108093559	108239826	13147
ENST00000452508.6	379.074	-0.418	0.433	0.631	OK	433.626	324.522	ATM	108093843	108239829	12954
ENST00000524792.5	9.246	1.363	NaN	NaN	Low	5.177	13.314	ATM	108158119	108236761	5912
ENST00000530958.5	6.391	0.137	0.818	NaN	Low	6.088	6.694	ATM	108093830	108107261	5718
ENST00000527805.5	0	NaN	NaN	NaN	Low	NaN	NaN	ATM	108093211	108158441	4841
ENST00000533690.5	13.051	-0.277	0.839	0.915	OK	14.301	11.801	ATM	108167701	108204635	3354
ENST00000533733.5	2.112	1.928	0.085	NaN	Low	0.879	3.344	ATM	108157905	108161394	1731
ENST00000531525.2	9.895	1.148	0.172	NaN	Low	6.155	13.635	ATM	108158327	108166723	1513
ENST00000525178.5	4.799	-1.974	0.512	NaN	Low	7.650	1.948	ATM	108217248	108236456	880
ENST00000527181.1	16.580	0.027	0.985	0.992	OK	16.422	16.738	ATM	108224483	108236465	740
ENST00000534625.1	0.164	1.435	0.648	NaN	Low	0.088	0.239	ATM	108170207	108173756	725
ENST00000533979.5	1.692	3.414	0.052	NaN	Low	0.290	3.094	ATM	108203489	108207135	705
ENST00000525056.1	1.968	1.465	0.429	NaN	Low	1.047	2.888	ATM	108203504	108206880	657
ENST00000532765.1	0.000	NaN	NaN	NaN	Low	NaN	NaN	ATM	108178719	108188194	610
ENST00000527891.5	0.660	3.999	0.201	NaN	Low	0.078	1.243	ATM	108093812	108114811	595
ENST00000525537.2	0.765	-2.042	0.088	NaN	Low	1.231	0.299	ATM	108198121	108199968	586
ENST00000601453.2	0.000	NaN	NaN	NaN	Low	NaN	NaN	ATM	108094805	108106470	582
ENST00000526567.5	0.872	0.240	0.832	NaN	Low	0.800	0.945	ATM	108094256	108100091	560
ENST00000531957.1	0.929	0.754	0.663	NaN	Low	0.692	1.167	ATM	108164112	108166097	537
ENST00000529588.5	0.027	1.263	0.689	NaN	Low	0.016	0.038	ATM	108173749	108186621	502
ENST00000532931.5	0.017	1.242	0.694	NaN	Low	0.010	0.024	ATM	108093817	108099999	481
ENST00000527389.2	0	NaN	NaN	NaN	Low	NaN	NaN	ATM	108198371	108200034	402
ENST00000419286.2	0	NaN	NaN	NaN	Low	NaN	NaN	ATM	108138067	108142079	305
ENST00000525012.5	0	NaN	NaN	NaN	Low	NaN	NaN	ATM	108123548	108124723	258
ENST00000533526.1	0	NaN	NaN	NaN	Low	NaN	NaN	ATM	108123548	108124723	234

Table 10. List of different ATM transcripts expression in the comparison of A-T cerebellum samples

Transcript ID	Base Mean	log ₂ Fold Change	p-value	p ^{adj}	status	control mean	comparison mean	gene	Transcript start	Transcript end	Transcript length
ENST00000278616.8	67.168	-0.761	0.645	0.857	OK	84.487	49.849	ATM	108093559	108239826	13147
ENST00000452508.6	298.546	0.008	0.969	0.991	OK	297.677	299.414	ATM	108093843	108239829	12954
ENST00000524792.5	1.718	-2.416	0.427	NaN	Low	2.894	0.542	ATM	108158119	108236761	5912
ENST00000530958.5	4.770	1.272	0.039	NaN	Low	2.794	6.745	ATM	108093830	108107261	5718
ENST00000527805.5	0.000	NaN	NaN	NaN	Low	NaN	NaN	ATM	108093211	108158441	4841
ENST00000533690.5	3.651	-1.269	0.382	NaN	Low	5.162	2.141	ATM	108167701	108204635	3354
ENST00000533733.5	0.744	1.391	0.363	NaN	Low	0.411	1.077	ATM	108157905	108161394	1731
ENST00000531525.2	5.726	1.873	0.004	NaN	Low	2.456	8.995	ATM	108158327	108166723	1513
ENST00000525178.5	11.910	-0.395	0.772	0.915	OK	13.533	10.288	ATM	108217248	108236456	880
ENST00000527181.1	7.398	-1.061	0.714	NaN	Low	10.002	4.793	ATM	108224483	108236465	740
ENST00000534625.1	0.000	NaN	NaN	NaN	Low	NaN	NaN	ATM	108170207	108173756	725
ENST00000533979.5	2.505	1.565	0.295	NaN	Low	1.266	3.744	ATM	108203489	108207135	705
ENST00000525056.1	0.316	1.690	0.572	NaN	Low	0.150	0.483	ATM	108203504	108206880	657
ENST00000532765.1	0.489	2.161	0.490	NaN	Low	0.179	0.799	ATM	108178719	108188194	610
ENST00000527891.5	0.380	-0.134	0.966	NaN	Low	0.397	0.362	ATM	108093812	108114811	595
ENST00000525537.2	0.905	1.316	0.392	NaN	Low	0.519	1.291	ATM	108198121	108199968	586
ENST00000601453.2	0.000	NaN	NaN	NaN	Low	NaN	NaN	ATM	108094805	108106470	582
ENST00000526567.5	1.114	3.308	0.032	NaN	Low	0.204	2.024	ATM	108094256	108100091	560
ENST00000531957.1	0.310	-1.144	0.715	NaN	Low	0.427	0.193	ATM	108164112	108166097	537
ENST00000529588.5	0.000	NaN	NaN	NaN	Low	NaN	NaN	ATM	108173749	108186621	502
ENST00000532931.5	0.060	0.203	0.949	NaN	Low	0.056	0.064	ATM	108093817	108099999	481
ENST00000527389.2	0.945	-1.891	0.521	NaN	Low	1.489	0.401	ATM	108198371	108200034	402
ENST00000419286.2	0.000	NaN	NaN	NaN	Low	NaN	NaN	ATM	108138067	108142079	305
ENST00000525012.5	0.000	NaN	NaN	NaN	Low	NaN	NaN	ATM	108123548	108124723	258
ENST00000533526.1	0.000	NaN	NaN	NaN	Low	NaN	NaN	ATM	108123548	108124723	234

Table 11. List of different ATM transcripts expression in the comparison of A-T cerebral frontal cortex samples

There are seven ATM protein-coding transcripts labelled with red colour in Table 7 and Table 8. ENST00000278616.8 is the longest transcript (13147bps), which is the MANE-selected canonical transcript for *ATM* gene. The second longest transcript, ENST00000452508.6 contains 12954 bps, which can also encode the full ATM protein with 3056 amino acid residues. The remaining protein-coding transcripts, ENST00000527805.5, ENST00000531525.2, ENST00000527891.5, ENST00000601453.2 and ENST00000532931.5 encode for truncated ATM peptide with 1369, 155, 154, 135 and 93 amino acid residues correspondingly. The *ATM*

transcripts with black colour could not be translated into protein peptides due to nonsense mediated decay, processed transcript and retained intron.

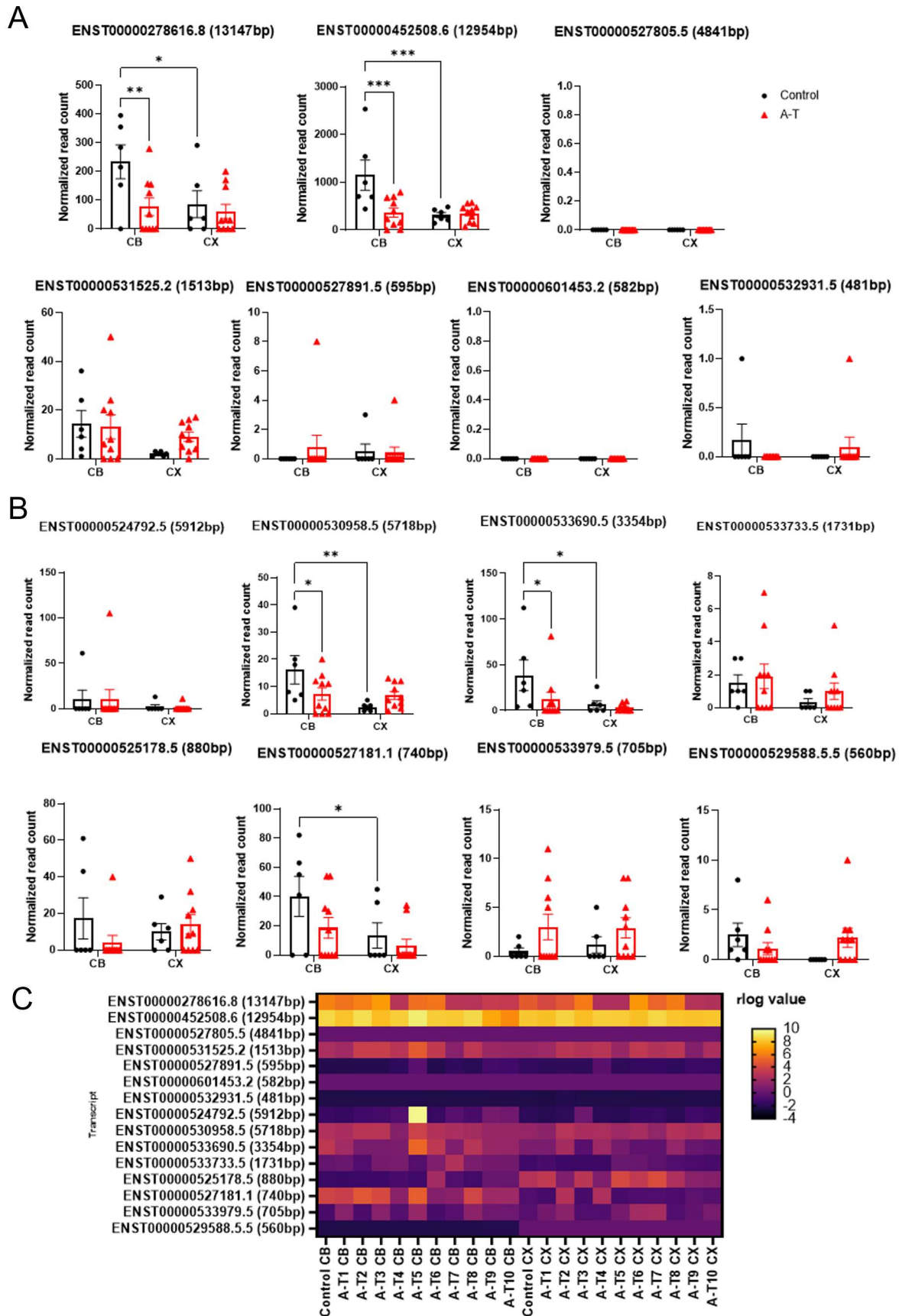


Figure 86. Quantification of different *ATM* transcripts in A-T cerebellum and cerebral frontal cortex sample.

A) Comparison of protein-coding transcript in A-T cerebellum and cerebral frontal cortex sample. B) Comparison of representative non-protein-coding transcript in A-T cerebellum and cerebral frontal cortex sample. (Two-way ANOVA with Fisher's LSD multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). C) Expression heatmap with rlog value of ATM transcripts across each A-T cerebellum and cerebral frontal cortex sample.

There was significant decrease of the full-length ATM protein-coding transcripts in A-T cerebellum compared to control (67.1% decrease in ENST00000278616.8, $p = 0.0077$; 68.4% decrease in ENST00000452508.6, $p = 0.0005$) (Figure 86A). Indeed, significant decrease was also shown in the full-length ATM protein-coding transcripts of control cerebral frontal cortex comparing to control cerebellum (63.3% decrease in ENST00000278616.8, $p = 0.0222$; 72.9% decrease in ENST00000452508.6, $p = 0.0008$) (Figure 86A). The expression level of full-length ATM protein-coding transcripts were similar among A-T cerebellum, control cerebral frontal cortex and A-T cerebral frontal cortex (Figure 86A). Yet, control cerebellum revealed a distinctly high expression of full-length ATM protein-coding transcripts. Other ATM protein-coding transcripts were not actively expressed except ENST00000531525.2.

In the comparison between non-protein-coding transcripts, control cerebellum usually had the highest expression level except ENST00000533733.5 and ENST00000533979.5 (Figure 86B). Compared to the control cerebellum sample, control cerebral samples were significantly less expressed in the ENST00000530958.5 (5718bp) (84.5%, $p = 0.0029$), ENST00000533690.5 (3354bp) (83.0%, $p = 0.0235$) and ENST00000527181.1 (740bp) (66.4%, $p = 0.0496$).

In general, reduced ATM transcript expression was only found in A-T cerebellum tissue but not cerebral frontal cortex. Expression of MANE-selected *ATM* transcript varied among A-T samples, but the expression heatmap revealed a consistent reduction of ENST00000278616.8 in A-T4, A-T9 and A-T10 (Figure 86C).

Alternative splicing of ATM transcript upon mutation

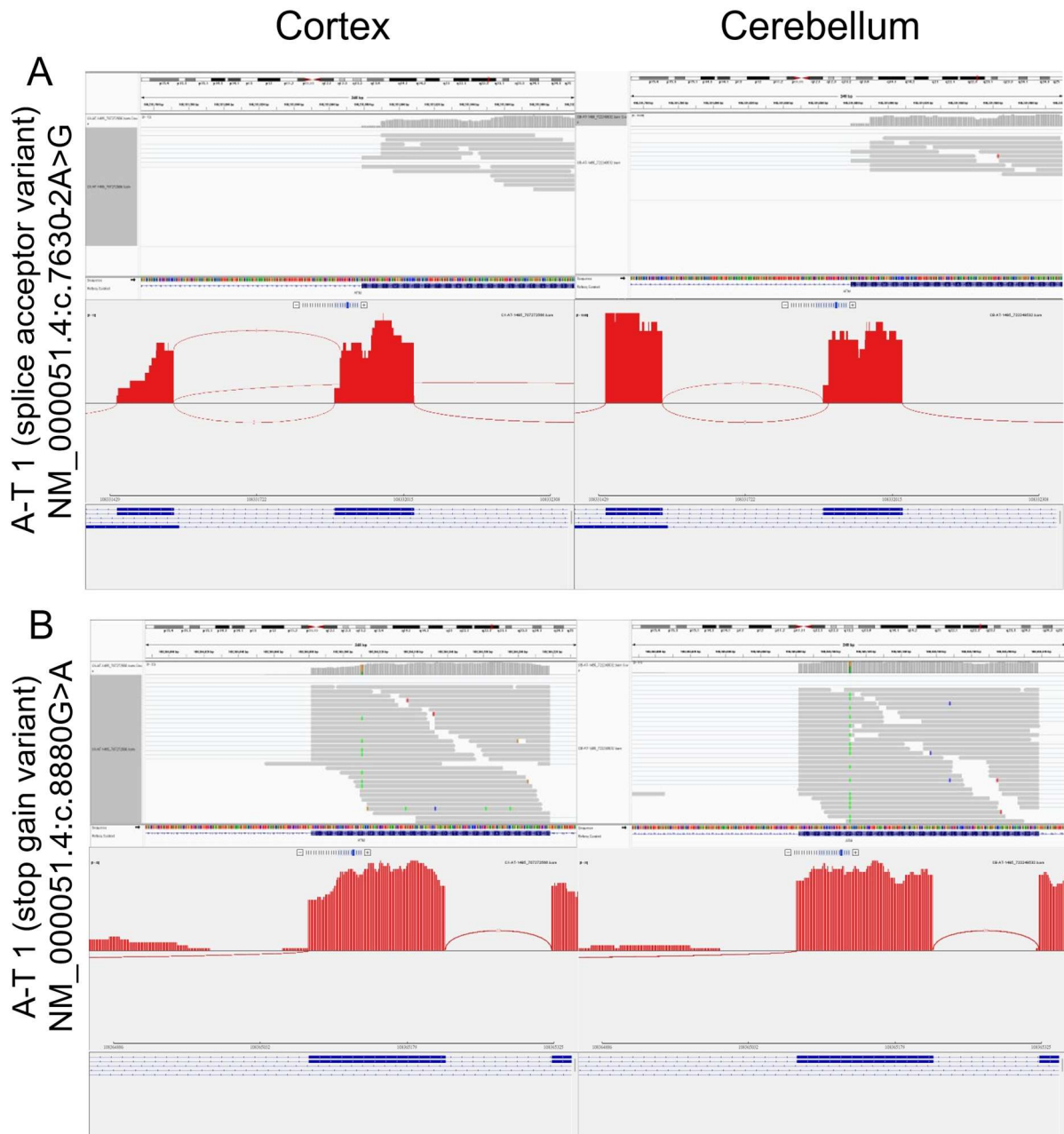


Figure 87. Gene coverage on pathogenic mutation site of A-T 1 detected with Illumina mRNA transcriptomic sequencing.

A) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.7630-2A>G pathogenic mutation of A-T 1. B) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.8880G>A pathogenic mutation of A-T 1.

Splice acceptor variant resulted in alternative 3' splicing site on both frontal cortex tissue and cerebellum tissue, but an exon skipping event was only found in the A-T 1 frontal cortex sample (Figure 87A). Stop gain variant did not result in alternation in splicing events (Figure 87B), but it terminated downstream protein translation.

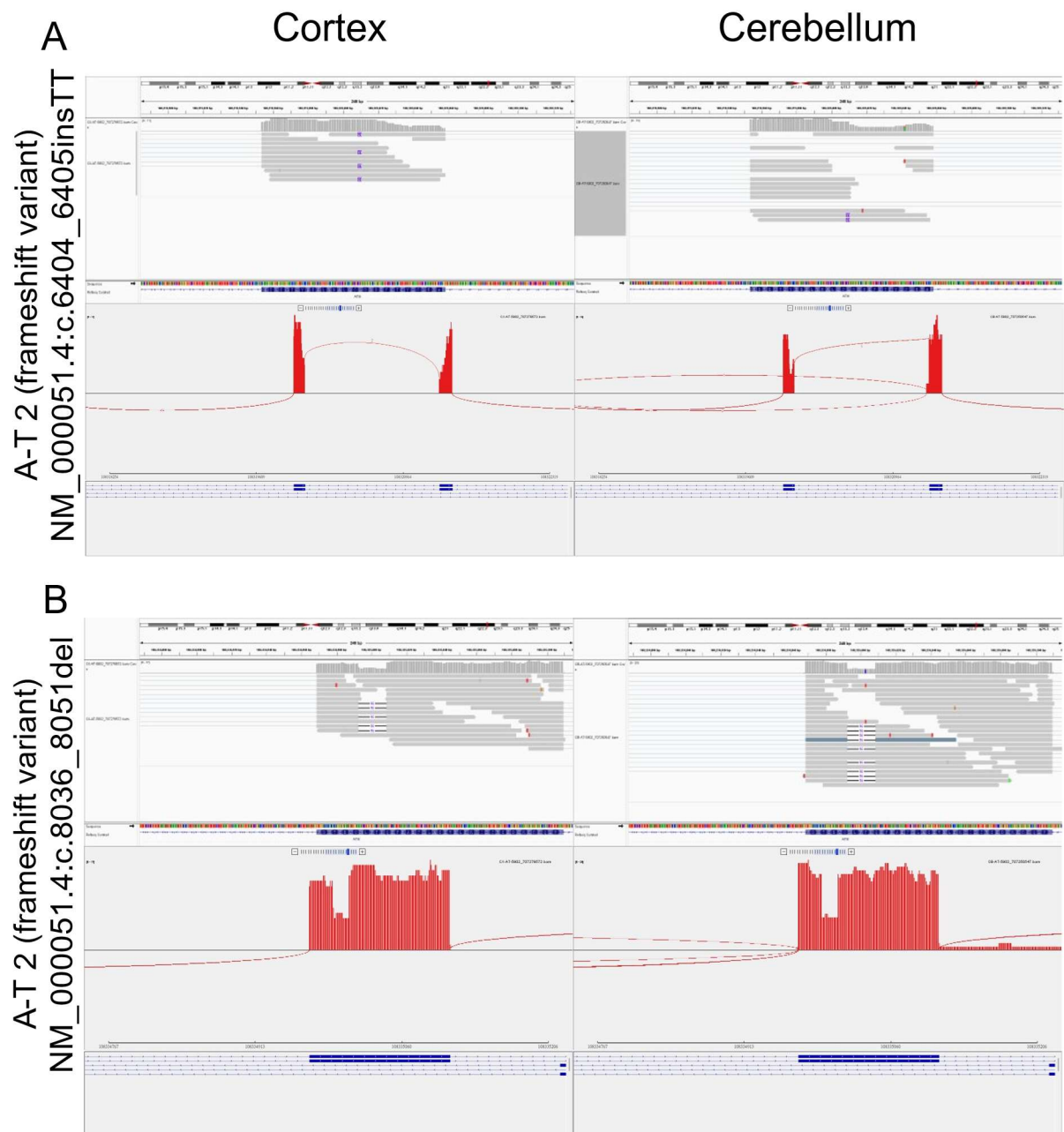


Figure 88. Gene coverage on pathogenic mutation site of A-T 2 detected with Illumina mRNA transcriptomic sequencing.

A) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.6404_6405insTT pathogenic mutation of A-T 2. B) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.8036_8051del pathogenic mutation of A-T 2.

The first frameshift mutation (NM_000051.4:c.6404_6405insTT) had no splicing effect on frontal cortex tissue, but caused exon 44 skipping in the cerebellum tissue (Figure 88A). The second frameshift mutation (NM_000051.4:c.8036_8051del) was causing 16 base pairs skipping in translation of exon 55 in both frontal cortex and cerebellum tissue (Figure 88B).

Moreover, alternative 3' splicing site was found in the cerebellum tissue at exon 55 (Figure 88B).

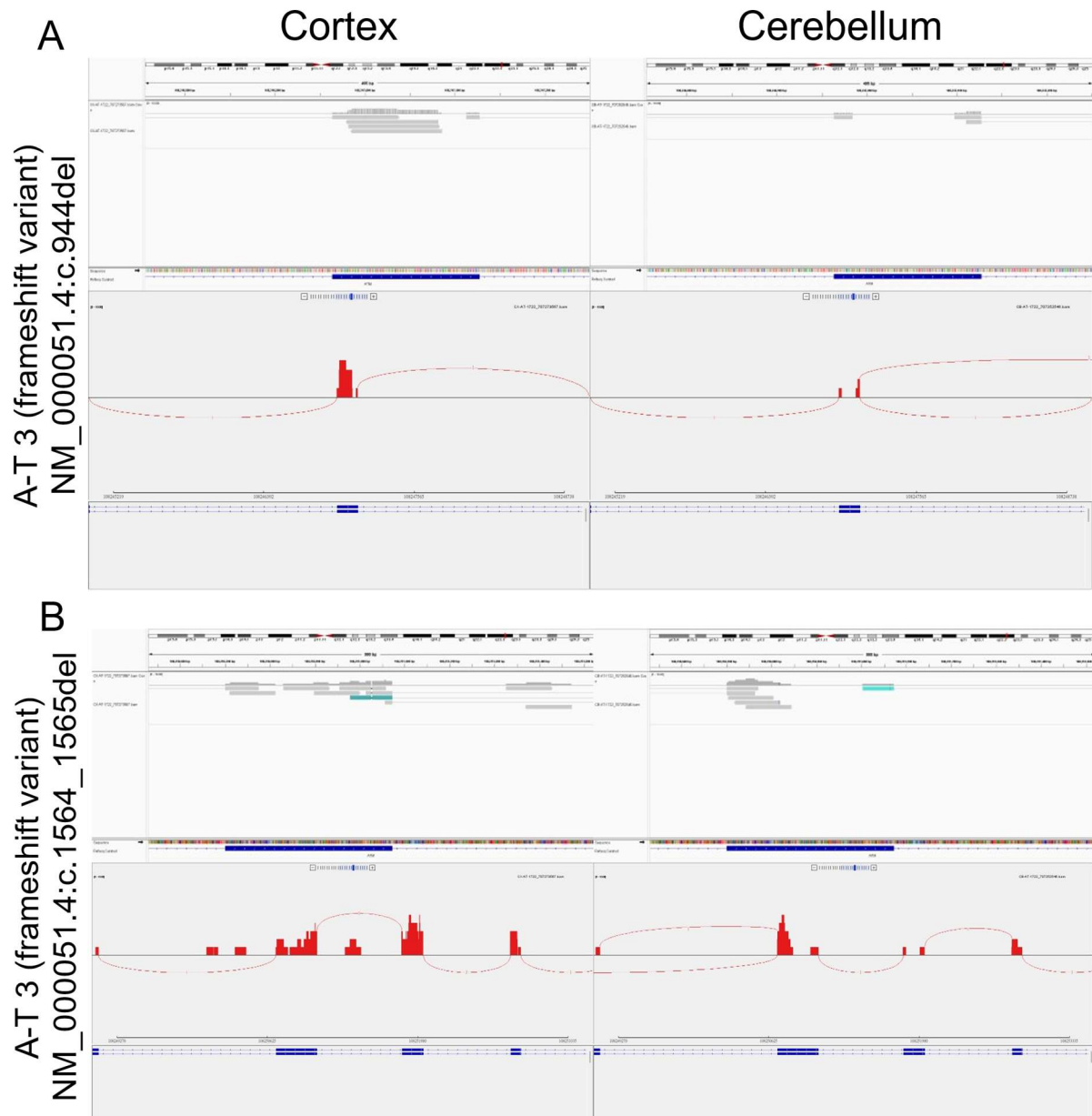


Figure 89. Gene coverage on pathogenic mutation site of A-T 3 detected with Illumina mRNA transcriptomic sequencing.

A) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.944del pathogenic mutation of A-T 3. B) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.1564_1565del pathogenic mutation of A-T 3.

The first frameshift mutation (NM_000051.4:c.944del) resulted in truncation of exon 8 in both frontal cortex and cerebellum tissue, while less read coverage was found with cerebellum tissue

(Figure 89A). The second frameshift mutation (NM_000051.4:c.1564_1565del) resulted in truncation of exon of exon 10 in both frontal cortex and cerebellum tissue (Figure 89B).

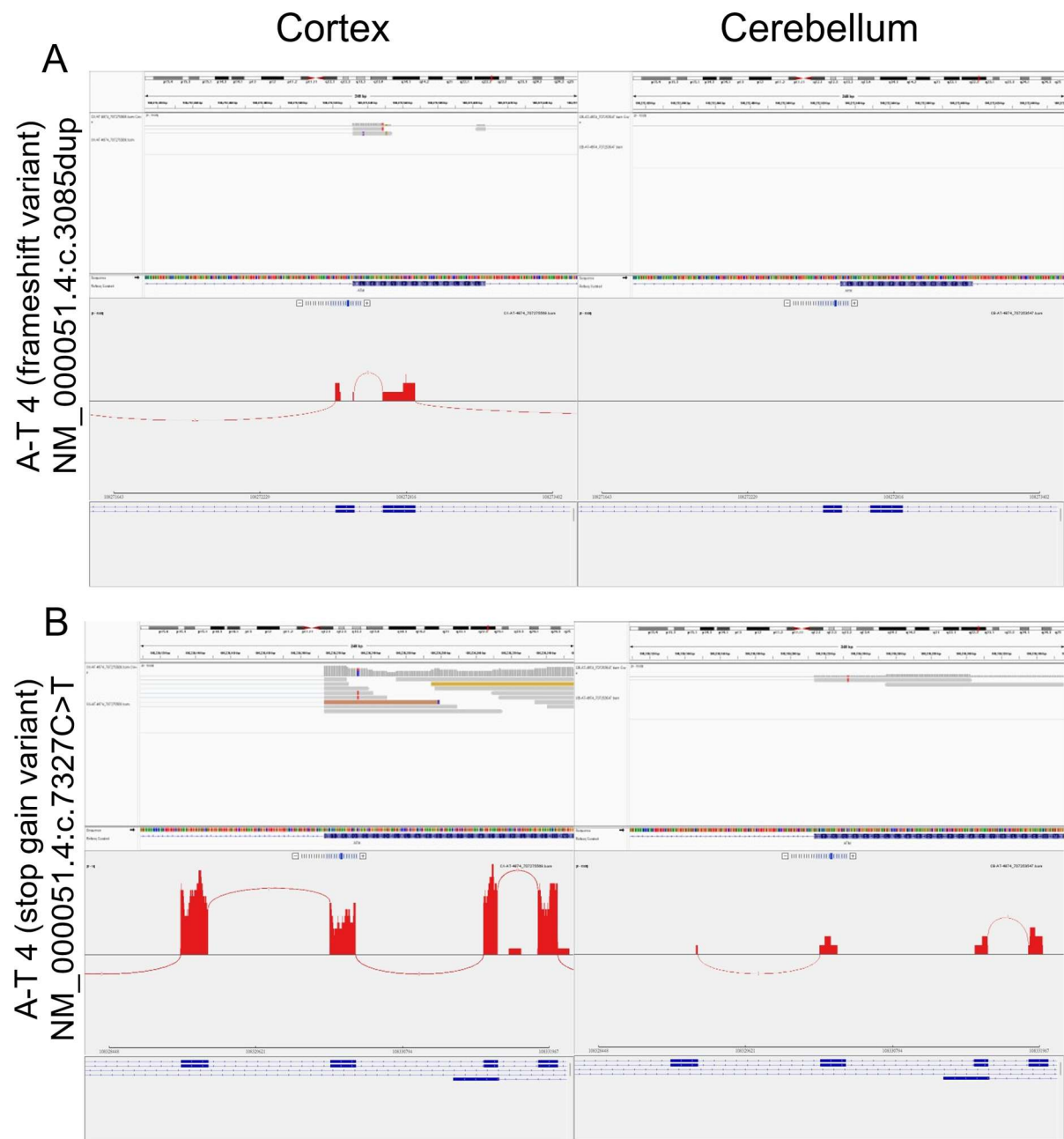


Figure 90. Gene coverage on pathogenic mutation site of A-T 4 detected with Illumina mRNA transcriptomic sequencing.

A) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.3085dup pathogenic mutation of A-T 4. B) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.7327C>T pathogenic mutation of A-T 4.

Frameshift variant resulted in truncation of exon 21 in frontal cortex tissue and null expression of exon 21 in cerebellum tissue (Figure 90A). Stop gain variant did not result in alternation in splicing events of frontal cortex but mis-splicing on 5' end of exon 50 in cerebellum tissue (Figure 90B).

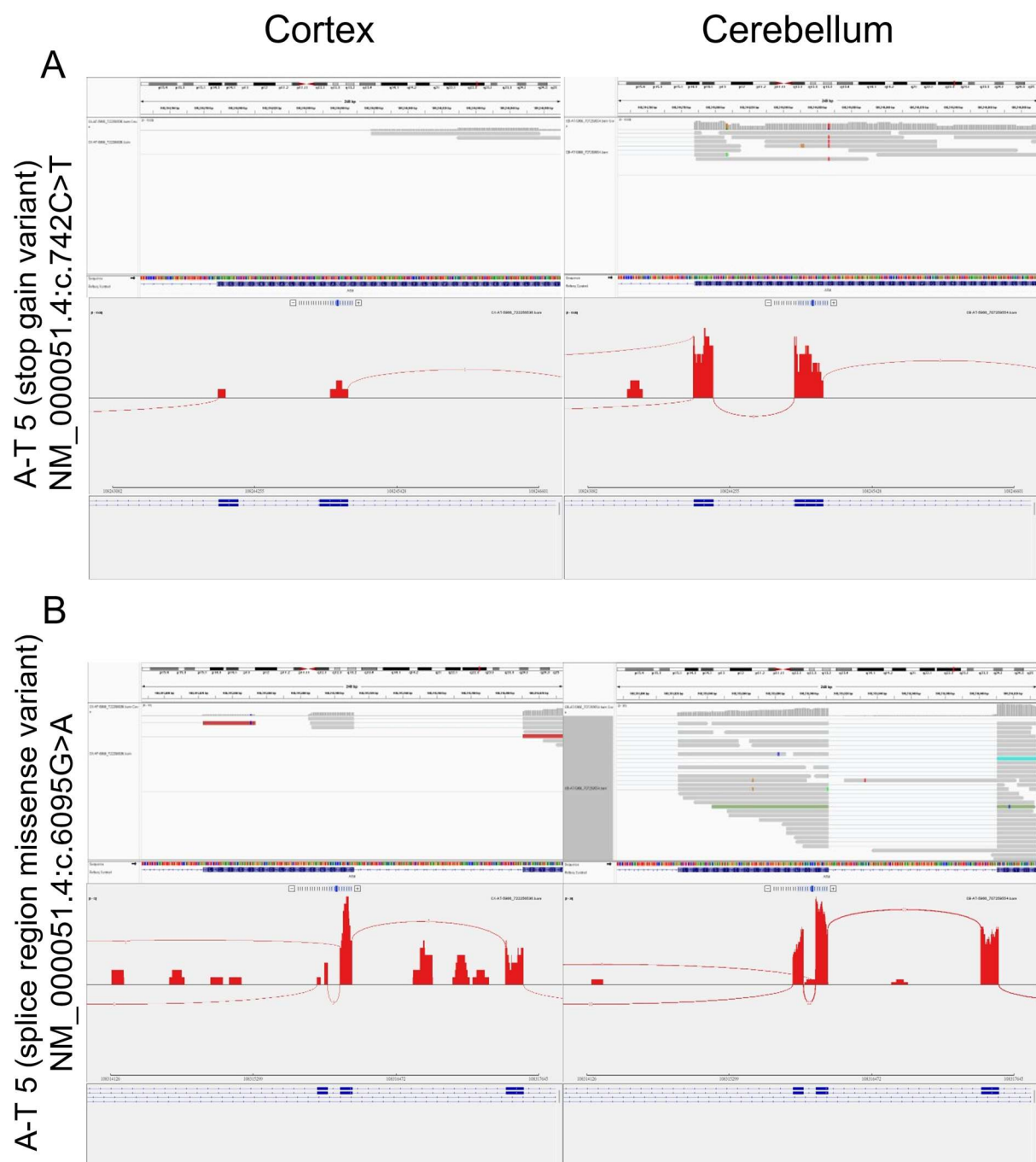


Figure 91. Gene coverage on pathogenic mutation site of A-T 5 detected with Illumina mRNA transcriptomic sequencing.

A) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.742C>T pathogenic mutation of A-T 5. B) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.6095G>A pathogenic mutation of A-T 5.

Stop gain variant only caused mis-splicing event in frontal cortex tissue but not cerebellum tissue (Figure 91A). Splice region missense variant caused exon 41 skipping in both frontal cortex and cerebellum tissue (Figure 91B).

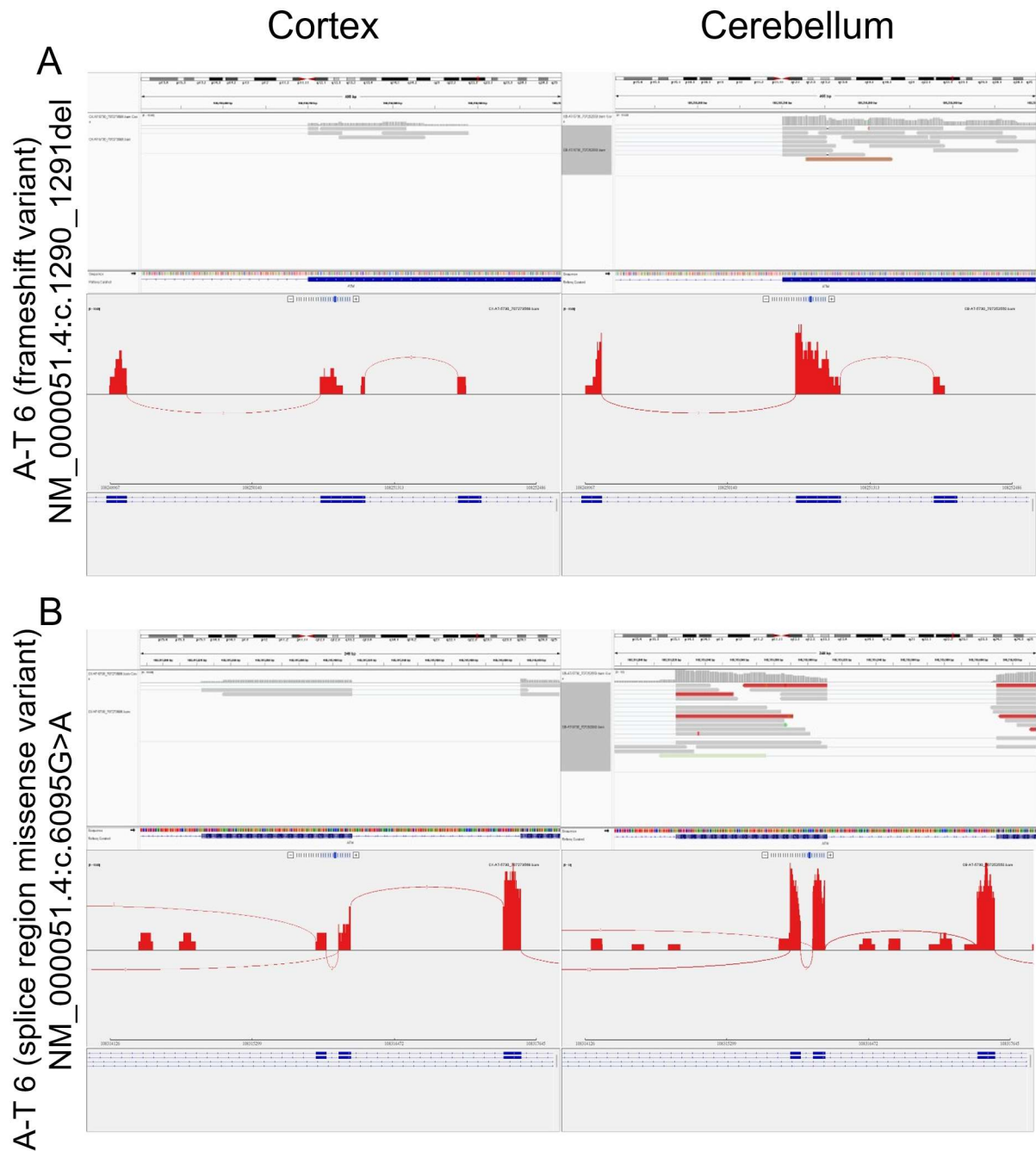


Figure 92. Gene coverage on pathogenic mutation site of A-T 6 detected with Illumina mRNA transcriptomic sequencing.

A) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.1290_1291del pathogenic mutation of A-T 6. B) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.6095G>A pathogenic mutation of A-T 6.

Frameshift variant resulted in exon 10 truncation of frontal cortex tissue but not cerebellum tissue (Figure 92A). Splice region missense variant caused exon 41 skipping in both frontal cortex and cerebellum tissue (Figure 92B).

A-T 7 (missense variant)
NM_000051.4:c.7875T>G, NM_000051.4:c.7876G>C

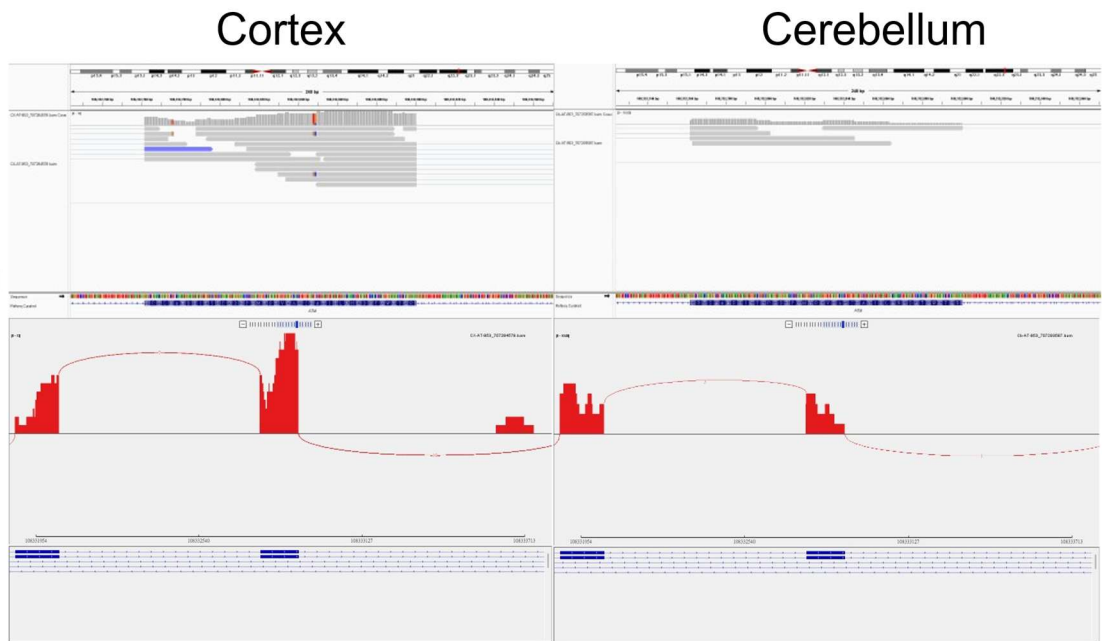


Figure 93. Gene coverage on pathogenic mutation site of A-T 7 detected with Illumina mRNA transcriptomic sequencing.

Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.7875T>G, and NM_000051.4:c.7876G>C pathogenic mutation of A-T 7.

Both missense variants did not cause alternation in splicing events in both frontal cortex and cerebellum tissue (Figure 93).

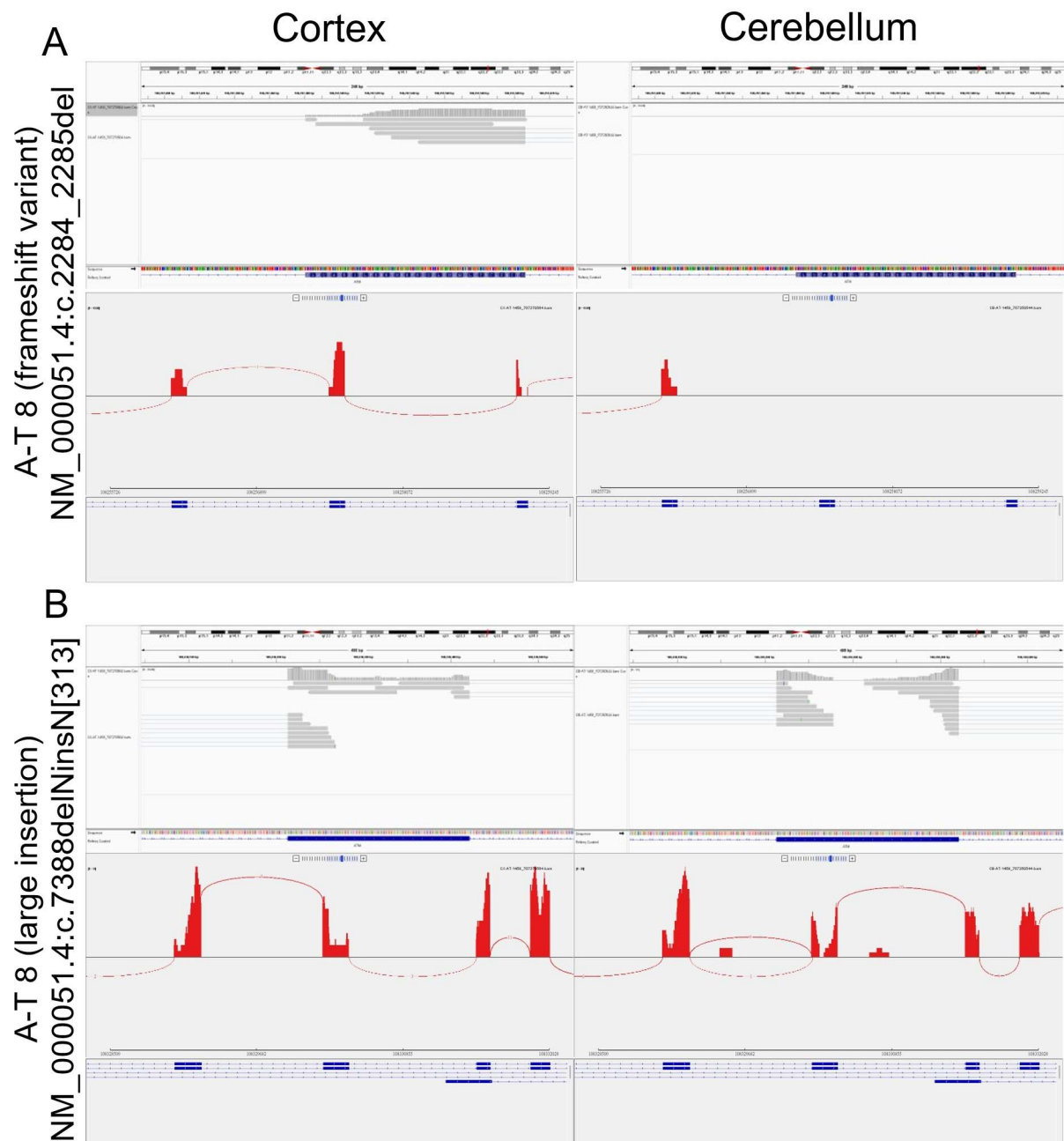


Figure 94. Gene coverage on pathogenic mutation site of A-T 8 detected with Illumina mRNA transcriptomic sequencing.

A) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.2284_2285del pathogenic mutation of A-T 8. B) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.7388delNinsN[313] pathogenic mutation of A-T 8.

Frameshift variant caused null expression of exon 15 in cerebellum but not frontal cortex tissue (Figure 94A). Large insertion caused truncated expression of exon 50 in cerebellum but not frontal cortex tissue (Figure 94B).

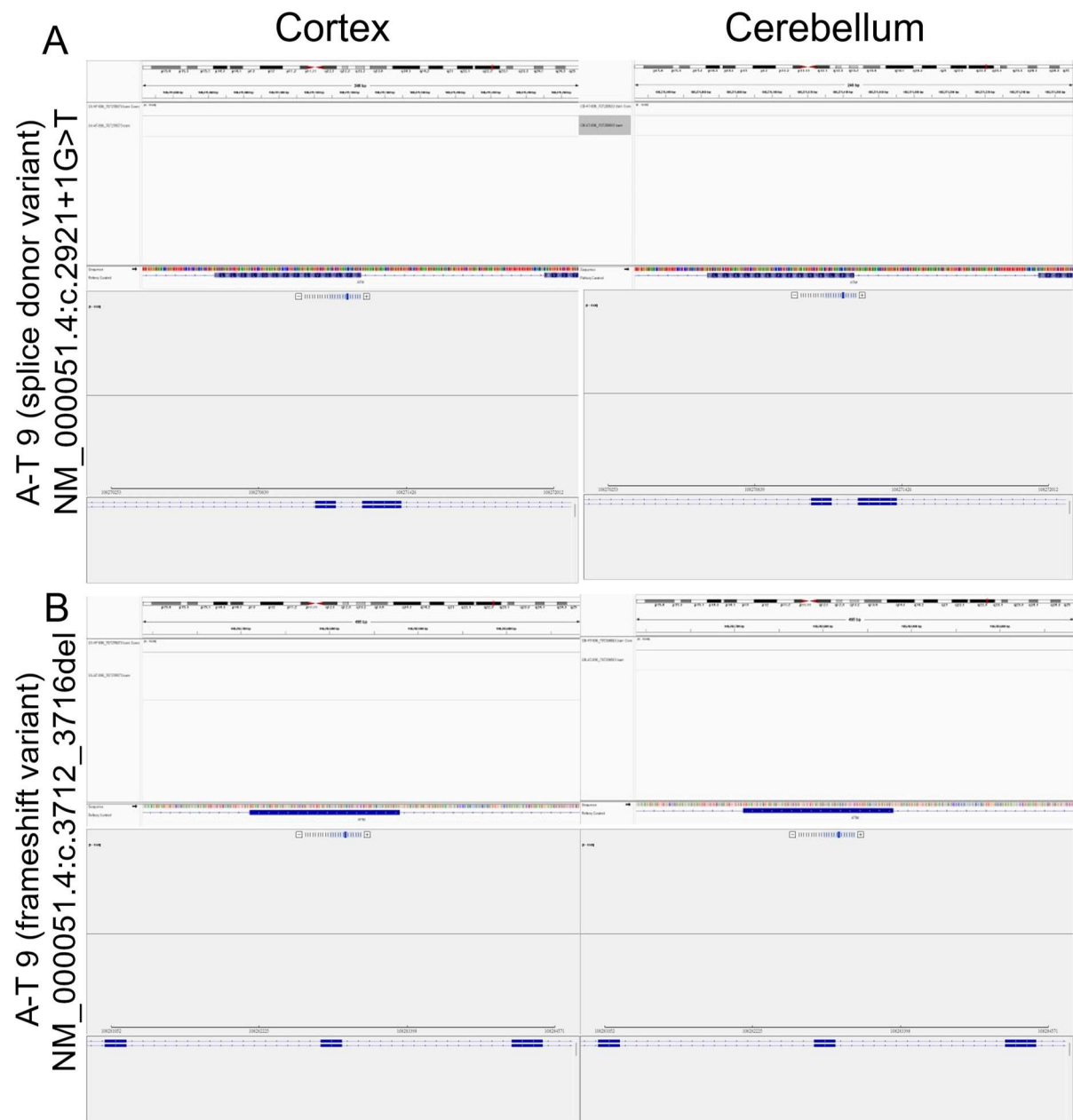


Figure 95. Gene coverage on pathogenic mutation site of A-T 9 detected with Illumina mRNA transcriptomic sequencing.

A) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.2921+1G>T pathogenic mutation of A-T 9. B) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.3712_3716del pathogenic mutation of A-T 9.

These two pathogenic mutations cause null expression of corresponding exons in both frontal cortex and cerebellum tissue (Figure 95).

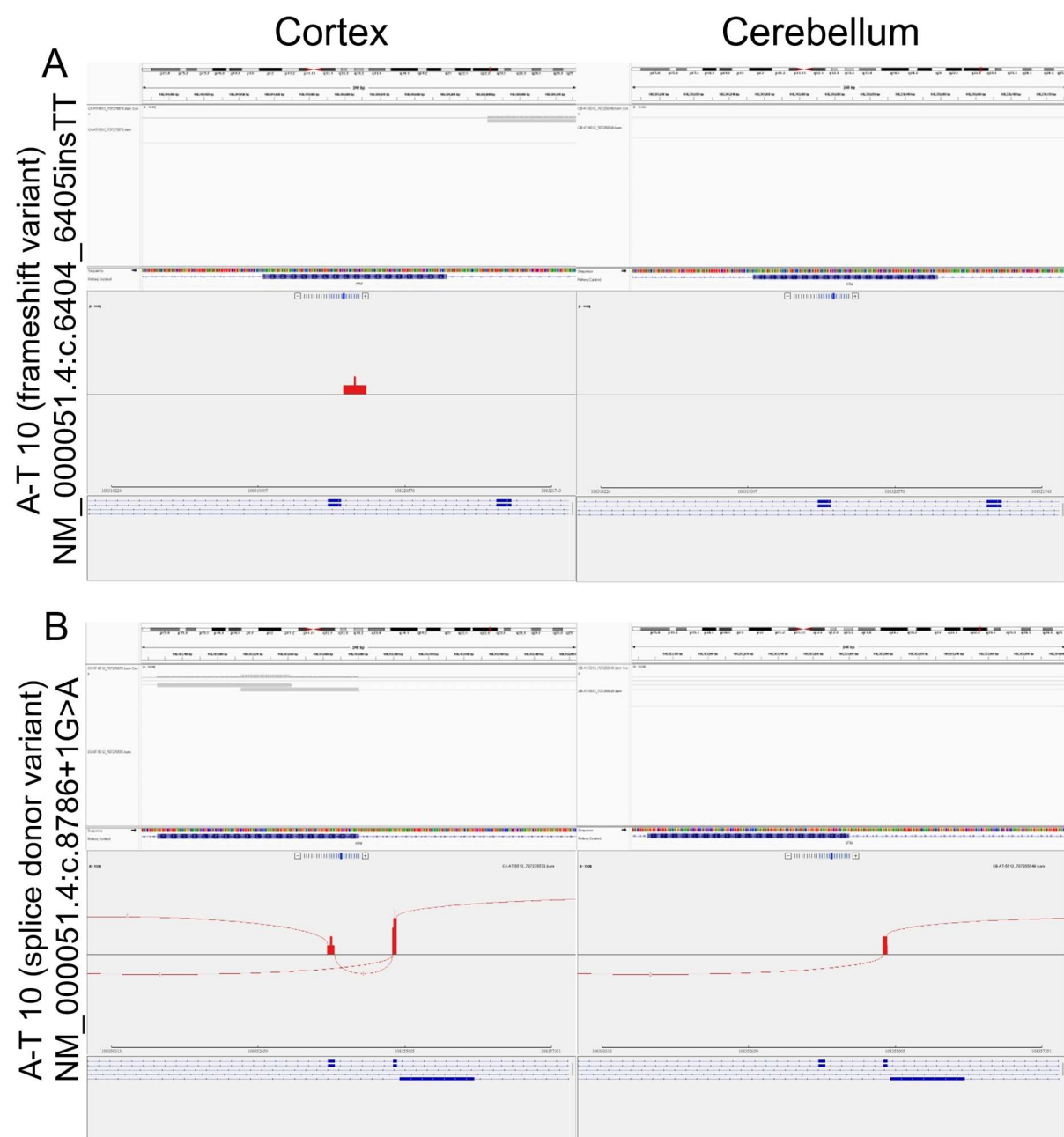


Figure 96. Gene coverage on pathogenic mutation site of A-T 10 detected with Illumina mRNA transcriptomic sequencing.

A) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.6404_6405insTT pathogenic mutation of A-T 10. B) Transcript coverage and sashimi plot reflecting transcript splicing of NM_000051.4:c.8786+1G>A pathogenic mutation of A-T 10.

Frameshift variant caused null expression of exon 44 in both frontal cortex and cerebellum tissue (Figure 96A). Splice donor variant caused exon 60 skipping event in frontal cortex tissue and null expression of exon 60 in cerebellum tissue (Figure 96B).

Protein truncation detected with western blotting

Large variations in *ATM* transcripts were found in the analysis of RNA sequencing. To expand the investigation of *ATM* gene mutation effect to protein transcription, western blotting results were re-analysed with different ATM protein isoform expressions. To unify the comparison results among cerebellum and cerebral frontal cortex, ATM isoform with molecular weight approximately at 235kDa, 180kDa, 150kDa, 130kDa and 105 kDa were found in both western blotting experiment and hence examined against control samples.

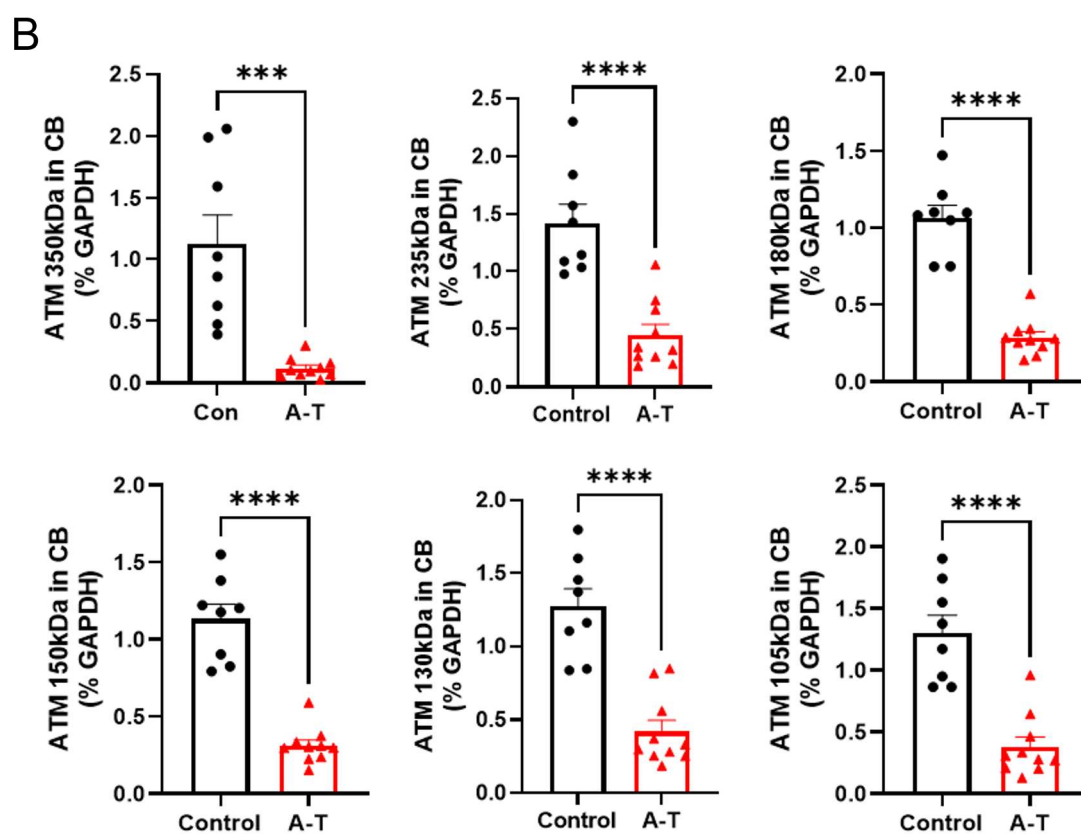
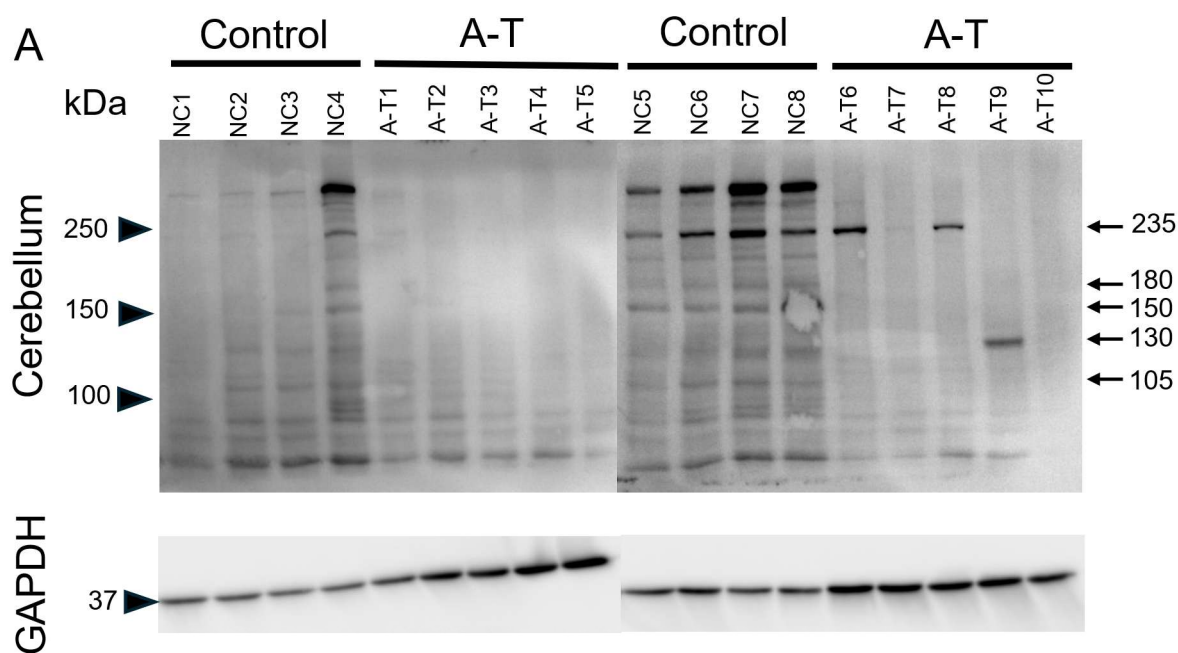


Figure 97. Quantification of different potential ATM protein isoform in A-T cerebellum sample.

The immunoblotting image was shown in figure 17 in chapter 9.2 already, this figure extended the analysis on the potential ATM isoforms. The tailed arrows indicate ATM isoforms to be examined in different approximate molecular weights. (Two-tailed unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Despite the decline in full-length ATM expression, all other five ATM protein isoforms experienced significant decrease in A-T subjects. (Figure 97). Expression of isoform at 235 kDa was decreased by 68.3% ($p < 0.0001$). Expression of isoform at 180 kDa was decreased by 72.4% ($p < 0.0001$). Expression of isoform at 150 kDa experienced decline of 72.6% ($p < 0.0001$). Expression of isoform at 130 kDa experienced decline of 66.9% ($p < 0.0001$). Expression of isoform at 105 kDa was decreased by 70.7% ($p < 0.0001$).

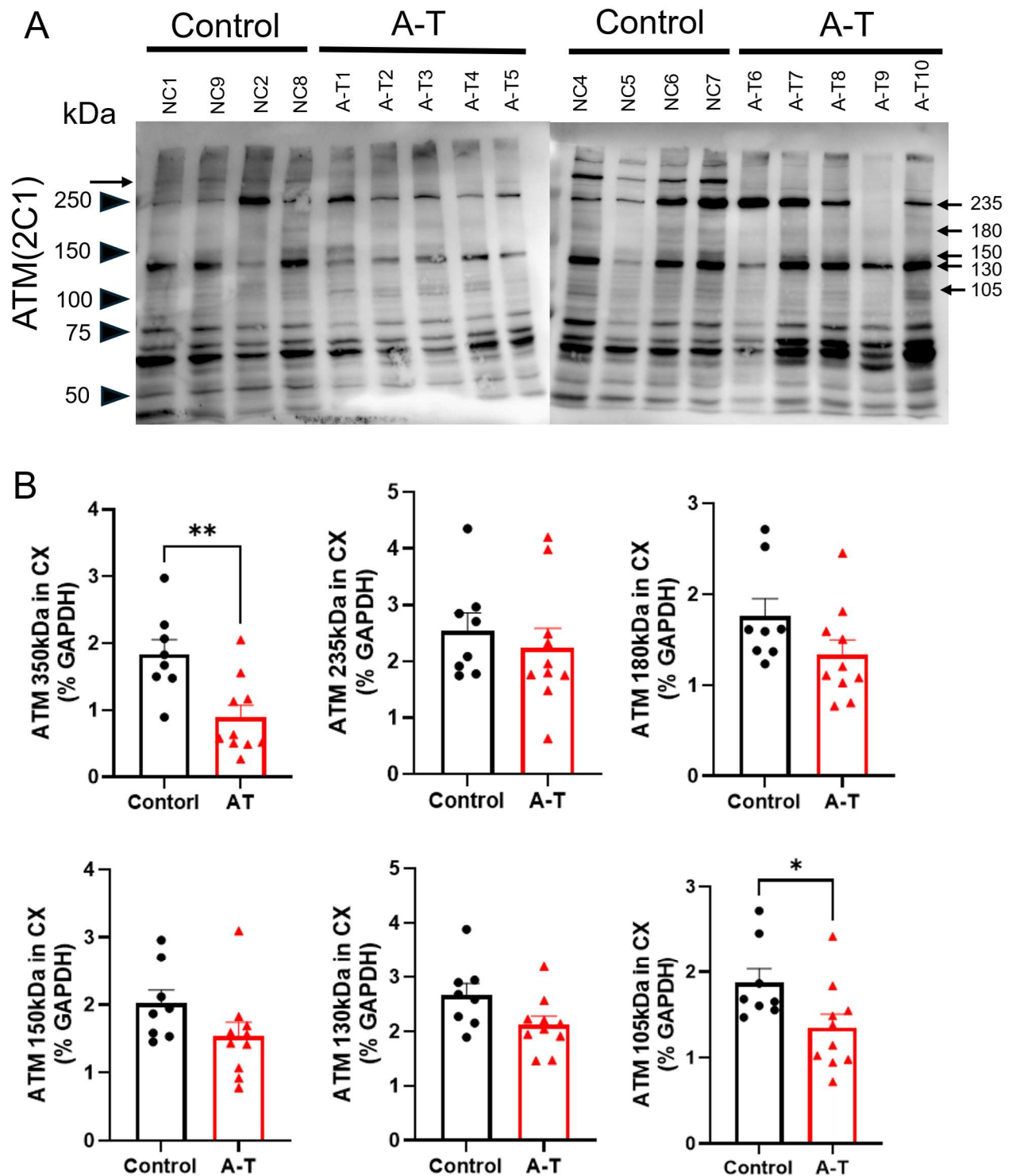


Figure 98. Quantification of different potential ATM protein isoform in A-T cerebral frontal cortex sample.

The immunoblotting image was shown in figure 19 in chapter 9.2 already, this figure extended the analysis on the potential ATM isoforms. The tailed arrows indicate ATM isoforms to be examined in different approximate molecular weights. (Two-tailed unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

In the comparison between control and A-T cerebral cortex samples, full-length ATM protein was experiencing the most significant decline among all ATM isoforms (Figure 98). As described in the previous chapter, full-length ATM protein expression was decreased by 51.2% ($p = 0.004$). However, only ATM isoform at 105 kDa was significantly decreased by 27.8% ($p = 0.0361$).

The difference in the protein isoform comparison of cerebellum and cerebral cortex samples was consistent with transcript-level analysis in RNA sequencing data. Both cerebellum and cerebral frontal cortex sample in A-T experienced minimum decline in transcription of full-length *ATM* transcript but significant decrease in full-length ATM protein expression. However, expression of *ATM* transcript variants and ATM protein isoforms were exclusively decline in cerebellum samples.

Correlation of ATM expression with pathological finding in different regions

There were large variations in histological findings, protein expression and gene expression among A-T patients. In theory, the loss of *ATM* gene and protein expression negatively correlated with the cell survival rate in histological findings. Here we demonstrated the correlation between *ATM* gene expression, protein expression and the histopathological investigation results in cerebellum and cerebral cortex of A-T patients.

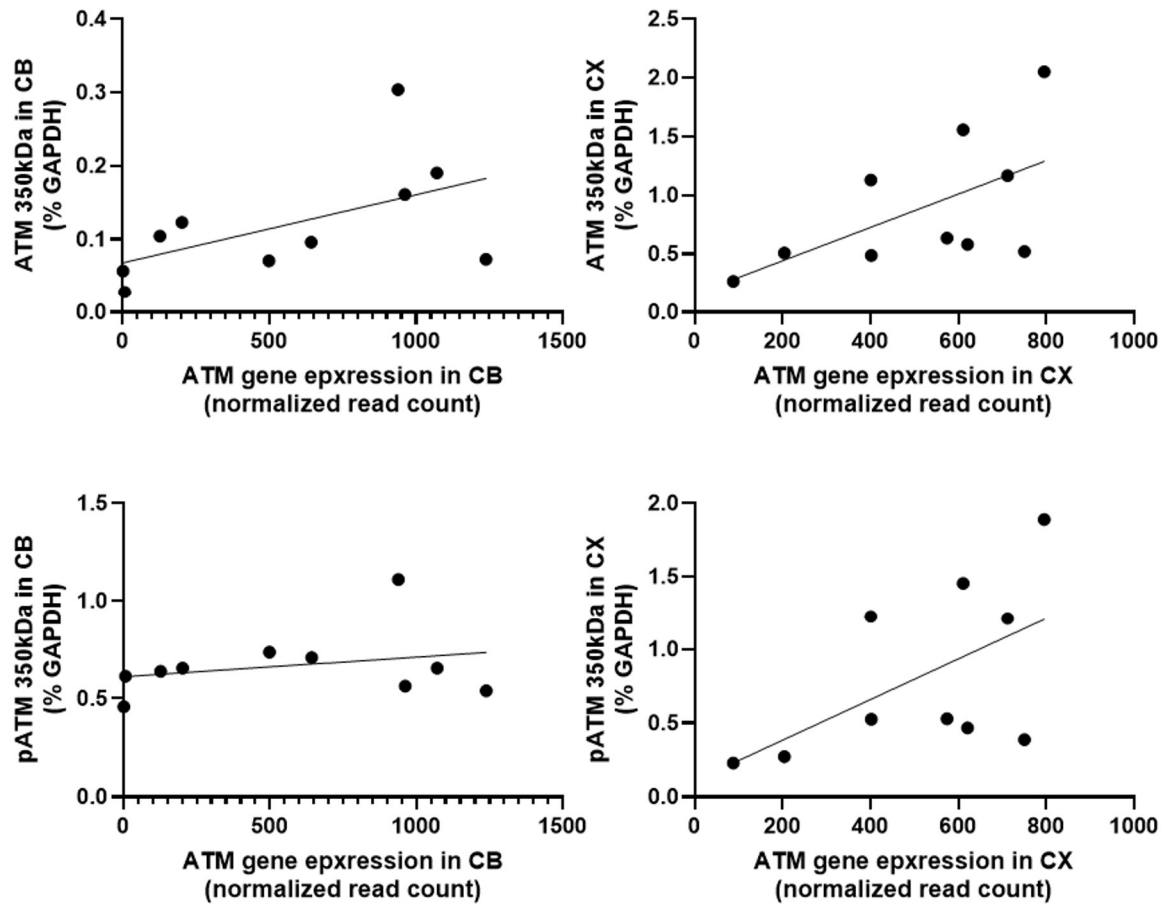


Figure 99. Correlation plotting between *ATM* gene expression and *ATM* protein expression or *ATM* protein activity in different brain regions.

(Two-tailed Pearson correlation; confidence interval: 95%; * $p=0.0332$)

Firstly, *ATM* gene expression from transcriptomic sequencing was plotted with *ATM* protein expression and pATM expression, representing *ATM* activity in A-T cerebellum and cerebral cortex. There were no significant correlations between *ATM* gene expression and *ATM* protein expression or *ATM* activity (Figure 99). However, the simple linear regression model simulations between *ATM* gene expression and *ATM* protein expression or *ATM* activity in cerebral cortex were showing larger inclination than cerebellum (Figure 99).

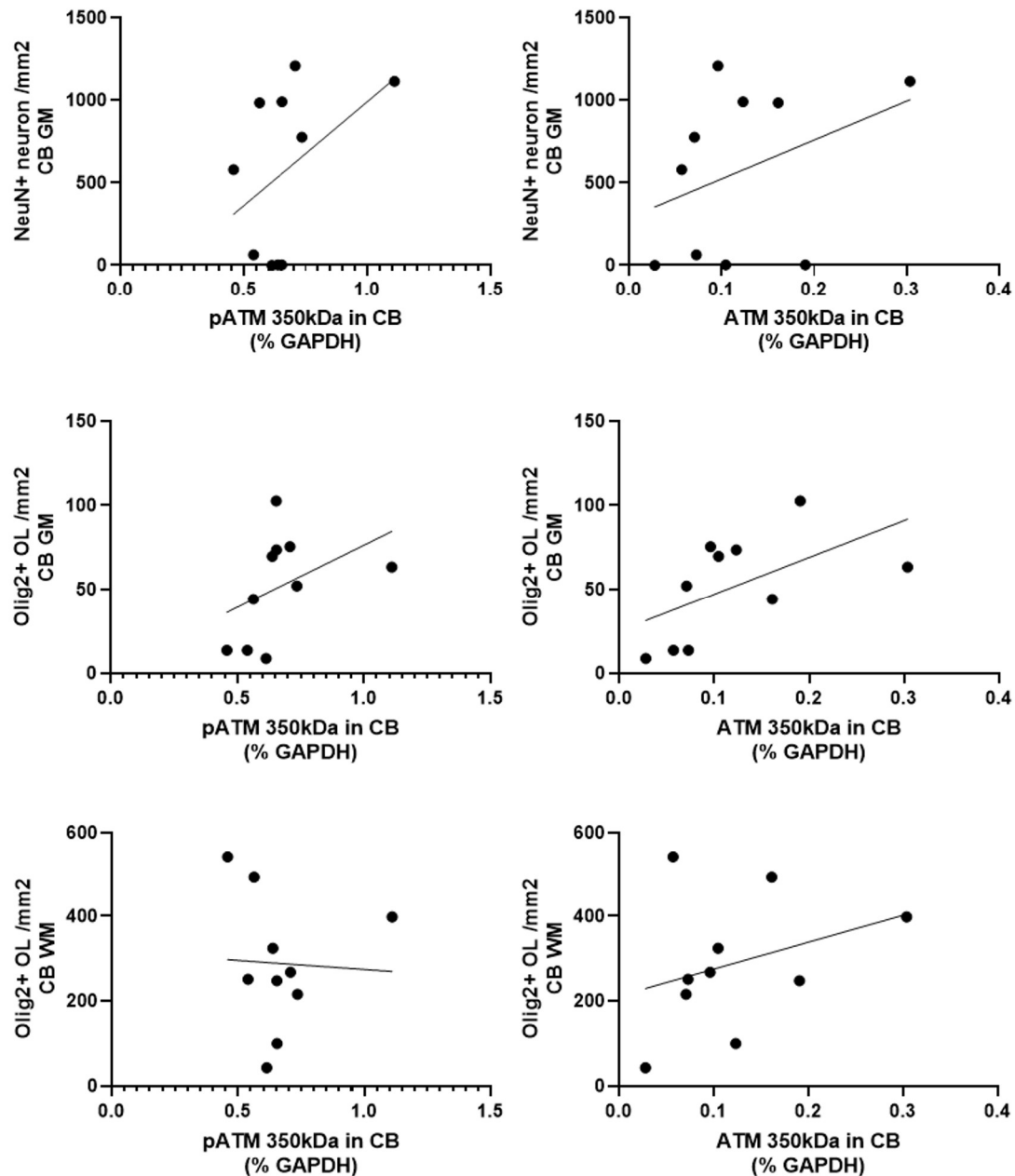


Figure 100. Correlation plotting between histological findings and ATM protein expression or ATM protein activity in cerebellum.

(Two-tailed Pearson correlation; confidence interval: 95%; *p=0.0332)

After that, histopathological findings in cerebellum were plotted against the ATM protein expression and ATM activity (Figure 100). There were no significant correlations between the cell density of NeuN+ neuron and Olig+ OL in cerebellar grey and white matter and the ATM protein expression and ATM activity in cerebellum (Figure 100).

