Developmental Splicing Deregulation in Leukodystrophies Related to EIF2B Mutations

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Abstract

Leukodystrophies (LD) are rare inherited disorders that primarily affect the white matter (WM) of the central nervous system. The large heterogeneity of LD results from the diversity of the genetically determined defects that interfere with glial cells functions. Astrocytes have been identiﬁed as the primary target of LD with cystic myelin breakdown including those related to mutations in the ubiquitous translation initiation factor eIF2B. EIF2B is involved in global protein synthesis and its regulation under normal and stress conditions. Little is known about how eIF2B mutations have a major effect on WM. We performed a transcriptomic analysis using ﬁbroblasts of 10 eIF2B-mutated patients with a severe phenotype and 10 age matched patients with other types of LD in comparison to control ﬁbroblasts. ANOVA was used to identify genes that were statistically signiﬁcantly differentially expressed at basal state and after ER-stress. The pattern of differentially expressed genes between basal state and ER-stress did not differ signiﬁcantly among each of the three conditions. However, 70 genes were speciﬁcally differentially expressed in eIF2B-mutated ﬁbroblasts whatever the stress conditions tested compared to controls, 96% being under-expressed. Most of these genes were involved in mRNA regulation and mitochondrial metabolism. The 13 most representative genes, including genes belonging to the Heterogeneous Nuclear Ribonucleoprotein (HNRNP) family, described as regulators of splicing events and stability of mRNA, were dysregulated during the development of eIF2B-mutated brains. HNRNP1H1, F and C mRNA were over-expressed in foetus but under-expressed in children and adult brains. The abnormal regulation of HNRNP expression in the brain of eIF2B-mutated patients was concomitant with splicing dysregulation of the main genes involved in glial maturation such as PLP1 for oligodendrocytes and GFAP in astrocytes. These ﬁndings demonstrate a developmental deregulation of splicing events in glial cells that is related to abnormal production of HNRNP, in eIF2B-mutated brains.

Introduction

Leukodystrophies (LD) represent a heterogeneous group of rare genetic disorders primarily affecting the white matter (WM) of the central nervous system (CNS). The WM is a complex structure composed of a vast number of axons unsheathed by a compact and lipid-rich membrane, the myelin. Beside myelinated axons, WM contains a variety of cells known as glial cells (astrocytes, oligodendrocytes and microglial cells) that play structural, metabolic and trophic roles for myelin and axons [1]. The large heterogeneity of LD results from the diversity of the genetically determined defects that interfere with glial cells functions. A common point in the LD physiopathology is the demyelination and/or development failure (hypomyelination) of CNS myelin from various origins [2]. The hypomyelinating group of LD includes diseases due to defects in myelin production or quality. The main causative gene of hypomyelinating LD controls by means of oligodendrocytes the production of the major brain myelin proteins, the proteolipid proteins (PLP). The demyelinating group of LD includes mainly defects in i) peroxisomal or lysosomal...
enzymatic activities important for myelin biogenesis and mainte-
nance, or ii) astrocytes functions responsible for progressive cystic
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EIF2B mutations have been initially described in childhood
ataxia with central hypomyelination (CACH/Vanishing white
matter (VWM) syndrome characterised in infants by a progressive
neurological deterioration exacerbated by episodes of febrile
infections or head trauma and a cerebrospinal fluid-like signal
intensity of the WM on brain magnetic resonance imaging [3,4].
EIF2B mutations have been subsequently observed in a wide
clinical spectrum from congenital rapidly lethal forms to slowly
progressive adult forms associated in some cases with ovarian
failure [5–9]. A correlation between age at disease onset and
disease severity has been established; disease onset <2, from 2 to 5
or >5 years are associated respectively to severe, classical or mild
phenotypes [10].

EIF2B is involved in the translation initiation regulation
particularly under cellular stress by activating the eIF2 complex
thanks to its nucleotide guanine exchange factor (GEF) activity. A
correlation between the eIF2B GEF activity and the disease
severity has been established particularly in the most severely
affected infantile group [11,12]. A hyper-activation of the
endoplasmic reticulum (ER)-stress response mediated by the
activating transcription factor 4 (ATF4) has been observed in rat
oligodendroglial-derived cells expressing mutated human EIF2B5
gene [13] as well as in primary fibroblasts [14] and cerebral WM
from eIF2B-mutated patients [15,16] but not in lymphocytes and
lymphoblasts from eIF2B-mutated patients [17] suggesting cell-
specific response to this genetic defect.

The neuropathological features of eIF2B related disorders show
an increased oligodendrocytic density [18,19] and a reduced
number of dystrophic astrocytes due to their abnormal maturation
[20], suggesting an abnormal glial cell maturation in the WM
susceptibility to eIF2B mutations. Moreover, mild transitory
abnormal myelination has been reported in mice homozygous
for a mutant Eif2b5 allele (Eif2b5<sup>R152H/R152H</sup> mice) with an early
defect of glial cells proliferation and maturation [21].

In order to identify genes and metabolic pathways specifically
involved in eIF2B-related disorders, we performed a differential
transcriptomic analysis using patients’ fibroblasts with or without
ER stress conditions. Fibroblasts of eIF2B-mutated patients with a
severe, early infantile form, were compared to age matched
fibroblasts of controls as well as patients with other types of LD
(OL-patients). We described here for the first time genes involved in
the mRNA splicing machinery as actors in the abnormal
maturation of glial cells observed in the eIF2B-mutated patient
brains.

**Results**

**70 Genes Specifically and Differentially Expressed in
eIF2B-mutated Fibroblasts**

In order to identify genes specifically involved in eIF2B-related
diseases, we performed a transcriptomic analysis comparing
fibroblasts of 10 eIF2B-mutated patients with 10 of OL-patients
and with to 10 age matched control fibroblasts (Table 1). ANOVA
was used to identify genes that were statistically significantly
differentially expressed between eIF2B-mutated and OL-cells at
basal state and after ER-stress by thapsigargin treatment.

Thapsigargin is a blocker of the ER calcium pump. The pattern
of differentially expressed genes between basal state and
thapsigargin treatment in the eIF2B-mutated, OL and control
cells did not differ significantly. These results suggest that ER-
stress does not enhance the effect of eIF2B mutations and is
probably not directly involved in the specific expression profile of
eIF2B-related disorders in cultured skin fibroblasts.

Two hundred fifty three genes were differentially expressed
(FDR<0.12%) between the eIF2B-mutated fibroblasts and control
fibroblasts whatever the stress conditions tested. Among these 253
genes, 70 were specifically differentially expressed in eIF2B-
mutated cells when compared with OL fibroblasts, and with
significant expression rate, considered as significant mean ratios
eIF2B-mutated/mean control values ≤0.9 or ≥1.05 (Table S1).
Sixty-seven of the 70 genes (96%) were under-expressed whereas
only three genes were weakly over-expressed (range over-
expression rate 1.06 to 1.07). These under-expressed genes are
involved in (a) transcription or mRNA stabilization and splicing
(25%), (b) mitochondria metabolism (15%), (c) development (12%),
(d) cell cycle (10%), (e) DNA compaction or repair (8%), (f)
cytoskeleton (5%), (g) protein synthesis (4%), and (h) other
metabolic pathways (21%) (Table S1).

