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Shyue-Fang Battaglia-Hsu, Rose Ghemrawi, David Coelho, Natacha Dreumont, Pauline Mosca, et al.. Inherited disorders of cobalamin metabolism disrupt nucleocytoplasmic transport of mRNA through impaired methylation/phosphorylation of ELAVL1/HuR. *Nucleic Acids Research*, 2018, 46 (15), pp.7844-7857. 10.1093/nar/gky634 . hal-01844820

**HAL Id: hal-01844820**

**<https://hal.univ-lorraine.fr/hal-01844820>**

Submitted on 4 Dec 2021

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# Inherited disorders of cobalamin metabolism disrupt nucleocytoplasmic transport of mRNA through impaired methylation/phosphorylation of ELAVL1/HuR

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Received May 04, 2017; Revised June 26, 2018; Editorial Decision June 29, 2018; Accepted July 12, 2018

## ABSTRACT

The molecular mechanisms that underlie the neurological manifestations of patients with inherited diseases of vitamin B12 (cobalamin) metabolism remain to date obscure. We observed transcriptomic changes of genes involved in RNA metabolism and endoplasmic reticulum stress in a neuronal cell model with impaired cobalamin metabolism. These changes were related to the subcellular mislocalization of several RNA binding proteins, including the ELAVL1/HuR protein implicated in neuronal stress, in this cell model and in patient fibroblasts with inborn errors of cobalamin metabolism and Cd320 knockout mice. The decreased interaction of ELAVL1/HuR with the CRM1/exportin protein of the nuclear pore complex and its subsequent mislocalization resulted from hypomethylation at R-217 produced by decreased S-adenosylmethionine and protein methyl transferase CARM1 and dephosphorylation at S221 by increased protein phosphatase PP2A. The mislocalization of ELAVL1/HuR triggered the decreased expression of SIRT1 deacetylase and genes involved in brain development, neuroplasticity, myelin formation, and brain aging. The mislocalization was reversible upon treatment with siPpp2ca, cobalamin, S-adenosylmethionine, or PP2A inhibitor

okadaic acid. In conclusion, our data highlight the key role of the disruption of ELAVL1/HuR nuclear export, with genomic changes consistent with the effects of inborn errors of Cbl metabolisms on brain development, neuroplasticity and myelin formation.

## INTRODUCTION

Vitamin B12, (Cobalamin, Cbl) is essential for normal neurologic and hematologic development and function. However, the signalling pathway linking Cbl to the frequent neurological manifestations in diseases associated with errors of Cbl metabolism remains unidentified despite decades of intensive clinical and basic science investigations (1). The clinical presentation includes megaloblastic anemia, mental retardation and encephalopathy in paediatric patients with the inborn errors (1), and also peripheral neuropathy as well as other central nervous system (CNS) manifestations such as loss of memory and cognition, and dementia in Cbl deficient adult patients. Untreated, the neurologic sequelae can become permanent; therefore, a thorough understanding of the molecular mechanisms underlying the broad spectrum of symptoms is paramount for the enlargement of therapeutic options to these diseases (2).

In brain, cellular Cbl uptake is mediated by its binding in the form of Cbl-transcobalamin (TC) complex to CD320/TCBLR receptor (3,4). Once inside the cytoplasm, Cbl transforms into methylcobalamin (MeCbl) and serves as the cofactor for methionine synthase for *de novo* syn-

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thesis of methionine and its downstream production of S-adenosylmethionine (SAM) (2). SAM is the universal methyl donor for methylation of DNA and key proteins involved in the control of gene expression (2,5). There are only a handful of adequate experimental models for detailed mechanistic studies to decipher the roles of Cbl in brain. *Cd320* knockout mouse is a model with brain specific Cbl deficiency causing DNA hypomethylation (3,6). The TC-oleosin (TO) expressing strategy applied to cultured neuronal cells is another suitable model in which impaired Cbl metabolism can occur when cells overexpress TC-oleosin fusion protein (7). Cbl sequestration is only possible when TC is attached to oleosin at N-terminal (TO) but not C-terminal of oleosin (OT) - as no binding of Cbl to OT (oleosin-TC) is possible (7). Compared with WT mouse and OT cells, *Cd320* KO and TO cells, respectively, show significantly lower methionine synthase activity and methylation potential (6,8).

Little is known of the signalling pathways associated with disrupted Cbl metabolism in brain. Methionine synthase reductase (*Mtrr*) mutation altered gene expression through decreased DNA methylation and induced tissue malformations including neural tube (9). The nutritional model where Cbl and folate were both eliminated from food intake during pregnancy and lactation was shown to alter gene expressions in pups through imbalanced methylation and acetylation of nuclear receptors and their partner, PPAR $\gamma$  coactivator 1 $\alpha$  (PGC1 $\alpha$ ) (10–14). Cbl impaired metabolism of the cultured TO neuroblastoma cells caused endoplasmic reticulum (ER) stress downstream of reduced SIRT1 expression (5). The latter result is in line with the previous proteomic results from fibroblasts with inborn errors of Cbl metabolism; these cells show large-scale changes in protein expression in ER and in ubiquitin-proteasome system with UPR (unfolded protein response) and anti-oxidative stress functions (15).

The presence of ER stress in cells deficient of Cbl may hold the key to understanding the Cbl-associated neurological manifestations as emerging evidences indicate that cellular stress can produce a subcellular mislocalization of RNA binding proteins (RBP) capable of disruption of mRNA processing, and lead to neurological diseases and brain ageing (16–18). Phosphorylation and methylation changes in RBPs such as ELAVL1/HuR, hnRNPA1 (heterogeneous nuclear ribonucleoprotein A1), SRSF1 (serine/arginine-rich splicing factor 1) and Y14 (RNA Binding Motif Protein 8A) are part of the mechanisms that regulate the nucleocytoplasmic transport of mRNAs in cancer cells and in replicative senescence (19,20).

Our goal was thus to determine whether the neuropathological outcomes of inborn errors of Cbl metabolism are the consequences of dysregulated subcellular distribution of RBP. To address this issue, we examined three experimental models carrying compromised cellular Cbl metabolism, TO/OT N1E115 cells, *Cd320* KO mouse, and fibroblasts from patients with inherited defects leading to reduced methionine synthase activity. The evidence presented herein revealed that Cbl influences mRNA nucleocytoplasmic transport through controlling subcellular localization of RBPs, in particular on ELAVL1/HuR, by altering their methylation and phosphorylation status.

## MATERIALS AND METHODS

### Cell culture and siRNA/plasmid transfections

N1E115 cell culture protocol and stable transfection of TO and OT chimeric proteins was as described previously (8). Details are provided in supplementary methods. In experiments where siRNAs (*siElavl1*, *siPpp2ca*, *siCarm1* and control siRNA) were used, the siRNAs were obtained from Life technologies (Saint Aubin, France). The sequences and details of transfection are given in supplementary methods. We used the pcDNA3-ELAVL1/HuR-cFLAG (gifted by Dr J.A. Steitz, Yale University) as the template for the creation of ELAVL1/HuR mutations using the QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies; see supplementary methods for details). Cell culture of patient and control fibroblasts was performed as described (21).

### Preparation of separate nuclear and cytoplasmic lysates

The separation of nuclear and cytoplasmic lysates was performed using Paris kit (Ambion, Life technologies). This kit was used according to the manufacturer's instructions.

### Western blot

To prepare cell lysate, live cells or frozen mouse tissues were lysed directly with a solution containing sodium phosphate anhydrous dibasic, potassium dihydrogen phosphate, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and Complete Protease Inhibitors (Roche, Boulogne-Billancourt, France). Lysates were then subjected to centrifugation at 12 000 rpm for 30 min. The protein concentration of the supernatant was determined using BCA Protein Assay kit (Pierce, Brebieres, France) and BSA as standard protein. In general, 40  $\mu$ g of the total protein was loaded per lane for SDS-PAGE. Proteins from lysates were electro-transferred onto PVDF membranes; the latter were then processed by incubation with primary and secondary antibodies as described in supplementary methods. The protein bands were quantified densitometrically, normalized with  $\beta$ -actin, and expressed as arbitrary units.