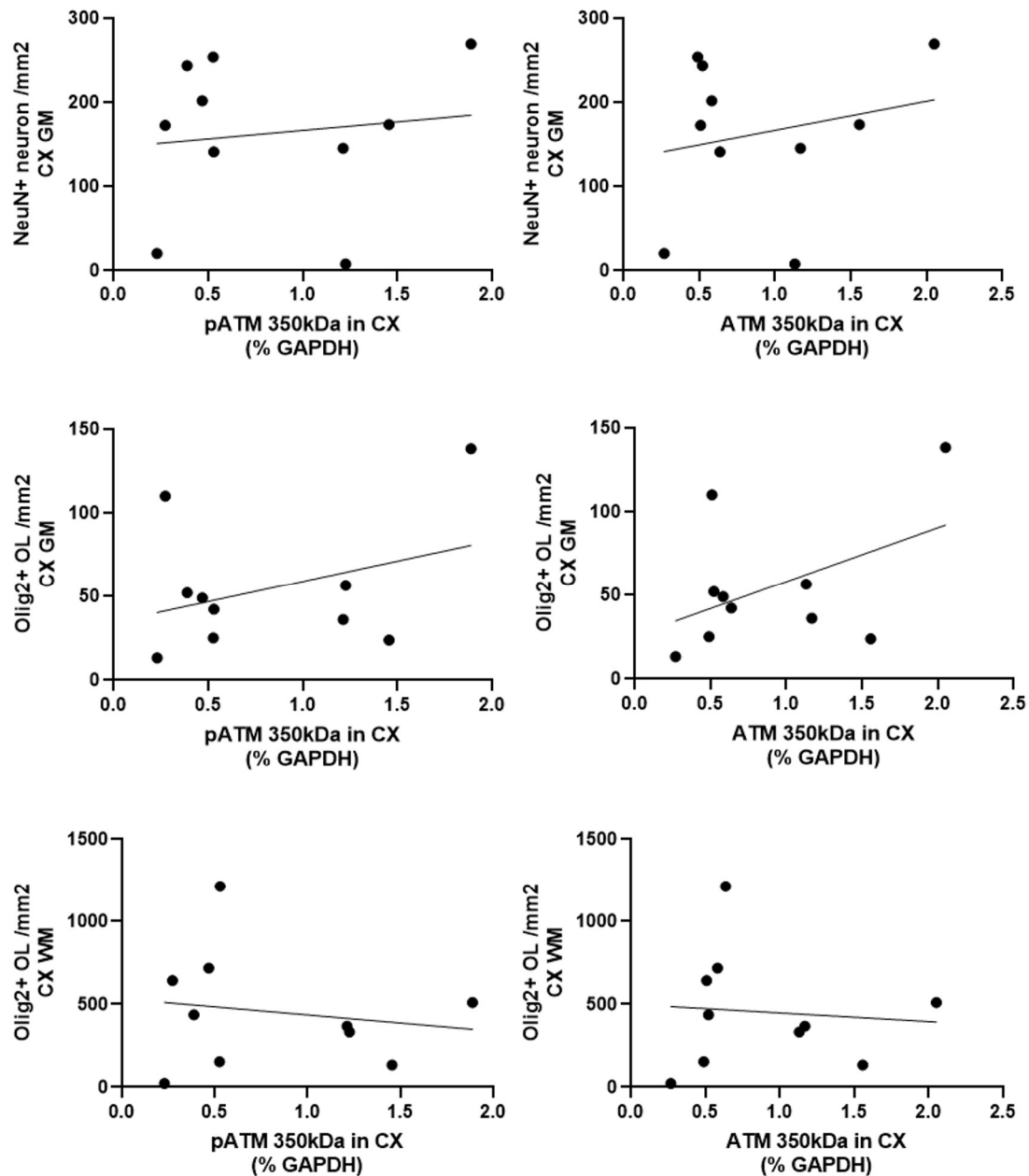


Figure 101. Correlation plotting between histological findings and ATM protein expression or ATM protein activity in cerebral cortex.

(Two-tailed Pearson correlation; confidence interval: 95%; *p =0.0332)

Moreover, histopathological findings in cerebral cortex were plotted against the ATM protein expression and ATM activity. However, there was no significant correlation found among the pairing of data sets (Figure 101). Compared to the correlation plotting of cerebellum tissue, cerebral cortex demonstrated less potential of correlation with less inclination in simulated linear regression model (Figure 101).

Effect of ATM mutation on pathological findings

NP_000042.3 12-NOV-2024

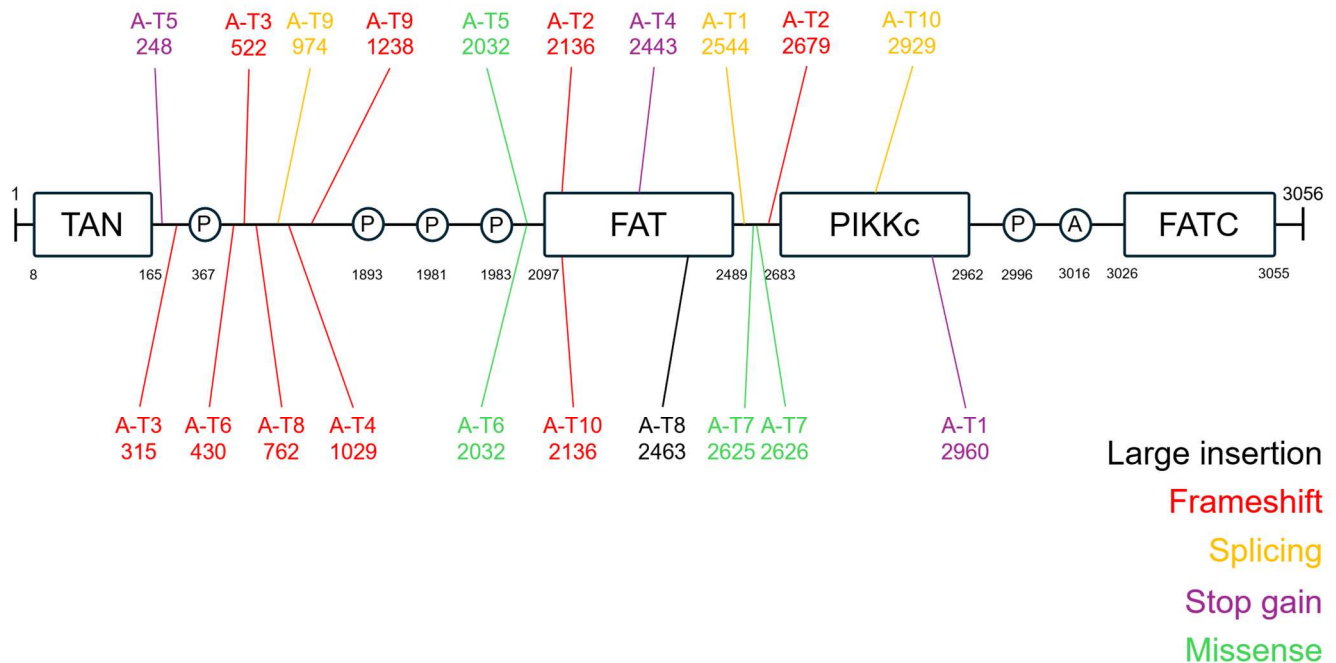


Figure 102. Pathogenic mutation mapping on ATM protein sequence.

TAN: Tel1/ATM N-terminal motif, for telomere-length maintenance and DNA damage repair; FAT: FRAP, ATM and TRRAP domain; PIKKc: phosphoinositide-3-kinase-related protein kinases catalytic domain of ATM; FATC: FRAP, ATM and TRRAP domain at C-terminal to the kinase domain; P: phosphorylation site; A: acetylation site

To determine the severity level of each pathogenic mutation on ATM protein integrity, each pathogenic mutation detected in A-T patients were mapped onto the ATM peptide sequence (NP_000042.3 [conserved regions], 12/11/2024 last edited) (Figure 102). Types of pathogenic mutation were identified with different colour on the figure. Frameshift mutation was the most frequent type of mutation discovered from A-T patients, while A-T2, A-T3, A-T4, A-T6, A-T8, A-T9 and A-T10 had at least one frameshift mutation (Figure 102). Then, they were queued by the mutation site on the ATM peptide sequence. The severity of the mutation was ranked by the earliness of the mutation location on ATM peptide. The earlier the mutation occurred in the sequence, the smaller the portion of the original ATM peptide that remained intact.

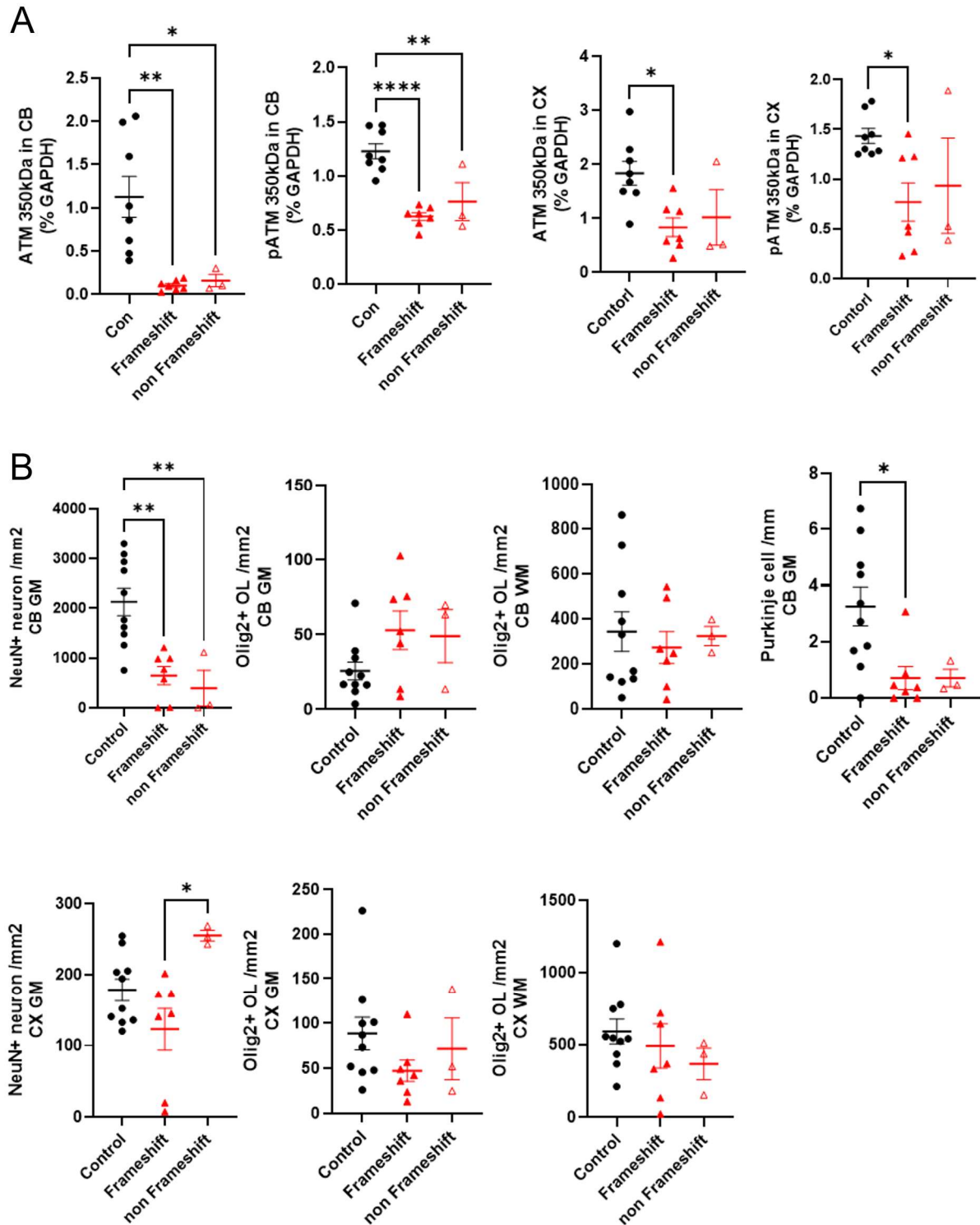


Figure 103. Differential effect of frameshift mutation on histological findings.

A) Residue ATM protein expression and activity of frameshift mutation group in cerebellum and cerebral cortex. B) Immunohistochemistry results of frameshift mutation group in cerebellum and cerebral cortex. (One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Firstly, A-T patients without pathogenic frameshift mutation on *ATM* were grouped as “non Frameshift”, remaining A-T patients with at least one frameshift mutation were identified as “Frameshift” group.

Nearly complete loss of ATM protein expression in cerebellum was found in both “Frameshift” and “non Frameshift” groups. Yet, significant decline of *ATM* expression in cerebral cortex (54.5%, $p = 0.0162$) was only found in “Frameshift” group (Figure 103A). More significant reduction of ATM activity in cerebellum was found in “Frameshift” group (48.9%, $p < 0.0001$) than that in “non Frameshift” group (37.9%, $p = 0.0052$) (Figure 103A). For ATM activity in cerebral cortex, significant reduction was only found in “Frameshift” group (46.3%, $p = 0.0369$) (Figure 103A).

Histological findings on cerebellum and cerebral cortex were compared among control, “Frameshift” and “non Frameshift” groups. Significant decline was found in NeuN⁺ neuron in cerebellum grey matter from both “Frameshift” and “non Frameshift” groups (Figure 103B). Purkinje cell number in cerebellum grey matter was only significantly reduced in A-T patients with pathogenic frameshift mutations (Figure 103B). Interestingly, significantly higher density of NeuN⁺ neuron was found in cerebral cortex of “non Frameshift” group samples comparing to “Frameshift” group (Figure 103B).

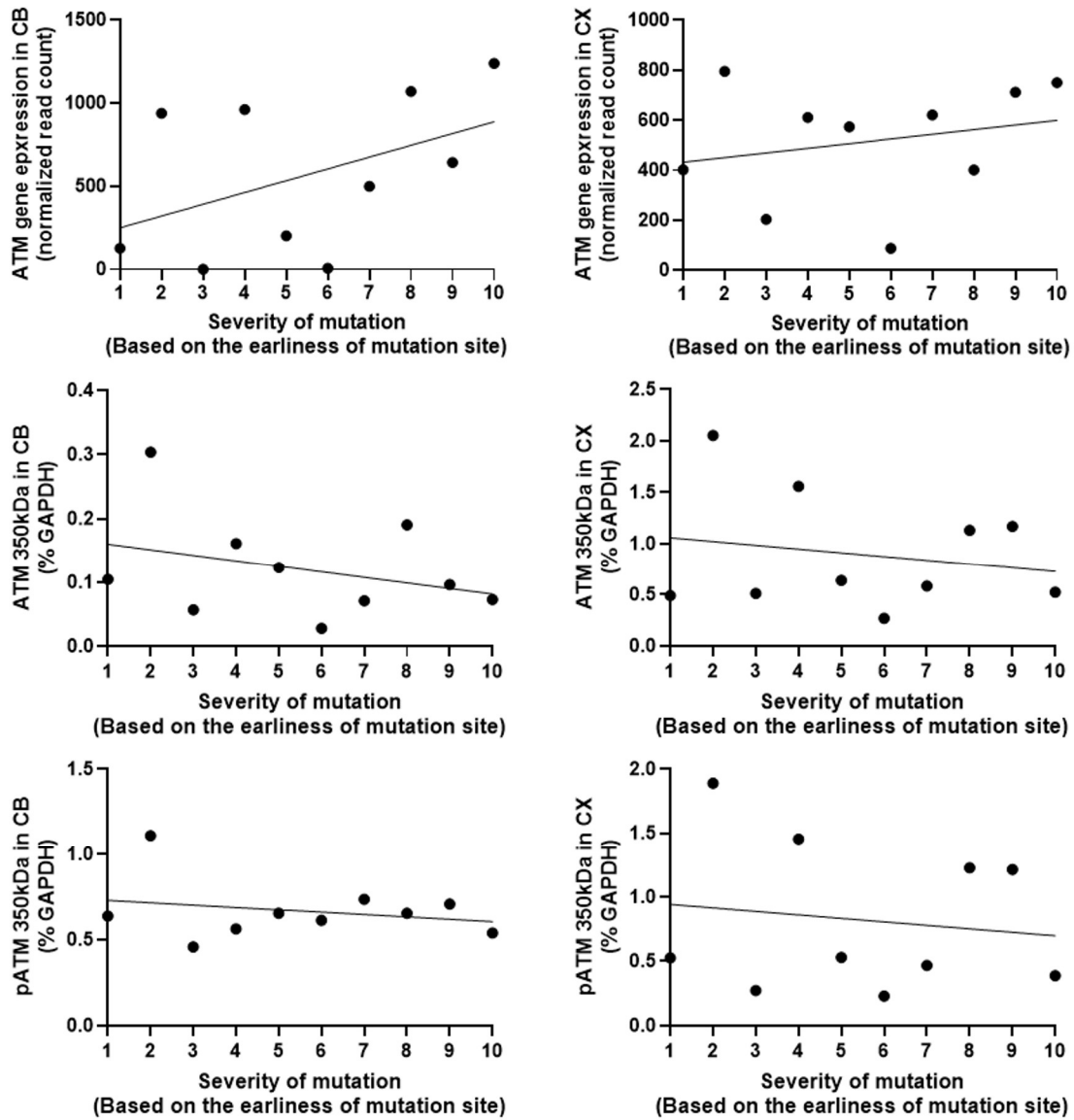


Figure 104. Correlation plotting between mutation severity level of A-T patients and ATM gene or protein expression.

(Two-tailed Pearson correlation; confidence interval: 95%; * $p=0.0332$)

Further the investigation on differential effect of *ATM* mutations, severity of mutations was classified according to their earliness of position occurred on ATM peptide. The estimated mutation-site-severity of each A-T patient was ranked as follow, from the most severe to the least severe (from 10 to 1), A-T5, A-T3, A-T6, A-T8, A-T9, A-T4, A-T2, A-T10, A-T1 and A-T7. Although all of the correlation tests did not reach significance, the result revealed an opposite trend between *ATM* gene and ATM protein expression or its activity (Figure 104).

There was a slight increasing trend between expression of *ATM* gene and the severity of mutations. However, the protein expression and activity were rather showing no changes or slightly negative trend in the comparisons (Figure 104).

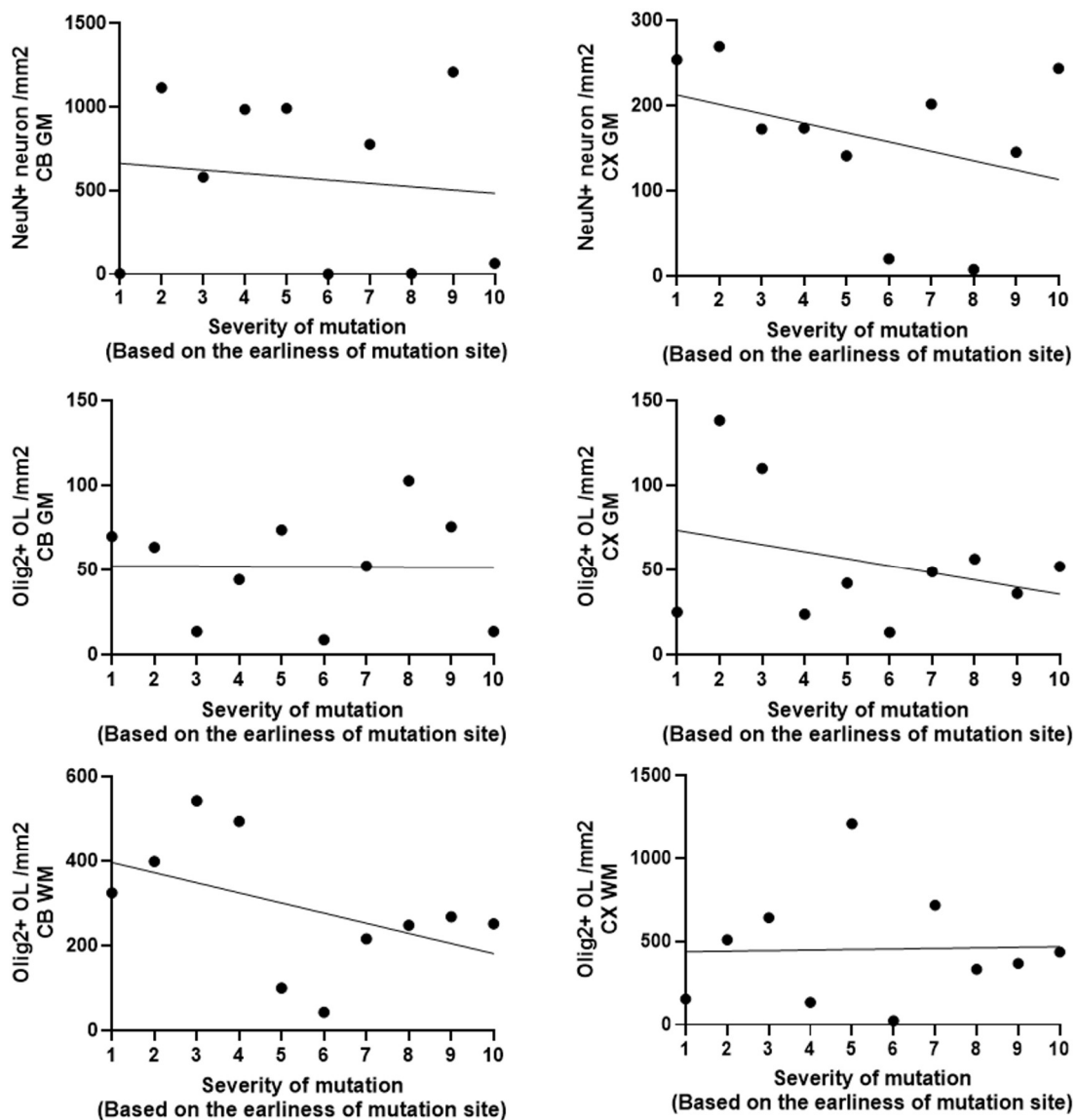


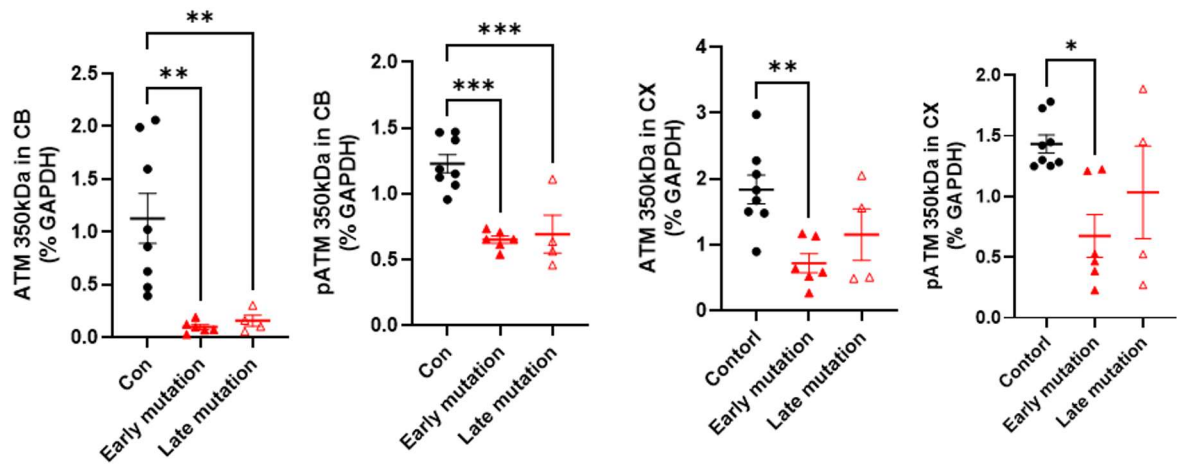
Figure 105. Correlation plotting between mutation severity level of A-T patients and histological findings.

(Two-tailed Pearson correlation; confidence interval: 95%; *p = 0.0332)

There were slightly reducing trend in NeuN+ neuron cell count with increasing severity of mutation in both cerebellum and cerebral cortex (Figure 105). Interestingly, slightly reducing

trend in Olig2⁺ OL cell count was only found with increasing severity of mutation in grey matter of cerebral cortex and white matter of cerebellum (Figure 105). For the Olig2⁺ OL cell count in grey matter of cerebellum and white matter of cerebral cortex, there were not even an incline or decline trend found in the comparisons (Figure 105).