**RNA Processing Deregulation in eIF2B-mutated
Fibroblasts**

The 70 genes were analysed using the BBSPE function of the
Genomatix software, in order to select biological processes mostly
and specifically involved in eIF2B-related disorders. We found that
the highest number of genes were all linked to RNA process:
mRNA metabolic process (z-score: 10.08), mRNA processing (z-
scores: 9.74 and 8.91) and mRNA splicing (z-scores: 9.54 to 8.03).
This group statistically diverges form the other biological processes
with a z-score difference of 1. As an example, 8.6% from these 70
deregulated genes are HNRNP genes.

**Expression Deregulation of Several Genes in eIF2B-
mutated Fibroblasts Confirmed by mRNA Quantification**

We selected 10 under-expressed genes for microarray data
validation according to their putative link with the physiopathol-
ogy of eIF2B-related disorders (Table 2). Among the group of 70
differentially expressed genes described above, we first chose three
genes involved in transcription or mRNA stabilization and
splicing: HNRNPH1, HNRNPL and HNRNPC due to the tight
regulation of the alternative splicing of the major myelin protein
mRNA during myelin formation and maintenance [13,16]. We also
selected the HNRNPF gene to the analysis, yet not selected using
our statistical approach (FDR>0.12 for this gene), due to the
particular role of hnRNPH1 and hnRNPF controlling the PLP1/
DM20 mRNA alternative splicing described recently in oligoden-
drocytes [26]. We also selected among our group of 70
differentially expressed genes five genes related to mitochondria
structure and metabolism, MRPS26 (Mitochondrial Ribosomal Protein
S26), MRPL28 (Mitochondrial Ribosomal Protein L28), HCCS (Holocy-
tochrome c Synthase), VDAC3 (Voltage-Dependent Anion-selective Channel
protein 3) and KIF5B (Kinesin Family member 5B) due to the potential
key role of mitochondria in WM homeostasis as suggested by the
involvement of mitochondrial defects in various neurodegenerative
disorders [27] including KIF5A in one inherited progressive
primary motor axonopathy (SPG10) [28]. One gene related to
follicle development and spermatogenesis, DLAPI3 (Dlaphanous
Homolog 3), the human homolog of the drosophila Dlaphanous
gene [29], was also selected due to the frequent ovarian dysfunction
observed in eIF2B-related disorders.
### Table 1. Characteristics of subjects and fibroblasts used in our differential transcriptomic analysis.

| Affected patient number | Type of LD | Age at disease onset | Disease duration* | Mutated gene | Molecular or biochemical defect | Gender | Age skin biopsy | Fb passages | Control subject number | Gender | Age at skin biopsy | Fb passages |
|-------------------------|------------|----------------------|-------------------|--------------|---------------------------------|--------|-----------------|-------------|-----------------------|---------|---------------------|-------------|
| 291-1 ERD               | 2.3 y      | 2.7 y                | EIF2B5            | c.338G>A/c.1160A>G | F                 | 9 y               | 5            | 1530-1                 | F                   | 9 y                 | 7            |
| 375-1 ERD               | 2.6 y      | 3 y                  | EIF2B5            | c.338G>A/c.1948G>A | F                 | 2.7 y             | 4            | 1545-1                 | F                   | 16 m                | 5            |
| 393-1 ERD               | 0.9 y      | 0.7 y                | EIF2B5            | c.925G>C/c.925G>C | F                 | 13 m              | 3            | 1540-1                 | F                   | 15 m                | 8            |
| 393-2 ERD               | 0.8 y      | 0.8 y                | EIF2B5            | c.925G>C/c.925G>C | F                 | 11 m              | 4            | 1541-1                 | F                   | 9 m                 | 6            |
| 431-1 ERD               | 1.5 y      | 3.5 y                | EIF2B5            | c.338G>A/c.1884G>A | F                 | 3.6 y             | 4            | 1178-1                 | F                   | 5 y                 | 10           |
| 432-2 ERD               | 1.5 y      | 2.5 y                | EIF2B5            | c.271A>G/c.1015C>T | M                 | 3.5 y             | 7            | 1596-1                 | M                   | 3 y                 | 8            |
| 590-2 ERD               | 1 y        | 3.5 y                | EIF2B5            | c.1028A>G/c.1153A>G | F                 | 3 y               | 3            | 1178-2                 | F                   | 3 y                 | 8            |
| 894-1 ERD               | 0.6 y      | 10 d                 | EIF2B5            | c.584G>A/c.584G>A | M                 | 7 m               | 3            | 1539-1                 | M                   | 8 m                 | 8            |
| 894-2 ERD               | 0.4 y      | 10 d                 | EIF2B5            | c.584G>A/c.584G>A | F                 | 5 m               | 3            | 1538-1                 | F                   | 6 m                 | 11           |
| 1036-1 ERD              | 0.8 y      | 6 m                  | EIF2B5            | c.967C>T/c.1280C>T | M                 | 12 m              | 3            | 1537-1                 | M                   | 10 m                | 9            |
| 351-1 AD                | 1 y        | NA                   | GFAP              | c.729C>T       | F                 | 7 y               | 4            | 1178-3                 | F                   | 8 y                 | 10           |
| 672-1 AD                | 6 m        | NA                   | GFAP              | c.243A>T       | F                 | 6.6 y             | 5            | 1594-1                 | F                   | 7 y                 | 6            |
| 1303-1 AD               | NA         | NA                   | GFAP              | c.249G>T       | F                 | 3.5 y             | 4            | 1178-2                 | F                   | 3 y                 | 8            |
| 256-1 MLC               | 3 y        | NA                   | MLC1              | c.249G>T/c.1555G>6T>G | M                 | 15 y              | 5            | 1178-4                 | M                   | 17 y                | 10           |
| 773-1 MLC               | 3.5 y      | NA                   | MLC1              | c.1351G>T/c.1351G>T | F                 | 4.7 y             | 3            | 1178-1                 | F                   | 5 y                 | 10           |
| 1143-1 SLS              | 2 m        | NA                   | ALDH3A2           | decreased FALDH activity | F                 | 4 y               | 6            | 1600-1                 | F                   | 4 y                 | 6            |
| 1179-1 SLS              | At birth   | NA                   | ALDH3A2           | decreased FALDH activity | M                 | 3 y               | 9            | 1596-1                 | M                   | 3 y                 | 10           |
| 771-1 KB                | 3.5 m      | NA                   | GALC              | decreased b-gal activity | F                 | 8 m               | 3            | 1539-1                 | F                   | 8 m                 | 9            |
| 167-1 PMD               | 1 m        | NA                   | PLP1              | PLP1 duplication | M                 | 4 y               | 7            | 1178-5                 | M                   | 5 y                 | 7            |
| 767-1 PMD               | At birth   | NA                   | PLP1              | c.454-1G>A/c.454-1G>A | M                 | 2 y               | 4            | 1598-1                 | M                   | 2 y                 | 5            |

LD: leukodystrophy, ERD: eIF2B-related disorder, AD: Alexander disease, MLC: Cystic Megalencephalopathy, SLS: Sjögren-Larsson syndrome, KB: Krabbe disease, PMD: Pelizaeus-Merzbacher disease, y: year, m: months, d: day, b-gal: beta-galactosidase, F: female, M: male, Fb: fibroblasts, NA: not available. *Disease duration in year, corresponding to the time between age at disease onset and death or confinement to bed with loss of neurodevelopment abilities and need of constant assistance.