### Immunofluorescence

**Neuroblastoma cells.** N1E115 cells were grown on slides for 48 h, then fixed with 4% paraformaldehyde for 10 min at 4°C and blocked with 3% BSA overnight. Cells were incubated with various primary antibodies as described in supplementary methods. The immunostained cells were imaged using Nikon C2 confocal microscope equipped with three laser lines (405, 488 and 543 nm). The images were obtained with 60x oil immersion lens.

**CD320 mice.** The inactivation of the transcobalamin receptor (CD320 KO) in mouse produced an impaired uptake of Cbl in the CNS (4,6). Sagittal brain sections (paraffin) were processed as described previously in the *immunofluorescence-Neuroblastoma cells* section (supplementary methods).

### Duolink (proximity ligation assay, PLA)

The proximity ligation assay (PLA; Duolink *in situ* PLA reagents; Olink Bioscience; Eurogentec, France) was performed to visualize and quantify interactions, as described previously (11,21). The PLA signals were counted as described in supplementary methods.

### FISH staining

Neuroblastoma cells were grown on slides for 48 h, fixed with 4% paraformaldehyde and permeabilized with PBS–0.1% triton. A prehybridization was performed followed by the hybridization; FISH probes were obtained from Eurogentec (Angers, France). The cells were imaged using Nikon C2. The detailed procedure is described in supplementary methods.

### ImmunoPrecipitation (IP)

Live cells or minced frozen mouse brain tissues from hippocampus and striatum were lysed directly with the IP lysis/Wash buffer. The total protein used per IP reaction (500  $\mu$ g) was combined with 5  $\mu$ g of affinity purified antibody and incubated overnight at 4°C to form the immune complex. This complex was added to the resin Protein (Thermo Scientific). After centrifugation followed by four washes with lysis/wash buffer, the final pellet was combined with laemmli buffer and loaded onto SDS-PAGE gel for separation via electrophoresis. Immunoblottings were then obtained by procedures as described in supplementary methods.

### Ribonucleoprotein immunoprecipitation (RIP)

The preparation of the immune complex (ELAVL1/HuR complex) was performed as described previously in the immunoprecipitation section above. Instead of adding laemmli buffer, 150  $\mu$ l IP lysis/Wash buffer, 7.5  $\mu$ l SDS 20% and 18  $\mu$ l Proteinase K (10 mg/ml) were added to the resin. This mixture was incubated for 30 min at 55°C with rotary agitation. Then a phenol–chloroform extraction was performed. Reverse transcription was done on the RNA by using the iScript cDNA Synthesis System (Bio-Rad, Marnes-la-Coquette, France) Quantitect Reverse Transcription kit (Qiagen, Paris, France). Quantitative PCR was performed as described in supplementary methods.

### Animals

The null *Cd320* KO mice were produced locally by cross breeding heterozygous C57BL/6N strain mice transported to France from SUNY Downstate Medical Center (Brooklyn, New York). The animal experiments were all performed in animal facilities authorized to carry out experimentation and followed the Directive 2010/63/EU revising the directive 86/609/EEC on the protection of animals used for scientific purposes.

### Transcriptome

RNA was purified and hybridized on Agilent 44k Array as described in supplementary methods (22).

### Statistics

Data were analyzed with Stata 12.0 software (StataCorp, College Station, TX, USA) and reported as means  $\pm$  S.E. Data were compared by one-way analysis of variance (ANOVA) with Fisher's test. We assigned a number of determination per study groups of cell ( $n = 8$  in each group) and animal experiments (12 males and 12 females), which corresponded to a study power calculation based on a 2-fold difference between groups, with  $1-\beta = 0.8$  and  $\alpha = 0.05$ . A  $P$ -value 0.05 was considered to indicate statistical significance. Results were denoted by asterisks in figures ( $*P < 0.05$ ;  $**P < 0.01$  and  $***P < 0.001$ ).

### Study approval

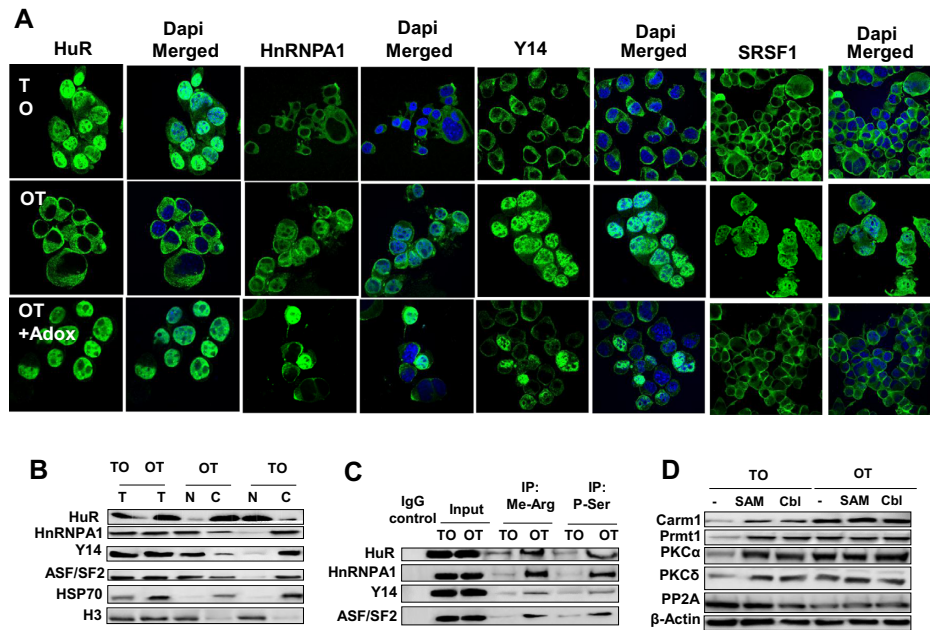
We obtained the approval of the ethical committee for animal experiments of the region Lorraine to perform the animal study in *CD320* mice (agreement # 5454722).

## RESULTS

### The impaired cellular Cbl availability produced dramatic transcriptomic changes associated with mislocalization of RNA binding proteins in TO and OT cells cultured in the presence of endoplasmic reticulum stress inducer G418

Reduced Cbl cellular availability influenced considerably the transcriptome of the cell model studied (TO versus OT cells; Supplementary Table S1 and S2 and Supplementary Figures S1 and S2). Metabolic processing of RNA, cellular response to stress, regulation of cell cycle, neurogenesis, neuron differentiation, neuroplasticity and neuron development were among the top molecular and cellular functions affected (Supplementary Table S1 and S2). Some of these changes were related to neuroplasticity, including altered expression in dopaminergic, GABAergic, serotonergic and cholinergic synapses (Supplementary Figure S1). The prominent changes related to metabolic processing of RNA and cellular response to stress led us to investigate potential deregulation of RNA transport mediated by RBPs, including ELAVL1/HuR, hnRNPA1, Y14, SRSF1 (also named ASF/SF2), FMRP, TDP43, and MLN51 (Figure 1 and Supplementary Figure S3). The transfected TO and OT cells were cultivated in the presence of G418. This aminoglycoside is used to maintain the expression of the *neo* gene to ensure stable transgene expression; it produces ER stress. We studied thus the effect of G418 on non-transfected N1E115 cells. The introduction of G418 in culture medium targeted ELAVL1/HuR into cytoplasm in both non-transfected N1E115 cells and OT control cells (Figures 1 and 2A); interestingly, G418 also caused a limited amount of hnRNPA1 shuttling into cytoplasm in control OT cells, but it had no effect on the nuclear localization of Y14 and SRSF1 (Figure 1A). In TO cells with the presence of G418, its Cbl deficiency blocked ELAVL1/HuR export and caused cytoplasmic retention of hnRNPA1, Y14 and SRSF1 (Figure 1A). This mislocalization was not observed in the other RBPs investigated (Supplementary Figure S3). These effects depended on protein methylation as the treatment in OT cells with AdoX, an antagonist of SAM and a general methylation blocker, triggered ELAVL1/HuR, Y14