A



B

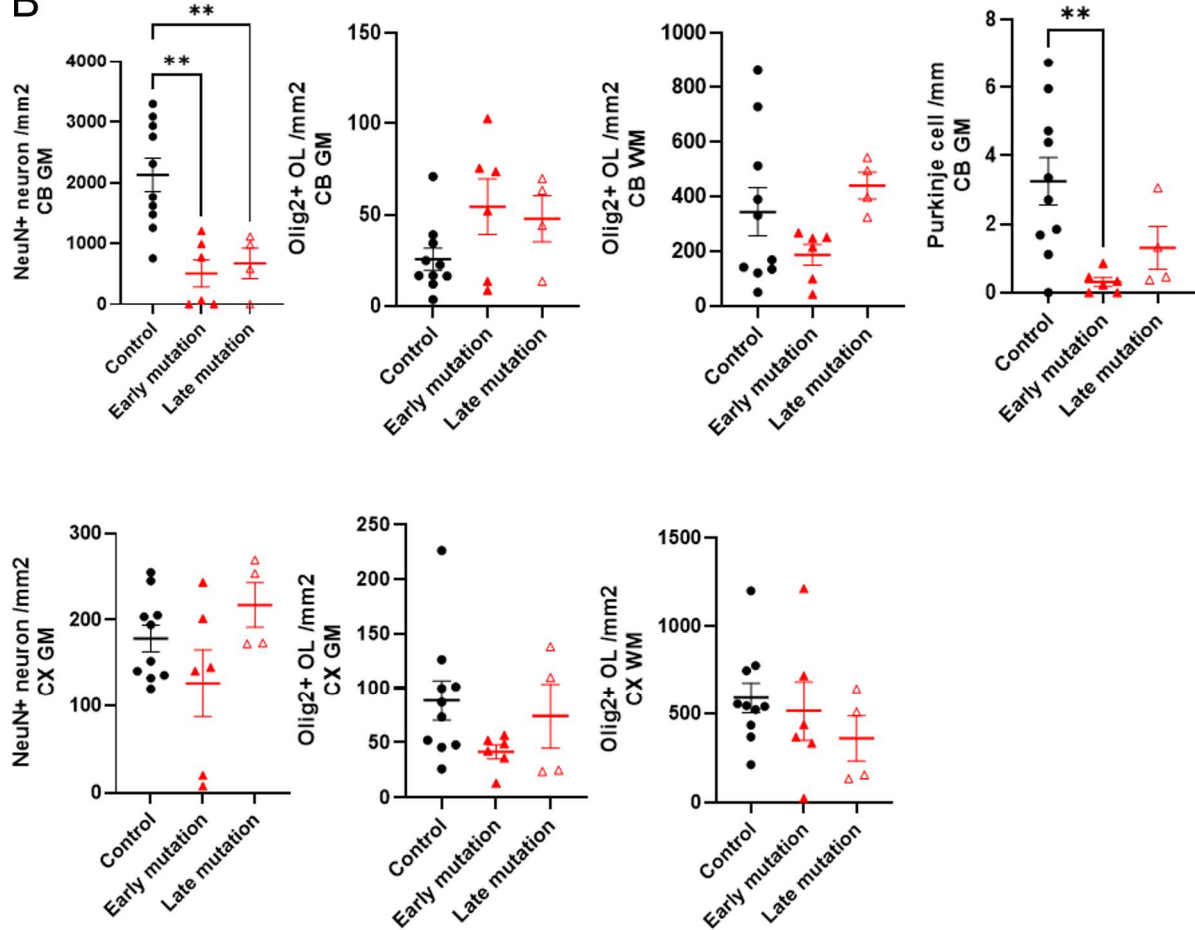


Figure 106. Differential effect of mutation position on histological findings.

A) Residue ATM protein expression and activity of early and late mutation group in cerebellum and cerebral cortex. B) Immunohistochemistry results of early and late mutation group in cerebellum and cerebral cortex. (One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

For better visualization of the differential effect on histological findings, A-T patients were grouped into “early mutation” and “late mutation”. Patient with mutations located in spiral and pincer region of ATM protein, representing aa 1-1890 in Figure 3, were considered “early mutation”. Otherwise, patients with mutations located in FATKIN region of ATM, representing aa 1891-3056 in Figure 3, were considered “late mutation”. By this definition, A-T3, A-T4, A-T5, A-T6, A-T8 and A-T9 were grouped as “early mutation”, while A-T1, A-T2, A-T7 and A-T10 were grouped as “late mutation”.

Nearly complete loss of ATM protein expression in cerebellum was found in both “early mutation” and “late mutation” groups. Yet, significant decline of *ATM* expression in cerebral cortex (60.9%, $p = 0.008$) was only found in “early mutation” group (Figure 106A). Significant reduction of ATM activity in cerebellum was found in both “early mutation” group (47.0%, $p = 0.0001$) and “late mutation” group (43.6%, $p = 0.0009$) (Figure 106A). For ATM activity in cerebral cortex, significant reduction was only found in “early mutation” group (52.9%, $p = 0.0173$) (Figure 106A).

Significant decline was found in NeuN⁺ neuron count of cerebellum grey matter in both “early mutation” (76.1%, $p = 0.0014$) and “late mutation” groups (68.5%, $p = 0.0097$) (Figure 106B). Purkinje cell number in cerebellum grey matter was also significantly reduced in “early mutation” group (90.4%, $p = 0.0092$) (Figure 106B).

Conclusion

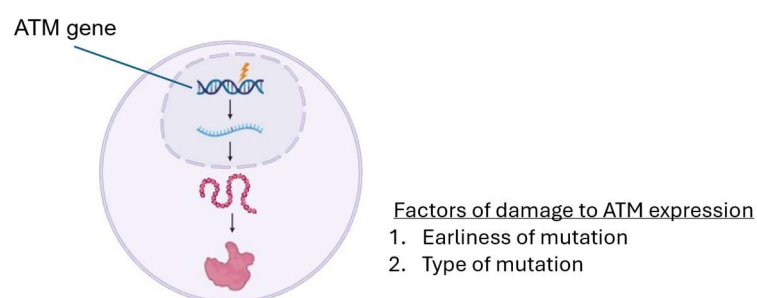


Figure 107. Conclusion for chapter 3: Factors affecting the transcription and translation of ATM

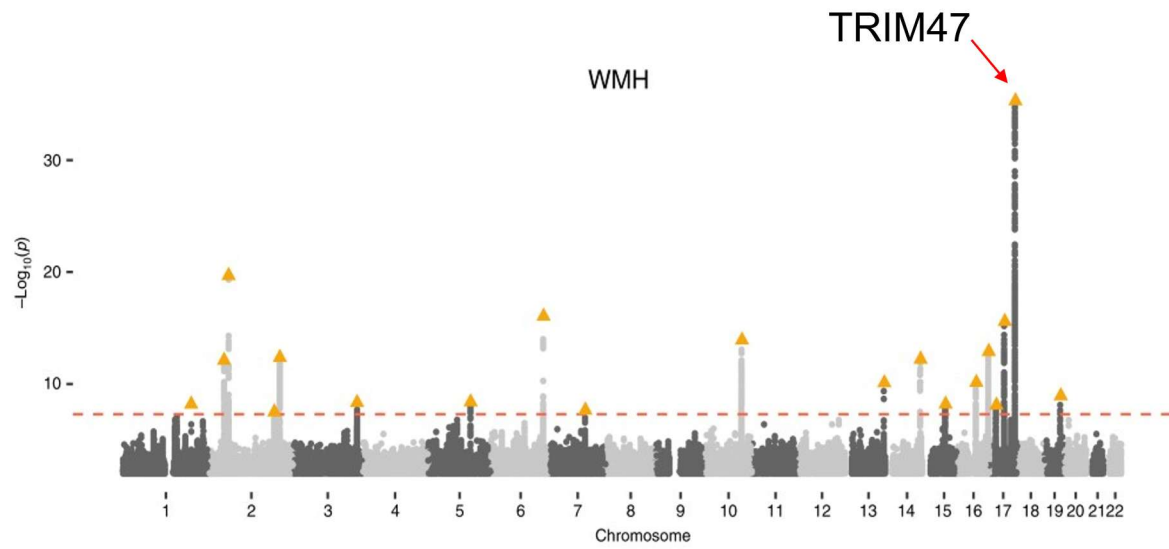
Chapter 4. The potential effect of ATM mutation on endothelial cell autophagy

Summary

Telangiectasia is a poorly studied pathological changes in A-T. As ATM regulates autophagy which in turn impacts on vascular endothelium, we hypothesize that *ATM* mutations in A-T patients may impact on TRIM family especially in white matter hyperintensity (WMH) formation. White matter hyperintensity (WMH) is one of the clinical manifestations of A-T patients that occurred in cerebral cortex. Age-related dementia and hypertension are strongly linked to white matter hyperintensity (WMH), though its underlying mechanisms remain unclear. Genome-wide association studies have identified *TRIM47* at the 17q25 locus as a primary genetic risk factor for WMH development. The TRIM family, a group of E3 ubiquitin ligases, plays crucial roles in autophagy, which is essential for brain endothelial cell (EC) remodeling during hypertension.

Central hypothesis

TRIM47 gene was identified by GWAS as a top genetic risk factor for WMH formation (Persyn et al., 2020), which is selectively expressed by brain endothelial cells in human and mouse. We propose that *TRIM47* modulates autophagy and that its dysfunction disrupts the cerebrovasculature (Figure 108). Therefore, we aimed to investigate the effect of *TRIM47* on endothelial cells and subsequent cerebrovascular pathology of A-T.



Adapted from Persyn, E. *et al*, 2020, Nat. Commun.

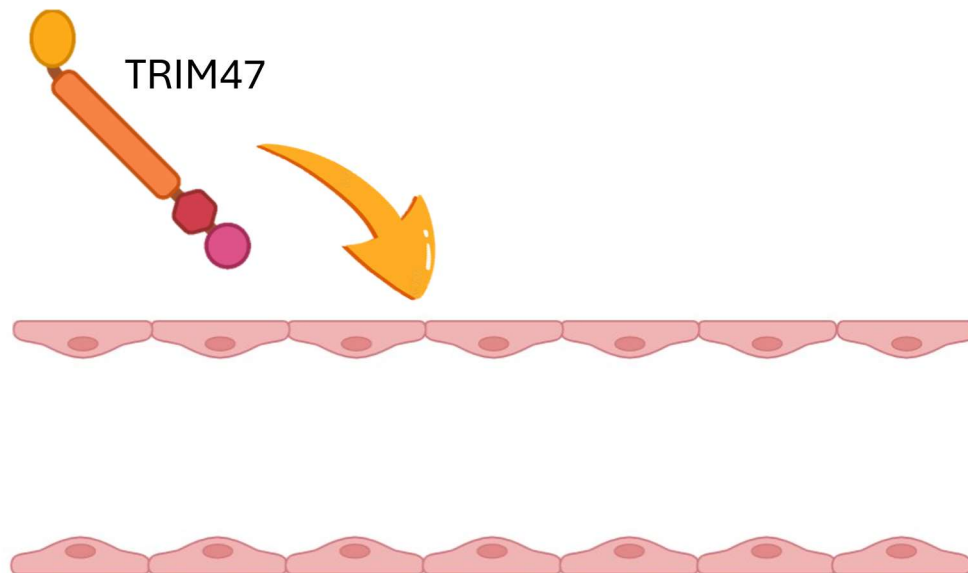


Figure 108. Central hypothesis 4: GWAS-identified WMH risk factor, TRIM47 modulates autophagic function in endothelial cells and its disfunction disrupts the cerebrovasculature

TRIM47 expression upon ATM deficiency

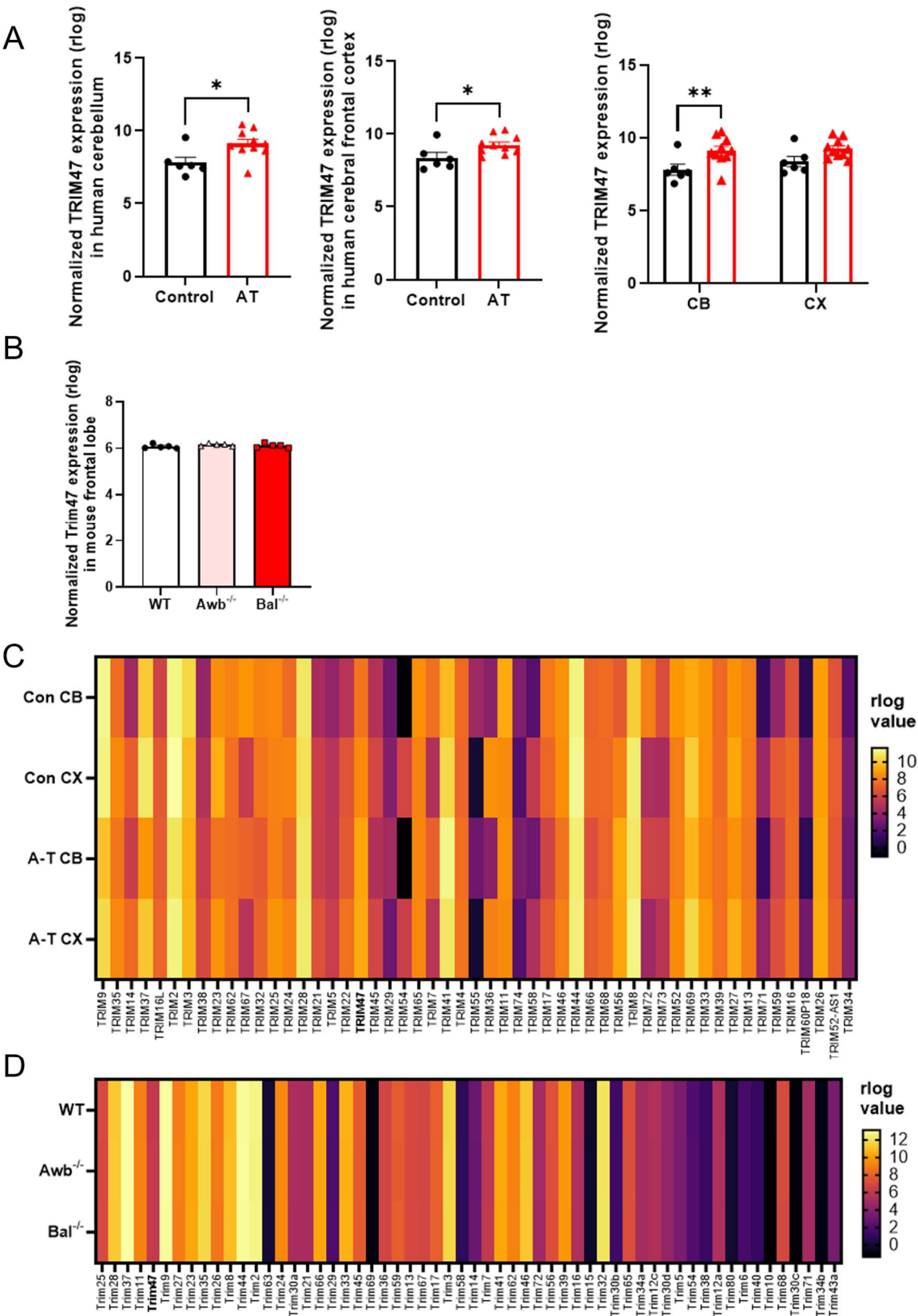


Figure 109. *TRIM47* expression upon ATM deficiency in transcriptomic sequencing

A) Quantification of *TRIM47* expression in A-T cerebellum and cerebral frontal cortex. (unpaired t-test; * $p < 0.05$, ** $p < 0.01$) Cross comparison of *TRIM47* expression among cerebellum and cerebral frontal cortex in control and A-T specimens. (Two-way ANOVA with Fisher's LSD multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) B) Quantification of *TRIM47* expression in frontal lobe transcriptome of ATM deficient mice. (One-way ANOVA with Tukey's multiple comparison test; * $p < 0.05$) C) Expression heatmap of TRIM family member with rlog value among cerebellum and cerebral frontal cortex in control and A-T specimens. D) Expression heatmap of Trim family member with rlog value among ATM deficiency mice.

Significant increase of *TRIM47* expression was found in A-T cerebellum (16.7%, $p = 0.0176$) and cerebral frontal cortex (10.4%, $p = 0.0366$) (Figure 109A). The significance of *TRIM47* expression elevation in A-T cerebellum sustained in the two-way ANOVA test of cross comparison of *TRIM47* expression among cerebellum and cerebral frontal cortex in control and A-T specimens ($p = 0.0056$) (Figure 109B).

To study the effect of ATM deficiency on *Trim47* expression, the RNA sequencing data of ATM deficient mice was examined again for the *Trim47* expression (Figure 109C). However, there was no observable change among wild type and ATM deficient mice.

TRIM47 expression in bEnd3 cell upon pharmaceutical regulation of autophagy

To further the investigation on the autophagic role of *TRIM47*, a mouse brain derived endothelial cell line, bEnd3, was employed. Autophagy was regulated in bEnd3 cell line using pharmaceutical approach for 24 hours. 3-Methyladenine (3-MA) was applied for suppression of autophagy while rapamycin (Rapa) was treated for promoting autophagy.

Quantification of TRIM47 expressing cell and LC3B-TRIM47 co-expressing cell. (One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

TRIM47 positive cell was slightly increased by 15.3% verse control under autophagy inhibition by 3-MA (Figure 110). Meanwhile, TRIM47 positive cell was reduced by 36.1% ($p < 0.05$) under autophagy activation by Rapa (Figure 110). LC3B and TRIM47 co-positive cell was also reduced by 48% ($p < 0.05$) (Figure 110).

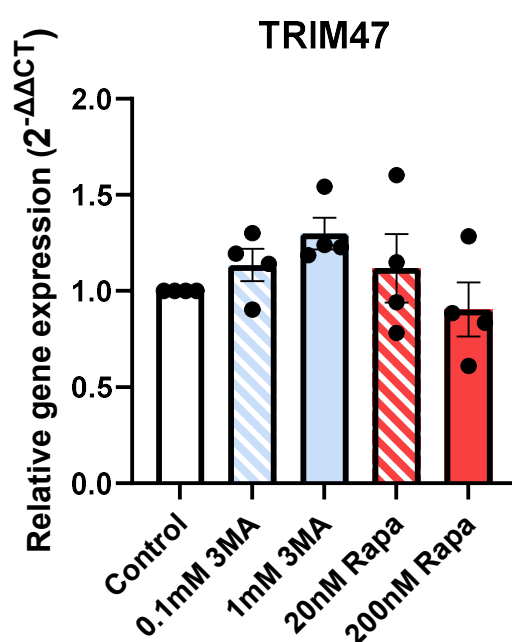


Figure 111. Quantification of TRIM47 gene expression in bEnd3 cell upon pharmaceutical regulation of autophagy.

(One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Trim47 gene expression upon pharmaceutical regulation of autophagy was further examined through quantitative PCR. High concentration 3-MA treatment (1mM) slightly increased *Trim47* expression by 30% (Figure 111). High concentration Rapa treatment (200nM) slightly decreased *Trim47* expression by 10% (Figure 111). However, the change in gene expression did not reach significance in p-value.

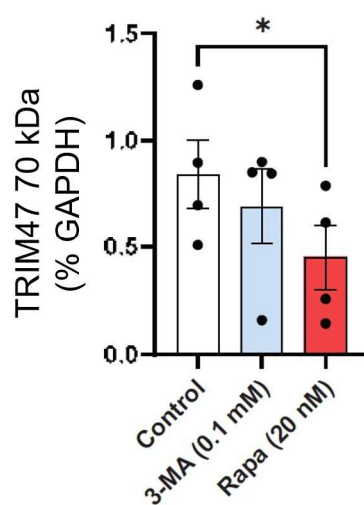
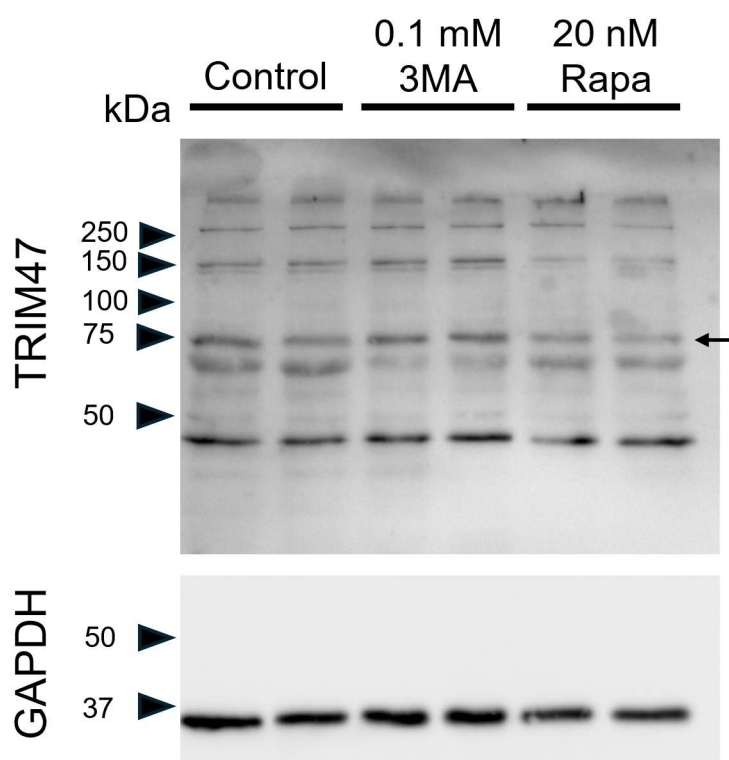


Figure 112. Quantification of TRIM47 protein expression in bEnd3 cell upon pharmaceutical regulation of autophagy.

Tailed arrow indicated targeted TRIM47 protein size at 70 kDa (One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

To extend the examination on ATM protein expression, western blotting experiment was performed on bEnd3 cell lysate upon pharmaceutical regulation of autophagy. There was around 47% decline ($p < 0.05$) in TRIM47 monomer protein expression level with Rapa treatment (20nM) (Figure 112).

As we have published recently, TRIM47 loss-of-function was found disturbing the cerebrovasculature (Yeung et al., 2024). Under ATM deficiency, there were also cerebrovasculature manifestation in A-T patients. Therefore, we decided to delineate the potential role of TRIM47 expression under ATM deficiency.

Conclusion

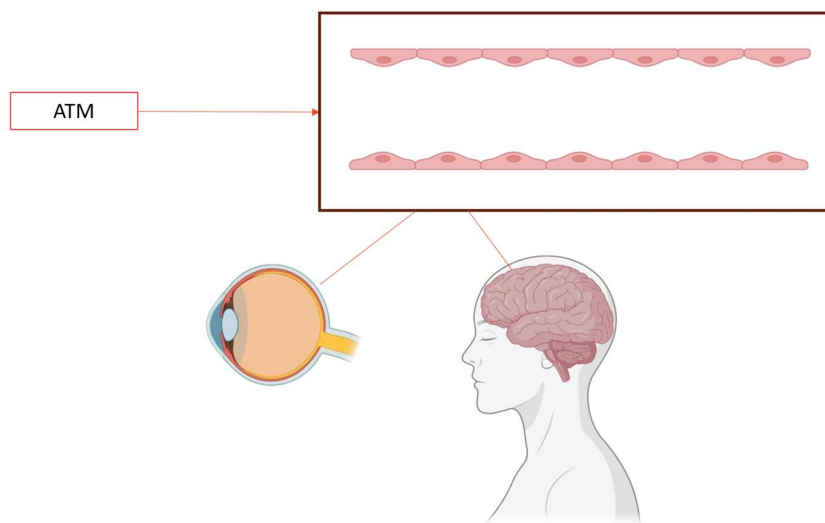


Figure 113. Conclusion for chapter 4: ATM potentially regulates TRIM47, an autophagic regulator for brain endothelial cell

10. Discussion

Chapter 1. ATM protein expression upon pharmacological inhibition and DSB induction in oligodendrocyte

The effect of ATM deficiency on cell cycle progression in OL lineage

The suppression of cyclin D1 and cyclin A2 was both found in primary OPC culture upon pharmacological ATM inhibition (Figure 9), reflecting the ATM-mediated G₁ phase entry and G₁ to S phase transition. Such dysregulation of cell cycles that OPCs barely proliferated upon ATM inhibition. As ATM regulates many cell cycle checkpoints, including p53 and chk2 (Westphal, 1997), this result is expected, but it is shown for the first time using pharmacological approach. The reason we performed immunocytochemistry instead of flow cytometry to study cell cycle progression was that CCD1 and CCA2 were not cell surface marker, which made them not ideal for performing flow cytometry. The advantage of using immunocytochemistry is that we could visualize nuclear localisation of CCD1 and CCA2 expression in OL lineage.

The rapid DSB formation in proliferating cell line arrests its cell cycle progression and potentially leading cancerous cell into a quiescent state (Ambrosio et al., 2016). The inhibition of G₁ phase and S phase entry upon DSB induction was potentially promoting OPC quiescence. The OPC cell line, Oli-Neu, showed a more resilient reaction to ATM inhibition compared to primary OPC culture (Figure 9). Pharmacological ATM inhibition might promote radiosensitivity of glioblastoma (Durant et al., 2018), which compensates for the inhibition of ATM. However, DSB induction inhibited its proliferation by suppressing cyclin D1 expression (Figure 9). Indeed, glioblastoma cell entered G₀ cell cycle arrest upon DSB lesion induction, allowing pro-survival mechanism to repair its DNA (Frosina et al., 2019).

