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To cite this version:

Laurent Villard, Pierre Cacciagli, Marie-Reine Haddad, Cecile Mignon-Ravix, Bilal El-Waly, et al.. Disruption of the ATP8A2 gene in a patient with a t(10;13) de novo balanced translocation and a severe neurological phenotype.. European Journal of Human Genetics, 2010, 10.1038/ejhg.2010.126 . hal-00563106

HAL Id: hal-00563106

https://hal.science/hal-00563106

Submitted on 4 Feb 2011

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Disruption of the *ATP8A2* gene in a patient with a t(10;13) *de novo* balanced translocation and a severe neurological phenotype.

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Running title : ATP8A2 haploinsufficiency and mental retardation.

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ABSTRACT

Mental retardation is a frequent condition that is clinically and genetically highly heterogeneous. One of the strategies used to identify new causative genes is to take advantage of balanced chromosomal rearrangements in affected patients. We characterized a *de novo* t(10;13) balanced translocation in a patient with severe mental retardation and major hypotonia. We found that the balanced translocation is molecularly balanced. The translocation breakpoint disrupts the coding sequence of a single gene, called *ATP8A2*. The *ATP8A2* gene is not ubiquitously expressed but it is highly expressed in the brain. In situ hybridization performed in mouse embryos at different stages of development with the mouse homologue confirms this observation. 38 patients with a similar phenotype were screened for mutations in the *ATP8A2* gene but no mutations were found. The balanced translocation identified in this patient disrupts a single candidate gene highly expressed in the brain. Although this chromosomal rearrangement could be the cause of the severe phenotype of the patient, we were not able to identify additional cases. Extensive screening in the mentally retarded population will be needed to determine if *ATP8A2* haploinsufficiency or dysfunction causes a neurological phenotype in humans.

Keywords : chromosome aberrations, mental retardation, Atp8a2, 10p12, 13q12.

INTRODUCTION

Mental retardation is a frequent condition that is clinically and genetically highly heterogeneous (1). Mutations in hundreds of different genes have been identified in patients suffering from mental retardation and these genes encode proteins performing a wide range of molecular functions inside and outside the central nervous system (2). Despite its important prevalence, ranging between 0,5 and 3% of the population, the majority of mental retardation cases remain unexplained.

The presence of *de novo* chromosomal rearrangements in patients with an abnormal phenotype can be used to identify disease-causing genes (3). Cloning of the breakpoints of balanced chromosomal translocations in affected patients has led to the identification of a large number of genes, including genes implicated in different types of mental retardation phenotypes (4).

Here, we describe a female patient with moderate mental retardation, severe hypotonia, and a *de novo* balanced translocation 46,XX,t(10;13)(p12.1;q12.13)dn. We cloned and sequenced the two translocation breakpoints and showed that the translocation is perfectly balanced at the molecular level. Analysis of the breakpoint regions reveals that the coding sequence of a P-type ATPase called *ATP8A2* on chromosome 13q12.13 is interrupted by the translocation whereas there is no known transcription unit located in the immediate vicinity of the chromosome 10 breakpoint. We analyzed the expression pattern of the *ATP8A2* transcript in mice using in situ hybridization and in human using RT-PCR. We show that *ATP8A2* is highly expressed in the brain and testis both in human and mouse. We hypothesize that haploinsufficiency of the *ATP8A2* gene could be involved in the severe neurological phenotype presented by the patient with the balanced translocation.

MATERIALS AND METHODS

Cell lines, karyotyping, FISH and array CGH

Karyotypes were determined by analysis of R-banded chromosomes at a minimum resolution of 550 bands using standard protocols. FISH was performed on metaphase chromosomes of the patient using BAC clones DNA (provided by the Wellcome Trust Sanger Institute, Hinxton, Cambridgeshire CB10 1SA, UK) and commercial probe CEP10 (Abbott Molecular Laboratories, USA). Hybridization was performed using the standard procedures. Array-CGH was performed using the Agilent Human Genome 244K CGH Microarray (Agilent Technologies, Inc., Santa Clara, CA). The microarray chip was scanned on an Agilent Microarray Scanner. Data were processed by Agilent Feature Extraction software 9.5 and analyzed with Agilent CGH Analytics 3.4 Software.