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We assayed the mRNA level of the 10 selected genes by QRT-PCR on the same RNA samples used for microarray analysis: 10 eIF2B-mutated patient/control fibroblasts couples and 10 OL-patient/control fibroblasts couples in stressed and non stressed conditions. For each gene, the determination correlation coefficient ($R^2$) between microarray normalized log ratios patient/control and QRT-PCR delta Ct (patient-control) have been calculated (Table 3). Among the ten eIF2B-mutated patient/control couples, lack of correlation between QRT-PCR and microarray data for 8 of the selected genes (except HNRNPC and MRPS26 genes) was found for two couples (393-2/1541-1 and 431-1/1178-1, data not shown); therefore these couples were subsequently excluded for the global correlation analysis. For six genes, the correlation coefficients were significantly higher (range of p-value 0 to 0.030) in the 8/10 eIF2B-mutated patient/control fibroblasts couples than in the 10 OL-patient/control fibroblasts couples (Table 3). The difference between eIF2B-mutated/control and OL-patient/control couples was high but not significant for the HCCS and HNRNPC genes (respective p-value = 0.152 and 0.144). No significant difference was observed for the KIF5B and MRPS26 genes (respective p-value = 0.876 and 0.736). These results confirm the specificity of the quantitative differential expression of 6/10 selected genes in the eIF2B-mutated patient's fibroblasts.

### Table 2. List of the genes selected for QRT-PCR analysis.

| Gene name | Description | Taqman Gene Expression Assay reference |
|-----------|-------------|---------------------------------------|
| HNRNP | Heterogeneous nuclear ribonucleoprotein F | Mm00456892_ml |
| DIAPH3 | Diaphanous homolog 3 | Hu01014497_ml |
| VDAC3 | Voltage-dependent anion-selective channel protein 3 | Hu01091534_ml |
| HNRPL | Heterogeneous nuclear ribonucleoprotein L | Hu00704850_ml |
| MRPL28 | Mitochondrial ribosomal protein 28 L | Hu00371771_ml |
| HNRPH1 | Heterogeneous nuclear ribonucleoprotein H | Hu01033845_ml |
| HCCS | Holocarboxylase C synthase | Hu00403938_ml |
| HNRPC | Heterogeneous nuclear ribonucleoprotein C | Hu01028912_ml |
| MRPS26 | Mitochondrial ribosomal protein 26 S | Hu00258287_ml |
| KIF5B | Kinesin family member 5 B | Hu00189659_ml |
| HNRNPU | Heterogeneous nuclear ribonucleoprotein U | Hu00244919_ml |
| HNRNPD | Heterogeneous nuclear ribonucleoprotein D | Hu00606052_ml |
| HNRNPR | Heterogeneous nuclear ribonucleoprotein R | Hu00195167_ml |
| PLP | Proteolipid protein 1 | Mm00456892_ml |
| PLP/DM20 | Proteolipid protein 1 | Mm00456894_ml |

Gene names, descriptions and Taqman Gene Expression Assay reference used for QRT-PCR quantitation are mentioned.

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We then focused our analysis on the protein levels of HNRNP, and particularly on HNRNPL and U by testing one eIF2B-mutated foetus brain (only one brain was available for protein extractions), six eIF2B-mutated children brains and two eIF2B-mutated adult brains in comparison to associated controls. As shown in Figure 1B, the relative amounts of these proteins seem to increase in the nuclear fraction of the eIF2B-mutated foetal brain, consistently with the previous results on transcripts found in two affected foetus brains. Moreover, we can observe an over-expression of HNRNPL in the nuclear fraction of the eIF2B-mutated foetus brain compared to cytoplasmic fraction, which is totally inverted in the control brains. Nevertheless, the protein analysis of only one foetal brain did not allow us to demonstrate a statistically significant deregulation (Figure 1B). Protein analysis of the children (Figure 1C) and adult brains (Figure 1D) using western blotting showed variability in HNRNL and U expressions that did not allow us to demonstrate a statistically significant deregulation between control and eIF2B-mutated brains at the protein level.

**Abnormal Splice Regulation of PLP1 and GFAP in Brains from eIF2B-mutated Patients**

We next wanted to know if the deregulation observed in the expression of genes implicated in the mRNA stabilization and splicing (e.g. HNRNP) has functional consequences. For this purpose, we first studied the PLP/DM20 mRNA alternative splicing, known to be regulated during oligodendrocytes matura-tion by hnRNP1 and hnRNPF: reduction in HNRNPC and F expression in differentiated mouse oligodendrocytes correlates...
Table 3. Recapitulative data of microarray and QRT-PCR experiments.

| Gene name | FDR (%) eIF2B-pathy | FDR (%) OL | Microarray expression rate eIF2B-pathy | Microarray expression rate OL | QRT-PCR expression rate eIF2B-pathy | QRT-PCR expression rate OL | R² eIF2B-pathy | R² OL | P-value |
|-----------|---------------------|------------|----------------------------------------|-------------------------------|-----------------------------------|----------------------------|----------------|--------|---------|
| HNRNPF    | 1.1                 | 6.93       | 0.88 ± 0.15                            | 1.16 ± 0.42                   | 0.76 ± 0.15                       | 1.23 ± 0.49                 | 0.888          | 0.05   | 0.000   |
| DIAPH3    | 0.12                | 77.39      | 0.84 ± 0.24                            | 0.97 ± 0.28                   | 0.60 ± 0.13                       | 1.01 ± 0.36                 | 0.711          | 0.203  | 0.009   |
| VDAC3     | 0.05                | 93.98      | 0.82 ± 0.19                            | 1.02 ± 0.43                   | 0.77 ± 0.17                       | 1.14 ± 0.30                 | 0.594          | 0.07   | 0.008   |
| HNRPL     | 0.5                 | 82.58      | 0.77 ± 0.22                            | 1.00 ± 0.21                   | 0.80 ± 0.18                       | 1.06 ± 0.26                 | 0.555          | 0.033  | 0.000   |
| MRPL28    | 0.07                | 61.09      | 0.86 ± 0.14                            | 1.01 ± 0.23                   | 0.76 ± 0.11                       | 1.13 ± 0.19                 | 0.497          | 0.004  | 0.004   |
| HNRPH1    | 0.05                | 5.02       | 0.66 ± 0.15                            | 1.01 ± 0.38                   | 0.75 ± 0.13                       | 1.09 ± 0.23                 | 0.479          | 0.051  | 0.030   |
| HCCS      | 0.01                | 12.91      | 0.84 ± 0.12                            | 0.93 ± 0.41                   | 1.01 ± 0.17                       | 1.03 ± 0.28                 | 0.337          | 0.062  | 0.152   |
| HNRPC     | 0.04                | 48.18      | 0.78 ± 0.23                            | 1.01 ± 0.24                   | 0.83 ± 0.20                       | 1.11 ± 0.27                 | 0.117          | 0.004  | 0.144   |
| MRPS26    | 0.07                | 15.24      | 0.86 ± 0.13                            | 1.09 ± 0.32                   | 0.94 ± 0.21                       | 1.22 ± 0.54                 | 0.016          | 0.001  | 0.736   |
| KIF5B     | 0.01                | 40.18      | 0.86 ± 0.11                            | 0.94 ± 0.43                   | 0.81 ± 0.16                       | 0.96 ± 0.25                 | 0.01           | 0.003  | 0.876   |