The effect of ATM deficiency on cell cycle progression of OL lineage is highly associated with DSB repair mechanism, and we demonstrated here that a direct causal relationship between ATM inhibition and pre-myelinating OL cell cycle dysregulation exists, and it may implicate in the A-T brain pathology.

Ectopic cell cycle protein re-expression in myelinating OL and the associated cell death

In previous research, a high level of G₁ phase protein expression in OL population with cell cycle entry was often associated with the lack of OL differentiation (Casaccia-Bonnet & Liu, 2003). Like mature neuron, myelinating OL remains in postmitotic stage after exiting cell cycle (Calver et al., 1998). Cell cycle re-entry found in postmitotic neuron cell was often related to cell death (Herrup & Yang, 2007). The increasing percentage of co-expression between MBP and cyclin D1 protein upon DSB induction revealed the re-entry of G₁ phase in mature OL (Figure 10). The result might suggest that the cell cycle re-entry is associated cell death of mOL upon DSB induction.

It was reported that the dedifferentiation of mature OL was accounted for re-expression of cell cycle marker upon growth factor treatment, followed by cell death (Grinspan et al., 1996). In our study, growth factor treatment activated re-expression of both cyclin D1 and cyclin A2 cell cycle marker, but significantly less cyclin A2 expression was found upon DSB induction (Figure 10). Dedifferentiation of mOL might therefore account for the re-expression of protein upon growth factor activation. However, as mOL arrested in G₁ phase without entering S phase upon DSB induction, this process is unlikely to be dedifferentiation. Indeed, cyclin D1 dependent apoptosis due to elevated cyclin D1 level could also be found in non-neuronal cell type (Kranenburg et al., 1996). Elevation of cyclin D1 in mOL resulted by arrested G₁ phase entry might potentially lead to cyclin D1 dependent apoptosis.

Ectopic cell cycle-associated cell death in aging and neurodegenerative disease

A significant proportion of OPCs remain in an undifferentiated and cycling state throughout adulthood, which act as reservoir for remyelination (Franklin & Ffrench-Constant, 2008). As ATM plays a crucial role in initiating cell cycle checkpoints following DNA damage, its inhibition might result in OPCs proliferation with unrepaired DNA damage. Dysregulated OPC cell cycle under ATM inhibition and DSB induction might prohibit proliferation of OPC with significant DNA damage. The potential ability of remyelination through OPC proliferation might also be diminished, which might eventually halt myelin repairment upon demyelination. Dysregulation of OPC cell cycle led to defective remyelination in many demyelinating diseases, such as multiple sclerosis (Kremer et al., 2016). Active cell cycle entry of OPC respond to CNS myelination injury, hence result in OPC proliferation and migration towards focal injury site (Hughes et al., 2013). Therefore, ATM deficiency might impose an inhibitory effect on OPC responding to myelin injuries. Meanwhile, cell cycle re-entry of mOL could result in directly associated cell death, which might directly associate with demyelination.

DSB is correlated with age-related functional decline and death (White & Vijg, 2016). While the defective DSB repairment could be attributed to ATM deficiency (Kühne et al., 2004), DSB induced cell cycle dysregulation in OL lineage might be the pathological pathway leading to the aging of brain. Myelination serves as the insulator for neuron axons and supports their survival. Demyelination resulted from mOL death and prohibited remyelination might eventually lead to neuronal degeneration.

Summary

In summary, OL is vulnerable to DSB induction and ATM inhibition throughout its lineage as OPC and mature OL. Accumulated DNA damage in OL lineage might disrupt its cell cycle regulation and lead to irreversible apoptotic cell death.

Chapter 2. ATM regulation on oligodendrocyte differentiation

Neuropathology of A-T not limited to cerebellum

The significant decline in granule cell and Purkinje cell density in cerebellum are the hallmarks of A-T disease (Bottini et al., 2012). We also performed immunohistology study of GFAP positive astrocyte on *Atm* deficient mice in our pre-print, which demonstrated increased astrocyte activation in cerebellar grey matter (Tse et al., 2021). Therefore, pathological changes in other brain regions are often underestimated in clinical studies. This study focused on the parallel comparison of the pathological changes in both cerebellum and cerebral cortex.

According to our immunohistology study, the significant loss of Purkinje cells and granule cells were found in A-T cerebellar grey matter (Figure 14). Moreover, pATM positive granule cells in A-T cerebellar grey matter was significantly reduced (Figure 14). There was no significant neuronal loss in A-T cerebral cortex region but there was a declining trend of pATM positive neuron (Figure 16). Decreased pATM and ATM expression was confirmed in both A-T cerebellar tissue and cerebral tissue with immunoblotting experiment (Figure 18, Figure 20). According to our targeted RNA sequencing result, the synaptic pathways were enriched with downregulated DEGs in both A-T cerebellum and cerebral cortex (Figure 24, Figure 28). Synaptic activity is dependent on neuronal function in the CNS. These findings demonstrated

a potential association between loss of ATM function in neuron and decline in CNS synaptic function. Recent study revealed that there were up to 40 % loss of synapse density under *Atm* deficiency (Cheng et al., 2018), which supported the analytic result from our RNA sequencing data.

In terms of myelination, there was slight decrease in *Olig2*⁺ OL in A-T cerebellar white matter, cerebral cortex grey matter and white matter (Figure 15, Figure 16, Figure 17), while the increase of *Olig2*⁺ cell with astrocytic morphology in A-T cerebellar grey matter could be attributed to astrogliosis (Figure 14). Compared to the change in neuron, the OL numbers were relatively stable in the brain of A-T patients.

A-T neuronal pathology was well known as neuronal loss responding to accumulated DNA damage without repairment (McKinnon, 2012). The defence mechanism pathway was enriched by commonly upregulated DEGs in cerebellar and cerebral cortex (Table 4), which might be contributing by the microglial viral response activation under ATM deficiency (Song et al., 2019). Indeed, *ATR* expression was decreased in both cerebellum and cerebral cortex under ATM deficiency (CB: $\log_2 \text{FC} = -0.46$; $p^{\text{adj}} = 2.93 \times 10^{-3}$, FCx: $\log_2 \text{FC} = -0.41$; $p^{\text{adj}} = 0.0313$) (Figure 33, Figure 36). *ATR* was suggested to have a complementary role with ATM in DNA repair mechanisms (Cimprich & Cortez, 2008). However, *ATR* gene expression was significantly reduced under ATM deficiency in this targeted RNA sequencing study. Meanwhile, cell cycle related gene expressions were greatly altered in A-T cerebellum and cerebral cortex (Figure 31, Figure 34), which revealed the cell cycle dysregulation in both regions. The targeted RNA sequencing study was tissue specific, but not cell type specific. Therefore, we could not delineate and compare the level of cell cycle and DSB repairment dysregulation between neuron and OL. However, both neuron and OL were vulnerable to DNA damage. Dysregulation of cell cycle and DSB repairment would effortlessly lead to apoptotic-associated consequence of neuron and OL in cerebellum and cerebral frontal cortex.

Motor function of cerebellum under ATM deficiency

Cerebellar degeneration in A-T is always associated with loss of certain motor function, as ataxia is the clinical symptom meaning impaired coordination of muscle motor activity (Ashizawa & Xia, 2016). According to a retrospective cohort study of 240 A-T patients, over 98% of them demonstrated cerebellar syndrome and 204 of them showed ataxia manifestation (Micol et al., 2011). In the classic study of *Atm* deficient mice (*Awb* mice), motor impairments were successfully identified with rota-rod, open-field, and hind-paw footprint tests (Barlow et al., 1996). However, in this study, mice with *Atm* deficiency did not show decline in motor function with Y-maze behavioural test. In the comparison of the mobile ability between wild type and *Atm* deficient mice, there was no significant difference in terms of their distance travelling, mobile time and average speed of moving (Figure 38). Traditionally, constant speed and accelerating rotarod tests were employed for examining mobility motor function of mice (Keshet et al., 2007). Paw grip endurance was utilized for assessing the grip strength of mice, while ink paw print test and open field activity test were designed to assess the walking pattern and unforced movement of mice (Hutter-Saunders et al., 2012). Motor function of mouse model should be examined with a series of comprehensive testing for drawing conclusions on the cerebellar motor performance.

Cognitive performance of cerebral cortex under ATM deficiency

Reduced A-T brain size was reported in different previous MRI studies (Lin et al., 2014; Volkow et al., 2014), while reduction of both cerebral and cerebellar brain size were reported in *Atm* deficient mice related study (Eilam et al., 1998). However, neither this study nor previous article focused on the actual weight loss of A-T brain or *Atm* deficient mice brain.

Cognitive impairments are found in A-T patients clinically (F. Hoche et al., 2014). Cerebrum, especially prefrontal cortex, is responsible for cognitive function in CNS (Friedman & Robbins, 2022). Therefore, we hypothesized that ATM deficiency affects cerebral cortex and leads to cognitive impairment.

In the targeted RNA sequencing study of A-T cerebellar and cerebral tissue, synaptic function pathways related genes, such as BDNF and SYP, were commonly downregulated DEGs (Figure 25, Figure 29). In the list of significantly enriched pathways from commonly downregulated DEGs, there were several pathways related to synaptic plasticity regulation, including regulation of neuronal synaptic plasticity (GO:0048168) and regulation of synaptic plasticity (GO:0048167) (Table 5). Such decline in synaptic plasticity regulation might reduce the function of corresponding brain regions. For instance, dysregulated synaptic plasticity in cerebellum might lead to motor learning deficit (Hirano, 2013). For cerebral frontal cortex, dysregulated synaptic plasticity could lead to decline in memory and cognitive functions and even psychiatric and neurological disorders, like schizophrenia and Alzheimer's disease (Goto et al., 2010). Indeed, cognition pathway was the third most significant pathway enriched with the downregulated DEGs of A-T cerebral cortex ($p = 0.00215$) (Figure 28). Memory related pathway was also significantly enriched with the downregulated DEGs of A-T cerebral cortex (Figure 28).

In agreement with the results of human targeted RNA sequencing, *Atm* deficient mice also revealed the deficit on cognitive performance in behavioural test, where a significant decline in reference special memory was shown in four-month-old *Bal^{-/-}* mice with complete loss of ATM kinase function (Figure 39). Our findings concur with a recent study which also demonstrated the cognitive defects of homozygous *Atm* knockout mice with Y maze (Focchi et al., 2022). Previous study suggested the association between *Atm* deficiency and dysregulated synaptic function of hippocampal neuron rather than cerebral neuron (Focchi et

al., 2022). In classical theory, cognitive abilities were believed to depend on neuronal activity within the prefrontal cortex (Friedman & Robbins, 2022). Therefore, in this section, we discuss the neuronal cell related effect on cognitive performance. Indeed, no neuronal or synaptic related pathway was enriched with DEGs of complete Atm knock-out mice, $Bal^{-/-}$ mice, in the transcriptomic study of its frontal lobe (Figure 57). However, transcriptomic study revealed that transmission of nerve impulse and neuronal action potential pathways were enriched by unique downregulated DEGs of $Awb^{-/-}$ mice (Figure 66). Previous study demonstrated the potential gain-of-function damage on genomic instability of kinase-dead Atm protein in mice, showing more harm than Atm null mutation (Yamamoto et al., 2012). The result of our transcriptomic study might also demonstrate a larger effect on neuronal function in cerebral cortex with truncated Atm protein in $Awb^{-/-}$ mice.

Recently, an interpretable deep learning model was developed to predict age-related cognitive decline using myelin histopathology (McKenzie et al., 2022), which indicated strong association between myelin pathology and cognitive performance. Moreover, proliferation and differentiation of OL in the prefrontal cortex had shown a strong correlation with working memory performance in vivo (Shimizu et al., 2023). The studies indicated that myelin and OL in prefrontal cortex also play significant role in cognitive performance. The linkage between ATM deficiency and myelination of mOL would be further examined in more detail in the following sections.

Cytoplasmic ATM activity associated with OL and myelin

Cytoplasmic ATM expression in neuron was found to be associated with synaptic function (Li et al., 2009). However, such cytoplasmic ATM function on synapse was reported being not associated with DNA repair mechanisms (Li et al., 2009). Earlier study showed that ATM

protein was predominantly cytoplasmic in Purkinje cell and neurons in mice primary culture (Barlow et al., 2000). However, in our immunohistology study, phosphorylated ATM protein was predominately expressed on the nuclei of Purkinje cells and granule cells in cerebellar tissue (Figure 14B). Predominate nuclei expression of phosphorylated ATM protein was also consistent with neuronal cell in cerebral tissue (Figure 16B). Nuclear ATM function is highly associated with DNA damage repair mechanism (Biton et al., 2006). Indeed, neurons maintaining viability under continuous DNA damage response activation (Fielder et al., 2017), which explained the highly activated ATM within neuronal cell for the DNA damage repair system in both cerebellum and cerebrum. However, cytoplasmic ATM did not involve self-phosphorylation at S1981, which was bind to VAMP2 and Synapsin-1 for modulating synaptic functions (Li et al., 2009).

Interestingly, in the staining pattern of cerebellar and cerebral white matter, phosphorylated ATM expression was found in threads like structure outside of the cell nucleus, which might be the neuronal axons or myelinated fibres (Figure 15B, Figure 17B). Coincident with the immunohistology finding in human cerebral cortex and cerebellar white matter, cytoplasmic phosphorylated Atm was shown in myelinating OL of primary glial culture (Figure 69A). Not only in the cytoplasmic region adjacent to Olig2 positive OL nuclei, but phosphorylated ATM was also co-expressed with MAG (Figure 69A), showing its potential association with myelination. Indeed, cytoplasmic phosphorylated Atm expressing mOL was significantly reduced in $Bal^{-/-}$ glial culture (Figure 69B). The potential association between Atm deficiency and myelin pathology might be attributed to the cytoplasmic function of Atm.

In summary, cytoplasmic ATM was highly phosphorylated in myelinating OL but not post-mitotic neuron. Cytoplasmic ATM was found predominately in neuron but not under phosphorylation.

The effect of ATM deficiency on OL differentiation

Earlier neuroimaging-based clinical investigation revealed significant demyelination in the cerebrum and cerebellum of A-T patient (Sahama et al., 2014). In the immunoblotting study of our preprint, significant decline of myelin protein expression was found in $Bal^{-/-}$ mice (Tse et al., 2023). In this thesis, we extend the investigation with histological study on *Atm* deficient mice. A significant myelin decline was found in frontal cortex, genu, hippocampus and cerebellar grey matter of one-month-old and four-month-old $Bal^{-/-}$ mice, but only found in hippocampus of four-month-old $Awb^{-/-}$ mice (Figure 40B, Figure 41B). Decreased myelin protein expression in cerebral cortex of four-month-old $Bal^{-/-}$ mice was also shown in the immunoblotting experiment of this study (Figure 48C).

Myelination is exclusively generated by differentiated mOL in CNS. In the immunohistochemistry study of our preprint, $CC1^{+}$ mOL was significantly reduced in both A-T cerebellar grey matter and white matter (Tse et al., 2023). In the immunoblotting study, a significant decline of MyRF protein expression was found in the cerebral cortex and cerebellum of $Bal^{-/-}$ mice, which implied the reduction of actively myelinating OL in both regions (Tse et al., 2023). According to our immunohistochemistry experiment, a significant reduction of $CC1^{+}$ mOL population was shown in frontal cortex, hippocampus and cerebellar grey matter of four-month-old $Bal^{-/-}$ mice as well as hippocampus of four-month-old $Awb^{-/-}$ mice (Figure 47B). Significant decline of CC1 protein expression was also shown in cerebral cortex tissue of four-month-old $Bal^{-/-}$ mice with immunoblotting experiment (Figure 48C).

Reduced mOL population under ATM deficiency might raise the question if it was an apoptotic consequence of unrepaired DSB damage, as OL is also vulnerable to DNA damage (Tse & Herrup, 2017). However, there was no significant change in gross OL population ($Olig2^{+}$ cell) in A-T cerebellar and cerebral tissue (Figure 14C, Figure 15C, Figure 16C, Figure 17C). Moreover, there were no significant change in gross OL population ($Olig2^{+}$ cell) among *Atm*

deficient mice in any age group (Figure 42B, Figure 43B). Immunoblotting experiment on cerebral cortex of *Atm* deficient mice also showed that there was no significant alteration in *Olig2* expression (Figure 48C). In vivo study on *Olig2* expression revealed minimal effect of OL loss upon ATM deficiency, which might reflect the potential role of ATM on regulation of OL differentiation. According to the transcriptomic sequencing result, the genes regulating cell cycle were differentially expressed in *Bal*^{-/-} mice instead of *Awb*^{-/-} mice in Figure 61, which suggested a shift in cell cycle regulation in *Bal*^{-/-} mice, which could lead to a delay or inhibition of cell differentiation (Li & Kirschner, 2014; Zhu & Skoultschi, 2001). These findings might explain the more severe loss of myelination and mOL population found in *Bal*^{-/-} mice instead of *Awb*^{-/-} mice.

Furthermore, the transcription in presynapse and postsynapse pathways were enriched by the upregulated DEGs in the comparisons of *Bal*^{-/-} versus WT mice and *Awb*^{-/-} versus WT mice (Figure 57, 59). This result indicated that a large portion of neuron related genes was upregulated in the frontal lobe of both *Atm* deficient mouse strain. However, lipid transportation related pathways were only enriched within the comparison between *Bal*^{-/-} and WT mice (Figure 57, 59, 65, 66). Indeed, myelin is the most lipid abundant part of the brain, containing approximately 70–85% lipid by its dry mass (Wang et al., 2018). However, the result of transcriptomic sequencing data was still not significant enough to draw solid conclusions due to the low threshold of fold change selected for DEGs.

A significant decline of the myelinating OL density was found in primary glial cell culture of both *Awb*^{-/-} and *Bal*^{-/-} mice (Figure 68B). There was no significant change in OL population of *Bal*^{-/-} culture, but significant decline in *Awb*^{-/-} culture (Figure 68B). To further investigate the activity of ATM, pATM was co-stained with markers of mass OL and myelinating OL in primary glial cell culture. Glial culture of *Awb*^{-/-} mice, but not *Bal*^{-/-} mice, demonstrated significantly reduced *Olig2*⁺ OL with pATM expression in both nucleus and cytoplasm (Figure

69B). Rather than null *Atm* expression, expression of inactive kinase-dead *Atm* might lead to a more severe DSB repair impairment in an A-T mouse model study (Tal et al., 2018). As OL was found to be uniquely vulnerable to oxidative DNA damage (Tse & Herrup, 2017), truncated *Atm* protein in *Awb*^{-/-} mice might have more chance leading OL to apoptosis caused by DNA damage. However, MAG⁺ mOL with cytoplasmic pATM expression was only reduced in *Bal*^{-/-} mice with significance (Figure 69B). Unlike *Awb*^{-/-} mice, *Bal*^{-/-} mice did not show significant reduction in OL population. Therefore, null *Atm* expression imposed a larger inhibitory effect on the differentiation of OL rather than the survival of OL. Moreover, the cytoplasmic pATM expression population in MAG⁺ mOL was stable across all genotypes at around 40%, showing a relatively higher expression percentage of cytoplasmic pATM in myelinating OL population. Especially for WT glial culture, percentage of cytoplasmic pATM expression population nearly doubled the percentage of nuclei pATM expression population in MAG⁺ mOL (Figure 69B). It demonstrated the importance of cytoplasmic *Atm* expression in mOL, potentially related to OL differentiation mechanisms.

Potential intrinsic inhibitory mechanism of OL differentiation was revealed under *Atm* deficiency in vitro. Accumulated loss of mOL without significant change in mass OL population and sustained myelin loss was shown in *Atm* deficient mice model in vivo. Concluding from the results of in vivo and in vitro study on the effect of *Atm* deficiency in OL differentiation and subsequent myelination, *Atm* might have a direct role in regulating OL differentiation pathway.

The potential mechanism(s) underlying myelin pathology of A-T

ATM protein participates in many different pathways, while cell cycle pathways and DNA damage repairment pathways were the most recognised pathways associated with ATM (Smith

et al., 2010; Westphal, 1997). ATM-Chk2 pathway is one of the most classic pathways associated with DNA damage repairment (Smith et al., 2010). A reduction of full-length ATM and phosphorylated ATM was confirmed in both cerebellar and cerebral cortex of A-T patient cohort in our immunoblotting result. Reduced expression of Chk2 without any significant change in phosphorylated Chk2 was found in the immunoblotting result of A-T cerebral tissue. Deficiency of ATM in cerebral cortex potentially altered Chk2 protein stability, but not phosphorylated Chk2. The stability of phosphorylated Chk2 through oligomerization might be the potential reason for the observation (Ahn et al., 2002). Yet, the western blotting result in A-T cerebellum might be variable, which was not able to reveal the complete effect on ATM-Chk2 pathway under ATM deficiency. Still, ATM deficiency still demonstrated the influence on its downstream effectors in A-T cerebral tissue.

In Chapter 2 we pointed out that there might be a potential mechanism for the effect of ATM deficiency on OL differentiation. In the transcriptomic study on *Atm* deficient mice, lipid transportation pathway was uniquely enriched by downregulated DEGs *Bal*^{-/-} mice (Figure 65). As myelin that is largely composed with lipid, this result demonstrated a potential role of *Atm* on myelin composition. Previous study suggested that MBP might play a role in reorganizing lipid diffusion (Steshenko et al., 2016).

To further investigate the potential role of ATM in myelinating OL, super-resolution structural illumination microscopy was utilised on wild type primary glial culture. In-focus co-localization between MBP and ATM was demonstrated in different z-plane under NSIM super-resolution microscopy (Figure 70). Images captured with 3D-SIM model was reconstructed a 3D image for better visualization of colocalising regions. The 3D image revealed an evenly distributed and abundant pATM expression on MBP sheath (Figure 71). In silico docking study in our preprint manuscript provided insight of interaction between ATM and MBP protein (Tse et al., 2023). Human ATM 3D model (PDB: 5NP0) was docked with highly conserved central

fragment of MBP (PDB: 2LUG). Three of the [S/T] Q sites of the MBP fragment formed putative hydrogen bonds with ATM protein, while Q100 of the MBP fragment formed the strongest (1.83 Å) hydrogen bond with K1387 of ATM (Tse et al., 2023). The result revealed a high potential for interaction between MBP and ATM protein.

To confirm the actual binding between ATM and MBP, immunoprecipitation experiment was performed with negative control. After pulling down ATM protein, MBP was successfully expressed in the residue on immunoblotting, which confirmed the interaction between ATM and MBP protein (Figure 72). The result of successful interaction between ATM and MBP further consolidated the direct regulatory function of ATM on OL myelination.

There was no definitive evidence showing whether myelin pathology would be a primary or secondary degenerative source for A-T. Primary cultural experiment from this study implicated that prohibited OL differentiation was found in the intrinsic Atm knock-out model. Result of immunoprecipitation experiment also provided the high potential mechanism for ATM affecting OL differentiation. Meanwhile, the loss of mOL and demyelination were also confirmed with immunohistology study in Atm knock-out. Concluding from the evidence above, we suggested that myelin pathology of A-T was independent of neuronal pathology.

Summary

There might be potential association between cognitive performance decline and demyelination in A-T cerebral cortex, as myelin plasticity was associated with learning and memory formation (McKenzie et al., 2014; Takeuchi et al., 2010). In short conclusion, ATM might play a role on interacting with myelin related protein in OL, which regulates the maturation of OL. Therefore, deficiency of ATM would possibly lead to myelin pathology in A-T.

