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In Cerebellar Atrophy of 12-Month-Old ATM-Null Mice, Transcriptome Upregulations Concern Most Neurotransmission and Neuropeptide Pathways, While Downregulations Affect Prominently *Itpr1*, *Usp2* and Non-Coding RNA

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Abstract: The autosomal recessive disorder Ataxia-Telangiectasia is caused by dysfunction of the stress response protein ATM. In the nucleus of proliferating cells, ATM senses DNA double-strand breaks and coordinates their repair. This role explains T-cell dysfunction and tumor risk. However, it remains unclear whether this function is relevant for postmitotic neurons and underlies the cerebellar atrophy, since ATM is cytoplasmic in postmitotic neurons. Here, we used ATM-null mice that survived early immune deficits by bone-marrow transplantation, and reached initial neurodegeneration stages at 12 months of age. Global cerebellar transcriptomics demonstrated ATM depletion to trigger upregulations in most neurotransmission and neuropeptide systems. Downregulated transcripts were found for the ATM interactome component *Usp2*, many non-coding RNAs, ataxia genes *Itpr1*, *Grid2*, immediate early genes and immunity factors. Allelic splice changes affected prominently neuropeptide machinery, e.g. *Oprm1*. Validation experiments with stressors were performed in human neuroblastoma cells, where ATM localized only to cytoplasm, similar to brain. Effect confirmation in SH-SY5Y cells occurred after ATM depletion and osmotic stress better than nutrient / oxidative stress, not after ATM kinase inhibition or DNA stressor bleomycin. Overall, we provide pioneer observations from a faithful A-T mouse model, which suggest general changes in synaptic and dense-core vesicle stress adaptation.

Keywords: cerebellar ataxia; cytoplasmic ATM; synaptic pathology

1. Introduction

The disease Ataxia Telangiectasia (A-T) is autosomal recessively inherited, shows a prevalence of 1:100,000 inhabitants, manifests in childhood and shortens lifespan to 25 years on average [1-3]. The diagnostic initial signs include problems of equilibrium (ataxia) and speech, together with uncontrolled eye movements, due to progressively impaired motor coordination in the cerebellar neural circuits, as well as a dilatation of capillary blood vessels (telangiectasia). Blood tests will reveal an abnormal elevation of the prenatal osmosis regulator AFP (alpha-fetoprotein), which should

normally be downregulated in postnatal life to be substituted by albumin [4, 5]. Recently, neurofilament light chain (NFL) has been described as a potential biomarker for neurodegeneration from early stages of A-T [6, 7]. In subsequent years, a combined immune deficiency will lead to infections of sinus and lungs, and over time to bronchiectasis [8]. Among classical A-T patients, IgA deficiency correlates with the poorest prognosis [9]. Gonadal atrophy will ensue, with gametogenesis undergoing meiotic arrest in early prophase, due to abnormal synaptonemal complex assembly resulting in fragmented chromosomes [10, 11]. Body weight and height decline with age, accompanied by deficient secretion of growth hormone (GH) and trophic factors such as blood IGF-1, suggesting age-associated nutrient regulation stress [12-14]. A-T patients are particularly vulnerable to ionizing radiation and ultraviolet B light (UVB wavelength is responsible e.g. for sunburns of skin), so their risk of cancer is elevated, manifesting particularly lymphoma and leukemia in childhood, and breast cancer in adulthood [15, 16]. Among these disease phenotypes, only immune deficits, infertility, and cancer risks have been mechanistically explained by the crucial role of nuclear ATM (the protein kinase Ataxia Telangiectasia Mutated, where nonsense or missense mutations usually trigger the A-T phenotype) for the detection and repair of DNA double-strand breaks (DSB) [17]. These DNA damage responses (DDR) coordinated by ATM are required to generate adequate antibody diversity in rapidly proliferating lymphocytes via V(D)J and class switch recombination [18, 19]. However, there is an ongoing debate (1) why the osmotic regulator AFP increases and blood vessels dilate, (2) why nutrients are inadequately controlled in growth, and (3) why selectively post-mitotic neurons in the cerebellum should undergo insidious atrophy [20-23].

More detailed insights about ATM cellular expression, its subcellular redistribution, its stable interaction partners and its transient phosphorylation targets, together with its downstream signaling effects, are urgently needed. Such knowledge would help to understand the cerebellar pathogenesis and to design therapeutic approaches. Currently we only know that cerebellar ATM is expressed mainly in excitatory glutamatergic granule neurons, but also in efferent inhibitory GABAergic Purkinje neurons [24], other cerebellar neurons and afferent neural projections from the brainstem (see <https://mouse.brain-map.org/gene/show/11706>), as well as glial and endothelial cells. Its expression levels change with stress / stimulus responses [25]. Immunohistochemical and ultrastructural evidence showed neuronal ATM to localize to the cytoplasmic more than the nuclear compartment [24, 26]. In immunoblots of nuclear versus cytoplasmic protein extracts from mice at maximal age 6 weeks, cytoplasmic ATM was solidly detected in cerebellum but not in spleen or in thymus, while nuclear ATM remained strongly predominant even in cerebellum at this young age [27]. However, this might change in adult animals when neuronal circuitry and myelination are complete. Regarding the ATM interactome, it is important to note that ATM is a member of the PIKK family (phosphoinositide 3-kinase-related kinases), which is anchored at membranes via the FATC domain [28, 29]. Most other PIKKs phosphorylate inositol lipids, while ATM and its homolog ATR were reported to target selectively serine or threonine followed by glutamine (SQ-TQ motif) amino acids within several hundred protein substrates identified so far [17]. ATM mutation affects the membrane interface between endoplasmic reticulum and mitochondria [30], as well as endosomes, peroxisomes, lysosomal and autophagic vesicles [24, 31, 32]. Upon endosomal association, ATM was found to interact with beta-Adaptin (AP1B1 / AP2B1) and Neuronal Adaptin-like beta-subunit Protein (beta-NAP) [33]. The cytoplasmic portion of ATM prompted different studies about altered pathways there, and about additional ATM functions [34-37], but a conclusive mechanistic scenario has not emerged yet. Association of ATM with presynaptic neurotransmitter-containing vesicles was also demonstrated [38], with a preferential binding to excitatory vesicles that contain VGLUT1 as glutamate transporter to control their quantal size [39, 40]. Pre- and post-synaptic swelling and loss of cytosolic texture were detectable by electron microscopy in ATM-null mouse cerebellar cortex already at age 2 months [41]. Cerebellar Purkinje pathology involves defects in calcium spike bursts and calcium currents, as well as the progressive reduction in spontaneous action potential firing frequency, from the age of 6 weeks to their maximal lifespan of 5 months in the absence of treatment [42]. Overall, the absence of ATM protein from its physiological membrane association in neuronal cytoplasm clearly triggers age-associated neurodegeneration, but it remains unclear to what degree

ATM acts via its physical interactions with membrane lipids and proteins, versus its protein kinase activity.

With regard to ATM presence as opposed to its kinase activity, it is important to know that mice expressing the kinase-deficient ATM exhibit an early embryonic lethality phenotype [43, 44], whereas ATM-null mice are viable and their affection becomes apparent only for the immune system at early adult ages. This might suggest that the absence of ATM is sensed and mostly compensated by cells, whereas substituting ATM function becomes much more difficult if it occupies the correct positions within its interactome, but fails to signal upon stress events. In mouse, the ATM deficiency usually results in shortened survival of hematopoietic cells, early frequent occurrence of lymphomas, and a lifespan over few months only, so the manifestation of ataxia and cerebellar atrophy is usually prevented by an untimely death due to the immune deficit [41, 45]. A dramatic extension of life expectancy from 4 to 12 months was achieved by bone-marrow transplants in ATM-null mice, and in such animals a decreased cerebellar size index was observed upon brain imaging at the age of 8 months [46].

Activation of normally inactive homodimeric ATM is differently regulated, when distinct stressors are applied. Variance in post-translational modifications and interaction partners of ATM exist. The DNA damage-dependent activation (e.g. by the DNA strand breaking drug bleomycin, or ionizing radiation) involves Ser1981 autophosphorylation, Lys3016 acetylation by KAT5, interaction with the MRN protein complex (MRE11, RAD50 and NBS1) and ATM monomerization [47-49]. In neuronal cells, strong excitation promotes immediate-early gene transcription via DNA-DSB, which are mediated by topoisomerase-1 cleavage complexes (TOP1cc), and have to be eliminated by ATM activation, otherwise toxic accumulation of R-loops will occur [50, 51]. It is thought that ATM senses TOP1cc / R-loops and organizes their removal, in a process that is impaired upon oxidative damage [17]. Indeed, elevated levels of R-loops were observed in ATM-null mouse testis, but not in brain tissue, at the age of 1 month [52]. Importantly, the R-loop activation of ATM promotes chromatin displacement of late-stage spliceosomes, so the alternative splicing in ATM mutants may be changed in genome-wide manner [53]. Some ATM-dependent changes in RNA processing were reported to be mediated by the nuclear splice regulator SAM68 [54]. Thus, RNA neurotoxicity via R-loops and SAM68, protein aggregation and unbalanced excitability have been proposed to underlie the ataxia and cerebellar atrophy in A-T, in view of similar clinico-pathological findings in other monogenic spinocerebellar ataxias where mutant AOA2, FRDA, ATXN2, ITPR1 trigger similar cytosolic pathways in pathogenesis [17, 55-57]. However, few other data are available to judge the overlap in pathogenesis between diverse monogenic ataxias, and to decide which other cerebellar ataxias are closest to A-T.

In contrast to these mechanisms following DNA and RNA damage, the activation of ATM upon osmotic stress (e.g. by the drug chloroquine, or hypotonic shock) involves its interaction with ATMIN [47, 58].

Furthermore, activation of nuclear ATM via nutrient deprivation (by 2-deoxyglucose exposure) is mediated by inefficient assembly of a protein complex between endoplasmic reticulum and mitochondrial membranes, which is composed by IP3R1 (gene symbol *ITPR1*), GRP75 (gene symbol *HSPA9*), and VDAC1. This inadequate assembly results in impaired release of Ca^{2+} and excitability in the human bronchial epithelial cell line HBEC3-KT [30].

Finally, the activation of ATM via oxidative stress (e.g. by the drug sodium arsenite, abbreviated as NaARS, or by hydrogen peroxide H_2O_2) involves Cys2991 disulfide bonds linking active ATM homodimers, but appears independent from the MRN complex [59]. Again, ATMIN plays a relevant role in the protection against oxidative stressors [60]. All these mechanistic insights were obtained in cell culture or in young adult animals. Thus, at present it remains completely unclear which of these stressors and molecular response mechanisms would play the prominent role in cerebellum when the age-associated pathology manifests.

For the present study, we analysed cerebellar homogenates from bone-marrow-transplanted, 12-month-old ATM-null mice, documenting their global transcriptome by oligonucleotide microarrays, in the hope to elucidate the impact of ATM for RNA-mediated stress responses. With this approach,

we hoped to answer the following questions: (1) Which impact exists on transcript levels of known ATM interactors, or known ATM phosphorylation substrates? (2) Which dysregulations occur in phosphoinositide pathway membrane factors, in vesicular factors, or in calcium homeostasis factors? (3) To what degree is the altered neuronal excitability reflected by dysregulations of neurotransmission factors or immediate early genes? (4) Were some dysregulations already observed in telangiectasia, in general growth deficit, or selective cerebellar atrophy, e.g. as known disease genes of other cerebellar ataxias? Such findings would define the mechanistic overlap with other genetic disorders. (5) Are there strong dysregulations of novel character outside these already explored pathways?

Validation work *in vitro* with further methods and samples was performed to answer additional questions: (6) Whether ATM in adult cerebellum is still mostly nuclear with solid cytosolic presence, and if the human neural SH-SY5Y cell line is a good model of ATM distribution, was assessed with differential detergent fractionation. (7) To understand if ATM kinase activity or ATM protein presence triggers such dysregulation events, we exposed the human neuroblastoma cell line SH-SY5Y either to the ATM kinase inhibitor drug KU-55933, or to stable ATM knockdown (KD) via shRNA, and quantified transcript alterations with RT-qPCR. (8) To identify which specific stressor agents provide the best model for the age-effect on dysregulated cerebellar transcripts in ATM-null mice, human SH-SY5Y cells with ATM-KD were assessed with RT-qPCR and quantitative immunoblots.

Overall, in ATM-null mice at advanced age, several strong cerebellar mRNA dysregulations were documented, and their reproducibility in cell culture after ATM depletion and stressor administration provided criteria to distinguish primary from secondary effects.

2. Materials and Methods

2.1. Animal model of Ataxia-Telangiectasia

To study the cerebellar atrophy of A-T, we used ATM-null mice (strain 002753 from the Jackson depository, also denominated as $Atm^{tm1Awb/F}$ or ATM-null or $Atm^{-/-}$) [45] in the 129/SvEV genetic background. Animal procedures were approved by the regional authority (RPDA number FK/2000). Mice were housed in accordance with the German Animal Welfare Act, Council Directive of 24 November 1986 (86/609/EWG) Annex II, ETS123, and the EU Directive 2010/63/EU, at the FELASA-certified Central Animal Facility (ZFE) of Frankfurt University Medical School, employing type II L cages (365 x 207 x 140 mm³, floor area 530 cm²), with mutants and wildtype (WT) controls being bred and aged in parallel, under controlled conditions of temperature, humidity, and light/dark cycles of 12 h, providing food and water *ad libitum*. Genotyping of ear-punch DNA was done by PCR procedures are described previously [61].

2.2. Intravenous transplantation of whole bone marrow cells

As conditioning regimen, the recipient mice received 0.125 mg/ml anti-CD4 antibody (clone GK1.5, Sigma, Steinheim, Germany) and 0.125 mg/ml anti-CD8 antibody (clone 53-6.7, Sigma) 7 days before bone marrow transplantation (BMT), and then a second dose of each antibody together with 200 mg/kg cyclophosphamide (80 mg/ml, Sigma-Aldrich, St. Louis, Missouri, USA) 1 day before BMT for nonmyeloablative conditioning. Bone marrow cells were harvested in sterile manner from CD-90.2 depleted ATM-competent donor animals on the day of BMT, and 5×10^6 bone marrow cells were injected intravenously into conditioned recipients [46, 62]. Ageing of mutants and sex- / age-matched WT animals until 12 months was closely monitored after the intervention, continuously assuring that lymphoma and immunological deficits were not threatening the mice. Dissection of 4 ATM-null versus 4 matched WT mice occurred after cervical dislocation, snap-freezing the fresh cerebellar tissue in liquid nitrogen for oligonucleotide microarray surveys and subsequent validation experiments by RT-qPCR.

2.3. Global transcriptome survey

Total RNA was extracted from frozen tissue using TRIzol reagent (Sigma-Aldrich, St. Louis, Missouri, USA), according to manufacturer's instructions. The RNA integrity number (RIN) was assessed using a 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, California, USA) and its concentration determined with NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Samples were kept at -80 °C until use. Then, 1 µg of RNA was pre-treated with DNase amplification grade (Invitrogen, Carlsbad, California, USA). The Gene Chip™ WT PLUS Reagent Kit (Applied Biosystems, Waltham, Massachusetts, USA) was used to generate single-stranded cDNA (ss-cDNA), which was fragmented and labeled right before hybridization to Clariom D arrays (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The signals were documented with the Affymetrix Gene Chip Scanner, and data were processed with the Transcriptome Analysis Console (TAC) 4.0.1 (Applied Biosystems, Waltham, Massachusetts, USA) software using default algorithm parameters. The complete gene expression data set was deposited publically in the Gene Expression Omnibus under accession number GSE241955.