Data obtained in eIF2B-mutated and OL fibroblasts for the 10 selected genes and correlation analysis between microarray and QRT-PCR data. Expression rate of microarray were calculated using the formula Mean ((Corrected G log value)i/(Corrected G log value)j) were (Corrected G log value) were calculated as explained in material and methods. i correspond to a patient’s fibroblasts and j to a control’s fibroblasts. The (Corrected G log value) between the ethanol and thapsigargin conditions were not different for the 10 selected genes which allowed us to mean them. Expression rate of QRT-PCR were calculated using the formula Mean (2^((delta (patient)- delta (control))) where delta (patient) correspond to (Ct gene x – Ct B2M) for one patient and delta (control) to (Ct gene x – Ct B2M) for one control. The 2^((delta (patient)- delta (control))) between the ethanol and thapsigargin conditions were not different for the 10 selected genes which allowed us to mean them. The two correlation coefficients are transformed with the Fisher Z-transform Zf = 1/2 * ln((1+R)/(1-R)) and the difference z = (Zf1 - Zf2)/SQRT(1/(N1-3)+1/(N2-3)) is approximately Standard Normal distributed. Then the p-value of the difference has been calculated. For six genes, the determination coefficient (R²) is significantly higher in 8/10 eIF2B-mutated patient/control fibroblasts couples than in the 10 OL patient/control fibroblasts couples. The difference between eIF2B-mutated/control and OL patient/control couples is high but not significant for the HCCS and HNRNPC genes and no significant differences is observed for the KIF5B and MRPS26 genes. FDR: False Discovery Rate value. doi:10.1371/journal.pone.0038264.t003
temporally with increased PLP/DM20 ratio [26]. DM20 results from an alternative splicing of the PLP1 gene resulting in exclusion of part of the exon 3 (Figure 2A). To gain insight into the splice representation of DM20/PLP showed no differences between the eIF2B-mutated and control foetal brains, and increasing in the post-natal brains compared to controls, in correlation with the hypomyelination observed in eIF2B-related disorders (Figure 2B, C). PLP and PLP+DM20 transcripts were undetectable or very low in the control foetal brains, and increasing in the children/adult brains consecutively to the myelination (Figure 2B, C). As a consequence, we observed an abnormal splicing or unstable regulation of the PLP/DM20 ratio was statistically significant in eIF2B-mutated foetal brains, reaching a peak during childhood and decreases by adult age (Figure 3B, C, D). The ratios pan-GFAP/GFAPalpha mRNA isoform was 4.5 and 7 times higher respectively in eIF2B-mutated children and adult brains.

The protein levels of PLP and DM20 and the quantification of the ratio PLP+DM20/PLP showed no differences between the control and patient brains at the children/adult stage, correlating with the QRT-PCR results (Figure 2F). PLP protein was undetectable in the foetal brains. These results showed a defect in the regulation of the PLP/DM20 mRNA splice or stability at an early stage of brain development.

Similarly, we quantified the astrocytic differentially expressed isoforms of GFAP, GFAPalpha and GFAPdelta in the same brain autopsy samples (Table 3). Pan-GFAP mRNA and the two isoforms GFAPalpha and GFAPdelta were amplified by QRT-PCR. The normal expression of the Pan-GFAP mRNA and its isoform alpha and delta is low at the foetal stage, reaches a peak during childhood and decreases by adult age (Figure 3B, C, D). Pan-GFAP and GFAPalpha mRNA are not differentially expressed between the control and eIF2B-mutated patient brains at all stages of development (Figure 3B, C). As a consequence, we observed an equivalent ratio pan-GFAP/GFAPalpha mRNA in the control and eIF2B-mutated patient brains (Figure 3E). However, GFAPdelta mRNA was significantly over-expressed in the eIF2B-mutated patient brains at every development stage (Figure 3D). The ratios pan-GFAP/GFAPdelta and GFAPalpha/GFAPdelta increased in the eIF2B-mutated children/adult brains compared to controls but not in the eIF2B-mutated foetus brains (Figure 3F, G). A specific upregulation of the GFAPdelta mRNA isoform was 4.5 and 7 times higher respectively in eIF2B-mutated children and adult brains.

Western blotting analysis confirmed that GFAPdelta was easier to detect in the eIF2B-mutated post-natal brains than in controls with a statistically significant difference in children samples (Figure 3H, antibody GFAPalpha not available). Similarly to PLP, GFAP proteins were undetectable in the foetal brains. These results demonstrate an abnormal splicing or unstable regulation of GFAP in the eIF2B-mutated patients brains that is more pronounced at the post-natal stage than in foetal stage.

### Table 4. Characteristics of brain samples used for microarray data validation on QRT-PCR (qPCR) and/or western blot (WB) analysis.

| Patient number | Age at autopsy | Gender | Mutated gene | cDNA mutation | Usage |
|----------------|----------------|--------|--------------|---------------|-------|
| 1327           | Fœtus 15w     | F      | WT           | WT            | qPCR, WB |
| 1437           | Fœtus 15w     | M      | WT           | WT            | qPCR, WB |
| 412            | Fœtus 19w     | M      | WT           | WT            | qPCR, WB |
| 46             | Fœtus 19w     | F      | WT           | WT            | qPCR, WB |
| 917            | Fœtus 19w     | M      | WT           | WT            | qPCR    |
| 311            | Fœtus 24w     | M      | WT           | WT            | qPCR    |
| 393            | Fœtus 14w     | M      | Eif2B5       | p.Val309Leu/p.Val309Leu | qPCR |
| 1767           | Fœtus 16w     | F      | Eif2B5       | p.Ala239Pro/p.Ala239Pro | qPCR, WB |
| 814            | Child 16m     | M      | WT           | WT            | qPCR, WB |
| 1500           | Child 7y      | M      | WT           | WT            | qPCR, WB |
| 1024           | Child 14y     | M      | WT           | WT            | qPCR, WB |
| 399-2          | Child 13m     | F      | Eif2B5       | p.Val309Leu/p.Val309Leu | qPCR, WB |
| 5103           | Child 16m     | M      | Eif2B4       | NA            | qPCR, WB |
| 357-1          | Child 4y      | M      | Eif2B5       | p.Arg136Cys/p.Arg339Trp | WB |
| 359-1          | Child 10y     | M      | Eif2B4       | p.Pro243Leu/p.Pro243Leu | qPCR, WB |
| NA             | Child         | NA     | Eif2B4       | NA            | qPCR    |
| 1203           | Child 7y      | M      | Eif2B5       | p.Gly386Val/p.Arg113His | qPCR, WB |
| 4993           | Child 12y     | M      | Eif2B4       | NA            | qPCR, WB |
| 1465           | Adult 17y     | M      | WT           | WT            | qPCR, WB |
| M3702M         | Adult 19y     | M      | WT           | WT            | qPCR, WB |
| 2804           | Adult 26y     | M      | WT           | WT            | qPCR, WB |
| 5017           | Adult 24y     | M      | Eif2B4       | NA            | qPCR, WB |
| 5106           | Adult 39y     | F      | Eif2B5       | p.Pro454Ser/p.Arg113His | qPCR, WB |