**Figure 1.** Subcellular mislocalization of RNA binding proteins (RBPs) in relation with their arginine methylation and serine phosphorylation and the expression of methyltransferases, kinases and protein phosphatase PP2A. (A) Subcellular localization of ELAVL1/HuR, hnRNPA1, Y14, SRSF1 examined using immunofluorescence technique in TO versus OT cells. In control OT cells, RBPs shuttling block could be induced by adding AdoX. (B) Western blots of nuclear and cytosolic ELAVL1/HuR, hnRNPA1, Y14, and ASF/SF2 (also named SRSF1) protein extracted from Cbl-lacking TO versus control OT cells. Histone 3 and heat shock protein 70 were used as internal controls; abbreviation: T, total protein extract; N, nuclear protein extract; C, cytosolic protein extract. (C) Immunoprecipitated RBPs were probed with anti-methyl-arginine and anti-phosphoserine antibody, respectively, to evaluate their methylation and phosphorylation status. (D) Expression of methyltransferases (CARM1 and PRMT1), kinases (PKC $\alpha$ , PKC $\delta$ ) and phosphatase PP2A in TO versus OT cells. Their expressions were cobalamin (Cbl) and SAM dependent.

and SRSF1 to localize to the same subcellular compartment as that observed in TO cells (Figure 1A). The mislocalization detected by immunofluorescence was later confirmed by Western blots (Figure 1B and Supplementary Figure S4). Western blots of the immunoprecipitated RBPs showed equally less arginine methylation and serine phosphorylation of ELAVL1/HuR, hnRNPA1, Y14 and SRSF1 in TO versus OT cells (Figure 1C and Supplementary Figure S4). Indeed, we found decreased expressions of CARM1 and PKCs, the enzymes involved in arginine methylation and serine phosphorylation of these RBPs, and an increased expression of PP2A phosphatase in TO versus OT cells (Figure 1D and Supplementary Figures S4 and S6).

Taken together, our data showed that impaired Cbl availability caused subcellular mislocalization and decreased arginine-methylation and serine-phosphorylation of RBPs, with ELAVL1/HuR as the most affected RBP in TO versus OT cells exposed to G418.

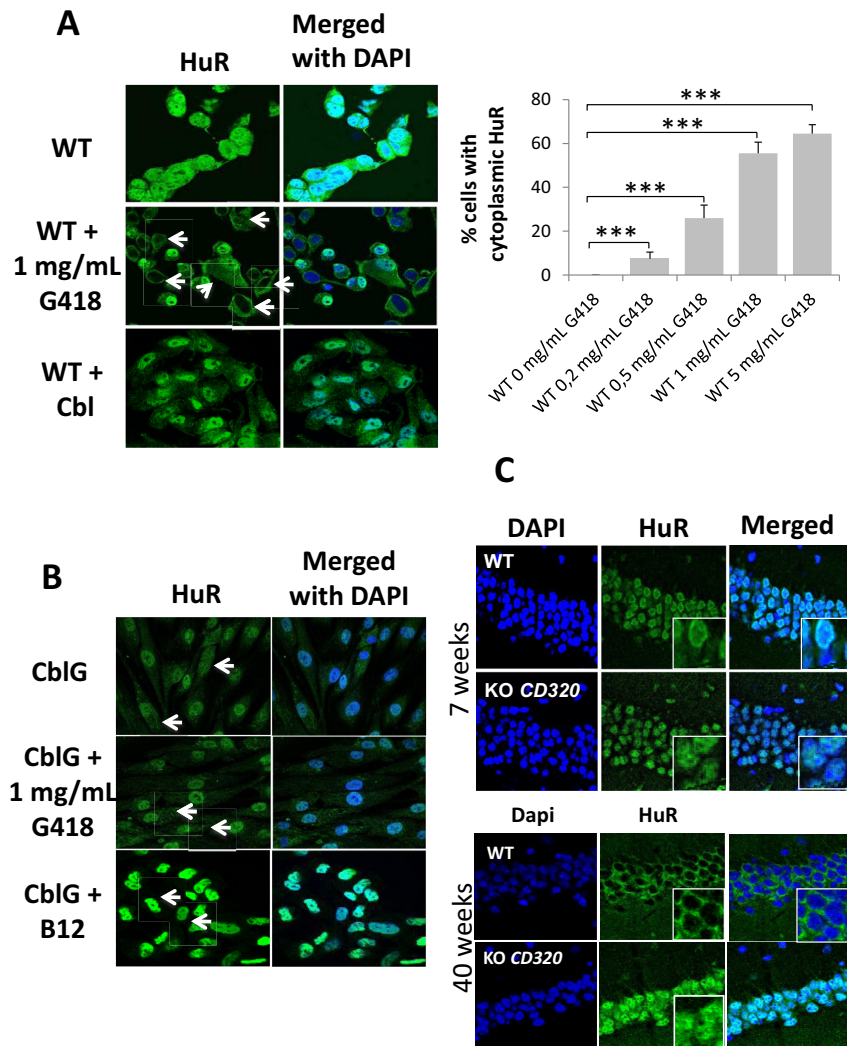
### The mislocalization of RNA binding proteins was also observed in fibroblasts of patient with inborn errors of Cbl metabolism and in Cd320 KO mice

In control wild type fibroblasts, we found ELAVL1/HuR localized exclusively to the nucleus. The exposure of these cells to G418 targeted ELAVL1/HuR into cytoplasm in more than 60% of the wild type cells. This effect was reversed with the addition of Cbl (Figure 2B). In contrast, in *CblG* fibroblasts, ELAVL1/HuR was localized to both the nucleus and cytoplasm, and this subcellular distribu-

tion pattern could not be modified further by the addition of G418 (Figure 2B). These results suggested that the nucleocytoplasmic shuttling of ELAVL1/HuR was partially blocked in *CblG* fibroblasts (even under ER stress with the presence of G418). In mice, the subcellular localization of ELAVL1/HuR changed with age. At 7 weeks old, no difference between wild type and *Cd320* KO mice was observed in ELAVL1/HuR subcellular distribution in brain, both being nuclear-bound. This changed with age; at 40 weeks in several neuronal types such as hippocampal neurons, ELAVL1/HuR was predominantly cytoplasmic in wild type mice, in contrast to the nuclear localization observed in *Cd320* KO mice (Figure 2C). Thus, we suggested that in WT mice the physiological stress of ageing is the driving force for ELAVL1/HuR export into the cytoplasm. In wild type neurons, ELAVL1/HuR successfully shuttled into the cytoplasm to exert anti-stress response. However, in *Cd320* KO mice, this failed due to the dysregulated ELAVL1/HuR export.

### The disruption of ELAVL1/HuR export triggered the nuclear retention of ELAVL1/HuR-specific AU-rich RNA including *Sirt1* mRNA

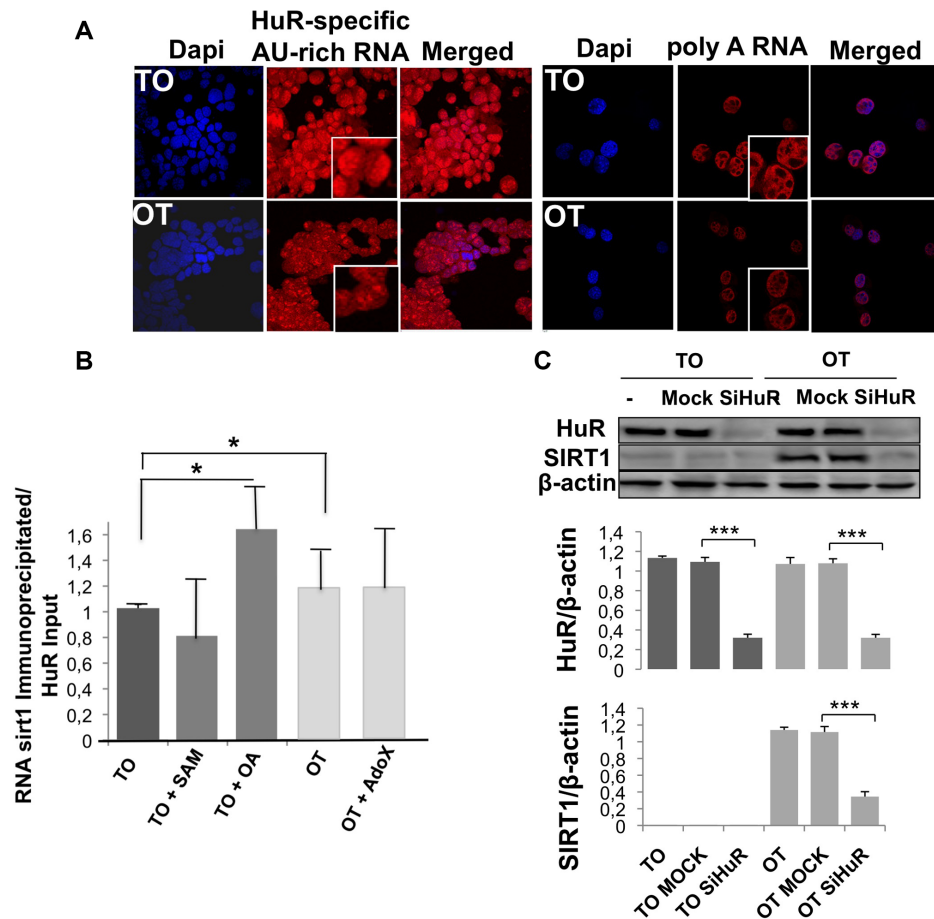
We subsequently asked whether the altered distribution of ELAVL1/HuR could block the nucleocytoplasmic mRNA transport in general and in particular *Sirt1*. Using degenerated RNA probes designed for ELAVL1/HuR (23), we observed a higher labeling intensity of the ELAVL1/HuR-specific AU-rich RNAs in the nucleus of TO versus OT