2.4. Bioinformatics analysis of global transcriptome data

The distribution of all microarray oligonucleotides that showed differential dysregulation with actual significance (false discovery rate FDR P-value < 0.05) in cerebella of 12-month old *Atm*-deficient mouse cerebella were displayed as volcano plot in Figure 2a (a logarithmic display where log2 values of fold change make downregulations in green color and upregulations in red color easily comparable on the X-axis, and -log10 of FDR P-values on the Y-axis enables graphic representation of outliers). The absolute numbers and percentages of downregulations and upregulations with nominal significance (gene level p-value < 0.05, fold change >1.2 or <-1.2) across the transcriptome, and the overrepresentation of Non-Coding transcripts among downregulations, versus overrepresentation of coding and Multiple-Complex transcripts among upregulations, are displayed as pie charts in Figure 2b. In the Clariom D microarray there are nine predefined oligonucleotide groups: Non-Coding, Multiple Complex (containing more than one of the other groups), Coding, Pseudogene, Precursor microRNA, small RNA, Ribosomal, Unassigned, and tRNA. All transcript dysregulations with nominal significance were subjected to Gene Ontology (GO)-enrichment analysis via PANTHER (<http://geneontology.org/>). Fisher's Exact was used for statistical evaluation, and correction was done by FDR. PANTHER Overrepresentation Test was carried out separately for upregulations (Figure 2d) and downregulations (Figure 2c), in each case calculating the enrichment for GO biological process (upper panel) and GO molecular function (lower panel). The resulting GO terms were sorted by Fold Enrichment, and the top 10 hits are displayed as bar graphs. Given that the Clariom D microarrays represent practically each exon of all coding transcripts, further analyses of alternative splicing were possible at genome-wide level (Figure 5). As filtering criteria, genes with exon splicing index > 5 or < -5, and significance with FDR P-value < 0.25 were selected (Figure 5a). Among them, pathway enrichment studies by the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) webplatform (<https://string-db.org/>) demonstrated an overrepresentation for the terms "Neuropeptide signaling pathway", "Regulation of neurotransmitter levels" and "Synapse organization" (shown as interaction plot in Figure 5b).

2.5. Neuroblastoma Cell Culture and Treatments

Parental SH-SY5Y human neuroblastoma cell line was cultured in high glucose DMEM (Thermo Fisher Scientific, Waltham, Massachusetts, USA, 21969-035) supplemented with 10% FCS (Thermo Fisher Scientific, Waltham, Massachusetts, USA, A3160802), 1% L-Glutamine (Thermo Fisher Scientific, Waltham, Massachusetts, USA, 25030-024) and 0.1% Penicillin / Streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA, 15140-122). ATM knockdown SH-SY5Y cells were kept in selection medium, as explained later.

Stable knockdown of *ATM* in SH-SY5Y was achieved via lentiviral transduction of five different MISSION short hairpin RNAs targeting *ATM* (shRNA, commercially available at Sigma-Aldrich, St. Louis, Missouri, USA) and one non-targeting control shRNA, targeting no known mammalian genes (Sigma-Aldrich, St. Louis, Missouri, USA, SHC002, hereafter referred to as NT CTRL, gift from Prof.

Dr. Kögel) in mammalian expression vector pLKO.1. The shATM sequences were: shATM#1-5'CCGGCCAAGGTCTATGATATGCTTACTCGAGTAAGCATATCATAGACCTGGTTTTG-3' (cat.no TRCN0000194861), shATM#2-5'CCGGTGGTCAAATACTTCATCAAATCTCGAGATTGATGAAGTATTGACCATTTG-3' (cat.no TRCN0000245108), shATM#3-5'CCGGTGATGGCTTAAGGAACATCTCTCGAGAGATGTCCTAAGACCATCATTGG-3' (cat.no TRCN0000010299), shATM#4-5'CCGGCCTTCATTAGCCTTAACTCGAGTTCTAAAGGCTGAATGAAAGGTTTG-3' (cat.no TRCN0000039948), shATM#5-5'CCGGGCCTCCAATTCTCACAGTAACACTCGAGTTACTGTGAAGAATTGGAGGTTTG-3' (cat.no TRCN0000039951). Stable KD cells were generated by transfecting 2 µg of the respective shRNA or NT CTRL plasmid DNA, 1.5 µg gag/pol plasmid DNA (psPAX2, Addgene #12260) and 0.5 µg VSV-G envelope plasmid DNA (pMD2.G, Addgene #12259) into HEK293T cells using FuGENE HD transfection reagent (Promega, Fitchburg, WI, USA, E2311) following the manufacturer's instructions. psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260; <http://n2t.net/addgene:12260>; RRID:Addgene_12260). pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259; <http://n2t.net/addgene:12259>; RRID:Addgene_12259). After 16h and 40h post-transfection, the viral supernatant was collected, pooled, sterile-filtered (0.45 µm) and applied to the SH-SY5Y cells in an 1:1 mixture with fresh medium supplemented with 3 µg/mL polybrene (Sigma-Aldrich, St. Louis, Missouri, USA, TR-1003). SH-SY5Y cells were transduced for 24h and selected via bulk selection using puromycin (Santa Cruz Biotechnology, Dallas, Texas, USA, sc-108071). To achieve this, the SH-SY5Y culture medium was supplemented with 1.25 µg/mL puromycin as determined by kill curve in parental cells. Cells were generally maintained in puromycin selection medium in order to reduce the probability of KD loss.

After expansion, shATM containing cells were assessed on protein and RNA level, and shATM#2 was selected for further experiments after achieving the best KD.

For stress experiments, parental and knockdown cells were treated with chloroquine (CQ, Sigma-Aldrich, St. Louis, Missouri, USA, C6628) for osmotic stress, bleomycin (BLEO, Merck Millipore, Burlington, Massachusetts, USA, 203408-250MG) for genotoxic stress, sodium arsenite (NaARS, Sigma-Aldrich, St. Louis, Missouri, USA, S7400-100G) for oxidative stress, and LY-294002 (LY, Cayman Chemical Company, Ann Arbor, Michigan, USA, 70920) for trophic stress via phosphoinositide 3-kinase (PI3K) inhibition. 20 µM chloroquine was administered for 24 h with sterile water as control. BLEO treatment was at 5 µM for 8 h, with DMSO as control condition. NaARS was delivered at 0.5 mM for 45 min, water serving as a control. LY was administered at a concentration of 10 µg/mL for 24 h, with DMSO as control. For pre-treatment of parental SH-SY5Y cells with the ATM inhibitor KU-55933 (KU, Selleckchem, Houston, Texas, USA, S1092), 10 µM were used over 30 min, prior to the cell stress exposure, with DMSO as a control.

Cells were harvested in Phosphate Buffered Saline (PBS) using cell scrapers. After centrifugation, pellets were frozen until usage in either nucleic acid analysis via RT-qPCR, or protein analysis via immunoblotting or subcellular fractionation.

2.6. Reverse Transcriptase real-time quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from either mouse cerebellum or cell pellets. RNA extraction was performed using TRI reagent (Sigma-Aldrich, St. Louis, Missouri, USA) following the manufacturer's protocol. To generate cDNA from RNA samples the SuperScript IV Kit (Invitrogen, Carlsbad, California, USA) was used. A total amount of 1 µg RNA was first digested with ezDNase enzyme (Invitrogen, Carlsbad, California, USA) for purification and finally reverse transcribed following the manufacturer's instructions. For gene expression analysis, RT-qPCR was performed using TaqMan Gene Expression Assays™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For this purpose, cDNA from 10 ng total RNA was used with 2x FastStart Universal Probe Master ROX (Roche, Basel, CHE) and the corresponding TaqMan Assay. The reaction was performed in a

StepOnePlus Real-Time PCR Cycler (Applied Biosystems, Waltham, Massachusetts, USA). Data was analysed using the $2^{-\Delta\Delta Ct}$ method [63].

The following TaqMan Assays were used for murine transcripts: *Atm* - Mm01177457_m1; *Atmin* - Mm01251229_m1; *Ecel1* - Mm00469610_m1; *Foxo3* - Mm01185722_m1; *Grid2* - Mm00515053_m1; *Grin2b* - Mm00433820_m1; *Grin2c* - Mm00439180_m1; *Grm4* - Mm01306128_m1; *Itpr1* - Mm00439907_m1; *Mme* - Mm00485040_m1; *Nr4a1* - Mm01300401_m1; *Nr4a2* - Mm01278507_g1; *Nr4a3* - Mm00450074_m1; *Oprm1* (Exon 2-3) - Mm01188089_m1; *Oprm1* (Exon 5-6) - Mm01188387_m1; *Per1* - Mm00501813_m1; *Rora* - Mm01173766_m1; *Slc17a6* - Mm00499876_m1; *Slc32a1* - Mm00494138_m1; *Sst* - Mm00436671_m1; *Tac1* - Mm00436880_m1; *Tacr1* - Mm00436892_m1; *Tbp* - Mm00446973_m1; *Usp2* - Mm00497452_m1.

The following TaqMan Assays were used for human transcripts: *ATM* - Hs01112311_m1; *ATMIN* - Hs00739820_m1; *CAMK2A* - Hs00947041_m1; *CAMK4* - Hs00174318_m1; *ECEL1* - Hs00191400_m1; *FOXO3* - Hs00818121_m1; *GRID2* - Hs00910017_m1; *ITPR1* - Hs00976045_m1; *MME* - Hs01115452_m1; *NR4A1* - Hs00374226_m1; *OPRM1* - Hs01053957_m1; *OPRM1* (Exon1-2) - Hs01053956_m1; *OPRM1* (Exon 3-4) - Hs00168570_m1; *PER1* - Hs00242988_m1; *RORA* - Hs00536545_m1; *RRAGD* - Hs00222001_m1; *SGK1* - Hs00178612_m1; *TBP* - Hs9999910_m1; *USP2* - Hs00275859_m1.

2.7. Immunoblotting

For protein analysis in cerebellum, the tissues were lysed, homogenized in urea lysis buffer and sonicated on medium power (three 10 second bursts). Lysates were centrifuged at 18,000 $\times g$ for 15 min. Protein content of the lysate was estimated using the Pierce 660nM protein assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Equal amounts of protein lysates (10 μ g) were separated by SDS-Polyacrylamide gel electrophoresis (PAGE) (Bio-Rad, Hercules, California, USA) and transferred to nitrocellulose membrane (Merck Millipore, Burlington, Massachusetts, USA). Non-specific binding was blocked using 5% nonfat dry milk/TBS-T for 1 h at room temperature and then the membrane was incubated with a primary antibody against ATM (#2873, Cell Signaling Technology, Danvers, Massachusetts, USA) or with β -Actin (ACTB, #4970, Cell Signaling Technology, Danvers, Massachusetts, USA) at 4°C overnight in 5% BSA/TBS-T. The next day, membranes were washed with TBS-T (3 \times 5 minutes each) and incubated with anti-rabbit IgG (H+L) (DyLightTM680 Conjugate) secondary antibody for 1h. Antibody binding was visualized on the LI-COR Odyssey NIR (near infrared) imaging system.

For protein analysis in SH-SY5Y cells, samples were first lysed in RIPA buffer (50 mM TRIS/HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate = SDS), containing HALT phosphatase inhibitors (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and cOmplete proteinase inhibitors (Roche, Basel, CHE) for 30 min on ice. Following that, the lysates were briefly sonicated and subjected to Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for determination of protein concentration following the manufacturer's instructions. For the SDS-PAGE, 25 μ g protein was used and denatured at 90 °C for 5 min. SDS-PAGE was done following standard procedures. Proteins were transferred on 0.2 μ m nitrocellulose membranes (Bio-Rad, Hercules, California, USA) and blocked in 5% bovine serum albumin (BSA, Carl Roth GmbH, Karlsruhe, GER) in TBS-buffer containing 0.1% Tween-20 (Sigma-Aldrich, St. Louis, Missouri, USA) for 1 h. Primary antibodies were rabbit anti-ATM (Cell Signaling Technology, Danvers, Massachusetts, USA, #2873), mouse anti-pATM (S1981, Cell Signaling Technology, Danvers, Massachusetts, USA, #4526), mouse anti- α -tubulin (=TUBA, Sigma-Aldrich, St. Louis, Missouri, USA, T9026), mouse anti-GAPDH (Calbiochem, St. Louis, Missouri, USA, CB1001), mouse anti-vinculin (=VCL, Proteintech, Rosemont, Illinois, USA, 66305-1-Ig), mouse anti-HSP60 (Santa Cruz Biotechnology, Dallas Texas, USA, sc-13115), rabbit anti-LAMIN-A/C (=LAMIN, Abcam, Cambridge, GBR, ab169532), rabbit anti-IP3 receptor (=ITPR1, abcam, Cambridge, GBR, ab5804), rabbit anti-PER1 (Proteintech, Rosemont, Illinois, USA, 13463-1-AP), rabbit anti-USP2 (Proteintech, Rosemont, Illinois, USA, 10392-1-AP). Incubation was performed over night at 4 °C. Membranes were incubated with the respective secondary antibody IRDye 800CW goat anti-rabbit (LI-COR, Lincoln,

Nebraska, USA, 926-32211), IRDye 680RD goat anti-rabbit (LI-COR, Lincoln, Nebraska, USA, 926-68071), IRDye 800CW goat anti-mouse (LI-COR, Lincoln, Nebraska, USA, 926-32210), IRDye 680RD goat anti-mouse (LI-COR, Lincoln, Nebraska, USA, 926-68070) for 1 h and subsequently imaged in LI-COR Odyssey Infrared Imager (Lincoln, Nebraska, USA).

2.8. Fractionation

Subcellular fractionation of cells was done as previously described [64]. Briefly, cell pellets were resuspended in cytosolic extract buffer (CEB; 250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) supplemented with 400 µg/mL digitonin (Sigma-Aldrich, St. Louis, Missouri, USA, D141-100MG), 100 µM PMSF (Carl Roth GmbH, Karlsruhe, GER, S367.1), 10 µg/mL leupeptin (AppliChem, Darmstadt, GER, A2183,0010) and 2 µg/mL aprotinin (Carl Roth GmbH, Karlsruhe, GER, A162.1). The cytoplasmic fraction was removed after centrifugation, and the mitochondrial fraction was generated from the pellets via incubation in mitochondrial lysis buffer (MLB; 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40) supplemented with 100 µM PMSF, 10 µg/mL leupeptin and 2 µg/mL aprotinin. Extracts were centrifuged and the mitochondrial fraction was removed, before nucleic lysates were prepared from pellets in RIPA buffer, which contained HALT phosphatase inhibitors (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and cOmplete proteinase inhibitors (Roche, Basel, CHE). The nuclear extracts were centrifuged to remove RIPA insoluble debris. Protein concentration in each fraction was quantified by BCA assay. Purity of the fractions was assessed via presence of GAPDH in cytosolic fractions, HSP60 in mitochondrial fractions and LAMIN-A/C in nuclear extracts via quantitative immunoblots.