y: years; m: months; w: weeks; NA: not available. M: male; F: female; WT: wild type.
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Discussion

Differential Transcriptomic Analysis in Rare Neurological Disorders needs Comparison by Variance Analysis of Related Groups of Disease

In rare inherited neurological disorders, DNA microarray experiments are directed to detect differential gene expression in order to identify genes or metabolic pathways important for diagnostic, prognostic or therapeutic approaches. The novelty of our study has been to compare transcriptomic profiles of fibroblasts from eIF2B-mutated patients with fibroblasts of control subjects in addition to fibroblasts from control subjects. Moreover, we consider that comparing the fold change results is a misleading information offered by microarray experiments [30,31]. We used analysis of variance with the GeneANOVA software [32] to compare the data taking into account different factors such as.

![Figure 1. Expression rates of the 13 selected mRNA and two hnRN proteins in eIF2B-mutated fetal and patients brains. A. RNA was isolated from brains and analyzed by quantitative RT-PCR for expression of the 13 selected genes. Expression rates were calculated using the formula Mean (2^(-[delta (patient)- delta (control)])) where delta (patient) correspond to (Ct gene x - Ct B2M) for one patient and delta (control) to (Ct gene x - Ct B2M) for the corresponding control. Errors bars represent standard error (s.e.m.) (*P<0.05, **P<0.01, ***P<0.001). B–D. To assess the expression of hnRNPL and hnRNPU proteins in the brain autopsy samples, a nuclear and cytoplasmic differential extraction were performed followed by western blot analysis using antibodies specific for hnRNPL and U. GAPDH served as loading control. H3K27 served as a nuclear control. Are represented in B the fetuses samples, in C the children samples and in D the adults samples. C: control, Mt: eIF2B-mutated.

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disease status, couple number and ER-stress treatment between the different groups of fibroblasts: eIF2B-mutated, OL and controls. This type of statistical analysis allowed us to maximize the use of available data (relative transcription level of the corresponding gene and systematic bias due to the protocol such as labelling efficacy).

ER-stress Activation is not Specific to eIF2B-related Disorders

A hyper-activation of ER-stress response has been reported in fibroblasts from eIF2B-mutated patients [14], or rat oligodendroglial-derived cells expressing mutated human EIF2B5 gene [13]. An UPR (unfolded protein response) hyper-activation has
Figure 3. Study of the splice GFAP in eIF2B-mutated patients brains. A. Transcripts of human GFAP splice variants. The boxes represent the exons that are transcribed for the different isoforms. The termination codon, indicated by a blocked round circle, is located in exon 9 for GFAPalpha, and in exon 7a for GFAPdelta. The black and grey arrows represent respectively common primers between GFAPalpha and GFAPdelta (forward primer between the exons 1 and 2, reverse primer between exons 2 and 3), and specific primers for each isoforms (forward primer between the exons 8 and 9, reverse primer in the exon 9 for the GFAPalpha isoform, forward primer in the exon 6, reverse primer between the exons 7 and 7a for the GFAPdelta isoform).
also been described in brains from eIF2B-mutated patient brains compared to control individuals [15] and to a single PMD case [16]. Our transcriptomic analysis showed that ER-stress induced by thapsigargin did not help to differentiate gene expression profile of eIF2B-mutated fibroblasts from leucodystrophic fibroblasts of other causes. We explain this discrepancy as our 10 eIF2B-mutated patients expressed a homogenous severe phenotype and were compared for the first time to a group of infantile severe forms of leukodystrophic patients from various other causes including patients with other type of vacuolating LD. This suggests that the ER-stress response observed in eIF2B-mutated cells has no specificity despite the role of the eIF2B complex in translation regulation after stresses. Stress, classically reported as an onset trigger or aggravating factor in eIF2B-related disorders [9,33], also worsened symptoms in other groups of WM disorders particularly in other types of vacuolating LD. The functions of the ER in proper folding of proteins, lipids biosynthesis and storage of calcium could explain the susceptibility of the WM to ER stress, particularly during the myelination process that requires rapid synthesis of large amount of proteins and lipids [34].

Specific Genes Involved in eIF2B-related Disorders are Under-expressed and Belong Mainly to mRNA Regulation and Mitochondrial Functions

Our transcriptomic analysis identified 70 genes that were specifically differentially expressed in eIF2B-related disorders and belonging to three main biological processes.

The first group gathers 25% of the selected genes which are involved in transcription or mRNA stabilization and splicing, with a high z-score for the associated biological processes, suggesting a major disturbance of mRNA regulation. We identified six genes belonging to the HNRNP family (HNRNP1I/C/D/L/U/F and R) and two MBNL genes (MBNL1 and MBNL2: Muscleblind like 1 and 2) which were involved in mRNA splicing or mRNA stability and transport suggesting that splicing and stabilization of a large panel of mRNA can be affected in the eIF2B-related disorders. Alternative splicing, widely used to generate protein diversity and to control gene expression, is highly abundant in brain relative to other tissues [35,36]. It may generate cell-specific combination of protein isoforms that define the functional properties of the cells and underline complex processes such as synaptic adhesion and CNS plasticity [37]. Most of the main specific myelin proteins are submitted to alternative splicing, such as PLP/DM20 [24,25], MAG (Myelin-Associated Protein) [22] or MBP (Myelin Basic Protein) [38] and the production of the different isoforms during myelination have to be tightly regulated. Indeed, it has been demonstrated that the hnrnpH1 and F proteins control the alternative splicing of the Plp/Dm20 mRNA in a mouse oligodendroglial cell line [26]. The knock-down of these hnRNP proteins by siRNA modified the Plp/Dm20 mRNA ratio by increasing the production of the Plp mRNA isoform. Moreover, a recent study demonstrated that over-expression of HNRNPC2 in a human neuroblastoma cell line up-regulated the message level of MBP [39]. The identification of an increase of such a number of HNRNP genes in foetal brains together with their decrease in children and adult brains suggests that eIF2B mutations may have a major effect on myelination timing and myelin maintenance. These results are in agreement with the recent microarray analysis performed in Eif2b3-mutated mice brains where authors described a down-regulation of oligodendrocyte-specific genes at specific time point during the development [40]. Analysis of the published microarray results of Eif2b3-mutated mice brains showed a decrease in Hnrnp genes (e.g. HnrnpH1) at postnatal day 18 and 21 (P18 and P21), corresponding to the peak period of myelin formation. Here we identified the first link between eIF2B mutations and a potential myelin defect due to HNRNP1I mRNA, a key regulator of the major myelin protein PLP/DM20 splicing and the dynamic of the myelin production during the development. It has also been demonstrated that several HNRNP shuttle between the nucleus and the cytoplasm, transporting mature mRNA for translation or regulating mRNA stability [41]. So, the differential localization of HNRNPL and U in the foetal brains between controls and eIF2B-mutated patients yield to the hypothesis of a functional consequence on target genes and processes of these hnRNP.