**Figure 2.** Localization of ELAVL1/HuR in wild type N1E115 cells, fibroblasts with inborn error of CblG, and Cd320 KO mice. (A) Effect of G418 on the nuclear localization of ELAVL1/HuR in control N1E115 (WT, wild type,  $P < 0.001$ , ANOVA). (B) Nucleus and cytoplasm localization of ELAVL1/HuR in fibroblasts of the CblG patient in the presence and absence of G418. Cobalamin increased the nuclear localization of ELAVL1/HuR in CblG cells. (C) In mice model, ELAVL1/HuR localization altered with age. In young animal, no difference was noted on the nuclear localization of ELAVL1/HuR; with age due to aging stress, in wild type mice ELAVL1/HuR became localized to the cytoplasm while in KO mice, ELAVL1/HuR remained nuclear. Here, hippocampal neurons were used to illustrate the mislocalization associated with aging stress of the Cd320 mice.

cells (Figure 3A). Consistent with this, significant transcriptomic changes in TO versus OT cells involved the known targets of ELAVL1/HuR (<http://starbase.sysu.edu>). In fact, among the 2447 orthologous ELAVL1/HuR targets in mouse, the transcription of as much as 82% of them were altered in TO versus OT cells (Supplementary Figure S2). The top functional annotations of these genes were related to RNA transport, ER stress, neuroplasticity, protein acetylation and cell cycle (Supplementary Figure S2, Supplementary Table S1). These results suggested that the cellular Cbl deficit caused nuclear sequestration of the AU-rich RNAs through impaired export of ELAVL1/HuR. Among the AU-rich targets of ELAVL1/HuR, we examined in particular the *Sirt1* transcript as we previously found decreased SIRT1 expression in TO versus OT cells (5). We performed RNP immunoprecipitation (RIP) of ELAVL1/HuR and we found a slight decrease of the amount of ELAVL1/HuR-

bound *Sirt1* mRNA in TO versus OT cells (Figure 3B). It should be noted that the real difference in the amount of cytoplasmic *Sirt1* mRNA bound to ELAVL1/HuR between TO and OT cells was underestimated since whole cell lysates were used for the RIP. The treatment of TO cells with SAM had no significant effect on the binding of ELAVL1/HuR to *Sirt1* mRNA. In agreement with this finding, AdoX had no effect in OT cells. These results showed therefore that the methylation of ELAVL1/HuR has a limited influence, if any, on the amount of ELAVL1/HuR-bound *Sirt1* mRNA in TO versus OT cells. In contrast, the treatment of TO cells with Okadaic acid, an inhibitor of protein phosphatase 2 (PP2A) increased the binding of ELAVL1/HuR to *Sirt1* mRNA. The effect of Okadaic acid suggests that the increased phosphorylation of ELAVL1/HuR may increase its binding to *Sirt1* mRNA. Conversely, the decreased phosphorylation of ELAVL1/HuR may explain the slight de-



**Figure 3.** Influence of ELAVL1/HuR nuclear cytoplasmic shuttling on SIRT1 expression and AU-specific mRNA transport. (A) FISH staining of the ELAVL1/HuR specific AU-rich RNAs and poly-A tail RNAs in the *Cbl* deficient TO versus OT cells. (B) *Sirt1* mRNA isolated from the RIP complex using anti-ELAVL1/HuR antibody and amplified using qPCR technique in TO versus OT cells. The ELAVL1/HuR/*Sirt1* mRNA binding was tested with and without treatment with SAM, AdoX and Okadaic Acid (OA) as described in supplementary informations. (C) SIRT1 expression in ELAVL1/HuR knockdown by siELAVL1/HuR in TO versus OT cells (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , ANOVA).

creased of its binding to *Sirt1* mRNA in TO versus OT cells (Figures 1C and 3B). To confirm the role of ELAVL1/HuR on *Sirt1* expression, *Elavl1* was knocked down using siRNA technique. The si*Elavl1* reduced both ELAVL1/HuR and SIRT1 protein expressions in TO and OT cells (Figure 3C). Providing quantifications assessing the effect of knocking ELAVL1/HuR on Sirt1 protein levels was not possible due to its very low levels in untreated TO cells (Figure 3C).

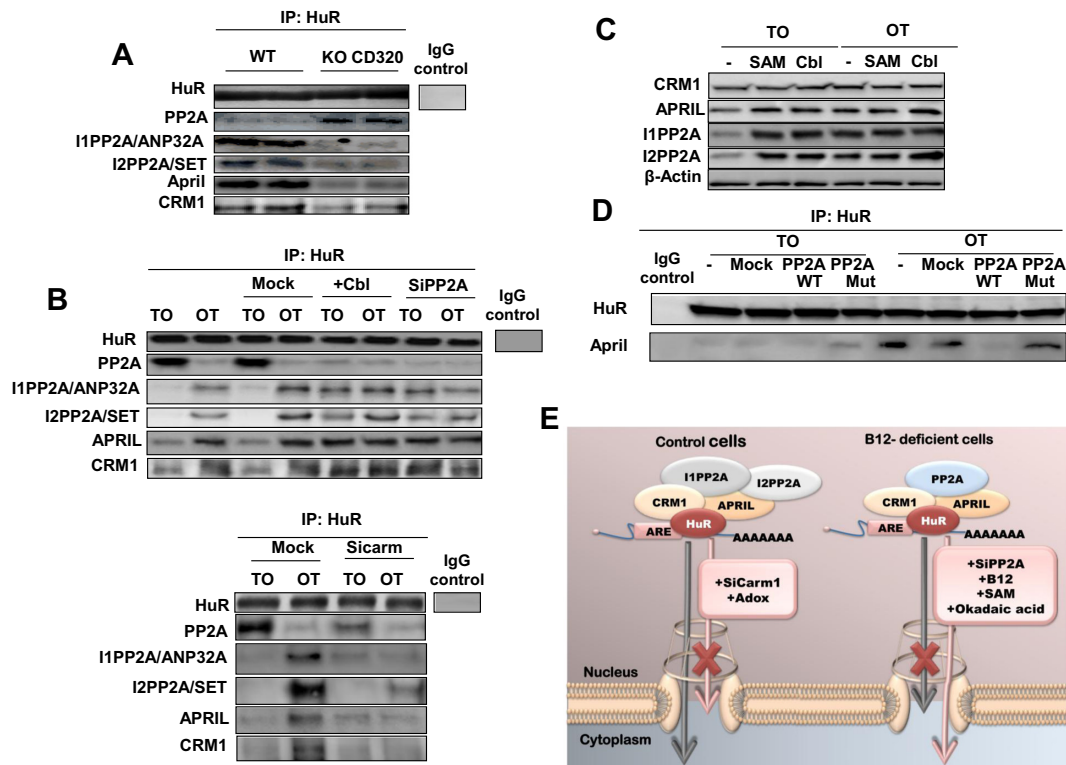
#### ELAVL1/HuR mRNA export was blocked by *Cbl* impaired metabolism through altered interactions of ELAVL1/HuR with its partners of the nuclear pore complex (NPC)