Subcellular fractionation of cerebellar tissue was performed as previously described [65]. In brief, one cerebellum was first homogenised in Buffer A (150 mM NaCl, 50 mM HEPES pH 7.4; 1 M hexylene glycol) supplemented with 400 µg/mL digitonin, 100 µM PMSF, 10 µg/mL leupeptin and 2 µg/mL aprotinin using a pestle motor mixer. Samples were further homogenised via centrifugation through a QIAshredder (Qiagen, Venlo, NLD). After a 10 min incubation period, samples were centrifuged to obtain the cytoplasmic fraction. Pellets were resuspended in Buffer B (150 mM NaCl, 50 mM HEPES pH 7.4, 1% NP-40, 1 M hexylene glycol) supplemented with 100 µM PMSF, 10 µg/mL leupeptin and 2 µg/mL aprotinin. Extracts were incubated for 30 min and centrifuged to generate mitochondrial fractions. Finally, pellets were incubated with 500 U benzonase nuclease (Sigma-Aldrich, St. Louis, Missouri, USA, E1014-25KU) to digest DNA. Nuclei were lysed by 10 min incubation with Buffer C (150 mM NaCl, 50 mM HEPES pH 7.4, 1 M hexylene glycol, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 100 µM PMSF, 10 µg/mL leupeptin and 2 µg/mL aprotinin, and nuclear extracts were harvested as supernatant after centrifugation. The fractions were subjected to BCA assay for determination of protein concentration. Purity of fractions was again assessed via presence of GAPDH in cytosolic fractions, HSP60 in mitochondrial fractions and LAMIN-A/C in nuclear extracts via immunoblotting.

2.9. Statistics

Data were statistically analysed using GraphPad Prism 8 Software. Grouped data was analysed via 2-way ANOVA followed by Sidak's post-hoc test for multiple comparisons. Independent data was analysed via 1-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Comparisons of two conditions were performed with unpaired t-test with Welch's correction. Asterisks represent significance (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001). P-values 0.05 < p < 0.10 were considered as statistical trend (T) and are displayed as exact values. Data are displayed as mean ± standard error of the mean (SEM) with or without additional single values. Protein and transcript ratios are displayed as fold changes, relative to the untreated control condition.

3. Results

3.1. The cerebellar transcriptome profile of ATM-null mice at 12 months of age

As shown in Figure 1a, the global transcriptome analysis of cerebellar tissue was performed in 4 WT versus 4 ATM-null mice, aged in parallel until 12 months in pairs of identical sex. The genotype of analysed mice was controlled by breeding protocols, PCR from ear-punch DNA, RT-qPCR of *Atm* mRNA and quantitative immunoblots of ATM protein (Figure 1b/c). The global transcriptome profile of the ATM-null cerebellum was documented in Table S1. To ensure data reproducibility among different organisms in this strongly affected tissue at advanced age, we compared our ATM-null mouse cerebellar transcriptome profile at age 12 months with a published [66] proteome survey of A-T patient cerebellar post-mortem samples (although distortions by altered tissue composition at end-stage will generate artefacts, and mass-spectrometry will detect maximally some 10,000 among all existing proteins), annotating the consistent findings in Table S1. The comparison of our 12-month-old ATM-null mouse cerebellar transcriptome profile with previous A-T patient cerebrospinal fluid proteome data [67] revealed parallel reductions for *Reln*, *Fat2*, *Omd*, *Cntn6* (down) and *C4b* (up). This transcriptome was then interrogated in the context of known ATM functions and phenotypes, as far as known in the current literature. Given the scarcity of 12-month-old ATM-null mice with cerebellar anomalies, and in view of the massive widespread transcriptome changes observed (which are probably a direct consequence of altered phosphorylation cascades that alert to membrane stress and modulate nuclear transcription), we also performed extensive validation work in stressed cell models to elucidate the role of prominent molecular events.

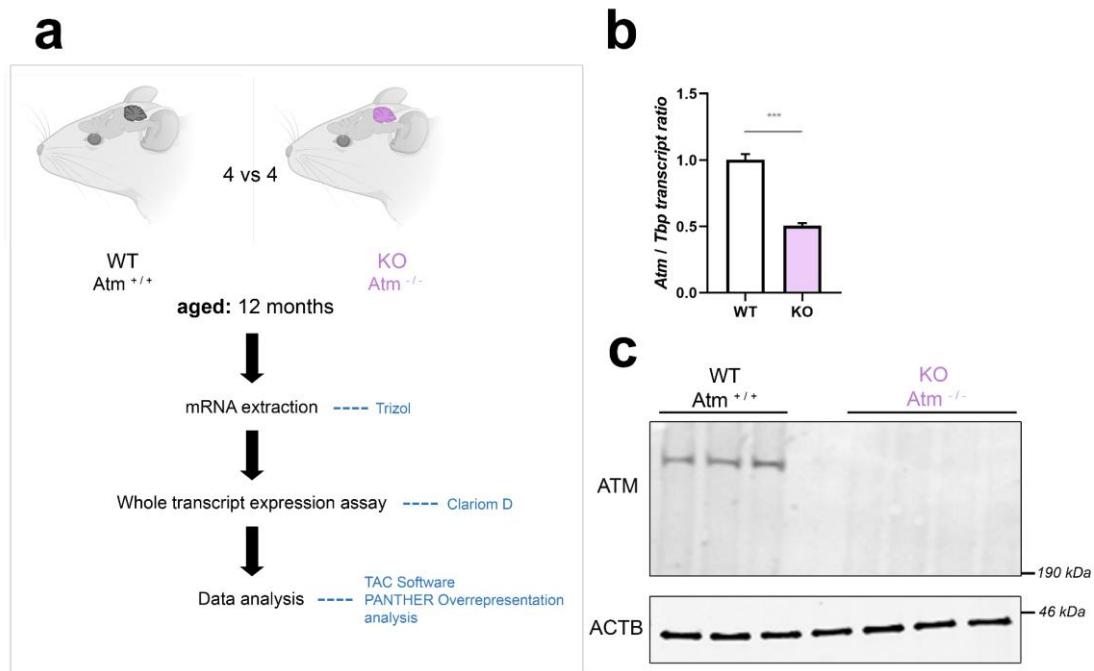


Figure 1. Workflow and quality control for genome-wide cerebellar transcriptome analysis from 12-month-old *Atm*-KO mice. (a) Schematic representation of the workflow performed in age and sex-matched *Atm*^{+/+} versus *Atm*^{-/-} mice (n = 4 vs 4). mRNA was extracted from cerebella of these animals using Trizol, and ClariomD microarray hybridisation was then performed. Data were analysed using the TAC Software provided by Affymetrix, and by PANTHER Overrepresentation analysis of pathway enrichments. (b) Mouse genotype validation via RT-qPCR, detecting the quantity of WT *Atm* transcript versus its reduction due to exon deletion and nonsense-mediated RNA decay in the *Atm*-KO samples, using *Tbp* transcript as normalizer (n = 4). (c) Mouse genotype validation via quantitative immunoblots, regarding ATM protein absence versus beta-actin (ACTB) as loading control (WT vs *Atm*-KO, 3 vs 4). Asterisks reflect significance: *** = p ≤ 0.001. Data are displayed as mean ± SEM.

In Figure 2a, a volcano plot displays the overall distribution of transcript dysregulations with actual significance (FDR < 0.05, corresponding to p < 0.0017), identifying particularly relevant coding transcripts by their gene symbols. Prominent upregulations of neurofilament medium and light chain

mRNAs (*Nefm* and *Nefl*) reflect the axon pathology at this disease stage and presumably represent cellular efforts to compensate the progressive neurofilament loss that is known to occur in A-T [6]. As noteworthy finding, ATM depletion was responsible for significant downregulations of mostly non-coding transcripts, whereas the upregulations concerned almost exclusively coding transcripts. The strongest downregulated microRNA was miR-495 (fold change -3.85, p=0.0002) as angiogenesis-hypoxia-autophagy-synaptic depression modulator [68-73]. An even greater downregulation was detected for the non-coding RNA TC0500000412.mm.1 (fold-change FC -10.77, p=6.30E-07) as a prime example for the massive impact of ATM-loss on non-coding RNAs in general. The cellular roles of TC0500000412.mm.1 are unknown at present. In Figure 2b, pie charts reflect this massive contrast between non-coding downregulations versus coding upregulations, providing absolute numbers and percentages. Extreme upregulations of several factors that are selectively expressed in the choroid plexus whose presence in the cerebellar samples was not controlled were interpreted as artefacts. A bioinformatics survey of gene ontology terms in biological processes and molecular functions by PANTHER software indicated prominent deficits in corticotropin-dependent stress responses as well as presynaptic machinery and vesicle priming (Figure 2c), versus prominent excess transcripts for neurotransmitter loading and channel activity (Figure 2d). The upregulations of neurotransmission components occurred without selectivity for any cell type, involving e.g. glutamate, GABA, glycine, muscarinic and nicotinic acetylcholine, as well as dopamine receptor transcripts. Notably, the neuropeptide signaling pathway (GO:0007218) was the 17th most enriched term among upregulations (FDR p=2.02E-05) and also showed a non-selective pattern in general, involving somatostatin, tachykinin, neuropeptides, endothelin, vasoactive intestinal peptide, encephalin, opioid mu and kappa3 signaling components. Further more detailed bioinformatics studies showed significant enrichment on the STRING webplatform for ataxia genes, vesicular factors, calcium homeostasis factors, and immediate early genes. The factors involved in these enrichments were annotated in Table S1, together with all ATM protein interactome components and the ATM kinase target proteins known at present.

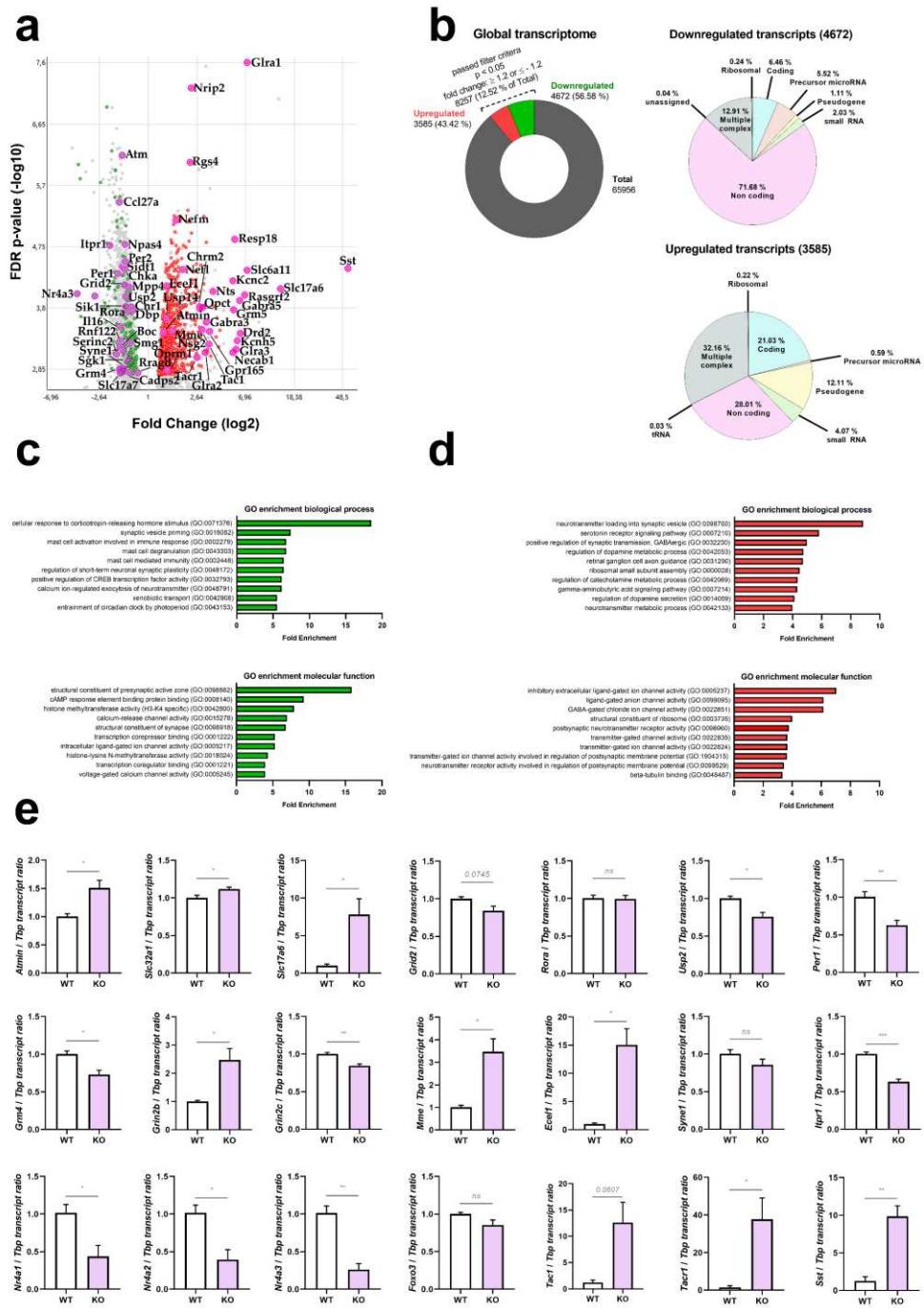


Figure 2. Genome-wide survey of transcript levels in cerebella from 12-month-old ATM-null mice.

(a) Global transcriptome documentation via Clariom D microarrays, visualized as Volcano plot with symmetry due to logarithmic scales, where the X-axis shows down- versus up-regulations (in green versus red color, respectively) while the Y-axis shows the significance of changes via false detection rates (FDR), identifying factors with relevance for pathway enrichments and for follow-up studies by their gene symbols. (b) Total amount of detected transcripts (65956) and ratio of transcripts that passed the filter criteria (8257, 12.52%). Of these, 43.42 % (3585) are upregulated and 56.58 % (4672) are downregulated. The upregulations and downregulation were further classified into different transcript categories, namely ribosomal, coding, precursor micro-RNA, pseudogene, small RNA, non-coding, tRNA and multiple complex, highlighting a prominent downregulation of non-coding RNAs. (c+d) Gene Ontology (GO) enrichment analysis of downregulated (green graphs) and upregulated (red graphs) transcripts, showing biological processes in the upper panel (prominent enrichment for cellular response to corticotropin-releasing hormone stimulus among downregulated transcripts, prominent enrichment for neurotransmitter loading into synaptic vesicle among upregulated

transcripts), molecular functions in the lower panel (prominent enrichment for structural constituent of presynaptic active zone among downregulated transcripts, prominent enrichment for inhibitory extracellular ligand-gated ion channel activity among upregulated transcripts). (e) Dysregulation validation via RT-qPCR in these 12-month-old mouse cerebella (WT vs *Atm*-KO, n = 4 vs 4) for key factors in ATM interaction, excitability, neurotransmission and neuropeptide signaling. For statistical trends, the precise p-value was shown. Asterisks represent significance: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, ns = non-significant. Data are displayed as mean ± SEM.

In the disease context, the transcriptome showed dysregulated expression with nominal significance for genes responsible for phenotypes of ataxia (compiled according to the Online Mendelian Inheritance of Man database, <https://www.ncbi.nlm.nih.gov/omim/>). Downregulations were observed for *Atm*, *Itpr1*, *Syne1*, *Grid2*, *Grik2*, *Fgf14*, *Rora*, *Gba2*, *Reln*, in good agreement with a previous proteome study of cerebrospinal fluid from A-T patients [67]; a significant enrichment was detected by the STRING webserver for “abnormal cerebellar granule neuron morphology” (q=0.0014) for the cluster of ATM, RORA [74] and GRID2 [75] proteins; an enrichment for “postsynapse” (q=0.0182) was detected for ITPR1 [66, 67, 75, 76], SYNE1, GRID2 [75] and GRIK2 [77]; upregulations were observed for the ataxia genes *Mme*, *Ebf3*, *Vamp1*, *Ppp2r2b*, *Svbp*, without significant enrichment, but VAMP1 being a vesicle-associated factor like ATM. Significant expression changes existed also for genes responsible for the pathogenesis of telangiectasia (upregulation of *Sst*, *Sstr1*, *Sstr2*, *Tac1*, *Tacr1*, *Svbp*) [78-81], and for general growth (*Sst*, *Sstr1*, *Sstr2*) [82].