RNA extraction, reverse transcription and PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). Reverse transcription of 5 µg of total RNA was performed in 20 µl of Superscript reaction buffer (Invitrogen Corp., Carlsbad, CA) containing 0.01M dithiothreitol (DTT), 7.5 ng/µl of dN6, 40 U of Rnase inhibitor (Invitrogen Corp., Carlsbad, CA), 0,5 mM dNTP and 200 U of Superscript reverse transcriptase (Invitrogen Corp., Carlsbad, CA). Real-time PCR reactions were performed in the LightCycler 480 system (Roche) using the SYBR Green I Master Kit (Eurogentec, Seraing, Belgium) with 2 µl of cDNA and 200 nM of each primer. Each reaction was performed in triplicate.

In situ hybridization on mouse embryos

The antisense and sense riboprobes used in this study correspond to nucleotides 2943-3637 of sequence NM_015803 (mouse *Atp8a2*). *In situ* hybridization was performed as

previously described (5). Control experiments were performed using corresponding sense riboprobes on adjacent sections, giving either no signal or a uniformly low background as expected.

RESULTS

Case report

The patient is the first child of non consanguineous, healthy parents. During pregnancy, the mother was given Zovirax for 5 days because she had been in contact with a patient suffering from chicken pox. The patient was born at term and with normal growth parameters (height 50cm, weight 3180g, OFC 36cm). The first clinical signs were noted after one month when she was noticed not to be able to hold her head. CT-scan, muscle enzymes, amino acids, organic acids and thyroid hormones were normal in the blood of the patient. At one year of age she had major axial hypotonia. She was not able to hold her head and could not sit. She was demonstrating incessant abnormal movements. She had a normal EEG. Nerve conduction velocity and electromyogram were normal, as were her visual and hearing parameters. The brain MRI performed at 13 months of age was reported to be normal. At three years of age, she had normal growth parameters but was still severely hypotonic and was not able to hold her head. She could speak a few words.

Cytogenetic and molecular characterization of the translocation

The routine karyotype revealed a translocation between chromosomes 10p12 and 13q12 (data not shown). Serial FISH experiments lead to the identification of two BAC clones overlapping the translocation breakpoints (supplementary figure S1). BAC clone RP11-

164A7 overlaps the chromosome 10 breakpoint at 10p12.1. BAC clones RP11-111G7 and RP11-467D10 overlap the chromosome 13 breakpoint at 13q12.13. A map of the translocation breakpoint regions on chromosomes 10 and 13 is presented in Figure 1. Array comparative genomic hybridization (a-CGH) was performed using an Agilent 244K CGH array and did not reveal any genomic imbalance at the resolution level provided by the array (data not shown).

To further refine the localization of the translocation breakpoints, Southern blots were prepared using the patient's DNA. Series of probes were prepared using PCR on the chromosome 13 breakpoint overlapping BAC DNA and the Southern blots were hybridized with these probes until a junction fragment could be identified. A 4.8 kb junction fragment was identified on the patient's DNA using the StuI restriction endonuclease and a probe located at position 70508-70999 on the RP11-164A7 BAC DNA sequence (supplementary figure S2). This junction fragment was cloned and sequenced (supplementary document S3). Analysis of the sequence of this fragment allowed us to map the chromosome 10 breakpoint at position 28,846,023 bp and the chromosome 13 breakpoint at position 25,231,633 bp (according to reference sequence Ensembl54) and to show that the translocation is perfectly balanced with no gain or loss of genomic material.