To dissect the molecular mechanisms of the ELAVL1/HuR-dependent mRNA export block, we tested whether *Cbl* deficiency altered the interactions of ELAVL1/HuR with its partners of NPC. In NPC, ELAVL1/HuR recruits CRM1/exportin via the adaptor proteins IPP2A and APRIL to shuttle the AU-rich element (ARE)-containing mRNAs (24). In *Cd320* KO versus WT mice, we found that less CRM1, I1PP2A, I2PPA and APRIL, and more PP2A proteins co-immunoprecipitated with ELAVL1/HuR (Figure 4A and Supplementary Figure

S4). The same difference was obtained in the co-IP of TO versus OT cells (Figure 4B). Two ELAVL1/HuR partners, I1PP2A and I2PP2A, were practically missing, and CRM1 and APRIL were significantly decreased in TO cells (Figure 4B and Supplementary Figures S4, S5). This was due in part to their decreased expression in TO cells (Figure 4C and Supplementary Figure S4). The reduced interaction of ELAVL1/HuR with CRM1 was counterbalanced by its interaction with PP2A (Figure 4B and Supplementary Figures S4 and S5). Treating TO cells with *Cbl* or SAM restored the expression of ELAVL1/HuR partners (Figure 4C). Decreasing PP2A expression via si*Ppp2ca* restored the interaction of ELAVL1/HuR with CRM1/exportin, while decreasing CARM1 expression via si*Carm1* produced the opposite effect (Figure 4B and Supplementary Figure S5). The increased expression of wild type PP2A by transfection in OT cells decreased the binding of APRIL to ELAVL1/HuR, showing that the interaction was affected by PP2A-mediated ELAVL1/HuR dephosphorylation (Figure 4D and Supplementary Figure S5).

Using Duolink (proximity ligation assay, PLA) we further studied the interaction between ELAVL1/HuR and CRM1, and the nucleocytoplasmic localization of





**Figure 4.** Influence of Cobalamin on the interaction of ELAVL1/HuR-containing mRNP complex with nuclear pore receptor CRM1. (A) Interaction of PP2A, I1PP2A/pp32/ANP32A, I2PP2A/SET, APRIL and CRM1 with ELAVL1/HuR in Cd320 KO versus WT mice. (B) Same interactions in TO versus OT cells. Knock-down of CARM1 disrupted these interactions while addition of Cbl, as well as knock-down of PP2A restored them. The interactions between ELAVL1/HuR and its partners CRM1, I1PP2A/pp32/ANP32A, I2PP2A/SET and APRIL were evaluated by the quantity of these proteins present in the ELAVL1/HuR Co-IP complex. (C) The levels of CRM1, APRIL, I1PP2A, I2PP2A in total cellular protein extracts in control and supplemented conditions with either SAM or Cbl. (D) Influence of PP2A mutant on the interaction between ELAVL1/HuR and April. (E) The proposed schematic of ELAVL1/HuR export and the effect of Cbl deficiency on ELAVL1/HuR shuttling. In control cells, the ELAVL1/HuR export complex includes I1PP2A/ANP32A, I2PP2A/SET, APRIL and CRM1. This export is blocked by treatment with AdoX or SiCarm1 transfection. In Cbl-deficient cells, the strong interaction between PP2A and ELAVL1/HuR appears to disrupt ELAVL1/HuR's interaction with its regular partners - I1PP2A/ANP32A, I2PP2A/SET, APRIL and CRM1 leading to an export block. This block is reversible with B12, SAM, okadaic acid supplementation or SiPP2A transfection.

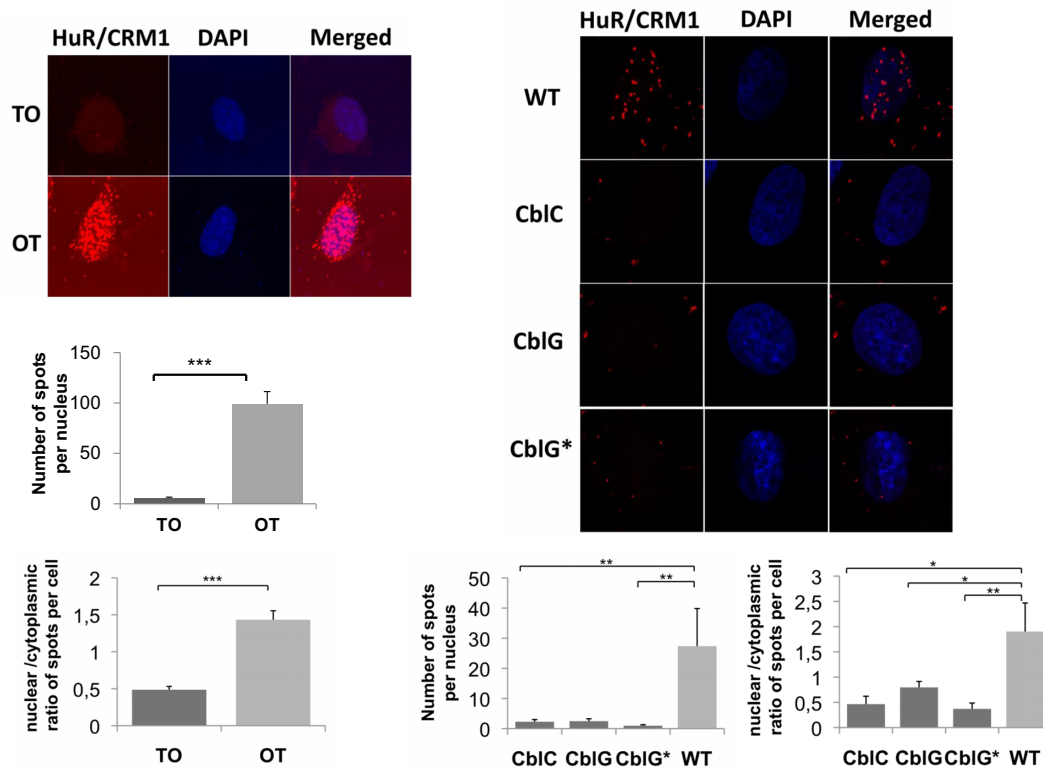
ELAVL1/HuR in fibroblasts of normal subject and patients with *CblC* (mutation of *MMACHC*), *CblG* (mutation of *MTR*), and *CblG*-variant inherited disorders. We observed a substantial decrease in ELAVL1/HuR-CRM1 interaction in the nucleus of patients vs. normal cells (Figure 5). Taken together these data indicated that cellular Cbl deficit in both cell and animal model disrupted the interaction of ELAVL1/HuR with CRM and its partners of NPC through PP2A- and CARM1-dependent mechanisms.

#### The disruption of ELAVL1/HuR shuttling by Cbl cellular deficiency was both SAM- and methylation-dependent

As Cbl serves for the *de novo* synthesis of methionine and, hence, for SAM, we asked if disrupted ELAVL1/HuR shuttling was the consequence of decreased SAM level in TO cells and patients' fibroblasts. We observed in TO cells but not OT cells a large reduction in the percentage of cells with nuclear ELAVL1/HuR staining when adding either Cbl or SAM (Figure 6A and B). Conversely, incubation of OT cells with SAM inhibitor AdoX led to ELAVL1/HuR nuclear retention (Figure 1A). The same treatment of TO cells produced no extra effect. The Duolink proximity

ligation assay of methyl-ELAVL1/HuR with antibodies against methyl-arginine and ELAVL1/HuR revealed that its localization was cytoplasmic (Figure 6C). CARM1 is known to catalyze the methylation of ELAVL1/HuR R217 (25). We used a custom-made affinity-purified antibody against methyl<sup>R217</sup>ELAVL1/HuR and a methylated peptide described previously (25) to answer whether cellular Cbl deficiency produced a R217 demethylated ELAVL1/HuR. Indeed, Western blots revealed lower methyl<sup>R217</sup>ELAVL1/HuR level in TO versus OT cells (Figure 7A). The addition of either Cbl or SAM to TO cells rescued the expression of methyl<sup>R217</sup>ELAVL1/HuR (Figure 7A and Supplementary Figure S6). Results of the immunofluorescence stainings with anti-methyl<sup>R217</sup>ELAVL1/HuR in the presence of blocking peptide, either methylated or non-methylated, indicated that methyl<sup>R217</sup>ELAVL1/HuR resided only in the cytoplasmic compartment of both TO and OT cells (Figure 7B). Data from knocking down CARM1 with *siCarm1* confirmed its involvement in ELAVL1/HuR cytoplasmic export (Figure 7C, Supplementary Figure S6). Together, the evidence showed that the decreased CARM1-dependent methyla-