The significant dysregulation of ATM interactome components *Atmin*, *Nr4a1* and *Foxo3* / *Foxo1* (but not the ATM interactome components *Mre11* / *Rad50* / *Nbs1*, nor its downstream effectors *Chk2* and *Tp53*) argued against neural ATM functions at this cerebellar age in DNA damage repair, instead suggesting osmotic / oxidative / nutrient stress [83-85]. Interestingly however, the deubiquitinase USP2 was reported recently to function in the ATM / NBS1 interactome [86], and showed strong downregulation within the ATM-null cerebellar transcriptome. Even the relatively weak *Kat5* induction observed may be relevant, in view of the known role of KAT5-dependent ATM Lys3016 acetylation.

Finally, among previously reported ATM phosphorylation target proteins [87-89] with significant dysregulation (see Table S1 annotations) in the 12-month-old ATM-null mouse cerebellar transcriptome, RTN4 (NOGO-A), DOCK10, FSCN1, SOX10, SEPT9 and CCNL2 were already implicated in glutamatergic synapse and dendrite effects [90-95]. Unexpectedly, the transcript upregulations concerned all neurotransmitter and neuropeptide pathways, rather than a signaling balance between glutamate-excitation on the one hand versus GABA-inhibition on the other hand [39].

Validation experiments by the independent method RT-qPCR in the remaining cerebellar tissue from these 12-month-old ATM-null and WT mice confirmed these dysregulations for practically all factors studied. These experiments focused on ATM interactors, ataxia genes, neurotransmitter loading factors, glutamate receptors, immediate early response components, and neuropeptide signaling molecules (Figure 2e). The RT-qPCR validation of these selected dysregulations was extended to cerebellar tissue from 1.5-3-month-old ATM-null versus age- / sex-matched WT mice, showing similar dysregulations to occur early on for *Nr4a1*, *Nr4a2*, *Oprm1*, and *Tacr1* (Figure S1). Furthermore, the 12-month-old ATM-null cerebellar transcriptome confirmed previous RT-qPCR results in ATM-null cerebellum at the age of 2 months [67] regarding the downregulations of *Itpr1*, *Atp2b2* and *Grin2c*, versus the upregulations of *Grin2b* and *Cyp46a1* mRNA levels.

3.2. In human neural cells with stable ATM-knockdown, cerebellar hallmark dysregulations are recapitulated best after osmotic stress, and partially after trophic stress

ATM-deficiency was studied further in cell culture, to assess the reproducibility of these findings in human and to identify the most suitable stressor *in vitro* that mirrors such age-associated dysregulations while enabling us to generate unlimited samples for mechanistic studies. We used the human SH-SY5Y neuroblastoma cell line, introduced various ATM shRNAs via lentiviral

transduction, and produced a stable ATM KD cell line that achieved high ATM protein and mRNA reduction for further analysis. The most efficient KD was produced by shRNA#2 (hereafter be referred to as shATM), triggering obvious changes in cellular morphology (Figure S2a), and reductions of ATM transcript to 36% (Figure S2b) and protein to 9.5% (Figure S2c), compared to the non-target shRNA control (NT CTRL) condition. In this human neural cell line, the application of the osmotic stressor chloroquine (CQ) did not alter the abundance of ATM protein, but induced phosphorylation at ATM residue S1981 (1.8-fold, with $p=0.1408$ in three biological replicates), an expected event for DDR-triggered autophosphorylation / activation of this stress sensor molecule (Figure S2d).

To assess whether SH-SY5Y neuroblastoma cells have a similar distribution of ATM in subcellular fractions as adult cerebellar tissue, differential detergent isolation of nuclear, mitochondrial, and cytoplasmic fractions was performed firstly in cerebellum from WT versus ATM-null mice at the age of 3.5 months (Figure 3a), and secondly in SH-SY5Y NT CTRL cells compared to shATM cells (Figure 3b). Even though there was some leakage from the GAPDH-immunopositive cytoplasmic (cyto) fraction to the HSP60-positive mitochondrial (mito) and the LAMIN-A/C-positive nuclear (nuc) fraction, ATM was clearly located in the cytoplasmic rather than the nuclear fraction in mouse cerebellum (Figure 3a). This finding is novel, since previous analyses until maximal cerebellar age of 6 weeks, after completion of Purkinje neuron maturation and granule cell precursor migration [96-98], had observed ATM more in nuclear than cytosolic fractions.

A localization in the cytosolic fraction was also clearly observed for ATM in SH-SY5Y cells, although the gels exhibited some leakage of the nuclear fraction to the mitochondrial fraction (Figure 3b). Importantly, this cytoplasmic localization of ATM *in vitro* was not altered by administration of the osmotic stressor CQ or the genotoxic stressor bleomycin (BLEO) in several independent experiments (Figure S3a/b). One experiment with LY stress, and one experiment with NaARS stress also failed to detect an ATM localization change. These results indicate that the ATM knockdown in SH-SY5Y neuroblastoma cells can be used as useful *in vitro* models for neural *Atm*-deficiency, regarding transcript and protein levels, stress induction and subcellular fractionation.

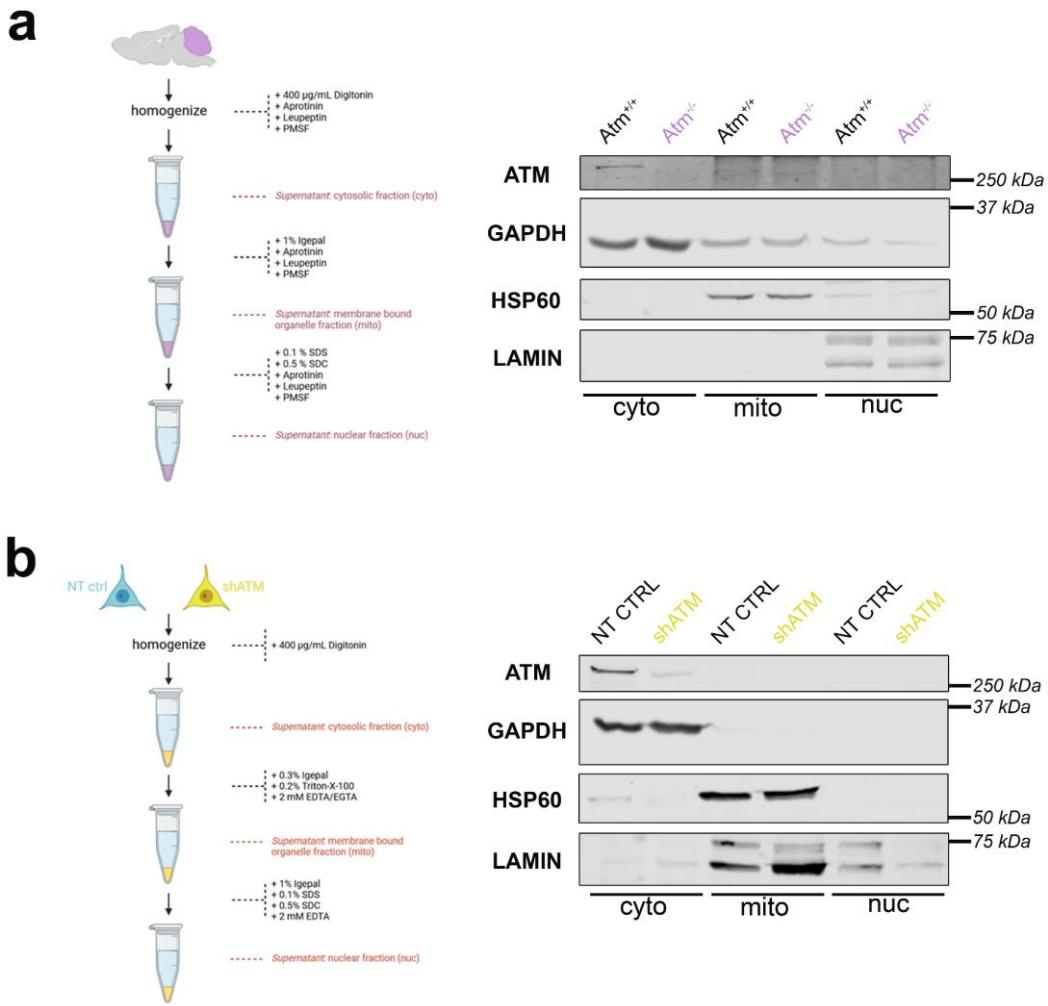


Figure 3. ATM localization in subcellular fractions by differential detergents. The workflow scheme is shown on the left side. On the right side, samples represent cell fractions of cytoplasm (cyto), mitochondria (mito) and nucleus (nuc). The purity of each aliquot was assessed by the markers GAPDH for cytoplasm, HSP60 for mitochondria, and LAMIN-A/C for nucleus. The size markers in kilodaltons (kDa) on the right margin of each gel confirm the expected molecular weight of each protein studied. **(a)** Immunoblot detecting ATM in WT versus *Atm*-KO mouse cerebella from 3.5-month-old mice. **(b)** Immunoblot detecting ATM in non-target shRNA transduced control (NT CTRL) versus shATM transduced mutant SH-SY5Y neuroblastoma cells.

3.3. The ATM-null cerebellar mRNA dysregulations are mimicked in SH-SY5Y cells by ATM knockdown rather than ATM kinase antagonism, and by CQ better than trophic / oxidative / genotoxic stress

To understand whether the cerebellar dysregulations of old ATM-null mice are due to ATM absence as platform for protein complex formation, or absent ATM kinase activity, we assessed if they are recapitulated after stress in neuroblastoma cells upon KD of ATM mRNA, or after treatment with KU-55933 (KU) as pharmacological inhibitor of ATM-mediated phosphorylation (scheme and control of ATM mRNA levels in Figure 4a/b). As representative transcripts under control of ATM, we chose upstream effectors such as *USP2* in view of its role within the ATM-interactome, and *PER1* as immediate-early transcript modulated by phosphorylation cascades (Figure 4c-f). Figure 4c shows the expected significant downregulation of *USP2* after CQ administration in the ATM-KD cells (to 65% of control after CQ, and further reduction to 48% and 38% in shATM cells with and without CQ stress), while in KU-treated cells downregulation of *USP2* was only generated by CQ-treatment but not the kinase inhibition. Similarly, Figure 4d shows significant ATM dependent CQ stressor effect,

with the significant 1.5 fold induction of *PER1* by CQ stressor being abolished to control levels in shATM cells. Again this effect was not reproduced in KU-treated cells. For *USP2* (Figure 4e), genotoxic and oxidative stress were unable to trigger the downregulation, only trophic stress by treatment with the PI3K-inhibitor LY-294002 resulted in a significant ATM-dependent reduction. For *PER1* (Figure 4f), all other stressors were ineffective. Exploiting the availability of a specific and sensitive anti-PER1 antibody, a reduction of PER1 protein was found in neuroblastoma cells with stable ATM KD even before the application of acute stress (Figure 4g). The administration of CQ resulted in a PER1 reduction in NT CTRL cells, but a converse PER1 protein induction in shATM cells. Thus, a combination of ATM-KD with CQ-stress appeared to represent the best *in vitro* modelling approach in SH-SY5Y neuroblastoma cells, to investigate the roles of cerebellar mRNA dysregulations in aged ATM-null cerebellum.

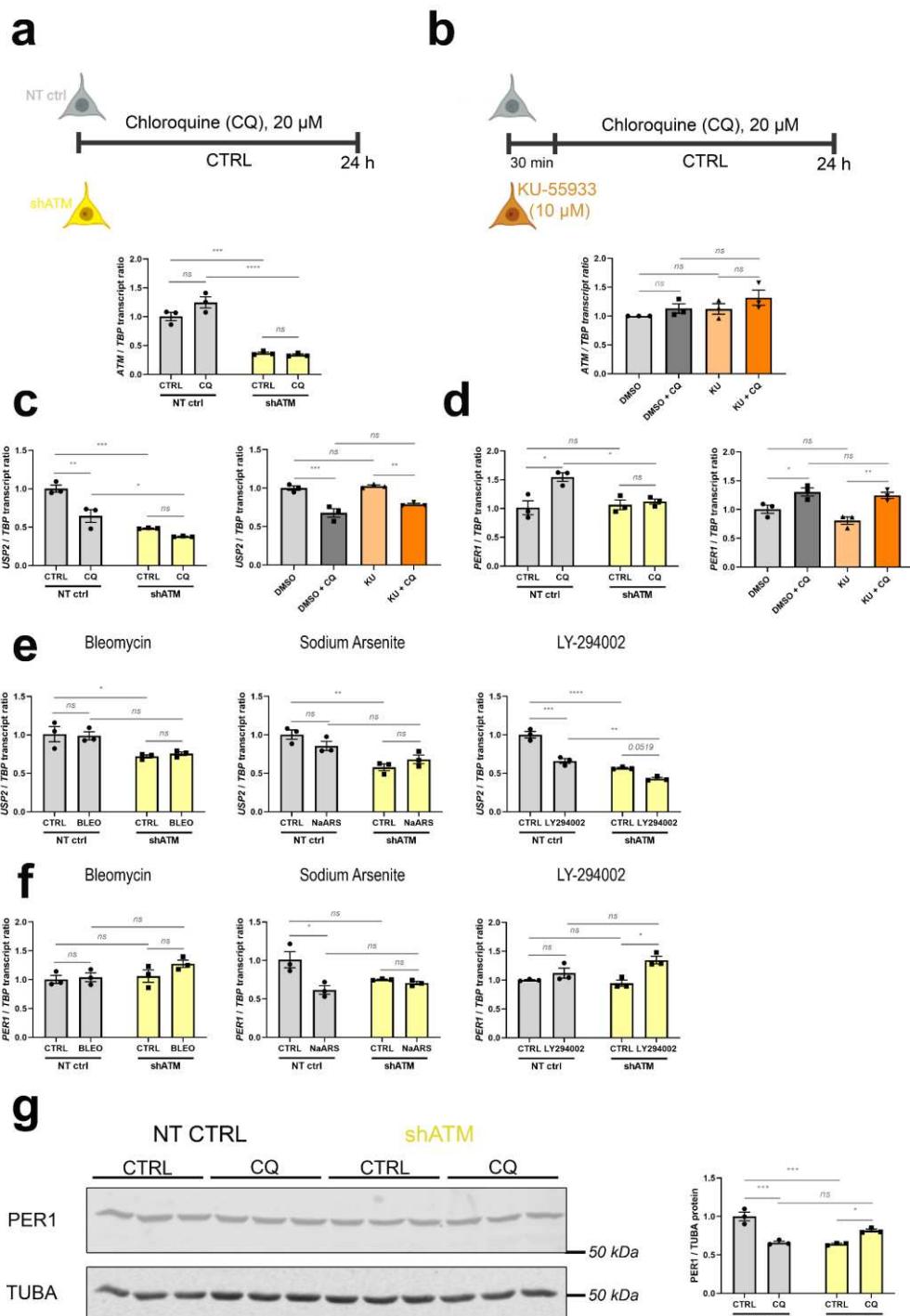


Figure 4. ATM-KD successfully models the dysregulations found *in vivo*, while inhibition of ATM kinase function is not effective. (a) Scheme of chloroquine (CQ) stressor treatment in ATM-KD SH-SY5Y cells (upper panel) and corresponding *ATM* transcript levels determined via RT-qPCR ($n = 3$, lower panel). (b) Scheme of CQ stressor treatment in parental SH-SY5Y cells with KU-55933 ATM kinase inhibitor pretreatment (upper panel) and corresponding *ATM* transcript levels determined via RT-qPCR ($n = 3$, lower panel). (c) Comparison of ATM interactor *USP2* mRNA and (d) immediate early transcript *PER1* levels in ATM-KD (left) vs ATM-kinase-inhibited parental cells (right) after CQ stress, as determined via RT-qPCR ($n = 3$). (e) Reduction of *USP2* transcripts upon CQ-stress and in ATM-KD is largely reproduced by LY-294002 stressor treatment, but not after BLEO and NaARS treatment of ATM-KD cells ($n = 3$). (f) BLEO, NaARS and LY-294002 stressor treatments do not reproduce the ATM dependent *PER1* transcript induction present in aged ATM-null cerebellum, which was however successfully modelled after CQ-stressor treatment of ATM-KD cells ($n = 3$) as seen in panel (d). (g) *PER1* protein is reduced by CQ-stress administration in NT CTRL cells and generally in shATM cells, as determined by quantitative immunoblots ($n = 3$). For statistical trends, the precise p-value was shown. Asterisks display significance. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = non-significant. Data were displayed as bar plots with data points, mean \pm SEM.