Chapter 3. Variant-specific and brain region-specific effect of ATM mutation on brain transcriptomes

Comparison of Illumina-based and Nanopore-based diagnosis

In this study, mutations of A-T patients were discovered with Illumina-based and Nanopore-based targeted sequencing. Illumina sequencing was often regarded as the golden standard of practical sequencing for diagnosis due to its accuracy and speed (Bentley et al., 2008). Although whole genome sequencing will be the state-of-art method for diagnosis in the future, targeted sequencing is beneficial for cost-effective diagnosis of patients with characterized symptoms (Rehm, 2013). Although the cost per sample for whole genome sequencing in research is reducing a lot these days, the translation of genomic sequencing into routine health care is still slow. There was a study in 2020, showing that £6841 was costed per cancer case and £7050 was per rare disease case (Schwarze et al., 2020). In a more updated study in 2024, €3700 was the suggested price in the Italy hospital for perform whole genome sequencing test on one case (Nurchis et al., 2024). In the future, whole genome sequencing could be more affordable. However, targeted sequencing could be a more decent way for diagnosis as it generates less useless data and provides less burden for bioinformatician to interpret the massive data generated from whole genome sequencing. However, practical Illumina targeted sequencing is usually limited to the exonal regions due to commercial or in-house panels designs which are unable to detect large gene insertions. It might act as effective screening for pathogenic mutations, but this method might not be able to deliver complete detection all the time. Indeed, Nanopore targeted sequencing demonstrates its potential for detection of splicing intron region and large insertion mutations with the comparable commercial panels which was primarily designed for Illumina sequencing. According to the gene coverage images from both targeted sequencing results, targeted Illumina sequencing showed a great decline of gene coverage in the edge of splice region but not shown in the result of targeted Nanopore

sequencing (Figure 76). However, the novel application of targeted panel on Nanopore sequencing was not tested in any clinical trial yet and it might demonstrate a higher error rate than Illumina sequencing (Leung et al., 2022), however its error rate has been greatly reduced recently (0.257 mismatch in 10,000 base pair) (Liu et al., 2024). In the recent comparison, Nanopore was already demonstrating 93.8% initial accuracy with R10 chips, while PacBio was still showing around 85% initial accuracy (Udaondo et al., 2021). In terms of corrected accuracy, both techniques achieved accuracy higher than 99.9%, while Nanopore sequencing could achieve 99.996% accuracy with 50x depth consensus sequence (Udaondo et al., 2021). Advantages of Nanopore targeted sequencing also include cheaper instrumental price and portability, but it is demerit in the lack of technical support and more demanding requirement on DNA input quantity and quality. Importantly, Nanopore sequencing is specially designed for real-time base-calling, which enables real-time adaptive sampling with computer modification. This function enables Nanopore-based targeted sequencing on region of interest and even methylation site of interest without additional library preparation, which also save a lot labour cost (Payne et al., 2021).

Illumina short-read RNA-seq may introduce systematic distortions that influence transcript quantification and downstream interpretation. Typical sources of error include uneven coverage caused by random hexamer priming, GC- and sequence-dependent biases introduced during library preparation and sequencing, and the inability of short reads to unambiguously resolve full-length isoforms (Hansen et al., 2010; Liu et al., 2022). Such bias could reduce the sensitivity of isoform level analysis to a certain extent. Yet, the great significance of differentially expressed ATM isoforms was still found between control and A-T patient group samples as shown in Figure 86.

There is a huge room for the AI development for whole genome sequencing diagnosis. By developing such platform for AI diagnosis, it would greatly improve the situation for diagnostic

odyssey of rare disease patients and it would further cut the cost for specific bioinformatician to analyze the data. However, we also must consider the balance of the patients privacy, as development of such powerful tool would take huge amount of patients data for AI training. Meanwhile, the whole genome sequencing analysis itself would also generate a lot “unwanted” data that we might want to avoid over-interpretation in AI-assisted diagnosis. In the past few years, AI-assisted variant calling methods and preliminary AI-assisted diagnostic models were already developed for rare genetic disease using whole genome sequencing data (Krenn et al., 2025; Poplin et al., 2018).

Regional difference of ATM transcript and ATM protein expression in the cerebrum and the cerebellum

The full-length ATM transcript and protein was declined in both A-T cerebellum and cerebral cortex. However, all ATM isoforms investigated in chapter three were significantly reduced in A-T cerebellum (Figure 97). Significant alteration of all ATM variant transcripts expression was only found in A-T cerebellum, but not cerebral cortex (Figure 98). The result was also further supported with the decline of ATM variant transcript expression in A-T cerebellum, but not cerebral cortex (Figure 86). They suggested regional specific ATM isoform expression and the potential function of ATM isoform in cerebellum.

Compared to other human brain regions, outstandingly high expression level of ATM was found in cerebellum (Deacon et al., 2024). According to the result from the Human Protein Atlas (HPA) Brain, white matter had the highest expression of ATM with averaged 60.8 normalized transcript per million (nTPM) (Sjöstedt et al., 2020). The second highest ATM expression region was cerebellum (nTPM: 47.1). Comparatively, cerebral cortex had a lower expression of ATM (nTPM: 42.3). According to our transcriptomic result, control frontal cerebral cortex was 63.3% ($p = 0.222$) less canonical ATM expression (ENST00000278616.8)

than control cerebellum (Figure 86). Compared to cerebellum, another ATM transcript expression that encodes for full length ATM protein (ENST00000452508.6) was also 72.9% reduced in cerebral cortex ($p = 0.0008$) (Figure 86). Moreover, significantly reduced full-length-ATM-protein-coding transcript (ENST00000278616.8, ENST00000452508.6) expression was found in A-T cerebellum but not A-T cerebral cortex (Figure 86). There were also larger deficits of full-length ATM (CB: 89.3%, $p = 0.0002$; CX: 51.2%, $p = 0.004$) and pATM (CB: 45.6%, $p < 0.0001$; CX: 42.9%, $p = 0.0115$) expression in A-T cerebellum than A-T cerebral prefrontal cortex detected with the immunoblotting experiment (Figure 18, Figure 20).

According to stereo-sequencing (spatial resolution $< 1 \mu\text{m}$) result on the HPA human cerebral cortex sample, the cell type with highest enrichment change (based on real measurements counts per million) of ATM protein coding transcript was microglia (Enrichment change: 352), followed by OL associated with myelin expression (Enrichment change: 134) (including PLP, CNPase and MAG) (Sjöstedt et al., 2020). However, the neuron cell type revealed zero enrichment change on ATM protein coding transcript in cerebral cortex (Sjöstedt et al., 2020). According to the correlation plotting between histological findings and ATM protein expression from our study, the Pearson R^2 value was higher in the neuronal cell count verses ATM protein expression of cerebellum (Pearson $R^2 = 0.140$) than that of cerebral cortex (Pearson $R^2 = 0.0477$) (Figure 100, Figure 101). Moreover, cerebral cortex showed a better correlation of ATM protein expression with Olig2+ OL cell count in grey matter (Pearson $R^2 = 0.215$) than that of NeuN+ neuronal cell count (Figure 101). The result implied that cell-type specific ATM expression pattern might be different in cerebellum and cerebral cortex.

According to another study profiling A-T cerebellum and prefrontal cortex with single nuclei RNA sequencing, OL associated with PLP and MBP expression was significantly increased in A-T cerebellum but significantly decreased in A-T prefrontal cortex (Lai et al., 2024).

Furthermore, the downregulated DEGs from OL of A-T prefrontal cortex enriched the myelination pathway with the lowest BH-adjusted p-value ($1.28e^{-5}$), but no myelin related pathways were enriched with DEGs from OL of A-T cerebellum (Lai et al., 2024). In the GO analysis of A-T cerebellum verses A-T prefrontal cortex, glial cell differentiation (BH-adjusted p-value: $2.22e^{-3}$) and myelination (BH-adjusted p-value: $2.77e^{-2}$) pathways were significantly enriched with the upregulated DEGs, which implied the pathway-related genes were significantly downregulated in A-T cerebral cortex (Lai et al., 2024). This result indicated the regional difference in the impact under ATM deficiency. OL in prefrontal cortex showed regional specific deficit in differentiation and myelination upon ATM deficiency.

In summary, there were significant difference in expression of ATM transcript and protein among different region of brain, especially cerebral prefrontal cortex and cerebellum. Cell type specificity and major function of ATM expression might also vary depending on the region of brain.

Mutation site dependent alteration of ATM transcript and ATM protein expression

Large variation was found in the histological findings among the A-T samples (Figure 14, Figure 15, Figure 16, Figure 17). Moreover, pathogenic ATM mutations were distributed on ATM peptide sequence evenly, not concentrated to neither earlier nor later position of ATM peptide and the conservative region or functional sites of ATM peptide (Figure 102). In fact, the region of protein kinase of ATM could be the hypothetically most crucial feature of ATM, since it serves as the substrate-binding domain for recognizing a few known substrates, including NBS1, p53 and BRCA1. Therefore, we hypothesized that different severity in A-T pathology was attributed to the difference of ATM mutation sites from each A-T patient.

Firstly, A-T patients were grouped by the nature of their corresponding ATM pathogenic mutations, “Frameshift” and “non Frameshift”. Frameshift mutations were generally associated with detrimental and deleterious effects by causing non-functional transcripts or proteins (Raes & Van de Peer, 2005). Therefore, we hypothesize that A-T patients with frameshift mutation might have more deleterious effect on histopathological findings. However, there was no significant difference between “Frameshift” and “non Frameshift” groups except for the NeuN+ neuron count in the grey matter of cerebral cortex (Figure 103). Compared to “Frameshift” group, significantly increased NeuN+ neuron count in the grey matter of cerebral cortex in “non Frameshift” group might indicate that frameshift mutations of ATM were more damaging to neuron in cerebral cortex (Figure 103). There were 27 over 77 A-T patients carried “Frameshift” ATM pathogenic mutation in the Supplementary Table 1, covering over 35% of the jointed database of A-T patients (Amirifar et al., 2021b; Huang et al., 2013; Podralska et al., 2014). Meanwhile, the group of “Frameshift” in our study was the majority, which might reflect a certain level of prevalence of “Frameshift” mutation in A-T patients. However, there were over 600 sites of mutation identified as the disease-causing mutation of A-T, with the majority of them are missense or nonsense variations (Stenson et al., 2003).

Then, the severity of pathological mutations was ranked according to the earliness of their location on ATM peptide. The earlier the mutation was located on the ATM peptide, there would be less region of original ATM peptide translated. Indeed, ATM protein expression slightly decreased with the severity of mutation in both cerebellum (Pearson R2: 0.108) and cerebral cortex (Pearson R2: 0.038) (Figure 104). However, the trend of gene expression verses the mutation severity level was opposite to the trend of ATM protein expression

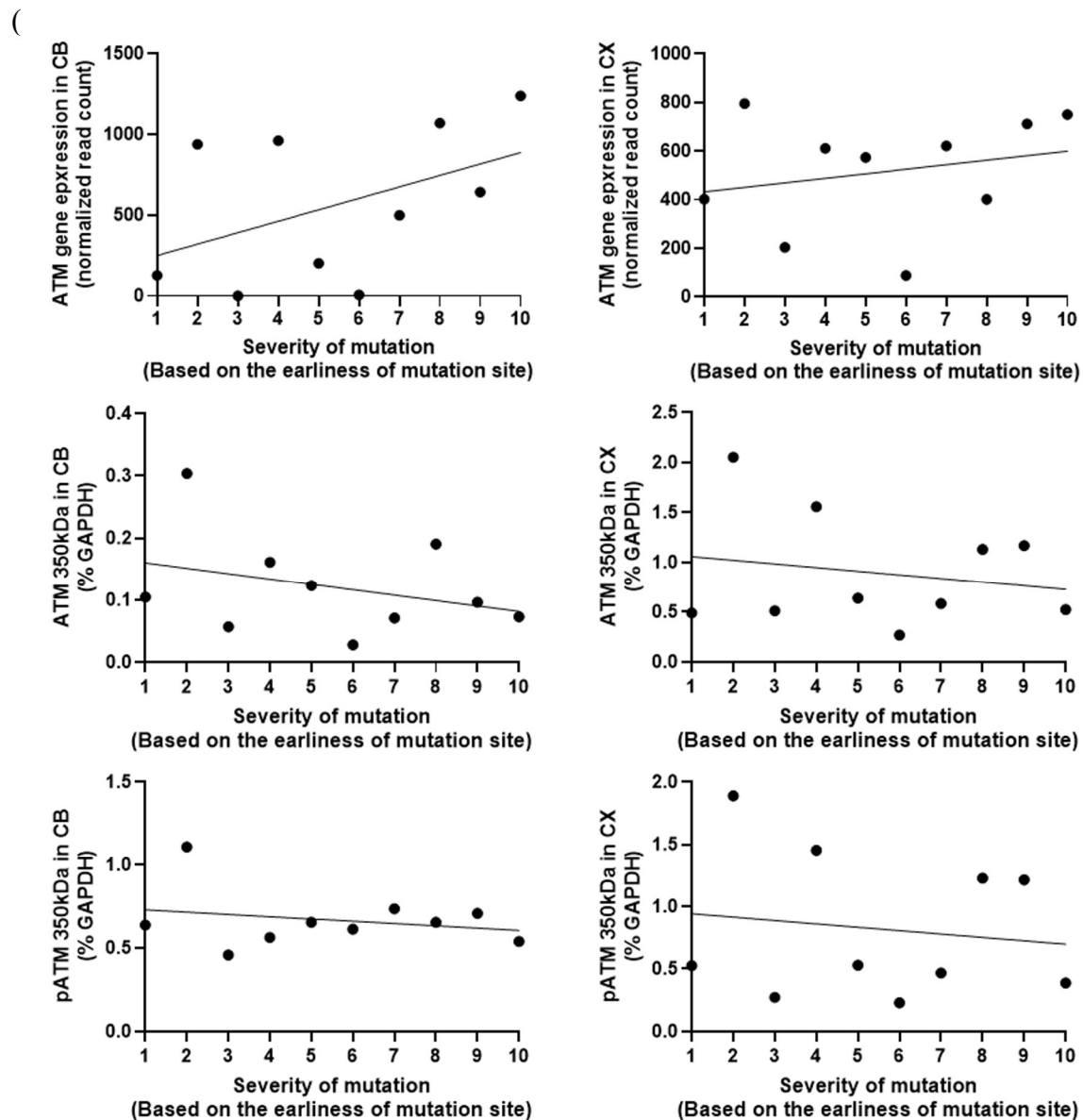


Figure 104). ATM expression and activity was reduced due to the pathogenic mutation, which might also result in the lack of ATM negative feedback mechanism related to p53 (Batchelor et al., 2008).

Eventually, ATM gene expression might be slightly promoted with more deleterious effect on ATM protein. Number of neurons in both cerebellum (Pearson R^2 : 0.014) and cerebral cortex (Pearson R^2 : 0.138) showed negative trend with the increasing level of mutation severity (Figure 105).

Number of OL in grey matter of cerebral cortex (Pearson R^2 : 0.103) and white matter of cerebellum (Pearson R^2 : 0.209) also showed negative trend with the increasing level of

mutation severity (Figure 105). The result reflected potential correlation between neuronal survival and the remaining function of ATM protein in both cerebellum and cerebral cortex. However, the increasing vulnerability of OL observed in grey matter of cerebral cortex and white matter of cerebellum with less conserved region in ATM protein might implied regional specific requirement of ATM on OL survival.

Indeed, there may not be a notable difference in ATM protein and transcript expression based on the nature or location of the ATM mutation. Due to the compound heterogeneous ATM mutations present in all A-T patients, we were also unable to discern the exclusive impact of each mutation on ATM transcript expression, ATM protein expression, and the associated immunohistochemistry outcomes. Yet, we still revealed potential trend of mutation nature and mutation site dependent alteration of histological findings, transcript and protein expression.

The association between ATM mutation and RNA splicing

Defective RNA splicing of ATM gene was found in great portion (30/62) of A-T patient in a 62 patients cohort study (Teraoka et al., 1999). Moreover, RNA splicing of ATM gene was found to be a therapeutic target with splice-switching antisense oligonucleotides (Kim et al., 2023). In Table 10, five mutations from A-T 1, A-T 5, A-T 6, A-T 9, A-T 10 were found to cause defective RNA splicing that potentially led to loss of ATM functionality. Figure 87, 91, 92, 95, 96 also demonstrated then actual RNA splicing effect of each ATM mutations. However, different level of ATM isoform was not considered the cause for the severity difference between A-T patients. Different level of neuropathology in A-T patients could be caused by how much remaining “functional portion” of ATM isoform compared to the intact ATM protein.

The association between ATM mutation and myelin pathology

Clinical reports revealed that WMH was frequently related to variant A-T (Schon et al., 2019; Tiet et al., 2021). Therefore, white matter pathology of A-T could occur with truncated ATM protein. However, there was no previous study on the association between ATM mutation sites and the A-T myelin pathology.

From an in vitro functional analysis study of six ATM variants, there was no direct correlation between position of ATM variants and the ATM activity on p53, Chk2, H2AX and BRCA1 phosphorylation (Baughan et al., 2022). Instead, the result suggested that markedly reduced activity of ATM in individual substrate was found in different ATM variants. For instance, S333F mutation reduced phosphorylation of Chk2 and H2AX, V2540I mutation reduced phosphorylation of p53 and BRCA1, but F1463C mutation showed no reduction of ATM activity (Baughan et al., 2022).

From the all twenty heterozygous pathological mutations discovered from ten A-T patients in this study, we could predict the malfunctioning or truncation site of ATM protein correspondingly in each patient. The in silico docking experiment in our manuscript revealed that ATM might possibly interact with MBP at K1387 position (Tse et al., 2023). The position fell on the pincer region of ATM protein (Figure 3). We hypothesized that the interaction between ATM and MBP might be potentially affected by the ATM mutation located earlier than K1387 position. Significantly reduced docking affinity (increased E_{balanced} score, $p = 0.011$) was found in the interaction between fragment MBP (2LUG) and ATM mutant with mutation located before K1387 (Tse et al., 2023).

The major function of MBP was associated with myelin compaction (Stadelmann et al., 2019). Yet, the microtubule-stabilizing activity of MBP was found essential for the late stages of OL differentiation (Galiano et al., 2006). In the immunohistochemistry study in our manuscript, number of CC1⁺ mOL was found significantly reduced in the grey and white matter of A-T

cerebellum (Tse et al., 2023). It suggested that prohibited OL differentiation could be the result of compromised interaction between ATM and MBP. Moreover, myelin compaction play roles in axonal conduction and myelin plasticity (Chapman & Hill, 2020). The degree to which MBP function was impaired by the defective ATM–MBP interaction was left for further investigation.

Summary

Indeed, targeted Nanopore sequencing could provide an insight of long read sequencing application into rare disease diagnosis, but there are technical limitations. The variant-specific binding affinity of ATM mutant protein toward MBP protein fragment was shown in silico experiment. Yet, variant-specific ATM protein expression, ATM transcript expression and histological findings were barely found in the correlation plotting, due to the heterogeneous mutations found in A-T patients. Generally, site-specific severity of mutation in pathogenic gene was expected in the rare disease(Rawat et al., 2022). However, A-T pathogenic mutations were spread all-over on the ATM gene. This phenomenon made us wonder whether the site-specific damage to particular region in ATM or the damage to the whole ATM protein structure would cause a more devastating symptom in A-T patients, while our data preferred the later explanation. A significantly higher ATM transcript expression was found in normal cerebellum, which reflected in a higher level of cerebellar neurodegeneration in A-T patients, in agreement with the literature.

Chapter 4. The potential effect of ATM mutation on endothelial cell autophagy

Function of TRIM family member on endothelial cell

There are over 70 TRIM family members in human with RING domain showing E3 ubiquitin ligase characteristic (Vunjak & Versteeg, 2019). The TRIM family generally functions as the regulators of various diseases, such as cancer, inflammatory diseases, infectious diseases, neuropsychiatric disorders and developmental diseases (Watanabe & Hatakeyama, 2017). Only some of the members function actively in endothelial cell, including TRIM14, TRIM27, TRIM28, TRIM46, TRIM47, TRIM59 and TRIM65 (Chen et al., 2024; Ma et al., 2024; Qian et al., 2022; Shen et al., 2022; Zeng et al., 2023).

TRIM14 was found with high expression in human vascular endothelial cell, which was promoting endothelial activation via NF- κ B signal pathway (Huang et al., 2020). TRIM27 was found in renal endothelial cell which showed protective effect through FoxO₁ signalling pathway (Liu et al., 2021). TRIM28 played crucial role in regulating the VEGFR-Notch signalling pathway for endothelial cell angiogenesis (Wang et al., 2020). TRIM46 showed protective effect to high glucose-induced ferroptosis in retinal capillary endothelial cells (Zhang et al., 2021). TRIM59 was associated with protection against atherosclerosis (Zeng et al., 2023). Moreover, TRIM59 was revealed having a role on autophagy regulation through ubiquitination of BECN1 (Han et al., 2018). TRIM65 showed inhibitory role on VCAM-1-mediated inflammatory response under atherosclerosis (Ma et al., 2024).

TRIM47 was suggested to play a significant role in the pathophysiology of cerebral small vessel disease (Mishra et al., 2022), which was also identified as GWAS-significant risk factor for WMH (Persyn et al., 2020). Recent research suggested potential role of TRIM47 on regulating blood brain barrier integrity in brain endothelial cell (Dupl  a et al., 2024). As telangiectasia of A-T patients likely involves the changes in endothelial cells, and ATM is a

key regulator of autophagy, we hypothesized that TRIM47 is an autophagy regulator in the brain endothelial cells and further hypothesized that TRIM47 might be affected in A-T patients.

Vascular pathology in ATM deficiency

The change in vasculature related pathways were significantly enriched with upregulated DEGs of A-T cerebellum (Figure 24). It revealed the potential vascular pathology in A-T cerebellum. Negative regulation of blood pressure was also the most significant pathway enriched with downregulated DEGs of *Awb^{-/-}* frontal lobe (Figure 59). Development of telangiectasia in A-T patient was associated with formation of aberrant vasculature (Schoenaker et al., 2018). Vascular endothelial growth factor (VEGF) mediated angiogenesis pathway was disrupted under ATM deficiency in endothelial cell (Okuno et al., 2012). To further investigate the effect of ATM deficiency on brain vasculature, VEGF expression was compared in the RNA sequencing data of A-T cerebellum and cerebral cortex. Expression of VEGF_c was significantly elevated in A-T cerebellum ($\log_2$ fold change: 1.23, adjusted p-value: 5.36×10^{-4}) but not cerebral cortex. VEGF_c was one of the essential components for fenestrated brain vessel formation (Parab et al., 2021). The result might reveal a more severe vascular pathology in A-T cerebellum.

In agreement to our hypothesis, TRIM47 expression was significantly increased in both A-T cerebellum (16.6%, $p < 0.0001$) and A-T cerebral cortex (10.4%, $p = 0.0133$). Aberrant TRIM47 expression might alter blood brain barrier integrity in both cerebellum and cerebral cortex. According to the clinical report summary, there were haemorrhage in cerebral vascular regions in some of the A-T patients, including A-T2, A-T7 and A-T8 (Table 7). The clinical finding of cerebral vasculature might be associated with the altered TRIM47 expression.

Potential role on autophagy under ATM deficiency

Although Trim47 expression shows no significant difference in the transcriptomic study of Atm deficient mice, it was found associated with autophagy in endothelial cell line upon autophagy activation.

Expression of TRIM47 was significantly reduced upon autophagy activation (Figure 110, Figure 112). The result revealed a potential role of TRIM47 as negative regulator on autophagy. Negative regulation of autophagy limits the intensity and duration of autophagic response (Liang, 2010). Loss-of-function of TRIM47 was found disrupting cerebrovasculature (Yeung et al., 2024). However, TRIM47 expression was promoted under ATM deficiency, which revealed enhanced inhibition of autophagy in brain endothelial cell. In our manuscript, potential interaction between TRIM47 and LC3B was demonstrated with in silico experiment (Yeung et al., 2024). Significantly reduced MAP1LC3B2 expression was found in A-T cerebellum ($\log_2$ fold change: -1.05, adjusted p-value: 1.43×10^{-2}). Inhibitory effect on autophagic activity was shown in A-T cerebellum.

Indeed, Purkinje cells in cerebellum are vulnerable to autophagy deficiency (Ko et al., 2005). For example, pharmaceutical inhibition of ATM on neuronal cell induced autophagy in vitro (Cheng et al., 2018). To further investigate the potential autophagic alteration under ATM deficiency, major autophagic gene expression was inspected from RNA sequencing data. The expression of ATG3 ($\log_2$ fold change: 1.18, adjusted p-value: 2.09×10^{-2}), BCL2 ($\log_2$ fold change: 1.82, adjusted p-value: 2.02×10^{-9}), GABARAP ($\log_2$ fold change: 0.91, adjusted p-value: 2.25×10^{-5}), GABARAPL1 ($\log_2$ fold change: -0.93, adjusted p-value: 1.77×10^{-3}) and MTOR ($\log_2$ fold change: -0.85, adjusted p-value: 1.40×10^{-3}) were significantly altered in A-T cerebellum. Meanwhile, expression of ATG7 ($\log_2$ fold change: -0.65, adjusted p-value: 1.45×10^{-2}), GABARAPL1 ($\log_2$ fold change: -0.97, adjusted p-value: 2.47×10^{-2}) and MTOR ($\log_2$ fold change: -0.56, adjusted p-value: 1.26×10^{-2}) were significantly altered in A-T cerebral cortex. The

significantly altered expression of specific genes revealed the potential alteration of macroautophagic function under ATM deficiency (Fradd et al., 2018; Grunwald et al., 2020; Høyer-Hansen et al., 2007; Pattingre et al., 2008). Although no definitive experiment was conducted to determine whether autophagy was upregulated or downregulated under ATM deficiency and where a direct consequence of TRIM47 expression is resulted, there was a markedly abnormal macroautophagy in A-T cerebellum and cerebral cortex. Macroautophagic function in brain endothelial cell plays a pivotal role in maintaining blood brain barrier integrity (Yang et al., 2021). Cerebral microbleeds under ATM deficiency might be ascribed to blood brain barrier disruption under abnormal autophagic activity (Tiet et al., 2021).