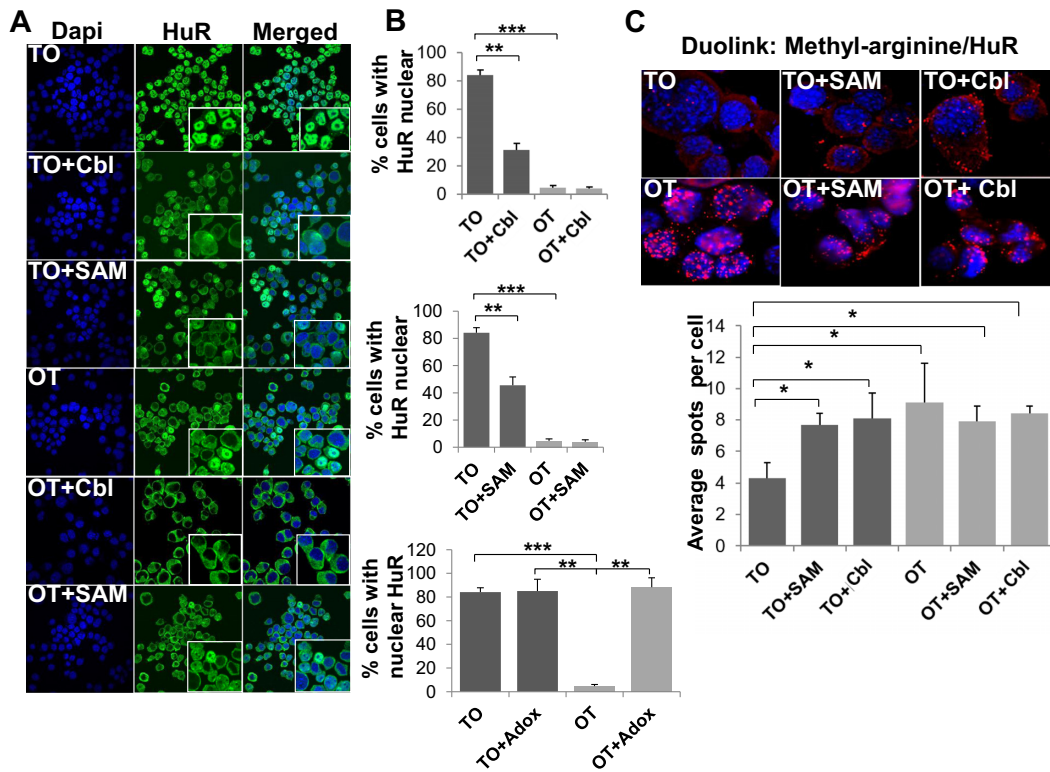
**Figure 5.** Duolink analyses of the interaction between ELAVL1/HuR and CRM1 in TO versus OT N1E115 cells and fibroblasts from patients with cobalamin inherited disorders. (A) Decreased ELAVL1/HuR-CRM1 interaction in the nucleus of TO and patient cells, compared to OT and control fibroblasts. (B) Decreased ELAVL1/HuR-CRM1 interaction in the nucleus of fibroblasts from patients with CblC, CblG and CblG-variant inherited disorders, compared with fibroblasts from control subjects (WT) (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , ANOVA).

tion of ELAVL1/HuR produced by Cbl cellular deficiency blocks the export of ELAVL1/HuR into cytoplasm.

### The dephosphorylation of serine 221 by PP2A and the methylation of arginine 217 by CARM1 in ELAVL1/HuR were two distinct mechanisms that predict the subcellular localization of ELAVL1/HuR

The presence of non-methylated ELAVL1/HuR in both cytoplasm and nucleus (see Figure 7B, TO versus OT in the presence of blocking methylated peptide) suggested that a second mechanism could influence the export of non-methylated ELAVL1/HuR. Recent data from the literature suggest that ELAVL1/HuR interacts with PP2A phosphatase (26) and that Cbl deficiency or DNA hypomethylation can lead to over-expression of PP2A (8,27). Consistently, we observed that the level of methylated and demethylated PP2A protein varied in a Cbl- and SAM-dependent manner in TO cells (Figure 8A). The Duolink assay shown in Figure 8B revealed that the impaired Cbl availability in TO cells reduced ELAVL1/HuR serine phosphorylation. This result was consistent with the Western blot of ELAVL1/HuR immunoprecipitated with an anti-phosphoserine antibody (Figure 1C). Both PP2A and ELAVL1/HuR were co-localized to the nucleus of TO cells and both were predominantly detected in the cytoplasm of OT cells (Figure 8C). The application of either PP2A inhibitor okadaic acid or knockdown of PP2A by *siPpp2ca*

independently changed the localization of both PP2A and ELAVL1/HuR from nucleus to cytoplasm in TO cells. This did not occur in OT cells (Figure 8C). The demethyl-PP2A was found in both nucleus and cytoplasm in TO cells while methyl-PP2A was mainly cytoplasmic (Supplementary Figure S6). Numerous serine phosphorylation sites have been previously identified in ELAVL1/HuR (28). We focused our attention here on S221 phosphorylation for two reasons: (a) S221 is located in the nucleocytoplasmic shuttling (HNS) domain of ELAVL1/HuR, and (b) PKC $\alpha$  and PKC $\delta$  have altered expression in TO versus OT cells (Figure 1D) and are both implicated in S221 phosphorylation. The decreased phosphorylation of ELAVL1/HuR in TO cells (Figure 1C) was consistent with the decreased expression of serine kinases, PKC $\alpha$  and PKC $\delta$  and the increased expression of PP2A (Figure 1D and Supplementary Figure S4). To decipher the respective and combined role of R217 methylation and S221 phosphorylation on ELAVL1/HuR shuttling, we created S221A, S221D, R217A single mutations and R217A-S221A and R217A-S221D double mutations in a human ELAVL1/HuR construct (29) and we overexpressed them in control OT cells. Transfection with R217A and S221A single mutants and R217A-S221A double mutants all led to fewer cells with cytoplasmic-only ELAVL1/HuR staining than those observed in mock transfected OT cells (Supplementary Figure S7). These results suggested that R217 methylation and S221 phosphorylation independently regulate the subcellular localization of



**Figure 6.** Influence of cobalamin and S-adenosyl methionine on the nucleocytoplasmic shuttling and methylation of ELAVL1/HuR. (A) The addition of cobalamin (Cbl) at 20  $\mu$ M or S-adenosyl methionine (SAM) at 75  $\mu$ M to the Cbl-lacking TO cells partially restored the shuttling block of ELAVL1/HuR in TO cells. (B) Influence of Cbl, SAM and AdoX at 20  $\mu$ M for 36 h on ELAVL1/HuR shuttling in control OT cells. (C) ELAVL1/HuR arginine methylation studied by Duolink proximity ligation assay in TO cells after the addition of SAM or Cbl. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (ANOVA).

ELAVL1/HuR. This conclusion was corroborated by the immunofluorescence staining of the R217A–S221D double mutant with only cytoplasmic staining in most of the transfected OT cells. However, it is worth noting that S221 phosphorylation alone is probably not sufficient for triggering ELAVL1/HuR export in regard to the results obtained by S221D overexpression in TO versus OT cells (Supplementary Figure S7).