3.4. Also in human cells, ATM-deficiency impacts key pathomechanism factors like interactor *ATMIN*, immediate-early mRNA *FOXO3*, osmotic regulator *RRAGD*, vasoconstriction regulator *ECEL1*, and ataxia transcripts *GRID2*, *ITPR1* and *MME*

Although the global transcriptome profile of old ATM-null cerebellum identified many novel pathogenesis events, it remained unclear to what degree these findings are conserved in human, and whether they can be explained by osmotic stress. Therefore, validation experiments were conducted with RT-qPCR and quantitative immunoblots to assess key factors in human SH-SY5Y cells with ATM-KD, unstressed or after CQ administration. For validation of individual dysregulations, we selected crucial effectors of ATM function and critical determinants of the phenotypes that characterize A-T.

In parallel to the desired reduction of *ATM* in SH-SY5Y knockdown cells documented in Figure 4a, these further studies (see Figures 5 and S4) confirmed strong genotype-dependent downregulations for *ATMIN* mRNA (to 71% and 76%) as mediator of ATM responses to osmotic and oxidative stress. *NR4A1* and *FOXO3* mRNA as immediate-early mediators of phosphorylation signals to the nucleus, both were found to be responsive to CQ-stressor treatment (1.5-fold and 1.6-fold increase, respectively), while displaying abrogated induction in shATM cells (Figure 5a, Figure S4a). Also the CQ-triggered inductions of calcium-dependent kinases *CAMK2A* and *CAMK4* mRNA (4.1 fold and 1.3 fold, respectively) were significantly impaired upon ATM-KD (Figure S4a). Importantly, an ATM-dependent mRNA downregulation (to 32% and 37% for unstressed and stressed condition, respectively) was also observed for the ataxia gene *ITPR1* and might therefore be interpreted as a loss-of-function that may have a primary role in the pathogenesis of autosomal recessive A-T, while the other ataxia genes *GRID2* [99, 100] and *MME* [101] showed ATM-dependent mRNA upregulations (2.4 fold and 3.4 fold for *GRID2*; 1.6 fold and 3.0 fold for *MME*, in unstressed and stressed ATM-KD cells) that may represent compensatory efforts, and the ataxia transcript *RORA* [102, 103] exhibited only a response to osmotic stress (1.8 fold increase in CQ treated cells; Figure 5a). As further evidence for compensatory reactions to osmotic stress, *RRAGD* mRNA (encoding Ras-related RagD amino acid sensor [104, 105]) showed significant upregulation after CQ treatment, and even bigger upregulation after ATM-KD (4.0 fold induction in NT CTRL cells and 8.1 fold increase in stressed shATM cells, Figure 5a). Also *SGK1* transcript was induced only upon CQ treatment in shATM cells (1.4-fold, $p = 0.3903$ and 2.4-fold, $p = 0.0007$) corroborates the presence of osmotic stress (Figure S4a). As putative modifier of vasodilatation, *ECEL1* mRNA [106, 107] was found upregulated after CQ stress in NT CTRL cells (1.4-fold, Figure 5a) and after oxidative stress in ATM-KD cells (1.4-fold), as well as after CQ stress during ATM kinase inhibition (1.2-fold) (Figure S4b).

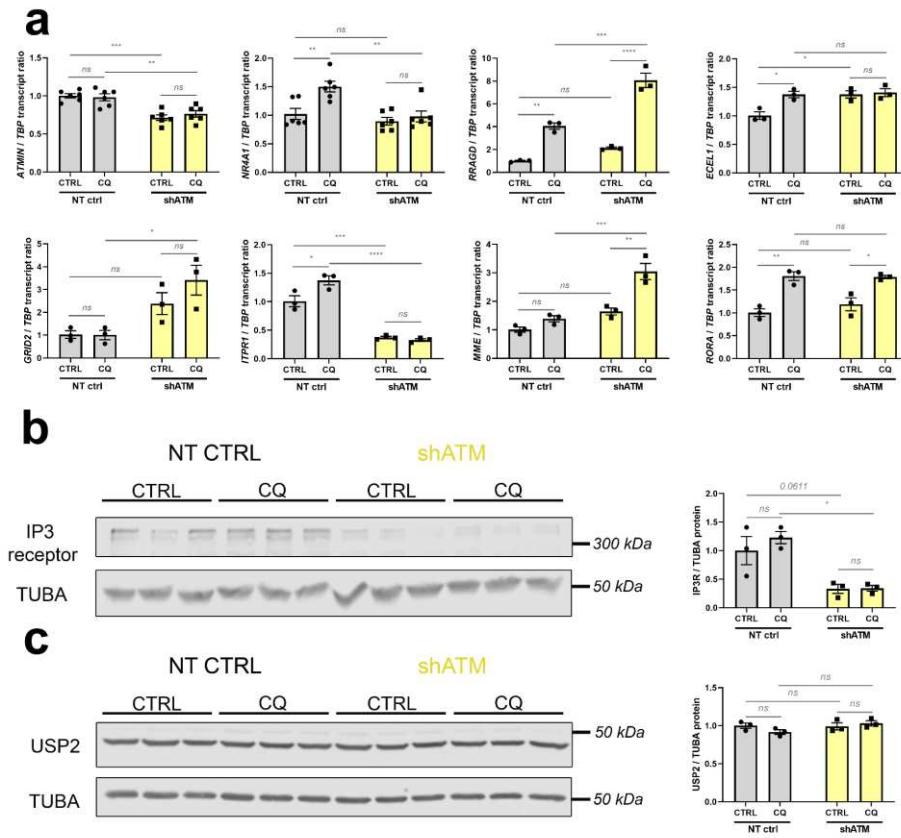


Figure 5. The stressor chloroquine provides the most effective model in SH-SY5Y ATM-KD cells, for representative strong dysregulations in different pathways, which were previously documented in aged ATM-null cerebellum. (a) *ATMIN* mRNA ($n = 6$) was analysed as interactome component of ATM. *NR4A1* ($n = 6$) was analysed as immediate early gene. *RRADG* ($n = 3$) transcript levels were analysed as positive controls for osmotic stress elicited by CQ. *ECEL1* ($n = 3$) and *MME* ($n = 3$) were analysed for the group of neuropeptide endopeptidases. mRNAs for *GRID2* ($n = 3$), *ITPR1* ($n = 3$) and *RORA* ($n = 3$) were analysed as known ataxia disease genes. **(b)** The protein Inositol-1,4,5-Trisphosphate receptor (IP3R, encoded by *ITPR1*) was also significantly reduced in shATM cells compared to NT CTRL cells as determined by quantitative immunoblots, while induction by CQ stressor treatment did not reach significance ($n = 3$). The double band around 315 kDa was quantified by densitometry. Tubulin A (TUBA) was used as sample loading control and normalizer, in view of its high abundance similar to IP3R. **(c)** USP2 protein appeared unchanged in quantitative immunoblots ($n = 3$) of shATM cells compared to NT CTRL cells, despite the transcript induction shown in Figure 3e. For statistical trends, the precise p-value was shown. Asterisks reflect significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = non-significant. Data were displayed as bar plots with data points, mean \pm SEM.

Quantitative immunoblots were conducted when commercial antibodies were available to us with sufficient specificity and sensitivity to detect the endogenous protein levels. For the human IP3R protein encoded by *ITPR1* transcript, these experiments confirmed a strong reduction of abundance (to about 35%) upon ATM-deficiency (Figure 5b). In contrast, the quantitative immunoblots indicated the protein levels of USP2 to be unchanged by CQ and by ATM-KD (Figure 5c), so apparently the significant *USP2* mRNA reduction after CQ and ATM-KD demonstrated in Figure 4c does not rapidly impact the steady-state immunoreactivity, and it may be that posttranslational control of USP2 and its MDM4/HDMX-MRN-complex-association [86, 108, 109] are more decisive for short-term USP2 activity regulation than its resynthesis. Still, our mRNA findings confirm USP2 as very consistently ATM-dependent factor whose expression is modified by cytosolic ATM in neural cells, and which

functions very upstream in the ATM-dependent stress response pathways – so it might be a useful target of novel preventive therapies.

3.5. ATM-null mouse cerebellum alternatively spliced mRNAs are enriched for neurotransmission and neuropeptide signaling factors

For rapidity, stress responses are conveyed through the cytoplasm by phosphorylation signals to adapt transcription of immediate-early genes in the nuclear chromatin, but even more directly stress signaling readjusts the alternative splicing of existing transcripts in the nucleus and the editing of actively translated transcripts in the cytosol. Since the ClariomD microarrays employed for cerebellar transcriptome profiling represent almost all exons, we also assessed splice dysregulations in this dataset from aged ATM-null mice, employing the “Alt Splice View” function in the Transcriptome Analysis Console from AppliedBiosystems. Interestingly, upon filtering for significant changes (p -value < 0.05 , FDR p -value < 0.25) with strong fold-change (Exon Splicing Index $>/< +/- 5$), a set of 40 alternatively spliced transcripts was identified. Of these, 31 displayed an overall increased exon splicing index, while 9 showed reduced exon splicing index (Figure 6a). More detailed assessment revealed that most of them represent altered quantities of a single exon within a dysregulated transcript, and because of concerns that not all oligonucleotide probes within a mRNA can be expected to exhibit parallel linear signal changes and might therefore mimic alternative splicing artificially, we decided to annotate such observations for the main candidates (Table S1, second datasheet). Again the neuropeptide signaling pathway (FDR $q=1.16e-6$), regulation of neurotransmitter levels and synapse organization were prominent, as identified by the STRING Web browser (Figure 6b).

Oprm1 stood out with an overall exon splicing index of 39.17, which is summarized graphically (Figure 6c) by the Transcriptome Analysis Console (Applied Biosystems) for each oligonucleotide probe along the transcript structure. Extrapolating probes within individual exons or at the junction between known exons or cryptic exons from these data in mouse onto known facts in human, we tested the credibility of these *Oprm1* splice changes by RT-qPCR in human neuroblastoma cells. It should also be taken into account that an oligonucleotide probe within an exon may show a differential increase or decrease, while a Taqman assay is usually quantifying the amplification product at the junction between two adjacent exons, so a splice change may be detectable by a specific RT-qPCR assay but not the neighboring assay, and the effects may not be conserved between species, with differing exon number nomenclature. As shown in Figure 6d, the exon 2-3 boundary of *Oprm1* exhibited a 15-fold signal increase in old ATM-null cerebellum, while the exon 5-6 junction signal was unaltered. In the human SH-SY5Y cells, the RT-qPCR results of exon 1-2 and exon 3-4 boundaries in *OPRM1* transcript also showed a massive dysregulation upon ATM deficiency (reduction to 5%), with no significant change after osmotic stress (Figure 6e). This downregulation was not detected upon ATM kinase inhibitor treatment (Figure S4c), where *OPRM1* transcript levels remained stable. *OPRM1* transcript downregulation exclusively in the ATM-KD condition was robustly reproduced also in experiments with BLEO, NaARS and LY-294002 (Figure S4c), reflecting stressor-independent effects of ATM deficiency itself. Thus, although the splicing details may differ between species and cell type, with cerebellar tissue even revealing opposite effects than cultured neuroblastoma cells, the mouse microarray data and validation experiments by RT-qPCR in mouse cerebellum and human neuroblastoma cells clearly identified the opioid mu receptor as mRNA under control of ATM.

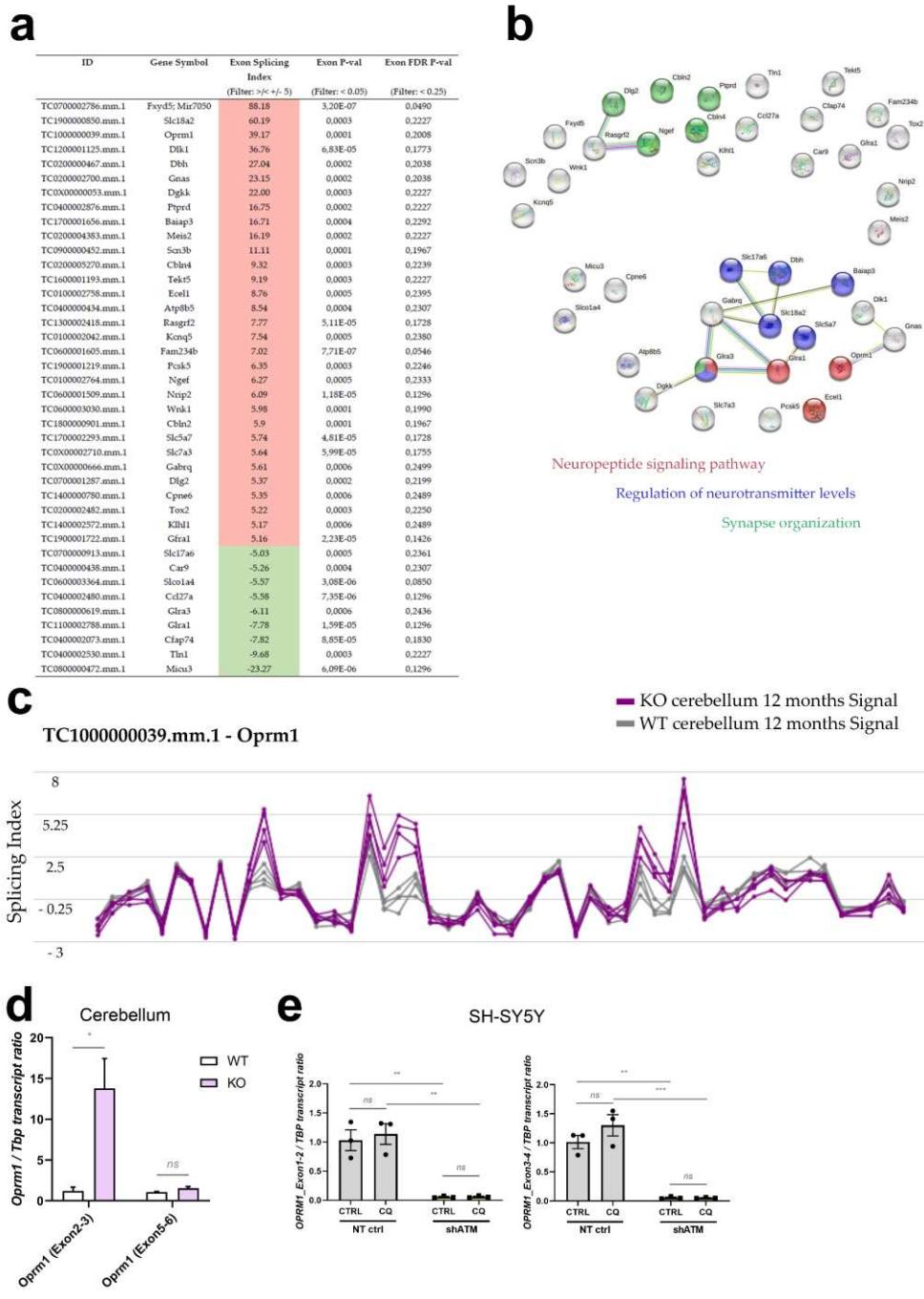


Figure 6. Genome-wide survey of alternative splicing in cerebella from 12-month-old ATM-null mice reveals enrichment for neuropeptide signaling pathways and neurotransmission. (a) Table of all transcripts that display excessive alternative splicing (Filter criteria: Exon Splicing Index $>/< +/- 5$, Exon p-value < 0.05 , Exon FDR p-value < 0.25 and Group: Multiple Complex and Coding). Increased exon splicing index is highlighted in red, decreased exon splicing index in green. (b) String functional connection networks (<https://string-db.org/>) of these alternatively spliced transcripts. Red buttons belong to the neuropeptide signaling pathway, blue buttons are implicated in regulation of neurotransmitter levels, and green buttons exert functions during synapse organization. (c) Structure view of the *Oprm1* transcript structure with splicing indexes displayed in line plots for WT (grey) and *Atm*-KO (purple) cerebellum. Validation experiments in (d) cerebellum of 12-month-old *Atm*-KO vs WT (4 vs 4) mice and (e) SHSY5Y-KD cells under CQ-stress. Asterisks reflect significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, ns = non-significant.