The chromosome 10 breakpoint does not interrupt a known transcription unit. A gene called *WAC* (WW domain containing adaptor with coiled-coil) is located 15,7 kb towards the centromere of chromosome 10. A gene called *MPP7* is located 235 kb away on the telomeric side of the chromosome 10 breakpoint. The chromosome 13 breakpoint is located between exons 27 and 28 of a gene called *ATP8A2*, recently shown to encode

a putative phosphatidylserine flippase (6). In order to test if a chimaeric transcript could

be produced between *ATP8A2* and *MPP7* on the der(13), we used primers located in *ATP8A2* (exon 24) and *MPP7* (exon 10) and tried to amplify a chimaeric transcript using cDNA prepared from RNA extracted from the lymphocytes of the patient. We were not able to detect any chimaeric transcript.

Expression of ATP8A2 in human and mouse tissues

In order to study the expression profile of *ATP8A2*, we performed RT-PCR on a panel of 13 human tissues. *ATP8A2* was found to be highly expressed in testis and brain (Figure 2A). To refine the brain expression profile, we next used a panel of RNAs prepared from 12 human brain areas to perform RT-PCR. This experiment revealed that *ATP8A2* is expressed similarly in the different brain regions tested.

The human ATP8A2 and mouse Atp8a2 proteins share 97% homology. We thus performed *in situ* hybridization in the mouse with a murine *Atp8a2* antisense probe. These experiments were performed in E14.5, E15.5, E18.5 embryos and at postnatal day 3 (P3). We observed a strong and homogeneous brain expression at all studied stages with the highest levels of *Atp8a2* transcripts detected before birth (Figure 2B).

Because *ATP8A2* is not expressed in lymphocytes, we were not able to quantify its expression in the cells of the patient. The expression of *MPP7* could not be tested either because it is not expressed in the lymphocytes (data not shown). We could quantify the expression of *WAC* in the lymphocytes of the patients, her two parents and a control cell line using real-time quantitative PCR. We found no difference in the amount of *WAC* transcript in the four samples tested (data not shown).

Screening of the ATP8A2 gene for mutations

We sequenced the 37 exons of the *ATP8A2* allele located on the normal chromosome 13 in the patient with the balanced translocation but found no mutation. We also sequenced all *ATP8A2* exons on 38 additional patients selected because they were suffering both from mental retardation and severe hypotonia. Again, we found no deleterious mutation (data not shown). This group of patient had been investigated using 105K array CGH
4 and no gross rearrangement was found (31 oligonucleotides cover the ATP8A2 gene on these arrays).

DISCUSSION

We have studied a patient with mental retardation, severe hypotonia and a de novo balanced translocation. We have cloned and sequenced the two translocation breakpoints and we have shown that the translocation is molecularly balanced. Based on the results obtained, we find it reasonable to hypothesize that the breakpoint on chromosome 10 is not involved in the phenotype of our patient. This hypothesis is based on the fact that this region of the genome does not contain any gene. The closest gene to the breakpoint on chromosome 10 is the *WAC* gene, located 15,7 kb away towards the centromere. The function of the protein encoded by the *WAC* gene is unknown. We have shown that the expression of *WAC* is not altered in the patient's lymphoblastoid cell line, ruling out a major position effect, although the expression in the brain of the patient is unknown.

If the phenotype of the patient is caused by the presence of the translocation, then we propose that it is due to the haploinsufficiency of the *ATP8A2* gene on chromosome 13.
3 A possibility exists that a chimaeric transcript could be produced using *ATP8A2* exons 1

to 27 and the *MPP7* exons, in the brain. In this later case however, *ATP8A2* dosage would also be altered. The interruption of the coding region of the gene by the translocation breakpoint is a strong argument in favour of the pathogenicity of the chromosomal rearrangement. Expression analysis in mouse and human shows that *ATP8A2* is strongly expressed in the brain and during development. Several studies have addressed the function of the *ATP8A2* gene. Injection of an antisense *ATP8A2* cDNA construct was shown to cause a loss of growth control and to convert non tumorigenic AIG cells into tumorigenic cells (7). A study of the mouse *Atp8a2* gene revealed that it was highly expressed in the central nervous system and that it could transport aminophospholipids (8). More recently, *ATP8A2* was confirmed to be involved in the energy-dependent translocation of phosphatidylserine across the cell membrane of photoreceptors (6). Phosphatidylserine plays important roles in several biological processes including apoptosis or cell signalling. Alteration of phosphatidylserine receptor (PSR) in the mouse reveals that it is necessary for proper brain development (9).