The Second Group of Selected Genes is Related to Mitochondria Metabolism and Transport

Interestingly, five genes encoding mitochondrial ribosomal subunits (MRPS26, MRPL23, MRPL38, MRPL16 and MRPS9) were under-expressed. This suggests that protein synthesis in mitochondria is probably also affected by eIF2B mutations. The identification of some genes involved in the electron transport pathway (HCCS or component of the mitochondrial membrane like VDAC3, mitochondrial respiratory chain (SDHD) and in mitochondrial transport (KIF5B), suggests that the global metabolism of mitochondria is affected in eIF2B-related disorders. In CNS, mitochondria are notably important for Ca\(^{2+}\) signalling in oligodendrocyte precursor cells which seems to be critical during their migration, proliferation and differentiation [42]. Mitochondria are also essential for neuronal activity [43] and defects in their functions have been involved in various neurodegenerative disorders [27], such as SPG10, due to mutations in the neuron-specific KIF5A gene. Moreover, a study showed that Kif1b is required for the localization of mhp mRNA to processes of myelinating oligodendrocytes in zebrafish [44].

Another interesting gene differentially expressed in eIF2B-mutated patient fibroblasts and brains is the DIAPH. The human homolog of the Drosophila Diaphanus gene, involved in cytokinesis [29]. Disruption of the Diaphanus gene in Drosophila is responsible for spermatids degeneration and abnormal follicle cells development leading to male and female infertility. Moreover, another human gene belonging to the Diaphanus family named DLA is disrupted in a patient with premature ovarian failure [45]. DIAPH3 has been recently involved in cell migration, axon guidance and neurogenesis [46]. We can therefore hypothesize that it may be also involved in folliculogenesis, and would explain the ovarian failure sometimes observed in mild cases of eIF2B-related disorders (ovarioleukodystrophy) [7].
The differences found in genes expression between foetal and children/adult eIF2B-mutated brains suggest that eIF2B mutations have different effects depending on the developmental stage. Nine of the 13 genes tested are significantly over-expressed in foetal brains but 10 and 8 are under-expressed in children and adult brains respectively (Figure 1), suggesting that eIF2B mutations have specific deleterious effects during embryonic life. Our results coupled to the various congenital abnormalities, observed in the “congenital” forms of eIF2B-pathies (glaucoma, cataract, dysmorphic features [45]) suggest a larger effect on the programmed cell differentiation.

Abnormal Splice Regulation of Genes Important for Glial Cells Maturation is Observed in eIF2B-mutated Brains

In order to further analyse the dysregulation of genes involved in mRNA stabilization and splicing during the CNS development, we focussed our attention on two genes with splice isoforms differently expressed during glial maturation, respectively PLP in oligodendrocytes and GFAP in astrocytes.

We found an up-regulation of the ratio PLP+DM20/PLP demonstrating an increased expression of the DM20 isoform specifically in the mutated brains at foetal age (Figure 2). This up-regulation of DM20 in the eIF2B-mutated foetal brains is comcomitantly associated with an increased expression of HNRNP [33], suggesting a splicing defect. Regulation of PLP alternative splicing and maintenance of the PLP/DM20 ratio are critical for oligodendrocytes differentiation and myelin maintenance. In mature myelinating oligodendrocytes, the PLP 5’ splice site is preferentially used, while in oligodendrocytes progenitor cells and in other cell types, DM20 is the preferred site [23]. Mutations that impair the PLP/DM20 ratio cause a spectrum of dysmyelinating disorders in humans [47,48]. Therefore, alteration of PLP/DM20 mRNA splicing or isoforms stability related to HNRNP dysregulation could account for the myelin paucity with substantial increase in the number of non myelinating oligodendrocytes observed in eIF2B-related disorders [18,49].

We also found that mutated brains over-express GFAPdelta at all the developmental stage but not pan-GFAP and GFAPalpha. As a consequence, the ratio pan-GFAP/GFAPdelta is down-regulated in the children/adult mutated brains, whereas it is not affected in the foetal brains (Figure 2). GFAPdelta results from an alternative splicing of GFAP gene, which replaces the two final exons of the predominant isoformal GFAPalpha with an alternative terminal exon [50]. In normal adult grey and WM parenchyma, GFAPdelta represents only a small fraction of total GFAP [51]. However, GFAPalpha and delta transcripts are both up-regulated in Alzheimer’s disease [52]. Moreover, the transient over-expression of GFAPdelta in a human astrocytoma cell line, destabilizing the ratio GFAP alpha/delta, results in the formation of cytoplasmic aggregates that often collapse the endogenous GFAP networks [51]. Similarly to the present study, an over-expression of the mRNA and protein isoform GFAPdelta have been recently reported in the brain WM of an eIF2B-mutated child (4.5 months) and two adult (12 and 29 years) patients compared to control brains without differences in the major GFAPalpha isoform and in the ratio GFAPalpha/pan-GFAP [53]. The abnormal morphology of mutated astrocytes has been invoked to explain the extensive WM cavitation and limited gliosis of affected tissues observed in eIF2B-mutated brains [20]. In the affected WM, astrocytes have coarse blunt processes instead of the fine arborisations observed in controls. In the LD related to GFAP mutations (Alexander disease), the inability to form proper astrocytic GFAP networks coexists with incomplete maturation of astrocytes and insufficient myelin formation [54]. Our findings suggest that assembly-compromised GFAPdelta could alter protein interactions of GFAP filaments, thereby contributing to the aberrant morphology of affected astrocytes. This may contribute to the clinical similarity found between eIF2B and GFAP-related disorders, both being a cavitared demyelinating LD with stress-induced acute phases [2].

We described here for the first time genes involved in the mRNA stabilization and splicing machinery as potential actors in the abnormal maturation of glial cells observed in the eIF2B-mutated patient brains. However, if the link between the hnRNP proteins and the abnormal splice of PLP/DM20 mRNA is clear, the relation between hnRNP and GFAP mRNA splicing remain to be elucidated.