4. Discussion

Overall, the novel transcriptome profile of 12-month-old ATM-null cerebellum shed light on the primary role of osmotic stress in A-T pathogenesis, identified molecular correlates of A-T phenotypes such as incipient ataxia / vasodilatation / growth impairment, defined dysregulations of interactor molecules of cytosolic ATM that may represent useful upstream targets of neuroprotection, and documented generalized affection of neurotransmission and neuropeptide signaling – presumably mediated by cytoplasmic vesicles that have ATM protein associated to them. Given that the validation experiments were able to reproduce faithfully many dysregulations in human cell culture models of A-T, it is worthwhile to take all cerebellar dysregulations seriously and discuss the novel evidence extensively.

4.1. The cerebellar transcriptome profile of ATM-null mice at 12 months of age

This cerebellar transcriptome profile was exceptionally informative, probably because protein kinases such as ATM are part of phosphorylation cascades that relay information on membrane events to the nucleus, governing transcriptional responses to stimuli versus stress. Before experiments in a cell culture model of ATM dysfunction validated whether individual dysregulations are reproducible in human, and how they depend on stress, it is important to understand the relevance of these key factors within the complex pathogenesis of cerebellar A-T. Thus, we feel the necessity to discuss the integration of all the strong dysregulations with multiple weaker effects within the same significantly enriched pathway, because often upstream events are small while subsequent signaling cascades will amplify the fold-changes of downstream molecular events. After identifying relevant changes in upstream coordinators and mechanisms of each affected pathway, it is important to explore how they are connected to ATM and how they overlap with other cerebellar ataxias. This discussion text aims to describe a coherent scenario where the failure of stress responses and the underlying toxic agents can be understood better.

To better understand the observations, it may help to consider the analogies between the cerebellar pathology in A-T on the one hand, versus the common sunburn on the other hand. Ionizing radiation and ultraviolet-B-light (UVB) light are typical causes of DNA-DSB in the nucleus, which are sensed by ATM to coordinate repairs. UVB is also the typical cause of sunburns in skin tissue, where not only DNA-DSB is known to ensue, but also cytoplasmic effects like calcium-dependent excitation with chemokine / cytokine release, vasodilatation, inflammation, pain, and keratinocyte death or carcinogenesis [110, 111]. It is already known that ATM is needed after sunburns to mitigate UVB damage and restore normal cell growth [112-114], so ATM deficiency is indeed expected to result to impact cytoplasmic homeostasis, including prolonged vasodilatation, edema and pain via peptide signaling. The present transcriptome data provide the molecular details for a similar scenario of pathomechanism in cerebellar tissue.

Regarding the prominent neurotransmission effects of cytosolic ATM, previous investigations had reported it to be key for glutamatergic excitation, while ATR was implicated in a complementary role for inhibitory GABAergic neurotransmission [39]. Indeed, our hypothesis-free microarray profiling observed a widespread profound affection of the glutamatergic pathway, reflected by downregulations of receptors *Grid2* (which is responsible for Spinocerebellar Ataxia type 18 [115]), *Grid2ip* [116], *Gria4*, *Grik2*, *Grin2c*, *Grm1* (responsible for autosomal recessive Spinocerebellar Ataxia type 13, and autosomal dominant Spinocerebellar Ataxia type 44 [117, 118]) and *Grm4*, the glial high affinity glutamate transporter *Slc1a3* (encoding EAAT1 / GLAST which is responsible for Episodic Ataxia type 6 [119, 120]) and *Slc1a6* (encoding EAAT4 which is involved in Spinocerebellar Ataxia type 5 [121, 122], the mitochondrial glutamate transporter *Slc25a22*, and an eye-catching contrast between downregulation of transporter *Slc17a7* (encoding VGLUT1 in parallel fibers of the cerebellar cortex), versus massive upregulation of *Slc17a6* (encoding VGLUT2 in climbing fibers of the deep cerebellum) [123]. Glutamatergic upregulations also affected *Grm5*, *Grm3*, *Grm8*, *Grin2b*, *Grin3a*, *Grid1*, *Slc1a1* (encoding EAAT3), *Slc1a2* (encoding EAAT2 / GLT1), *Slc1a4* (encoding ASCT1), the AMPA-receptor interactor *Nsg2* [124], the glutamate receptor interactor *Grip1* [125], and excitation-repressing *Cnr1* [126].

While extending our notions about a glutamate-focused ATM role, this transcriptomic approach permitted the additional insight that ATM loss also upregulates receptors in the inhibitory GABA-pathways (*Gabra3*, *Gabra5*, *Gabra2*, *Gabrg1*, *Gabrg3*, *Gabrg2*, *Gabrq*, *Gabre*, *Gabrb1*, *Gabarapl1* (contrasted by downregulation only for *Gabra6*), together with upregulation of GABA-transporters *Slc6a11* and *Slc32a1*, as well as receptors in the inhibitory glycine (*Glra1*, *Glra3*, *Glra2*, *Glra4*), and in the dopamine (*Drd2*, *Drd5*), acetylcholine (*Chrm2*, *Chrna4*, *Chrm3*, *Chrna7*, *Chrna6*, *Chrm5*, *Chrb4*, *Chrb3*) systems. These data suggest that ATM plays a main role for stress adaptation of synaptic vesicles not only for excitatory but also for inhibitory signaling, possibly via the regulation of vesicle availability / loading / release / recycling.

Regarding neuropeptides and their receptors, additional general affection of these signaling pathways was documented, with upregulations of somatostatin (*Sst*, *Sstr1*, *Sstr2*), neuropeptidin (*Nts*, *Ntsr1*, *Ntsr2*), tachykinin (*Tac1*, *Tacr1*, *Tacr3*), neuropeptide-Y (*Npy*, *Npy1r*, *Npy4r*) mRNAs, and *Resp18* mRNA encoding a factor responsible of neuropeptide packing in dense core vesicles [127-129], as well as *Qpct* encoding a factor responsible for the N-terminal pyroglutamyl residues of neuropeptides and cytokines [130]. Also *Oprm1* as mu-type (morphin-type), *Oprl1* as kappa3-type (nociception-type), and *Oprk1* as kappa1-type (for alpha-neoendorphins and dynorphins) of opioid receptors showed upregulated transcripts. These cerebellar findings identify molecular mechanisms how ATM deficits trigger not only excessive changes in neurotransmission, but may come to impact vasodilatation / telangiectasia and inflammatory edema (via tachykinins, *Ecel1*), growth and fertility (via somatostatin and neuropeptide-Y), immunity and lipid metabolism (via neuropeptidin), as well as pain perception (via opioids). As one of the strongest upregulated transcripts, *Rgs4* is a known regulator of G-protein signaling downstream from mu- and kappa-opioid signaling [131-134]. Even the neuropeptide activator *Pcsk1* and its inhibitor *Pcsk1n*, as well as neuropeptide inactivators like *Mme* (which is responsible for Spinocerebellar Ataxia type 43 [101]) and *Ecel1* were upregulated. These data suggest that ATM plays a stress adaptor role in general also for dense core vesicles where neuropeptides are stored.

Regarding the atrophy of aged cerebellum in A-T, it is plausible to pay attention to neurotrophins and other cytokines, which are stored in large dense core vesicles (LCDV) before their release and where ATM might play a similar role as for neuropeptides. Indeed, upregulations of neurotrophin receptors *Gfra1* and *Gfra2* [135, 136], inhibitory neurite growth modulators *Slitrk3* [137, 138], *Slitrk5* [139] and *Slitrk6* [140], neuronal sorting receptors *Sorcs1* and *Sorcs2* [141-144], stress-dependent transcription factor *Jun* with its kinase *Mapk9* / *Jnk2* [145, 146], and heavy-metal-toxicity-inducible death executor *Ngrap1* [147, 148] suggest at first glance that LCDV pathology might contribute to a trophic imbalance in the cerebellum. Furthermore, a downregulation of the ligands *Nrg1* and *Nrg3* with converse upregulation of their receptor *Erbb3* and *Erbb4* transcripts was observed [149]. However, while systematic interrogation confirmed exclusively upregulations as in neurotransmission and neuropeptide pathways, a similar systematic interrogation of cytokine receptors and their ligands did not reveal a similarly uniform effect. Crucial downregulations of neurotrophin *Ntf3* with its receptor *Ntrk3*, as well as the sorting receptor *Sor1l* [150] were observed. On the one hand, increased transcript levels were documented for *Tgfb1*, *Tgfb2*, *Pdgfra* (with ligand *Pdgfc*), *Epha4*, *Epha5*, *Epha5* (with ligands *Efna2*, *Efna3*, *Efna5*), *Fgfr3* (with ligands *Fgf18* and *Fgf5*), *Fgfr1op2*, *Csf1r*, *Bmpr1b*, *Lifr*, *Atp2b4*, *Tnfrsf13c*, *Tnfrsf21*, *Fzd8*, *Sfrp5*, *Kdr*, and ligands *Bmp2*, *Fgf13*, *Efnb3*, *Il18*, *Il33*, *Il34*, *Igf1* without their receptors. On the other hand, a smaller number of conversely decreased transcript levels were documented for *Fzd4* (with downregulated ligands *Wnt3*, *Wnt7a*), *Igf2r* (with downregulated ligand *Igf2*), *Igflr1*, *Epha3*, *Pdgfrl*, *Sfrp1*, *Kit*, *Socs7*, *Il20rb*, and ligands *Bmp1*, *Bmp7*, *Fgf14*, *Il16* without their corresponding receptor. Thus, while a systematic effect of ATM on all cytokines and neurotrophins is doubtful, specifically the deficits of *Ntf3* and *Ntrk3* are relevant for the survival of cerebellar granule neurons in a mechanism via Phospho-inositol-3'-kinase (PI3K). Importantly, the vesicle release of neurotransmitters, neuropeptides and neurotrophin-3 has a common upstream mediator in *Cadps2*, which showed decreased cerebellar mRNA levels [151-156]. The balance between neurotrophin support and glutamate neurotoxicity is known to be critical also for the survival of Purkinje neurons [157-160].

What other pathways were impacted in several components across the cerebellar transcriptome profile of ATM-null mice, given that the hypothesis-free global transcriptomics approach might give novel clues to understand A-T pathogenesis better? Second messengers downstream from neuropeptide receptors appeared altered, in view of the G-protein signaling factors *Rgs4*, *Rasgrf2* and *Gpr165* upregulations [131, 161, 162], and the dysregulation of calcium modulators *Necab1* (up) [163] versus *Itpr1* (down, its loss-of-function being the cause of autosomal dominant Spinocerebellar Ataxia types 15 and 29, as well as autoimmune cerebellar ataxia) [57, 164-166]. The decreased mRNA levels of inositol-trisphosphate receptor *Itpr1*, and of *Cadps2* (the Ca^{2+} -dependent release activator for neurotransmitters, neuropeptides and neurotrophins), might be underlying contributors to this generalized pathology, given that loss-of-function of both downregulated factors results in cerebellar ataxia [154, 164, 167-169]. As further potentially upstream coordinators of pathology, the deficits of inositol-triphosphate-associated *Astn2*, *Sorl1*, and *Mpp4* levels could lead to inappropriate localization of membrane proteins away from the tip of neural processes [170, 171].

Regarding upstream factors within the protein interactome of ATM, the following dysregulations deserve discussion: As a key modulator much farther upstream in the pathomechanism, the increased levels of ATM interactor *Atmin* mRNA probably represent a compensatory response to ATM dysfunction, possibly affecting synaptic adhesion [172, 173]. The *Atmin* upregulation upon ATM deficiency was unexpected, since both factors were thought to stabilize each other, with ATM levels being reduced upon ATM decrease, and vice versa [60, 83]. The ATM-interacting MRN complex responsible for DNA damage signaling did not show any changed mRNA levels, but the NBS1 stabilizing factor *Usp2* mRNA displayed a downregulation of similar effect size and significance as *Atm* mRNA [86]. USP2-null mice show impaired motor coordination and equilibrium [174], so its deficiency in ATM-null cerebellum might contribute to ataxia pathogenesis. This deubiquitinase is also known as regulator of circadian clock components [175], and indeed several USP2 effectors also showed deficient transcript levels, such as *Cry1* and *Cry2*. In view of the role of KAT5 for the regulation of ATM activity it may also be relevant that a transcript reduction in our dataset was observed for the KAT5-dependent kinase *Chka*, which is responsible for phospholipid biosynthesis [176, 177].

Overall, excitation and growth stimuli in the ATM-null mouse cerebellum appeared to elicit deficient nuclear responses, in view of the downregulation of immediate-early genes *Nr4a3* / *Nr4a2* / *Nr4a1*, *Dusp1*, *Fos* / *Fosl2*, *Npas4*, *Per1* / *Per2* / *Per3*, *Foxo3* / *Foxo1*, and *Homer1*. A parallel downregulation of the NPAS4 protein interactor *Arnt* and its binding partner *Hif3a* mRNA were observed, as well as reduced transcript levels of downstream factors *Slc2a12* and *Rora* (which is responsible for ataxia and intellectual deficits [178, 179]), contrasting with upregulation of the alternative interactor *Arnt2* mRNA [180-184]. With relevance to the osmotic homeostasis in ATM-null cerebellum, a strong downregulation was observed for *Dbp* as the transcription factor that controls the expression of alpha-fetoprotein and albumin (whose dysregulation is characteristic for A-T [4, 5]), and is crucial for circadian regulation of synaptic plasticity [185-187]. The notion of changed nutrient and osmotic regulation was also supported by the downregulation of amino acid-sensing *Rragd* [104]. Downregulation was prominent for immune-regulating and damage-responsive protein kinase transcripts *Smg1*, *Sik1* and *Sgk1* [188-190], findings that also implicate altered RNA surveillance, osmotic and nutrient homeostasis in A-T pathology.