Summary

This study provided insight on vascular pathology under ATM deficiency, which was barely investigated. TRIM47 was potentially associated with the abnormal autophagic activity under ATM deficiency. However, further experimentation is required for confirming the mechanism behind vascular pathology in A-T patient.

11. Significance and limitation of the study

This study unravelled a novel potential mechanism of myelin pathology in A-T patient. It provided the underlying understanding on demyelination process in neurodegenerative disease. Region specific and variant specific pathology was found in A-T patients, indicating the complexity of A-T pathology. More detailed diagnosis could lead to a more appropriate health caring and treatment for A-T patients. Alternative clinical diagnostic procedure was also tested in this study, which demonstrated the applicability of targeted Nanopore sequencing on rare disease diagnosis. Improvement of diagnostic efficiency and convenience could be achieved with adaptive sampling on Nanopore sequencing for diagnosis.

This study was limited by the sample size of A-T subjects. Variation within A-T cohort was greatly affecting statistical analysis. To further the discussion, there were only heterogenic mutations in A-T cohort, which hinder the direct investigation on the sole mutation effect with A-T samples. Due to the limitations of experimental design, we focused on the region with highest novelty in our study. 1-month-age of ATM deficient mice was also our target for potential next batch of sequencing samples. However, we chose 4-month-age of ATM deficient mice due to the combined significance of changing in behavioral test and immunohistology studies. Indeed, the choice of cortex for sequencing experiment was that there were OL changes and cognitive changes. Therefore, we wondered if these observations were cerebellum independent. The examination of cerebellum and at the 1 month old would be completed in the future. Cell type specificity in transcriptomic analysis was another great limitation in this study as OL-specific molecular pathology might not be interpreted with the RNA sequencing result in this study. To further study on the OL-specific transcriptomic shift in the brain region of interest, we might employ the Drop-seq or flow cytometry sequencing in the future. Another reason for unexpectedly low DEG number in the sequencing result was that size of constrained

gene in targeted sequencing panel was too small. Moreover, it might be challenging to perform the pull-down experiment of ATM to MBP due to the huge size difference between MBP (18-22kDa) and ATM (350kDa) as the immunoprecipitation experiment between large protein size differences is more prone to cross-contamination (Qoronfleh et al., 2003). To better understand the complete role of ATM in OL differentiation, we planned to study the ATM level change from OPC differentiated into myelinating OL through primary culture and immunohistology study on developmental stages of mouse.

Full investigation of ATM mutation effect on downstream transcription and translation required multi-omics technology, such as mass spectrometry peptide analysis. ATM mutation uniquely detected by Nanopore targeted sequencing method should also be further confirmed with traditional Sanger sequencing method.

For the TRIM47 direction, mouse models of A-T can be stained with TRIM47 marker to determine the region of focal lesions in the future.

12. Conclusion

This report covered genetic mutation variants discovery, transcriptomic, histological, immunoblotting study on human A-T specimens with thorough comparison in different region and variants. Myelin pathology under ATM deficiency was validated with in vivo translational study. Loss of mOL and cognitive decline is noticed in 4MO Atm deficient mice, while mice model with complete loss of Atm activity showed more severe phenotype and histological change. Based on the results in this report, ATM deficiency play a role in interrupting OL lineage, while the severity depends on the effect of mutation on ATM activity. Cell cycle protein expression was disrupted in both OPC and mOL under Atm deficiency. There might be detrimental effect on OL lineage from multiple aspects under ATM deficiency. Differential effect of ATM deficiency was found in different brain regions, which accounted for different subsequence neurological manifestations. The severity of brain pathology varied in A-T patients with different heterogenic ATM pathological mutation. The severity of myelin pathology in A-T patient was potentially associated with the site of ATM- MBP interaction. The vascular pathology of A-T was found associated with macroautophagic dysregulation. Meanwhile, TRIM47 played a significant role in the autophagic regulation in brain endothelial cell. Nanopore targeted sequencing method was tested for potential clinical application, which was advantageous for intronic mutations and large segment inserts detection.

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14. Supplementary Table

Patient ID	Ethnicity	Sex	Age	DNA mutation	Type	Protein effect	Zygosity	Location	Ataxia (age)	Telangiectasia (age)	Immunodeficiency		
CHAT1	Chinese	F	13	c.1464G>A ins56-1G>A	Nonsense Splicing	p.W448X Splicing	Compound hetero	Exon10 Exon57	+	(2)	+	(3)	↓IgG, ↓IgA
CHAT2	Chinese	F	4	c.1464G>A ins56-1G>A	Nonsense Splicing	p.W448X Splicing	Compound hetero	Exon10 Exon57	+	(1.5)	+	(2)	↓IgG, ↓IgA
CHAT3	Chinese	F	14	c.2680delG c.7166C>G	Frameshift Nonsense	p.D894fsX4 p.S2389X	Compound hetero	Exon18 Exon49	+	(2.5)	+	(3)	↓IgA
CHAT4	Chinese	F	7	c.2680delG c.7166C>G	Frameshift Nonsense	p.D894fsX4 p.S2389X	Compound hetero	Exon18 Exon49	+	(1.5)	+	(1)	↓IgA
CHAT5	Chinese	F	13	c.3174G>A Exon 63 deletion	Nonsense large genomie deletion	p.W1058X N/A	Compound hetero	Exon22 Exon63	+	(4)	+	(1)	Normal
CHAT6	Chinese	M	8	c.2152_2154delinsAAAC c.8713_8714insCA	Frameshift Frameshift	p.C718KfsX19 p.V2906QfsX32	Compound hetero	Exon14 Exon60	+	(1.5)	+	(2)	Normal
CHAT7	Chinese	M	8	c1402_1403delAA c.2413C>T	Frameshift Nonsense	p.K468EfsX17 p.R805X	Compound hetero	Exon10 Exon16	+	(2)	+	(2)	↓IgG, ↓IgA
CHAT8	Chinese	F	7	c.6885G>T c.3742_3743insGGAGGTTCT	Missense Frameshift	p.V1248F p.Y1248WfsX10	Compound hetero	Exon46 Exon25	+	(2)	+	(2)	↓IgA
AT-1	Iranian	N/A	N/A	Deletion EX62-63	Frameshift		Homo	Exon62-63	+		+		↓IgG, ↓IgA
AT-2	Iranian	N/A	N/A	c.67C>T	Nonsense	p.R23	Homo	Exon2	+		+		
AT-3	Iranian	N/A	N/A	c.1537C>T c.8050C>T	Nonsense Nonsense	p.Q513X p.Q2689X	Compound hetero	Exon10 Exon55	+		+		
AT-4	Iranian	N/A	N/A	c.3244-3245insG	Frameshift	p.H1082RfsX14	Homo	Exon22	+		+		
AT-5	Iranian	N/A	N/A	c.3895delG	Frameshift	p.A1299PfsX50	Homo	Exon26	+		+		
AT-6	Iranian	N/A	N/A	c.6658C>T	Nonsense	p.Q2220X	Homo	Exon46	+		+		↓IgG, ↓IgA
AT-7	Iranian	N/A	N/A	c.3600-3601delTT	Frameshift	p.F1201WfsX3	Homo	Exon25	+		+		
AT-8	Iranian	N/A	N/A	c.8907T>G c.8050C>T	Nonsense Nonsense	p.Q2684X p.Q2969X	Compound hetero	Exon55 Exon62	+		+		
AT-9	Iranian	N/A	N/A	c.6452G>C	Missense	p.R2151T	Homo	Exon44	+				
AT-10	Iranian	N/A	N/A	Deletion EX37-48	Frameshift		Homo	Exon37-48	+		+		
AT-11	Iranian	N/A	N/A	c.5585delA	Frameshift	p.S1863LfsX54	Homo	Exon37	+		+		
AT-12	Iranian	N/A	N/A	c.6658C>T	Nonsense	p.Q2220X	Homo	Exon46	+		+		↓IgG, ↓IgA
AT-13	Iranian	N/A	N/A	Deletion EX1	Frameshift		Homo	Exon1	+		+		
AT-14	Iranian	N/A	N/A	c.6259delG c.6658C>T	Frameshift Nonsense	p.E2087KfsX9 p.Q2220X	Compound hetero	Exon43 Exon46	+		+		↓IgG, ↓IgA
AT-15	Iranian	N/A	N/A	c.5552_5553insC	Frameshift	p.Q1852PfsX5	Homo	Exon37	+		+		
AT-16	Iranian	N/A	N/A	c.6047A>G	Missense	p.D2016G	Homo	Exon41	+		+		
AT-17	Iranian	N/A	N/A	c.6198+1G>A c.6047A>G	Splicing error Missense	Splice donor p.D2016G	Compound hetero	Intron42 Exon41	+		+		
AT-18	Iranian	N/A	N/A	18-61 duplication c.7788G>A	Frameshift synonymous	- p.E2596=	Compound hetero	Exon18-61 Exon52	+		+		
AT-19	Iranian	N/A	N/A	c.9097_9101dupAATTT	Frameshift	p.L3035fsX8	Homo	Exon63	+		+		
AT-20	Iranian	N/A	N/A	c.7308-6T>G	Splicing error	-	Homo	Intron 49	+		+		
AT-21	Iranian	N/A	N/A	c.8046-8047delTA	Frameshift	p.I2683TfsX4	Homo	Exon55	+		+		
AT-22	Iranian	N/A	N/A	c.6658C>T	Nonsense	p.Q2220X	Homo	Exon46	+		+		↓IgA
AT-23	Iranian	N/A	N/A	c.829G>T	Nonsense	p.E277X	Homo	Exon7	+		+		
AT-24	Iranian	N/A	N/A	c.6658C>T	Nonsense	p.Q2220X	Homo	Exon46	+		+		↓IgA
AT-25	Iranian	N/A	N/A	Deletion EX62-63	Frameshift		Homo	Exon62-63	+		+		↓IgG, ↓IgA
AT-26	Iranian	N/A	N/A	Deletion EX62-63	Frameshift		Homo	Exon62-63	+		+		↓IgG, ↓IgA
AT-27	Iranian	N/A	N/A	c.3600_3601delTT	Frameshift	p.F1201WfsX3	Homo	Exon25	+		+		
AT-28	Iranian	N/A	N/A	c.5003A>G	Missense	p.L1668P	Homo	Exon33	+		+		
AT-29	Iranian	N/A	N/A	c.3102T>G	Nonsense	p.Y1034X	Homo	Exon21	+		+		
AT-30	Iranian	N/A	N/A	Deletion EX62-63	Frameshift		Homo	Exon62-63	+		+		
AT-31	Iranian	N/A	N/A	c.5712dupA	Frameshift	p.S1905fsX12	Homo	Exon38	+		+		
AT-32	Iranian	N/A	N/A	c.1159A>C	Missense	p.K387Q	Homo	Exon9	+		+		
AT-33	Iranian	N/A	N/A	Deletion EX62-63	Frameshift		Homo	Exon62-63	+		+		↓IgA
AT-34	Iranian	N/A	N/A	Deletion EX61-62	Frameshift		Homo	Exon61-62	+		+		Normal
AT-35	Iranian	N/A	N/A	c.6807+1G>C	Splicing error	-	Homo	Intron46	+		+		
AT-36	Iranian	N/A	N/A	c.829G>T	Nonsense	p.E277X	Homo	Exon7	+		+		
AT-37	Iranian	N/A	N/A	Deletion EX62-63	Frameshift		Homo	Exon62-63	+		+		↓IgG, ↓IgA
AT-38	Iranian	N/A	N/A	c.6199-1G>T	Splicing error		Homo	Intron42	+		+		

AT-39	Iranian	N/A	N/A	c.664C>T	Nonsense	p.Q222X	Homo	Exon7	+	+	
AT-40	Iranian	N/A	N/A	c.8741T>A	Missense	p.I2914N	Homo	Exon60	+	—	
AT-41	Iranian	N/A	N/A	c.1834C>A	Missense	p.L612I	Homo	Exon12	+	—	
AT-42	Iranian	N/A	N/A	c.6067G>C	Missense	p.G2023R	Homo	Exon41	+	—	
AT-43	Iranian	N/A	N/A	Deletion EX37-48	Frameshift		Homo	Exon37-48	+	+	
AT01	Polish	M	11	c.8441delG c.6095G>A	Truncation Aberrant splicing	p.Glu2814LysfsTer43 Exon 43 skipped	Compound hetero		—	—	↓IgG
AT02	Polish	F	7	c.3402+30_3402+32delATC			Compound hetero		+(1.8)	—	↓IgA
AT06	Polish	M	7	c.6145T>G c.434T>G	Missense Missense	p.Tyr2049Asp p.Leu145Arg	Compound hetero		+	+	↓IgA, ↓IgG, ↑IgM
AT07	Polish	F	5	c.6754_6754delA c.6095G>A	Truncation Aberrant splicing	p.Thr2252ProfsTer5 Exon 43 skipped	Compound hetero		+(1.8)	—	↓IgA, ↓IgG
AT7.1	Polish	M	5	c.6754_6754delA c.6095G>A	Truncation Aberrant splicing	p.Thr2252ProfsTer5 Exon 43 skipped	Compound hetero		+(1.8)	—	↓IgA, ↓IgG
AT08	Polish	F	6	c.7630-2A>C	Aberrant splicing	Exon 54 skipped	Hetero		+	+	↓IgA, ↓IgG2
AT10	Polish	M	14	c.6095G>A	Aberrant splicing	Exon 43 skipped	Compound hetero		+(1.3)	+(9.5)	↓IgA, ↓IgG2
AT12	Polish	M	3	c.7630-2A>C	Aberrant splicing	Exon 54 skipped	Compound hetero		+(1.4)	+(3)	↓IgG3
AT13	Polish	F	6	Deletion EX62-63 c.5932G>T	Truncation Truncation	p.Glu1978Ter	Compound hetero	Exon62-63	+(1.4)	+(3)	↓IgA, ↓IgG2
AT15	Polish	M	17	c.1179_1180delGG	Truncation	p.Trp393Ter	Compound hetero		+(1.5)	+(7)	↓IgA
AT19	Polish	M	2	c.6095G>A	Aberrant splicing	Exon 43 skipped	Compound hetero		+(1.2)	—	↓IgG3
AT21	Polish	F	9	c.7010_7011delGT c.5932G>T	Truncation	p.Glu1978Ter	Compound hetero		+		↓IgA
AT23	Polish	M	4	c.381_381delA c.3402+30_3402+32delATC	Truncation	p.Thr127ThrfsTer2	Compound hetero		+(2.1)	+	↓IgA, ↓IgG
AT24	Polish	M	16	c.4007_4008insA	Truncation	p.Phe1336PhefsTer3	Homo		+	+	↓IgA
AT26	Polish	F	21	c.7606G>A	Truncation	p.Gly2536Ter	Compound hetero		+		Normal
AT27	Polish	M	4	c.3402+30_3402+32delATC c.5932G>T	Truncation	p.Glu1978Ter	Compound hetero		+(1.8)	—	↓IgA
AT28	Polish	M	9	c.5932G>T c.7630-2A>C	Truncation Aberrant splicing	p.Glu1978Ter Exon 54 skipped	Compound hetero		+	+	↓IgA
AT30	Polish	F	9	c.6095G>A	Aberrant splicing	Exon 43 skipped	Compound hetero		+(2)	+(6)	↓IgA, ↓IgG2, ↓IgG4
AT31	Polish	M	13	c.2250G>A c.7630-2A>C	Aberrant splicing Aberrant splicing	Exon 54 skipped	Compound hetero		+(1.3)	+(2)	↓IgA, ↓IgG2
AT33	Polish	M	3	c.7630-2A>C c.5932G>T	Aberrant splicing Truncation	Exon 54 skipped p.Glu1978Ter	Compound hetero		+(1)	+(2)	↓IgA
AT33.1	Polish	M	3	c.7630-2A>C c.5932G>T	Aberrant splicing Truncation	Exon 54 skipped p.Glu1978Ter	Compound hetero		+(1)	+(2)	↓IgA
AT34	Polish	F	5	Deletion of exons 19 and 20 Deletion of exon 63	Truncation Truncation		Compound hetero		+(2)	—	↓IgA, ↓IgG2, ↓IgG3, ↓IgG4
AT35	Polish	M	12	c.5932G>T	Truncation	p.Glu1978Ter	Compound hetero		+(1.5)	+(8)	↓IgA, ↓IgG2
AT36	Polish	M	4	c.3802_3802delG	Truncation	p.Val1268Ter	Compound hetero		+(1)	—	↓IgA, ↓IgG2, ↓IgG3, ↓IgG4
AT37	Polish	F	8	c.9021_9022insA	Truncation	p.Arg3008ThrfsTer54	Homo		+	+	↑IgG
AT38	Polish	M	5	c.7010_7011delGT c.7630-2A>C	Truncation Aberrant splicing	p.Gly2337SerfsTer35 Exon 54 skipped	Compound hetero		+	+	↓IgG

Supplementary Table 1. A-T patient profile from literature

Note. Data in this table was collected from three different studies, describing the specific mutation site and corresponding clinical manifestation in 77 A-T patients from Chinese, Iranian and Polish. From “Ten new ATM alterations in Polish patients with ataxia-telangiectasia.”, by Marta Joanna Podralska, 2014, Mol Genet Genomic Med, 2(6), p.504-511; “The spectrum of ATM gene mutations in Iranian patients with ataxia-telangiectasia.”, by Parisa Amirifar, 2021, Pediatr Allergy Immunol.; “Twelve Novel Atm Mutations Identified in Chinese Ataxia Telangiectasia Patients”, by Yu Huang, Neuromolecular Med, 15(3), p.536-540.

Case ID	Sex	Brain Weight (g)	Age
A-T1	Female	980	24
A-T2	Male	1572	27
A-T3	Male	1400	17
A-T4	Male	1380	26
A-T5	Male	1180	36
A-T6	Male	1370	25
A-T7	Male	1565	35
A-T8	Female	1153	19
A-T9	Female	1410	28
A-T10	Female	N.D.	26

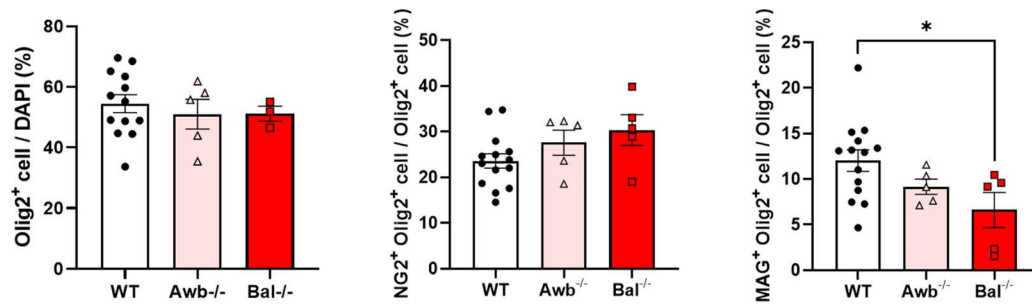
Supplementary Table 2. Details of A-T patients brain acquired from NeuroBioBank.

Case ID	Mutation	Forward Primer	Reverse Primer	Product size	Annealing temperature (°C)	Exon / Intron
A-T1	NM_000051.4:c.7630-2A>G	CAGGCATACACGCTCTACCC	TCTGCTTCTTCTGGCTACCTC	244	67	51(I)
A-T1	NM_000051.4:c.8880G>A	GTTTGTTCCTCCCTCCCATC	ATGGCCTGCTGTATGAGCAA	500	68	62(E)
A-T2	NM_000051.4:c.6404_6405insTT	ACCAGGCAGAGTATCTGAGTA	CTGATCACAGCCACCAAGTCT	450	65	44(E)
A-T2	NM_000051.4:c.8036_8051del	ACACCCGGCCTAAAGTTGTA	CTCTCCTTGCCATCGGAACC	274	67	55(E)
A-T3	NM_000051.4:c.944del	ATGGAAGGTTGGACCAGGTG	TAAAAGCCCCAAAATGCCCAGT	364	66	8(E)
A-T3	NM_000051.4:c.1564_1565del	CGATGCCTTACGGAAGTTGC	TCTCGGCCAAACAAGAAAAGC	385	67	10(E)
A-T4	NM_000051.4:c.3085dup	GCCATTCTATGGTAGCCCCC	GCCATCTGCAGCATTCCAAA	623	67	21(E)
A-T4	NM_000051.4:c.7327C>T	GCTTGGGGAGAGTCCCCTTT	AGCCAGAGGGAACAAAGTCG	389	68	50(E)
A-T5	NM_000051.4:c.742C>T	TGTTTTAGCAGGAGGGAGAGC	GGGTTTTGGCTCCTTTCGGA	471	68	7(E)
A-T5	NM_000051.4:c.6095G>A	ACAGAAGTATAGGGGAGCCA	AGGGGATTGCTGTTTCGAGG	243	66	41(E)
A-T6	NM_000051.4:c.1290_1291del	CCACCTGGCCCAAATATTGC	CTGCAGGCTGACCCAGTAAA	639	68	10(E)
A-T6	NM_000051.4:c.6095G>A	ACAGAAGTATAGGGGAGCCA	AGGGGATTGCTGTTTCGAGG	243	66	41(E)
A-T7	NM_000051.4:c.7875T>G	ATAGGATCGAACAGAGGCTGC	AAGTCCCAGGGCAGTTTTAGT	508	67	53(E)
A-T7	NM_000051.4:c.7876G>C	ATAGGATCGAACAGAGGCTGC	AAGTCCCAGGGCAGTTTTAGT	508	67	53(E)
A-T8	NM_000051.4:c.2284_2285del	TCTTGGTAGTACTCTGTCACTGG	ACGAGTGGACACCAAAACAGT	629	66	15(E)

A-T8	NM_000051.4:c.7388delNinsN[313]	GCGAGAGCTGGAGTTGGATG	GCCAGAGGGAACAAAGTCGG	462 (149 for control)	69	50(E)
A-T9	NM_000051.4:c.2921+1G>T	GCTTTTAAAGGAGCTTCCTGGAG	CCCTTGTGTTCTCAGAGTCCAT	295	67	19(I)
A-T9	NM_000051.4:c.3712_3716del	GACTTTTTGGTTCGTGCAGGT	GTAGGCTCCCACACACACAT	311	67	25(E)
A-T10	NM_000051.4:c.6404_6405insTT	TCTCTGCATGTCCCTTGTTAC	ACATCTGATCACAGCCACCAA	638	67	44(E)
A-T10	NM_000051.4:c.8786+1G>A	GTGTTGCTTTTGAACAGGGCA	GGGATGCTTCCAGAGGAGGT	521	65	60(I)

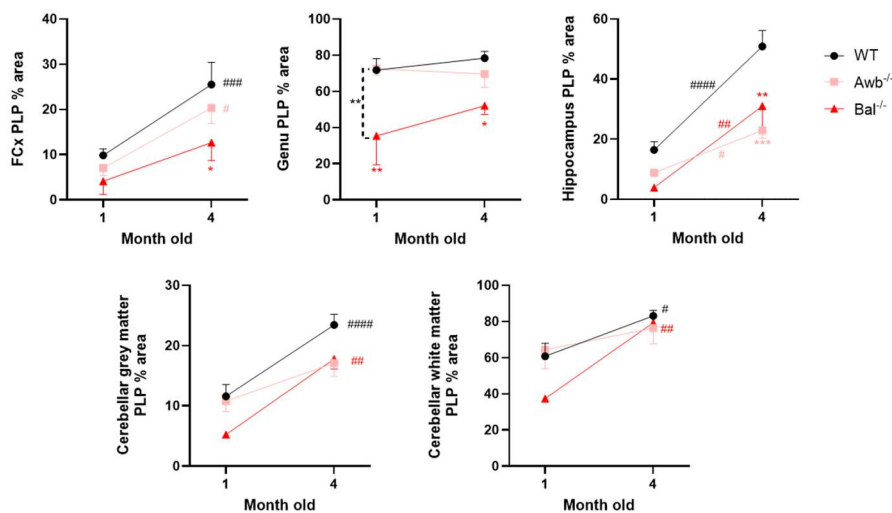
Supplementary Table 3. Primer list of PCR confirmation

15. Supplementary Figure



Supplementary Figure 1. Purity of OL primary culture

Quantification of Olig2⁺ OL, NG2⁺ OPC and MAG⁺ mOL cell population percentage. (One-way ANOVA with Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)



Supplementary Figure 2. Percentage area of PLP staining in *Atm* deficient mice

Age-dependent change of PLP percentage area in each region of Awb^{-/-} and Bal^{-/-} mice. (Two-way ANOVA with Tukey's multiple comparison test; p-value for row factor, i.e. mice genotype: *, hyphen-line * indicates p-value from comparison between Awb^{-/-} and Bal^{-/-} mice group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; p-value for column factor, i.e. mice age: #, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$)