Terminal deletions of chromosome 13 are rare but they represent a well known clinical entity (10). Deletions of 13q14 have extensively been studied in the context of retinoblastoma (11). On the contrary, proximal deletions affecting the 13q12 region have not been frequently reported. An interstitial 13q12.1q14.1 deletion was reported in a patient with developmental delay, dysmorphic features and hypotonia (12). The rearrangement was inherited from a normal mother with a complex chromosomal rearrangement. This later case was not characterized molecularly and it is not known if the *ATP8A2* gene was involved. Deletions in the 13q13-q31 region cause growth retardation but are usually not associated with major malformations (13).

The screening of additional patients revealed no mutations in the *ATP8A2* gene. However, it is very difficult to select patients for mutation screening because the phenotype of the patient carrying the balanced translocation is not very specific, although severe. Mental retardation and severe hypotonia are found in several other syndromes such as Prader-Willi patients when they are young. However, in many instances, the clinical course of these disorders when the patients grow older usually reveals additional features. Given that our patient is still rather young, we were not able to refine the clinical diagnosis enough to select a clinically matched patient cohort for molecular screening.

ACKNOWLEDGEMENTS

We thank the Centre de Ressources Biologiques of La Timone Children's Hospital for access to the biological samples used in this study, and the microarray core facility of the Department of Medical Genetics of La Timone Children's Hospital. We thank Dr. Jean-Christophe Roux for helpful discussions. The authors declare no competing interest.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

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TITLES AND LEGENDS TO FIGURES

Figure 1

Top panel : ideograms of human chromosomes 10 (Hs.10) and 13 (Hs.13). Middle panel : BAC clones and gene content in the two regions investigated by FISH and Southern
1 blotting. Arrows indicate the direction of transcription for each gene in the region. The size of each gene and the number of exons are also indicated. Bottom panel : position of the two translocation breakpoints with respect to the exons of the *WAC* and *ATP8A2* genes (large white arrows). The location and number of exons contained in the breakpoint region is indicated for each gene (exons 1 to 3 for the WAC gene, exons 26 to 28 for the ATP8A2 gene). The chromosome 10 breakpoint is located at nucleotide 28,846,023 and the chromosome 13 breakpoint at position 25,231,633 (according to reference sequence Ensembl54).

Figure 2

A. Expression of *ATP8A2* (top panels) and *GAPDH* (bottom panels) in 13 human tissues (left panels) and in 12 regions of the human brain (right panels). *ATP8A2* is strongly expressed in all regions of the brain, in the retina and testis. A faint signal is also detected in the foetal kidney. **B**. In situ hybridization performed with a mouse *Atp8a2* probe on sections of embryos at E14.5 (a-c), E15.5 (d-e) and E18.5 (g-h). *Atp8a2* is strongly expressed in the central nervous system. A faint background staining only is visible on the sections hybridized with an *Atp8a2* sense probe (f and i). **C**. In situ hybridization performed on postnatal day 3 (P3) and day 10 (P10) mouse brain

revealing a strong expression of *Atp8a2* in the cerebral cortex and the hippocampus. Sections hybridized with an *Atp8a2* sense probe are shown (b and d).

SUPPLEMENTARY FIGURES AND DOCUMENTS

Figure S1.