A deficit in inflammatory responses was also evident from the downregulated transcripts of *Ccl27a*, *Sidt1*, *Il16*, *Rnfl22* and *Serinc2* [191-195]. The deficiency of immunoglobulin/fibronectin-domain-containing *Boc* may contribute to the observed upregulation of many protocadherin, cadherin, and contactin pathway members (*Cdh6*, *Cdh9*, *Cdh10*, *Cdh19*, *Pcdh7*, *Pcdh10*, *Pcdh11x*, *Pcdh17*, *Pcdh18*, *Pcdh19*, *Cntn4*, *Cntn5* and *Cntnap5a*) [196].

In conclusion, pathway enrichment analyses of the transcriptome profile supported the novel concept that failure of ATM-mediated adaptation to osmotic / nutrient and perhaps oxidative stress, via altered USP2 / ATM signals, leads to a generalized abnormality in neurotransmitter-neuropeptide signals from synaptic and dense core vesicles, with reduced immediate-early signals and impaired synaptic adhesion.

4.2. The alternative splice profile of ATM-null mouse cerebellum at 12 months of age

Previous studies of ATM dysfunction demonstrated its impact on alternative splicing [53], and of course the stimulus- / stress-dependent changes in phosphorylation cascades would first impact the splice apparatus before topoisomerase-dependent immediate-early reactions and more cumbersome chromatin unpackaging events permit the subsequent transcription adaptations. Overall, our interrogation of the transcriptome for factors with strong exon splicing index effects confirmed significant enrichments in three pathways. Neurotransmission (*Slc17a6*, *Slc18a2*, *Slc7a3*, *Slc5a7*, *Slco1a4*, *Scn3b*, *Kcnq5*, *Gabrq*, *Glra1*, *Glra3*, *Dbh*, *Wnk1*, *Car9*, *Micu3*), neuropeptides / neurotrophins (*Oprm1*, *Baiap3*, *Gnas*, *Dlk1*, *Dlg2*, *Rasgrf2*, *Ngef*, *Ecel1*, *Dgkk*, *Ccl27a*, *Pcsk5*, *Cpne6*, *Ptprd*, *Gfra1*) and synaptic adhesion (*Fxyd5*, *Cbln4*, *Cbln2*, *Nrip2*) modulation was prominent. *Baiap3* splice changes could contribute to impaired biogenesis of secretory vesicles, with consequences for the Ca^{2+} stimulated release of neurotransmitters and neuropeptides [197]. While cerebellar tissue has a broad expression profile, a neural cell culture model would express only a small subset of these molecules, so further validation experiments were focused on *Oprm1*, which exhibited the exceptional exon splicing index of 39.7, and acts to dampen glutamatergic neurotoxicity in the contacts between cerebellar granule neuron projections (parallel fibers) and Purkinje neuron dendrites [198-200], which is the cerebellar site most vulnerable to ataxia pathogenesis [201, 202]. With a prominent negative exon splicing index of -5.03 ($p=0.005$), *Slc17a6* (encoding VGLUT2) also displayed evident adaptation of its exon structure, providing additional evidence that also glutamatergic climbing fiber signaling is modulated by ATM. In contrast, *Slc17a7* (VGLUT1 in parallel fibers) showed a change only for its 3'-exon with nominal significance and an exon splicing index of 1.6. While the ClariomD microarray has oligonucleotides to detect sequences within most exons, the Taqman RT-qPCR assays in contrast are optimized to detect exon-exon-boundaries, so a validation experiment by RT-qPCR can only confirm the dysregulation of a specific mRNA overall, and may detect whether it disappears for a specific exon, but will not quantify the selective inclusion / exclusion of an exon. Overall, it is important to be cautious regarding the value of this splicing profile, because dysregulations of a complete mRNA may not be equally represented by every oligonucleotide, mimicking true alternative splice changes, and because experimental quantitative validation across species is cumbersome. Thus, we consider these data as preliminary screen.

4.3. Differential detergent fractionation of adult mouse cerebellum and SH-SY5Y neuroblastoma cells detects ATM mainly in cytosol

For validation of these mouse findings in the human species, a knockdown of ATM in neuroblastoma SH-SY5Y cells was employed, taking into account the previous usefulness of such human *in vitro* modelling projects in autophagy and chemoresistance studies of ATM [203, 204]. Neuroblastoma cell lines are known to represent a mixed population, termed N-type (neural) and S-type (substrate-adherent, epithelial like) cells. While N-type cells are neuroblast-like with little cytoplasm and few neuritic processes, the S-type cells have bigger cytoplasm and flattened morphology with strong attachment to the substrate [205-208]. Unexpectedly, ATM-KD SH-SY5Y cells displayed gross alterations in appearance, with larger and flattened cell bodies without processes, while the non-targeting control (NT CTRL) cells retained an overall neuroblast-like appearance and displayed short neuritic processes (Figure S2). Thus, flattened cells without processes that are predominant for ATM-KD cells could reflect a shift in neuroblastoma cell populations towards S-type cells. The remodelling of Ca^{2+} signaling was already demonstrated to be altered in S-type cells [206]. Given that SH-SY5Y cells were already shown to require ATM mediated phosphorylation of CREB protein at serine 133, to enable retinoic acid induced differentiation [209], this change of gross morphology in ATM-KD cells may represent a loss of differentiation. Indeed, dysregulation of retinoic-acid dependent differentiation regulators was also evident in the 12-month-old ATM-null mouse cerebellar transcriptome, where most components of this well characterized pathway [210-212] were strongly dysregulated, displaying increased *Nrip2* (Fig. 2a), *Nrip3*, *Rorb*, *Creb5*, *Crebl2* and *Zflhx3* mRNA levels versus decreased expression of *Tcf4*, *Crtc2*, *Foxo3* and the ataxia disease gene *Rora*, as well as downstream *Itpr1* [213, 214]. A more proliferative state of the ATM-KD

cells may result in reduced expression of many neurotransmission factors, potentially explaining the drastic downregulation of *OPRM1* in ATM-KD neuroblastoma cells, as opposed to increased *Oprm1* in ATM-null cerebellum (Figure 6d). Although some dysregulations of neuropeptide signaling were massive in the old ATM-null mouse cerebellum and were very relevant for phenotypes of A-T, e.g. the increases in mRNA of growth hormone inhibitor somatostatin (*Sst*), vasodilator preprotachykinin (*Tac1*) and the tachykinin receptor (*Tacr1*), as well as the glutamate-excitability factor VGLUT2 (encoded by *Slc17a6*), the SH-SY5Y neuroblastoma line did not express these genes. It is also important to consider that glutamate availability is very restricted for excitable neurons *in vivo*, but provided constantly in overdose to cultured neuroblastoma cells. Thus, our *in vitro* model had very limited value to model and study the ATM-dependent stress-adaptations of neurotransmitter- and neuropeptide-containing vesicles, but seemed quite helpful for the study of upstream factors in the interactome of ATM and of immediate-early responses in the nucleus.

The use of differential detergents to achieve subcellular fractionation of the old adult mouse cerebellum and of SH-SY5Y neuroblastoma line localized ATM mainly to the cytosol, both in unstressed and in stressed conditions, in WT cells and ATM-KD cells (Figure 3, Figure S3). The absence of ATM from the nucleus is in excellent agreement with subsequent findings that characteristic ATM-null cerebellar transcriptome profile anomalies such as *USP2* and *PER1* downregulations could not be elicited robustly by the DNA DSB stressor bleomycin *in vitro*, but were mirrored best by osmotic stress instead (Figure 4). Our observations that ATM is almost exclusively found within cytoplasmic fractions of cerebellum and SH-SY5Y cells are in excellent agreement with a previous immunohistochemical study that localized ATM in the cytosol of cerebellar tissue Purkinje neurons from this mouse mutant [32], but they contrast with human reports and with the immunohistochemical observation of ATM in the nucleus of cerebellar Purkinje and granule neurons, once they are dissected and kept in organotypic slice cultures [215, 216]. While nuclear ATM clearly has a role for DNA repair in proliferating cells, these fractionation findings emphasize the urgent need to understand what the functions of cytosolic ATM in postmitotic neurons are, and how impaired stress adaptation there might trigger a neurodegenerative process. Given the inefficiency of ATM kinase inhibition by KU-55933 to reproduce transcript changes observed in cerebellar tissue and upon ATM-KD *in vitro*, we consider the possibility that cytoplasmic ATM acts as a protein scaffold and interaction platform, rather than a kinase in cytoplasmic signaling. This was already proposed when cytoplasmic ATM was demonstrated to serve as docking site for PP2A to dephosphorylate AKT and thereby regulate cell death upon ER-stress via an ATM-AKT-GSK3 β - α NAC/ γ TX signaling axis [217]. Further research is necessary to elucidate the potential functions of ATM as a protein scaffold in the cytoplasm.

4.4. Validation work in SH-SY5Y cells shows ATM-deficiency to impair the CQ-triggered regulation of postsynaptic calcium release channel ITPR1, in parallel to immediate-early transcripts PER1/NR4A1

The mechanistic validation experiments (Figure 4, 5, S4) focused on A-T-phenotype-related factors, documenting consistent reductions for ITPR1 levels upon ATM deficiency. Given that ATM is a member of the phosphatidylinositol 3' kinase-like kinase (PIKK) enzyme family, it may have functional interaction with the inositol-1,4,5-trisphosphate-receptor IP3R, so this decrease of *ITPR1* mRNA and IP3R abundance may also represent a crucial primary loss-of-function event in autosomal recessive A-T. Genetic loss-of-function of IP3R has a profound impact on calcium-dependent excitability of Purkinje neurons and was repeatedly observed as sufficient to cause hereditary progressive cerebellar neurodegeneration, with deletion of one *ITPR1* gene copy via haploinsufficiency triggering ataxia inheritance in autosomal dominant manner [218, 219]. Thus, our observation of IP3R abundance reduction below 50% in ATM-KD neuroblastoma cells (Figure 5b) emerges from the transcriptome profile validation work as arguably the most important molecular event, which might explain the preferential affection of cerebellar neurons [57]. In this context it is important to note that parallel loss of IP3R together with two more ataxia-responsible proteins was previously demonstrated in cerebellar tissue of A-T patients [66], namely the calcium homeostasis factor INPP5A (also known as Type I Inositol 1,4,5-Trisphosphate 5-Phosphatase) [220] and the CA8

(also known as Carbonic Anhydrase 8) [221, 222]. Furthermore, a protein complex of ITPR1 as endoplasmic reticulum Ca^{2+} homeostasis regulator with the mitochondrial HSP70-family member / chaperone GRP75 (=HSPA9) and the mitochondrial voltage-gated Ca^{2+} homeostasis channel VDAC1 was previously shown to mediate ATM dysfunction in bronchial cells after nutrient stress [30]. A very similar expression alteration in the ATM-null mouse cerebellar transcriptome may therefore be relevant, where downregulation of the ataxia gene *Itpr1* occurred in parallel with downregulation of the HSP70-family member / chaperone *Hspa12a* and several voltage-gated Ca^{2+} homeostasis channels in the plasma membrane (*Cacna1a*, *Cacna1c*, *Cacna1d*, *Cacna1g*). Cytosolic HSPA12A protein has a very specific role, modulating its interactor SORL1 with downstream GFRA1/2 [223, 224], all of which showed downregulated mRNA levels in our ATM-null mouse cerebellar transcriptome profiling study, suggesting that the sorting of trophic signaling receptors is abnormally regulated. Also the receptor tyrosine kinase ERBB2 is regulated both by SORL1 and by USP2 [225, 226], and this may underlie the reduced cerebellar levels of *Erbb2ip* as a factor responsible for the surface localization of glutamate receptors [227]. In analogy to the impact of nutrient deficits as stressors via ATM onto endoplasmic reticulum and mitochondria homeostasis as previously published [30], the transcriptional dysregulation of ITPR1, HSPA12A (instead of HSPA9/GRP75) and its interactors SORL1 and ERBB2 in ATM-null cerebellum may therefore constitute the primary pathogenesis pathway and explain why neurotransmitter receptors / transporters / neuropeptide modulators are not in the right position in polarized processes, trophic signaling deficits ensue, and tissue shrinkage ensues over time. Given that SORL1, GFRA1/2, ERBB2, are poorly or not expressed in SH-SY5Y neuroblastoma cells according to the Human Protein Atlas, we made no attempt to model this trophic pathogenesis cascade *in vitro*.

The reduction of IP3R would also mediate a postsynaptic excitability deficit, and contribute to the diminished transcriptional response of immediate-early transcripts such as *PER1* and *NR4A1*.

5. Conclusions

Overall, our genome-wide RNA profile provided useful knowledge to identify factors that might underlie the growth deficit (somatostatin and neuropeptide-Y) and vasodilatation phenotypes (tachykinins and *Ecel1*) of A-T, and to define the mechanistic overlap of A-T with the *Itpr1*-triggered monogenic variants of cerebellar ataxia. The data in this project suggested that the presence of cytosolic ATM in postmitotic cerebellar neurons serves an important modulator of the transcriptional regulation of excitability factors in response to ageing, and to osmotic stress more than nutrient or oxidative stress. Validations in neuroblastoma culture could largely reproduce crucial insights and the prominent alterations found in the cerebellar transcriptome: Strong reduction of *Atm* levels was reflected by similar strong decreases of its interactor *Usp2*, the mainly Purkinje-neuron-expressed Ca^{2+} -excitability modulator *Itpr1* mRNAs, and immediate-early signaling factors such as *Per1* and *Nr4a1*.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: RT-qPCR validation of cerebellar transcript dysregulations in 1.5-3-month-old ATM-null mice shows early onset of immediate-early mRNA reductions, suggesting insufficient excitability; Figure S2: Modeling stable ATM-deficiency *in vitro* via knockdown in SH-SY5Y neuroblastoma cells; Figure S3: Subcellular localization of ATM to SH-SY5Y cytoplasm is unchanged after CQ and BLEO stress; Figure S4: RT-qPCR analysis of the modelling of cerebellar dysregulations in SH-SY5Y cells, testing different stressors, as well as ATM deficiency versus kinase inhibition; Table S1: The global transcriptome profile of the ATM-null cerebellum.