#### The arginine-methylation and serine-phosphorylation of ELAVL1/HuR were also impaired in fibroblasts of patients with *CblC* and *CblG* inborn errors of Cbl metabolism

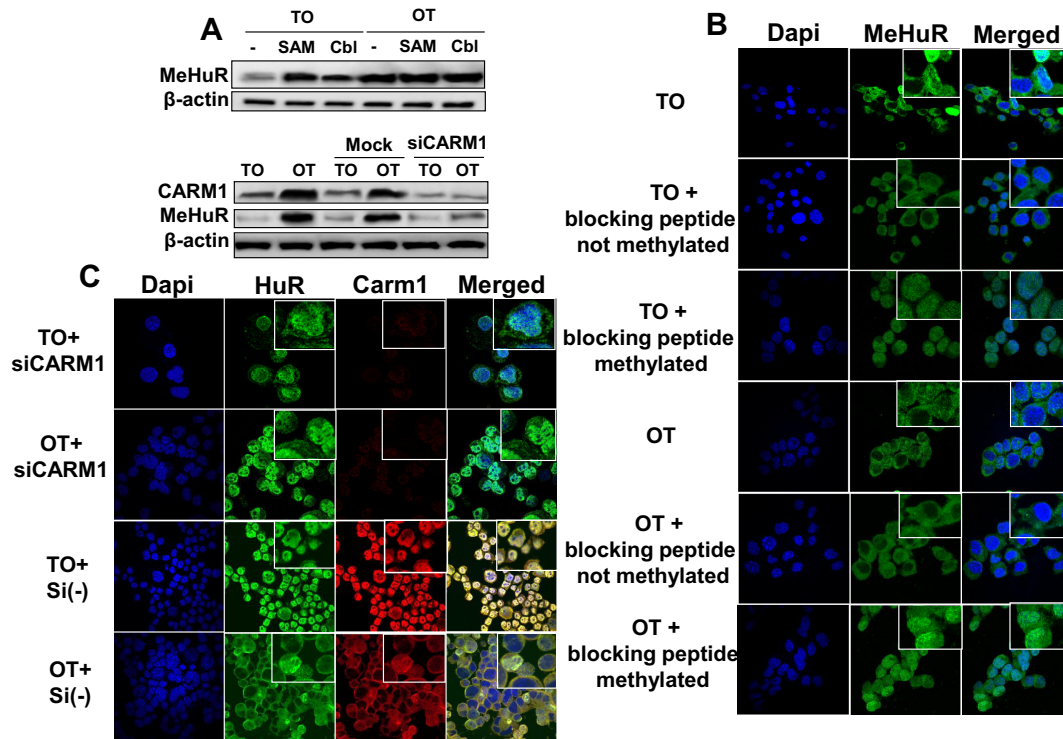
Duolink proximity ligation assay of the fibroblasts from patients with *CblC* and *CblG* inherited disorders showed also decreased protein arginine-methylation and protein serine-phosphorylation of ELAVL1/HuR (Figure 9A). These results were confirmed by Western blots on immunoprecipitated ELAVL1/HuR protein using antibodies against methyl-arginine and phosphoserine in *CblC* and *CblG* fibroblasts. The treatment of *CblC* and *CblG* fibroblasts with PP2A inhibitor okadaic acid restored the serine phosphorylation of ELAVL1/HuR, and the treatment with Adox blocked the methylation of ELAVL1/HuR (Figure 9B). In contrast to ELAVL1/HuR, Y14 is not mislocalized and is neither phosphorylation nor methylation in patient fibroblasts (Supplementary Figure S8). Taken together, these data showed that similar mechanisms as those observed in both

cell and animal models regulate ELAVL1/HuR shuttling in fibroblasts of patients with *CblC* and *CblG* inherited disorders of Cbl metabolism.

## DISCUSSION

The mechanisms at the origin of the highly variable clinical presentation of inherited Cbl disorders are far from being understood (1,2). Part of the explanation may lie in the complexity of dysregulated methylation dependent signaling pathways downstream of Cbl deficiency. In fibroblasts of patients with Cbl inborn errors, the production of reactive oxygen species (ROS) has been found increased and the defense against oxidant stress decreased (28,29). In Cbl deficient cell and animal models, ER stress has also been evidenced downstream of reduced SAM synthesis and SIRT1 activity (5). Here, impaired Cbl metabolism is shown to alter RNA binding proteins shuttling between nucleus and cytoplasm in N1E115 neuronal cells, patient fibroblasts and neurons of the Cd320 KO mouse. It is thus plausible that altered nucleocytoplasmic shuttling of mRNAs represents one key element underlying the generation of oxidative and ER stress which gives rise to the neurological disorders of the Cbl-deficient patients.

Our investigation revealed that Cbl may impact a large scale of RBPs as a number of RBPs such as ELAVL1/HuR, hnRNPA1, Y14 and SRSF1 are found mislocalized in Cbl-deficient neuronal cells, all linking to altered

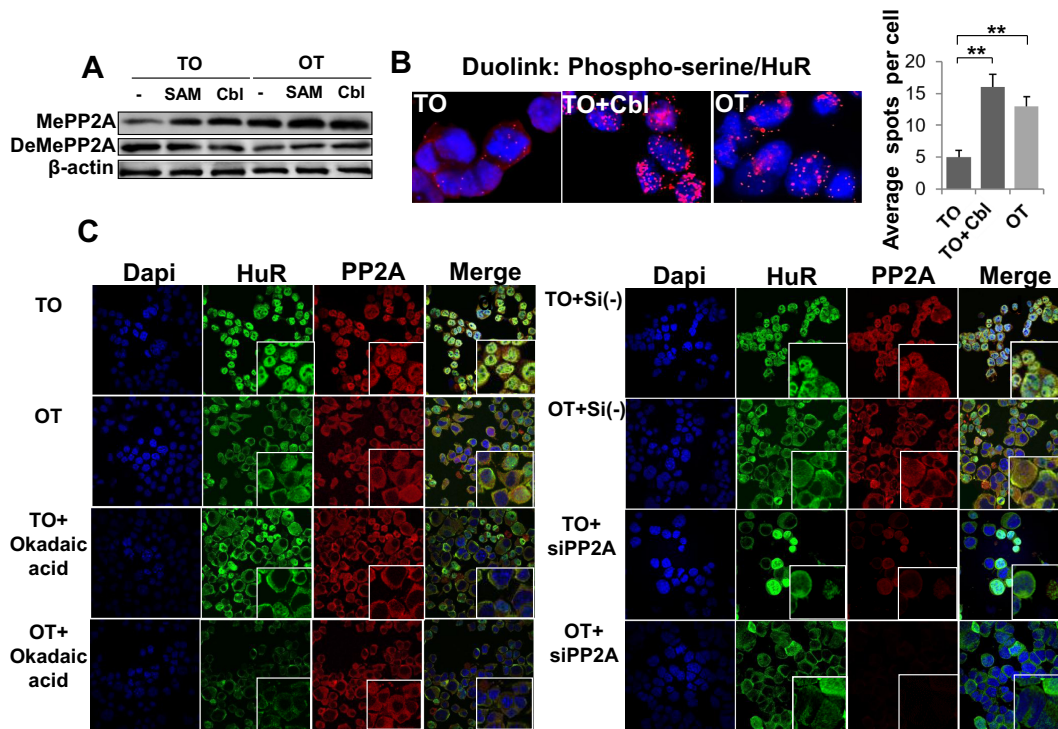


**Figure 7.** CARM1-dependent methylation of ELAVL1/HuR on Arginine 217. (A) Western blot with anti-methylR217 ELAVL1/HuR antibody in TO versus OT cells treated or not with cobalamin (Cbl), S-adenosyl methionine (SAM) and siCarm1. (B) Immunofluorescence staining using anti-methylR217 ELAVL1/HuR antibody, in the presence of either methylated ELAVL1/HuR peptide or non-methylated ELAVL1/HuR peptide. (C) CARM1 and methylation of ELAVL1/HuR in TO versus OT cells. The CARM1 knockdown by siCarm1 reduced the R217 methylation and blocked the ELAVL1/HuR export.

protein arginine-methylation and serine-phosphorylation. ELAVL1/HuR, a key regulator of neuronal stress, is the most affected RBP, and its mislocalization hinted disrupted export of AU-rich element (ARE)-containing mRNA targets from nucleus to cytoplasm. We indeed observed dramatic transcriptomic changes related to stress response. More importantly, the gene functions related to neurogenesis, synapse transmission, brain development, and neuroplasticity are equally touched. Remarkably, these alterations are consistent with the neurological manifestations associated with both Cbl deficiency and Cbl inherited diseases (1,2). Cobalamin deficiency additionally caused cytoplasmic accumulation of hnRNPA1, a RBP known to be responsive to osmotic shock stress and capable of shuttling between nucleus and cytoplasm. Our evidence suggests this to be linked to dysregulated serine phosphorylation at the C-terminal domain of the protein which blocked its nuclear importation (30). Equally important is the mislocalization of Y14 and SRSF1. As these proteins participate in constitutive and alternative splicing needed to maintain cellular homeostasis, survival, and DNA repair, their mislocalization weakens cellular stress response to DNA damage, oxidative stress, and perturbed one-carbon metabolism. Given the number of RBPs examined that are mislocalized in the N1E115 cells, this dysregulation of RNA metabolism may represent a prominent mechanism associated with the neurological manifestations of inherited disorders of cobalamin metabolism metabolic diseases.