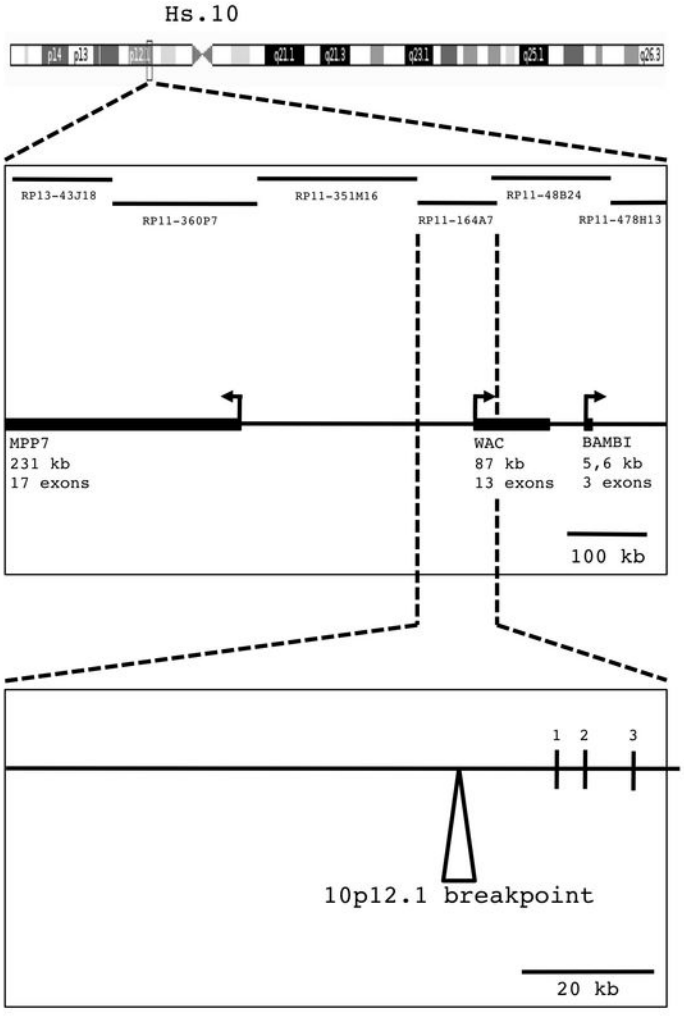
Fluorescent in situ hybridization performed on the patient's metaphase chromosome with BAC clones overlapping with the translocation breakpoint : RP11-164A7 on chromosome 10 (**A**), RP11-111G7 (**B**) and RP11-467D10 (**C**) on chromosome 13. BAC clones are labelled with a Cy5 (red). A chromosome 10 centromeric probe (CEP10) was co-hybridized and labelled with Cy3 (green).

Figure S2.

Southern blot prepared using the DNA of the patient and her father digested by the *StuI* restriction endonuclease. A 4.8 kilobases abnormal restriction fragment (arrow) is detected in the patient's DNA only.

Supplementary document S3.