EIF2B-mutated fibroblasts lack a specific transcriptomic ER-stress profile but they differentially express genes important for mRNA regulation (HNRNP), mitochondrial metabolism/transport and gonadogenesis when compared to controls. Moreover, the abnormal HNRNP mRNA production observed in eIF2B-mutated brains varies during cerebral developmental and is comcomitantly with the splice dysregulation of the main genes involved in glial maturation. This suggests that the brain susceptibility of abnormal translation regulation related to eIF2B mutations involves a developmental expression deregulation of proteins implicated in mRNA splicing and stabilization machinery like the hnRNP which play a major role in other neurodegenerative disorders.

Materials and Methods

Ethics Statement

Studies have been performed with the ethical agreement of the “Centre de protection des personnes Sud-Est VI”, France and a signed informed consent of the parents. All patients or their legal guardians gave their written informed consents.

Samples from Fibroblasts of Leukodystrophic Patients

For the differential transcriptomic study, we analysed the leukodystrophic fibroblasts from ten eIF2B-mutated patients and from ten patients affected by OL coupled with 20 sex and age-matched control subjects without neurological signs (Table 1).

In the eIF2B-mutated group, all patients have a severe phenotype characterised by (i) an early infantile age of onset (mean 1.2±0.7 years, range: 5 months to 2.7 years), and (ii) a rapid progression leading to death or absence of motor and cognitive capacities in a mean of 1.7±1.4 years (range: 10 days to 3.5 years) (Table 1). We selected patients with a severe, early infantile form, because the impacts of the eIF2B mutations on the phenotype seem greater than in milder forms in which other environmental factors are also involved [33].

The 10 patients with OL have also an infantile age of onset (mean 0.9±1.3 years, from birth to 3.5 years). They included (i) five patients with other types of vacuolating LD (three patients with Alexander disease relative to GFAP mutation and two patients with megalencephalic leukoencephalopathy with subcortical cysts related to MLC1 mutations), (ii) two patients with a severe congenital form of hypomyelinating LD caused by PLP1 mutations PMP22 deficiency without motor acquisition, (iii) three patients with a demyelinating LD related to lipid metabolism enzymatic defect with a rapid progression in one case of Krabbe disease and a slower disease evolution in two cases of SLS (Table 1).

Samples from Patients’ Brains

Affected brain tissues used for quantitative real-time PCR gene analysis (Tables 4 and 5), provided from the brains samples of two eIF2B-mutated fœtuses (393 and 1767), were obtained after the
therapeutic abortions performed at respectively 14 and 16 weeks of gestation due to the affected status of the foetuses found by DNA/CVS analysis and immediately frozen in liquid nitrogen. In addition, brain samples of affected patients were identically frozen immediately after the brain autopsy performed between 13 months and 39 years. Control brain samples (fetus and children/adults) were provided by the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, Maryland (USA) and the fetopathologic unit of the Clermont-Ferrand University Hospital, (Dr AM Beaufre), Clermont-Ferrand (France).

Cell Culture, Thapsigargin Treatment and RNA Isolation
Primary fibroblasts were obtained from skin biopsy. They were grown on RPMI 1640 L-Glutamine medium (Gibco) supplemented with 1% Penicillin 1000U/ml, Streptomycin 10mg/ml, 0,001% Amphoterin B 2,5mg/ml and 10% FBS (Foetal Bovine Serum) at 37°C and 5% CO₂. Twenty-four hours after treatment, fibroblasts at nearly confluence were trypsinized with 0.1% trypsin-EDTA and cells were equally divided. One half of the cells were treated with 1μM thapsigargin and the other half with 0.05% ethanol during 4h at 37°C and were harvested with two PBS washes just after thapsigargin treatment. Thapsigargin raises cytosolic calcium concentration by blocking the ability of the cell to pump calcium into the endoplasmic reticulum which causes these stores to become depleted. Finally, fibroblasts from 6 control subjects used as reference were grown, harvested, lysed and RNA extracted identically to the others. Total RNA from fibroblasts and from all brain samples were extracted using the RNaseqy Lipid Tissue Mini Kit (Qiagen), according to the manufacturer’s instructions and stored at −80°C.

RNA Preparation for Microarray Experiments
For each sample, RNA quality and concentration were assessed using a Bioanalyser 2100 (Agilent Technologies, Waldbronn, Germany), according to the manufacturer’s instructions. For microarray hybridizations, 500 ng of total RNA were directly labelled by reverse transcription using the Low RNA input linear amplification (Agilent Technologies) and were purified using the RNeasy mini kit (Qiagen) according to the manufacturer’s recommendations. This reaction has been performed for RNA of each fibroblast of eIF2B-mutated patients, OL-patients and age- and sex-matched control subjects with Cy3 (green) incorporation.

The reference sample (pool of 6 control subject’s fibroblasts) was labelled with Cy5 (red) incorporation. All the dye incorporation rates were checked by ND-1000 spectrophotometer (Nanodrop Technologies). Fragmentation of cDNA was performed by denaturing the samples at 60°C during 30 min in order to obtain cDNA fragments of 50 to 200 nucleotides.

cDNA Microarray Hybridizations and Scanning
Two microarray experiments have been performed with and without ER-stress conditions: i) M1: cDNA samples of 10 eIF2B-mutated fibroblasts versus matched coupled controls fibroblasts; ii) M2: cDNA samples of 10 OL fibroblasts versus matched coupled controls fibroblasts. 

750 ng cDNA (M1) or 825 ng cDNA (M2) of mutated and controls cells, treated with thapsigargin or ethanol and labelled respectively with Cy3 and Cy5, were mixed into a single pool with the hybridization buffer. They were cohybridized on the same microarray slide (Human pangenomic 44K from Agilent Technologies) in an Agilent hybridization platform (Imaxio, Diagnogen division, Saint-Beauzire, France) at 65°C during 17h. Microarray slides were then scanned with the following parameters: i) M1 experiment: Cy3 Photo Multiplier Tube (PMT): 100, and Cy5 PMT: 100 with a 10 μM resolution; ii) M2 experiment: parameters for Cy3 and Cy5: Xdr High 100% –Xdr Low 10% with a 5 μM resolution, the Xdr corresponding to two scans (one at PMT 100 and one at PMT 10). These processes were repeated for each of the 40 hybridized slides. The images were analyzed with the Feature Extraction 9.1 software (Agilent Technologies) using the GE2-v4_91 (M1) or GE2-v5_95 (M2) protocols according to the manufacturer’s instructions.

cDNA Microarray Data Analysis
The statistical analysis was performed for characterized genes. EST and chromosomal location (LOC) have been excluded.

Correction and normalization. Transformation and normalization of hybridization data were performed to minimize variations arising from technical differences in RNA quality, probe labelling, and hybridization conditions between experiments. The reparation of the gene expression rates is asymmetric with a small number of high values leading us to perform a logarithmic transformation and standardisation for each signal intensity (giving the “log values”). Correction was next performed for differences in the variability across the range of gene expression levels using the formula : (corrected G log value)i = (G log value)i − (R log value)i + (mean R log value); where “G log value” represents sample signal intensity, “R log value” represents the reference signal intensity, and “mean R log value” represents the mean of all R values obtained for the gene “i” in reference across the different conditions.