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Abbreviations

ACTB	β-Actin
AFP	Alpha-Fetoprotein
AKT	AKT serine/threonine kinase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AOA2	Ataxia with oculomotor apraxia type 2
AP1B1	Adaptor Related Protein Complex 1 Subunit Beta 1
AP2B1	Adaptor Related Protein Complex 2 Subunit Beta 1
ARNT2	Aryl Hydrocarbon Receptor Nuclear Translocator 2
ASTN2	Astrotactin 2
A-T	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia Mutated
ATMIN	ATM Interactor
ATP2B2	ATPase Plasma Membrane Ca ²⁺ Transporting 2
ATP2B4	ATPase Plasma Membrane Ca ²⁺ Transporting 4
ATR	Ataxia Telangiectasia And Rad3-Related Protein
ATXN2	Ataxin 2
BAIAP3	BAI1 Associated Protein 3
BCA	Bicinchoninic acid
beta-NAP	Neuronal Adaptil-like beta-subunit Protein
BLEO	Bleomycin
BMP1	Bone Morphogenetic Protein 1
BMP2	Bone Morphogenetic Protein 2
BMP7	Bone Morphogenetic Protein 7
BMPR1B	Bone Morphogenetic Protein Receptor Type 1B
BMT	Bone arrow transplantation
BSA	Bovine Serum Albumin
C4B	Complement Component 4B
CA8	Carbonic Anhydrase 8
CACNA1A	Calcium Voltage-Gated Channel Subunit Alpha1 A
CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha1 C

CACNA1D	Calcium Voltage-Gated Channel Subunit Alpha1 D
CACNA1G	Calcium Voltage-Gated Channel Subunit Alpha1 G
CADPS2	Calcium Dependent Secretion Activator 2CAMK2A
CAMK4	Calcium/Calmodulin Dependent Protein Kinase IV
Car9	murine Carbonic Anhydrase 9
CBLN2	Cerebellin 2 Precursor
CBLN4	Cerebellin 4 Precursor
CCL27A	C-C Motif Chemokine Ligand 27
CCNL2	Cyclin L2
CDH10	Cadherin 10
CDH19	Cadherin 19
CDH6	Cadherin 6
CDH9	Cadherin 9
cDNA	complementary DNA
CEB	cytosolic extract buffer
CHK2	Checkpoint Kinase 2
CHKA	Choline Kinase Alpha
CHRM2	Cholinergic Receptor Muscarinic 2
CHRM3	Cholinergic Receptor Muscarinic 3
CHRM5	Cholinergic Receptor Muscarinic 5
CHRNA4	Cholinergic Receptor Nicotinic Alpha 4 Subunit
CHRNA6	Cholinergic Receptor Nicotinic Alpha 6 Subunit
CHRNA7	Cholinergic Receptor Nicotinic Alpha 7 Subunit
CHRNB3	Cholinergic Receptor Nicotinic Beta 3 Subunit
CHRNB4	Cholinergic Receptor Nicotinic Beta 4 Subunit
CNR1	Cannabinoid Receptor 1
CNTN4	Contactin 4
CNTN5	Contactin 5
CNTN6	Contactin 6
CNTNAP5A	Contactin Associated Protein Family Member 5
CPNE6	Copine 6
CQ	Chloroquine
CREB5	CAMP Responsive Element Binding Protein 5
CREBL2	CAMP Responsive Element Binding Protein Like 2
CRTC2	CREB Regulated Transcription Coactivator 2
CRY1	Cryptochrome Circadian Regulator 1
CRY2	Cryptochrome Circadian Regulator 2
CSF1R	Colony Stimulating Factor 1 Receptor
CYP46A1	Cytochrome P450 Family 46 Subfamily A Member 1
DBH	Dopamine Beta-Hydroxylase
DBP	D-Box Binding PAR BZIP Transcription Factor
DDR	DNA Damage Response
DGKK	Diacylglycerol Kinase Kappa

DLG2	Discs Large MAGUK Scaffold Protein 2
DLK1	Delta Like Non-Canonical Notch Ligand 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOCK10	Dedicator Of Cytokinesis 10
DRD2	Dopamine Receptor D2
DRD5	Dopamine Receptor D5
DSB	Double-Strand Breaks
DUSP1	Dual Specificity Phosphatase 1
EBF3	EBF Transcription Factor 3
ECEL1	Endothelin Converting Enzyme Like 1
EFNA2	Ephrin A2
EFNA3	Ephrin A3
EFNA5	Ephrin A5
EFNB3	Ephrin B3
EPHA3	EPH Receptor A3
EPHA4	EPH Receptor A4
EPHA5	EPH Receptor A5
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ERBB3	Erb-B2 Receptor Tyrosine Kinase 3
ERBB4	Erb-B2 Receptor Tyrosine Kinase 4
FAT2	FAT Atypical Cadherin 2
FATC	FRAP, ATM, TRRAP C-terminal
FCS	Fetal Calf Serum
FDR	False Discovery Rate
FGF13	Fibroblast Growth Factor 13
FGF14	Fibroblast Growth Factor 14
FGF18	Fibroblast Growth Factor 18
FGF5	Fibroblast Growth Factor 5
FGFR1OP2	FGFR1 Oncogene Partner 2
FGFR3	Fibroblast Growth Factor Receptor 3
FOS	Fos Proto-Oncogene
FOSL2	FOS Like 2
FOXO1	Forkhead Box O1
FOXO3	Forkhead Box O3
FRDA	Friedreich's Ataxia
FSCN1	Fascin Actin-Bundling Protein 1
FXYD5	FXYD Domain Containing Ion Transport Regulator 5
FZD4	Frizzled Class Receptor 4
FZD8	Frizzled Class Receptor 8
GABA	Gamma-aminobutyric acid
GABARAPL1	GABA Type A Receptor Associated Protein Like 1
GABRA2	Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha2

GABRA3	Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha3
GABRA5	Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha5
GABRA6	Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha6
GABRB1	Gamma-Aminobutyric Acid Type A Receptor Subunit Beta1
GABRE	Gamma-Aminobutyric Acid Type A Receptor Subunit Epsilon
GABRG1	Gamma-Aminobutyric Acid Type A Receptor Subunit Gamma1
GABRG2	Gamma-Aminobutyric Acid Type A Receptor Subunit Gamma2
GABRG3	Gamma-Aminobutyric Acid Type A Receptor Subunit Gamma3
GABRQ	Gamma-Aminobutyric Acid Type A Receptor Subunit Theta
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GBA2	Glucosylceramidase Beta 2
GFRA1	GDNF Family Receptor Alpha 1
GFRA2	GDNF Family Receptor Alpha 2
GH	growth hormone
GLRA1	Glycine Receptor Alpha 1
GLRA2	Glycine Receptor Alpha 2
GLRA3	Glycine Receptor Alpha 3
GLRA4	Glycine Receptor Alpha 4
GNAS	GNAS Complex Locus
GO	Gene Ontology
GPR165	G Protein-Coupled Receptor 165
GRID1	Glutamate Ionotropic Receptor Delta Type Subunit 1
GRID2	Glutamate Ionotropic Receptor Delta Type Subunit 2
GRID2IP	Grid2 Interacting Protein
GRIK2	Glutamate Ionotropic Receptor Kainate Type Subunit 2
GRIN2B	Glutamate Ionotropic Receptor NMDA Type Subunit 2B
GRIN2C	Glutamate Ionotropic Receptor NMDA Type Subunit 2C
GRIN3A	Glutamate Ionotropic Receptor NMDA Type Subunit 3A
GRIP1	Glutamate Receptor Interacting Protein 1
GRM3	Glutamate Metabotropic Receptor 3
GRM4	Glutamate Metabotropic Receptor 4
GRM5	Glutamate Metabotropic Receptor 5
GRM8	Glutamate Metabotropic Receptor 8
GRP75 / HSPA9	Heat Shock Protein Family A (Hsp70) Member 9
GSK3β	Glycogen Synthase Kinase 3 Beta
H ₂ O ₂	Hydrogen peroxide
HDMX	human ortholog of mouse MDMX (also known as MDM4)
HIF3A	Hypoxia Inducible Factor 3 Subunit Alpha
HOMER1	Homer Scaffold Protein 1
HSP60	60 KDa Heat Shock Protein, Mitochondrial
HSP70	Heat Shock 70 KDa Protein 4
HSPA12A	Heat Shock Protein Family A (Hsp70) Member 12A
IGF1	Insulin Like Growth Factor 1

IGF2	Insulin Like Growth Factor 2
IGF2R	Insulin Like Growth Factor 2 Receptor
IGFLR1	IGF Like Family Receptor 1
IgG	Immunoglobulin G
IL16	Interleukin 16
IL18	Interleukin 18
IL20RB	Interleukin 20 Receptor Subunit Beta
IL33	Interleukin 33
IL34	Interleukin 34
INPP5A	Type I Inositol 1,4,5-Trisphosphate 5-Phosphatase
ITPR1 / IP3R1	Inositol 1,4,5-Trisphosphate Receptor Type 1
JUN	Jun Proto-Oncogene
KAT5	Lysine Acetyltransferase 5
KCNQ5	Potassium Voltage-Gated Channel Subfamily Q Member 5
KD	knockdown
KDR	Kinase Insert Domain Receptor
KIT	KIT Proto-Oncogene
KO	knockout
KU	KU-55933
LCDV	Large Dense Core Vesicles
LIFR	LIF Receptor Subunit Alpha
LY	LY-294002
MAPK9 / JNK2	Mitogen-Activated Protein Kinase 9
MDM4	MDM4 Regulator Of P53
MICU3	Mitochondrial Calcium Uptake Family Member 3
miR	microRNA
MLB	mitochondrial lysis buffer
MME	Membrane Metalloendopeptidase
MPP4	MAGUK P55 Scaffold Protein 4
MRE11	Double-Strand Break Repair Protein MRE11
MRN	MRE11-RAD50-NBS1
mRNA	messenger RNA
NaARS	Sodium Arsenite
NBS1	Nibrin
NECAB1	N-Terminal EF-Hand Calcium Binding Protein 1
NEFL	Neurofilament Light Chain
NEFM	Neurofilament Medium Chain
ng	nanogram
NGEF	Neuronal Guanine Nucleotide Exchange Factor
NGFRAP1	Nerve Growth Factor Receptor Associated Protein 1
NPAS4	Neuronal PAS Domain Protein 4
NPY	Neuropeptide Y
NPY1R	Neuropeptide Y Receptor Y1

NPY4R	Neuropeptide Y Receptor Y4
NR4A1	Nuclear Receptor Subfamily 4 Group A Member 1
NR4A2	Nuclear Receptor Subfamily 4 Group A Member 2
NR4A3	Nuclear Receptor Subfamily 4 Group A Member 3
NRG1	Neuregulin 1
NRG3	Neuregulin 3
NRIP2	Nuclear Receptor Interacting Protein 2
NRIP3	Nuclear Receptor Interacting Protein 3
NSG2	Neuronal Vesicle Trafficking Associated 2
NT CTRL	Non-targeting control shRNA
NTF3	Neurotrophin 3
NTRK3	Neurotrophic Receptor Tyrosine Kinase 3
NTS	Neurotensin
NTSR1	Neurotensin Receptor 1
NTSR2	Neurotensin Receptor 2
OMD	Osteomodulin
OPRK1	Opioid Receptor Kappa 1
OPRL1	Opioid Related Nociceptin Receptor 1
OPRM1	Opioid Receptor Mu 1
PBS	Phosphate Buffered Saline
PCDH10	Protocadherin 10
PCDH11X	Protocadherin 11 X-Linked
PCDH17	Protocadherin 17
PCDH18	Protocadherin 18
PCDH19	Protocadherin 19
PCDH7	Protocadherin 7
PCSK1	Proprotein Convertase Subtilisin/Kexin Type 1
PCSK1N	Proprotein Convertase Subtilisin/Kexin Type 1 Inhibitor
PCSK5	Proprotein Convertase Subtilisin/Kexin Type 5
PDGFC	Platelet Derived Growth Factor C
PDGFRA	Platelet Derived Growth Factor Receptor Alpha
PDGFRL	Platelet Derived Growth Factor Receptor Like
PER1	Period Circadian Regulator 1
PER2	Period Circadian Regulator 2
PER3	Period Circadian Regulator 3
PI3K	Phosphoinositide 3-kinase
PIKK	Phosphoinositide 3-kinase-related kinases
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein phosphatase 2A
PPP2R2B	Protein Phosphatase 2 Regulatory Subunit Bbeta
PTPRD	Protein Tyrosine Phosphatase Receptor Type D
QPCT	Glutaminyl-Peptide Cyclotransferase
RAD50	RAD50 Double Strand Break Repair Protein

RASGRF2	Ras Protein Specific Guanine Nucleotide Releasing Factor 2
RELN	Reelin
RESP18	Regulated Endocrine Specific Protein 18
RGS4	Regulator Of G Protein Signaling 4
RIN	RNA integrity number
RNA	Ribonucleic acid
RNF122	Ring Finger Protein 122
RORA	RAR Related Orphan Receptor A
RORB	RAR Related Orphan Receptor B
RRAGD	Ras Related GTP Binding D
RTN4	Reticulon 4
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SAM68	Src-Associated In Mitosis 68 KDa Protein
SCN3B	Sodium Voltage-Gated Channel Beta Subunit 3
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SEPT9	Septin 9
SERINC2	Serine Incorporator 2
SFRP1	Secreted Frizzled Related Protein 1
SFRP5	Secreted Frizzled Related Protein 5
SGK1	Serum/Glucocorticoid Regulated Kinase 1
shATM	shRNA targeting ATM
shRNA	Short hairpin RNA
SIDT1	SID1 Transmembrane Family Member 1
SIK1	Salt Inducible Kinase 1
SLC17A6 / VGLUT	Solute Carrier Family 17 Member 6
2	
SLC17A7 /	Solute Carrier Family 17 Member 7
VGLUT1	
SLC18A2	Solute Carrier Family 18 Member A2
SLC1A1	Solute Carrier Family 1 Member 1
SLC1A2	Solute Carrier Family 1 Member 2
SLC1A3	Solute Carrier Family 1 Member 3
SLC1A4	Solute Carrier Family 1 Member 4
SLC1A6	Solute Carrier Family 1 Member 6
SLC25A22	Solute Carrier Family 25 Member 22
SLC2A12	Solute Carrier Family 2 Member 12
SLC32A1	Solute Carrier Family 32 Member 1
SLC5A7	Solute Carrier Family 5 Member 7
SLC6A11	Solute Carrier Family 6 Member 11
SLC7A3	Solute Carrier Family 7 Member 3
SLCO1A4	Solute Carrier Organic Anion Transporter Family Member 1A2
SLTRK3	SLIT And NTRK Like Family Member 3

SLTRK5	SLIT And NTRK Like Family Member 5
SLTRK6	SLIT And NTRK Like Family Member 6
SMG1	SMG1 Nonsense Mediated mRNA Decay Associated PI3K Related Kinase
SOCS7	Suppressor Of Cytokine Signaling 7
SORCS1	Sortilin Related VPS10 Domain Containing Receptor 1
SORCS2	Sortilin Related VPS10 Domain Containing Receptor 2
SORL1	Sortilin Related Receptor 1
SOX10	SRY-Box Transcription Factor 10
ss-cDNA	single-stranded cDNA
SST	Somatostatin
SSTR1	Somatostatin Receptor 1
SSTR2	Somatostatin Receptor 2
SVBP	Small Vasohibin Binding Protein
SYNE1	Spectrin Repeat Containing Nuclear Envelope Protein 1
TAC	Transcriptome Analysis Console
TAC1	Tachykinin Precursor 1
TACR1	Tachykinin Receptor 1
TACR3	Tachykinin Receptor 3
TBP	TATA-Box Binding Protein
TBS	Tris Buffered Saline
TBS-T	TBS with 0.1% Tween-20 detergent
TCF4	Transcription Factor 4
TGFBR1	Transforming Growth Factor Beta Receptor 1
TGFBR2	Transforming Growth Factor Beta Receptor 2
TNFRSF13C	TNF Receptor Superfamily Member 13C
TNFRSF21	TNF Receptor Superfamily Member 21
TOP1cc	Topoisomerase-1 cleavage complexes
TP53	Tumor Protein P53
tRNA	transfer RNA
TUBA	α -tubulin
USP2	Ubiquitin Specific Peptidase 2
UVB	Ultraviolet B
V(D)J	Variability – Diversity – Joining Rearrangement
VAMP1	Vesicle Associated Membrane Protein 1
VCL	Vinculin
VDAC1	Voltage Dependent Anion Channel 1
WNK1	WNK Lysine Deficient Protein Kinase 1
WNT3	Wnt Family Member 3
WNT7A	Wnt Family Member 7A
WT	Wildtype
ZFHX3	Zinc Finger Homeobox 3
α NAC	Nascent Polypeptide Associated Complex Subunit Alpha
γ TX	γ -taxilin

μ g	microgram
μ m	micrometer

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