Sequence of the 4864 bp *StuI* junction fragment.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p13
p12.1
q21.1
q21.3
q23.1
q25.1
q26.3
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RP11-360P7
RP11-351M16
RP11-164A7
RP11-48B24
RP11-478H13
MPP7
231 kb
17 exons
WAC
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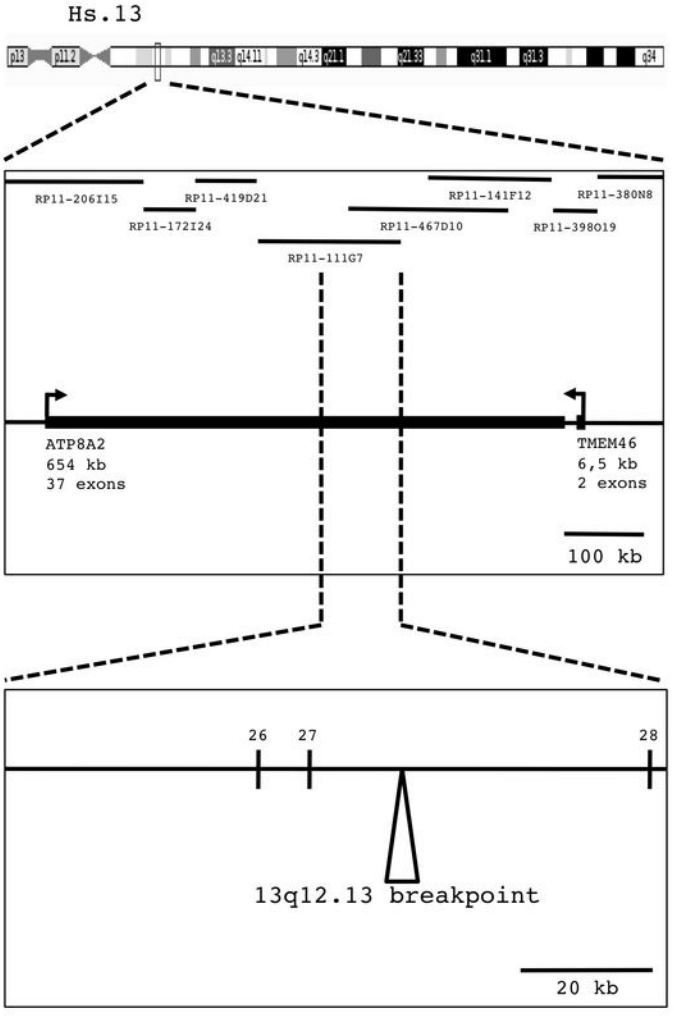
13 exons
BAMBI
5,6 kb
3 exons
100 kb
1
2
3
10p12.1 breakpoint
20 kb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q13.3
q14.11