Identification of Genes Specific of eIF2B-related Disorder
Genes differentially expressed (i) between the eIF2B-mutated patients and the control subjects for the M1 and (ii) between the OLs-patients and the control subjects for the M2, were determined statistically by variance analysis using the GeneANOVA software [32]. For M1 and M2 analyses, we constructed a statistical model including 3 factors: disease status (patients or control subjects), patients’ couple (couple 1 to 10) and treatment (thapsigargin or ethanol). Then we identified genes specific of eIF2B-related disorder by comparing the experiments M1 and M2 and the genes differentially expressed between the eIF2B-mutated patients and the OLs-patients were determined statistically by variance analysis using the GeneANOVA software [32]. We constructed a statistical model including 2 factors: disease status (patients or

**Table 5. List of the genes quantified by QRT-PCR analysis.**

| qPCR Primers | Sequence (5’ -3’) |
|--------------|------------------|
| Total GFAP, forward | AGAAGCTTACGATGAAACC |
| Total GFAP, reverse | TTCTCTGCTTCGTCATAGG |
| GFAPalpha, forward | AGAGCTTACAGGATCGCA |
| GFAPalpha, reverse | CACATCTTGCTTCGTC |
| GFAPdelta, forward | CCTTACAGGATGCTAGAG |
| GFAPdelta, reverse | GGGTTTCACTGACGAGT |
| GAPDH, forward | CTTCCTGCTTCGTCGAGC |
| GAPDH, reverse | TGGGAGCTTGCAGAGCCT |
| HPRT, forward | ATGGGGACCGCATCAGT |
| HPRT, reverse | ATGAATCCAGCAGGTAGCA |

Transcript-specific primers and their sequences for SYBR Green quantitation are mentioned.
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control patients) and treatment (thapsigargin or ethanol). The variance across each group of patients was included in residual.

In Silico Analyses of the Biological Processes Involved in eIF2B-related Disorders

Biological processes were analysed using the Genomatix software, available at http://www.genomatix.de. Using the BBSPE (Bibliosphere) function, we analysed and selected biological processes involving a maximum of the 70 selected genes. For each biological process, a z-score (or z-value) is calculated: it expresses the divergence of the experimental result from the most probable result as a number of standard deviations. The larger the value of z, the less probable the experimental result is due to chance. When z-score is over 3, one can consider that the result is significantly not due to chance.

Gene Expression Analysis of Selected Genes by Quantitative Real-time PCR (QRT-PCR)

Total RNA extracted from fibroblasts and from the brain samples was used for gene expression analyses by QRT-PCR of 13 selected genes, as well as PLP1/DM20 and GFAP isoforms (Tables 2 and 3). RT for each sample has been performed with 1 μg of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Two independent RT were performed for each sample and quantitative PCR was performed on Taqman 7300 using the Taqman Probe technology (Applied Biosystems) according to the manufacturer’s instructions. Two independent RT were performed for each RT sample and quantitative PCR was carried out on Taqman 7300 using the Taqman Probe technology (Applied Biosystems) with specific probes and primers (Taqman Gene Expression Assay) for each 13 selected genes and PLP1/DM20 isoforms, as pre-designed to overlap exon-exon boundaries and then prevent genomic DNA amplification. Quantitation was carried-out regarding the housekeeping β2-microglobulin (B2M) mRNA. For each selected gene, 4 μl of 1/10 cDNA dilution were amplified with 1X Taqman Gene Expression Master Mix (Applied Biosystems) and 1X of Taqman Gene Expression Assay (Tables 2 and 3) in a final volume of 20 μl. The program included an initial step of UDG incubation at 50°C for 2 min, a step of enzyme activation at 95°C for 10 min followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 min. The QRT-PCR were carried out in duplicates for each RT sample and each selected gene. The cycle of threshold value (Ct) was used to calculate the relative expression of the gene of interest and normalized to the transcript for the housekeeping gene B2M. Expression rate of QRT-PCR were calculated using the formula Mean (1 - (ΔCt(patient) - ΔCt(control))) where ΔCt(patient) correspond to (Ct gene x - Ctb2m) for one patient and ΔCt(control) to (Ct gene x - Ctb2m) for the corresponding control.

For the GFAP isoforms quantifications, transcript-specific primers were designed to overlap exon-exon boundaries to prevent genomic DNA amplification [53] (Table 5). The QRT-PCR was carried out using a sample volume of 20 μl containing Power SYBR Green PCR Master Mix (Applied Biosystems), 10 μM primers and 4 μl of 1/10 cDNA dilution. The relative abundance of transcript expression was calculated using the cycle of threshold value and normalized to the endogenous controls GAPDH (Glyceraldehyde Phosphate Deshydrogenase) and HPRT (Hypoxanthine-guanine Phosphoribosyl Transferase). The results were expressed as mean ± S.E.M. and compared using the Mann-Whitney test.

Protein Extraction and Western Blot Analysis

To assess the expression of hnRNP proteins in the brain autopsy samples, we performed a nuclear and cytoplasmic differential extraction, due to the major nuclear localisation of these proteins, using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manufacturer’s instructions. We validated the fractionation procedure using the anti-trimethyl-histone H3 (Lys27) (H3K27) antibody, that recognizes a histone mark specific to the nucleus. The cytoplasmic fractions were also used for PLP and GFAP detection. Protein concentration was then assessed by the Bradford method. 10 μg of protein lysates were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). Unspecific binding were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.1% Tween 20 overnight at 4°C. Incubations with primary antibodies were carried out at room temperature using mouse anti-GAPDH (1:5000, Abcam, ab8245), mouse anti-hnRNPL (1:3000, Sigma Aldrich, R005), mouse anti-hnRNPU (1:3000 for cytoplasmic fraction and 1:12000 for nuclear fraction, Sigma Aldrich, R6278), rabbit anti-GFAP (1:1500, Chemicon, ab5304), rabbit anti-GFAP delta (1:500, Abcam, ab28926), rat anti-PLP (1:200, gift from W Macklin) and rabbit anti-H3K27 (1:200, Millipore, #07-4449). After washing, membranes were incubated with an accordingly HRP-conjugated secondary antibody: anti-mouse (1:5000, Amer sham), anti-rabbit (1:5000, GE Healthcare) or anti-rat (1:5000, Rockland) at room temperature. Protein expression was detected by ECL reagent (GE Healthcare).

Bands on the films were quantified with ImageQuant TL software and compared using the Mann-Whitney test.

Supporting Information

Table S1 List of the 70 genes specifically dysregulated in eIF2B-mutated Fb obtained by microarray analysis. (DOC)

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Author Contributions

Conceived and designed the experiments: A. Huyghe LH OBTH AF. Performed the experiments: A. Huyghe LH MG AF. Analyzed the data: A. Huyghe LH A. Hénaut YD. Wrote the paper: A. Huyghe LH A. Hénaut AF. Reviewed the paper: DR EB RS.

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