q14.3
q21.1
q21.33
q31.1
q31.3
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RP11-172I24
RP11-419D21
RP11-111G7
RP11-467D10
RP11-141F12
RP11-398O19
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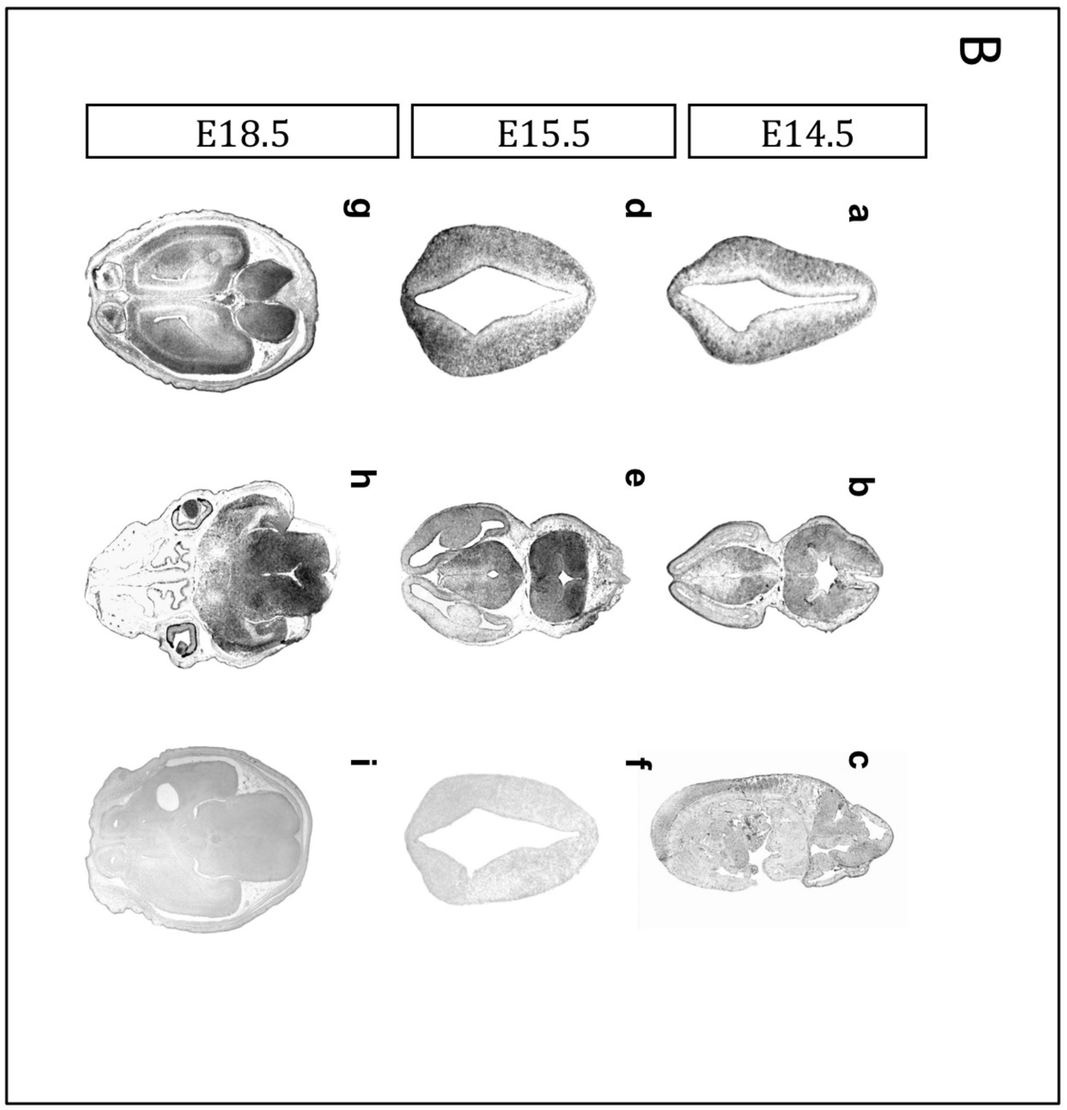
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a
b
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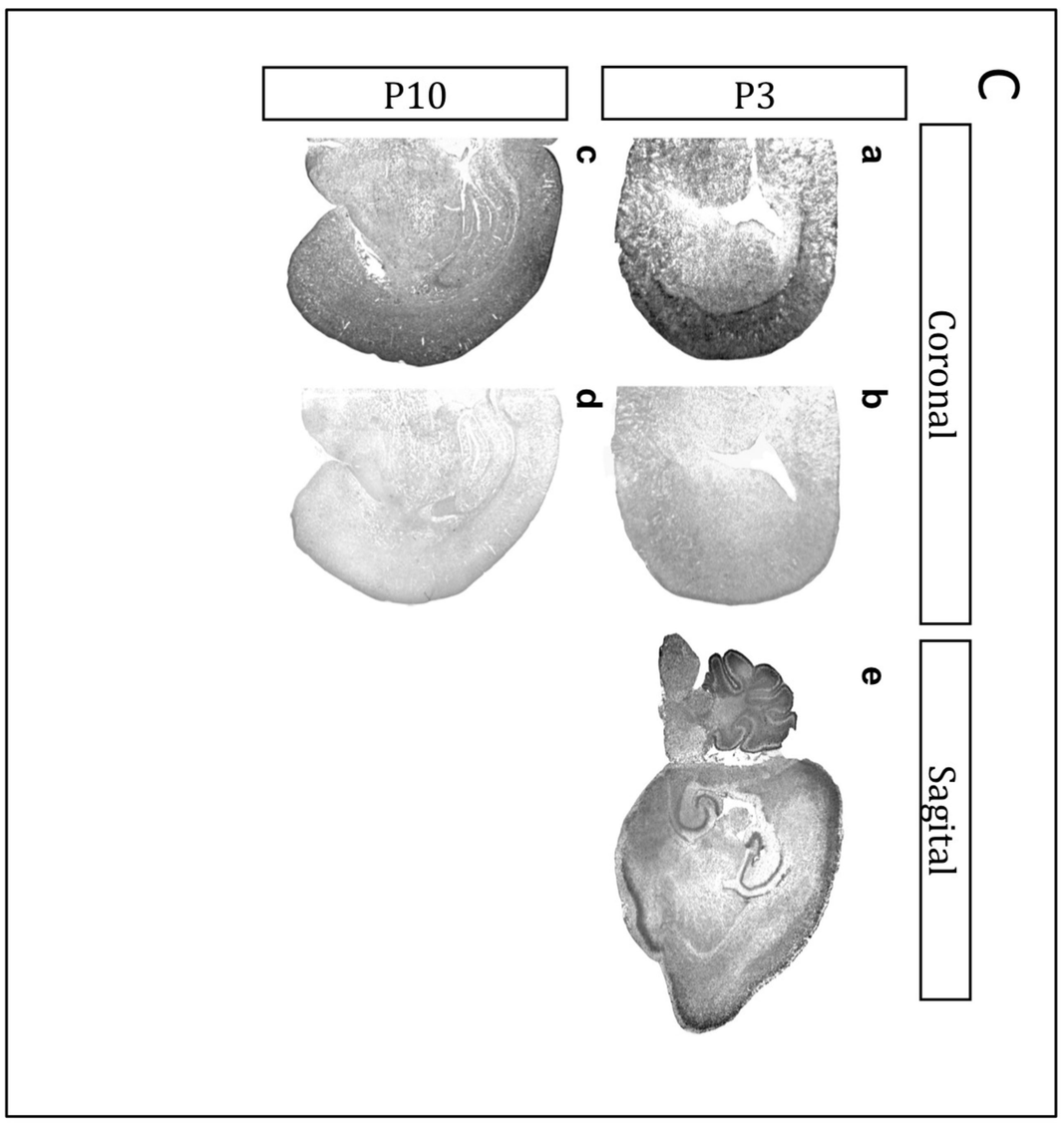
i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P10
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b
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d
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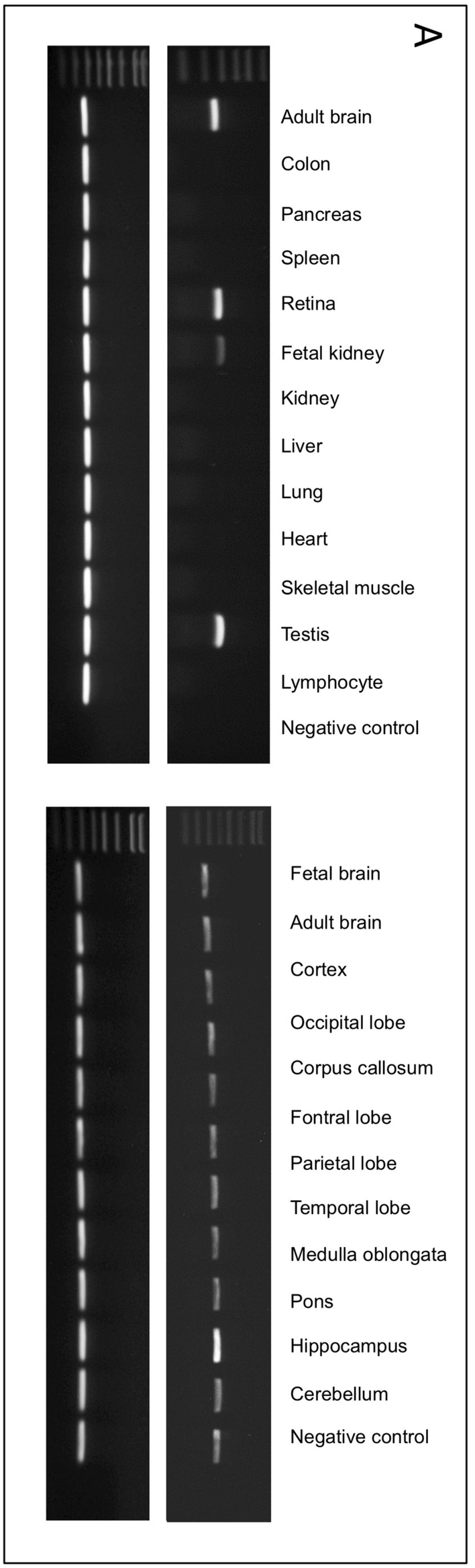
A
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Colon
Pancreas
Spleen
Retina
Fetal kidney
Kidney
Liver
Lung
Heart
Skeletal muscle
Testis
Lymphocyte
Negative control
Fetal brain
Adult brain
Cortex
Occipital lobe
Corpus callosum
Fontral lobe
Parietal lobe
Temporal lobe
Medulla oblongata
Pons
Hippocampus
Cerebellum
Negative control