It is worth noting that to deprive the intracellular Cbl, the neuronal cell model N1E115 has to be grown in the presence of G418 to maintain the stable expression of transgene TC-Oleosin (TO) or Oleosin-TC (OT). It is with the presence of G418, ELAVL1/HuR is confined to the nucleus in Cbl-deficient TO cells, in contrast to the cytoplasmic localization observed in OT cells. This indicates that while under stress in non-deficient cells, ELAVL1/HuR can be exported to the cytoplasm, in Cbl deficient cells the exportation is blocked. Interestingly, cellular stress - manifested downstream often in the form of oxidative and ER stress - is an integral part of disease mechanisms, affecting maladies like inborn errors of Cbl metabolism. Cellular stress is expected to trigger ELAVL1/HuR export from nucleus to cytoplasm. Considering that ELAVL1/HuR export is also altered in patient fibroblasts and in hippocampal neurons of the Cd320 KO mice (the experimental systems without the presence of G418), we conclude that cellular response to stress mediated by ELAVL1/HuR-containing hnRNP complex shuttling is impaired in both human and mouse under aberrant Cbl metabolism. Together with the previous evidence that in TO and OT model cell system, cellular Cbl deficit produced non-adapted unfolded protein response due to decreased SIRT1 activity (5,13,31), our data here provide a mechanistic link between anti-stress responses mediated by ELAVL1/HuR and SIRT1 activity. A major consequence of ELAVL1/HuR nuclear retention is the confinement of ELAVL1/HuR target mRNAs like Sirt1





**Figure 8.** PP2A-dependent dysregulation of nucleocytoplasmic transport of ELAVL1/HuR. (A) Western blot with anti-methyl PP2A and anti-demethyl PP2A in TO versus OT cells with and without treatment with cobalamin (Cbl) and S-adenosyl methionine (SAM). (B) ELAVL1/HuR serine phosphorylation in TO versus OT cells evaluated by Duolink proximity ligation assay with anti-ELAVL1/HuR and anti-phospho-serine antibodies. (C) PP2A subcellular co-localization with ELAVL1/HuR in TO versus OT cells. The PP2A inhibitor okadaic acid (20 nM for 6 h) and siPP2a knockdown influence ELAVL1/HuR export in TO cells;  $**P < 0.01$  (ANOVA).

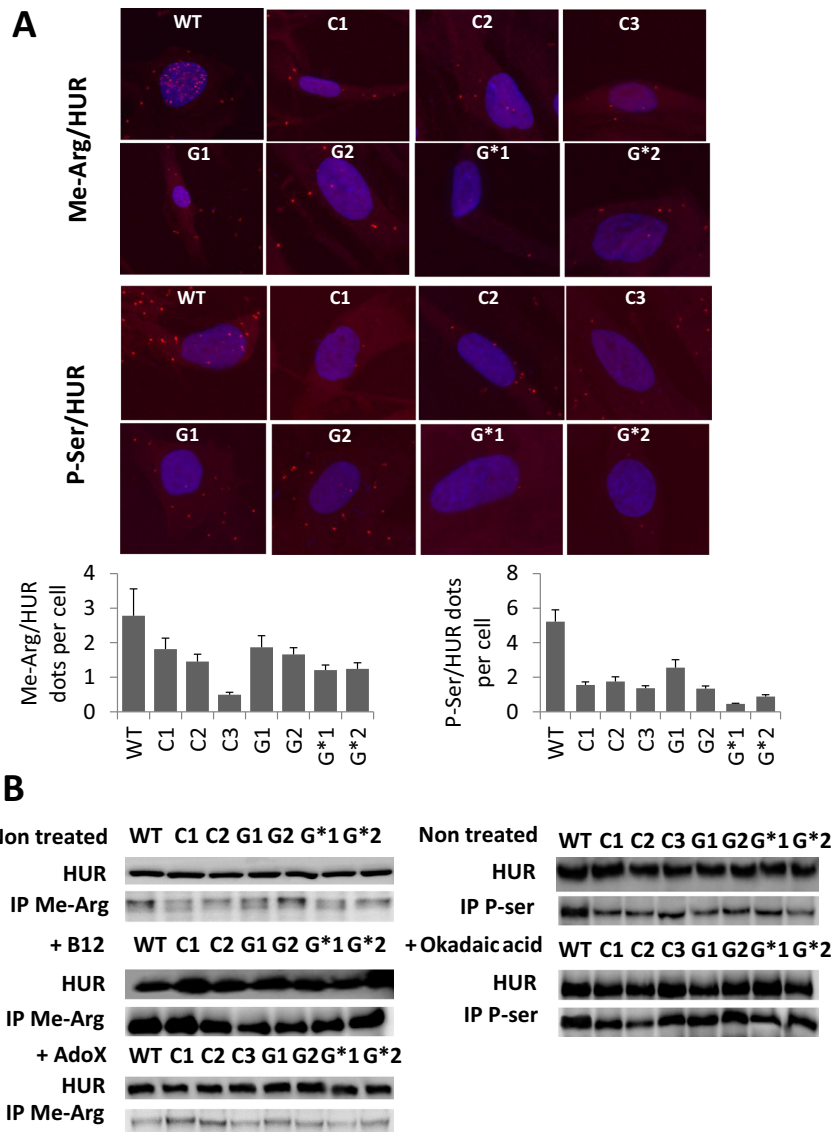
in the nucleus, hence reduced translation of ELAVL1/HuR-relevant stress response proteins. A decreased expression and activity of SIRT1 is a key component of the non-adapted response to the ER stress of the TO cells and the rats subjected to Cbl and folate deficient diet (5,12,13,31), as reduced SIRT1 protein expression leads to HSF1 hyperacetylation, the latter in turn reduces the expression of heat shock proteins (HSPs) (32). Besides HSPs, other gene function may also be affected as decreased SIRT1 activity can contribute to dysregulated nuclear receptor co-regulation via deacetylated PGC1 $\alpha$ , as observed in the brain, heart and gut of rats deficient in Cbl and folate (10–14,31).

We traced the cause of the disrupted ELAVL1/HuR exportation to altered post translational methylation and phosphorylation of ELAVL1/HuR that affect the affinity of ELAVL1/HuR to its nuclear receptor CRM1/exportin. CRM1/exportin is a general export receptor that mediates ARE-containing mRNAs export in the cytoplasm (33). Under Cbl deficiency, the binding of ELAVL1/HuR to CRM1/exportin and to other partners of the NPC machinery like I1PP2A and APRIL (33,34) were found all reduced. Furthermore, we found that in N1E115 TO/OT cell model, while R217 methylation helps to localized ELAVL1/HuR to the cytoplasm, serine dephosphorylation of demethylated ELAVL1/HuR causes its nuclear retention. These changes in post translation modifications of ELAVL1/HuR correlate with decreased levels of SAM, CARM1 and PKCs, and increased level of PP2A, all resulting from of perturbed Cbl metabolisms. The altered phosphorylation of

ELAVL1/HuR was influenced by PP2A and the subsequent disruption of the interaction between ELAVL1/HuR and CRM1/exportin could be reversed by Cbl and siPP2a cell transfection. These data are consistent with the up-regulation of PP2A previously observed in TO versus OT cells (8). The methylation of ELAVL1/HuR on the R217 residue has been shown to increase its association to *Sirt1* mRNA (35,36). The effect of impaired cobalamin metabolism on the methylation of ELAVL1/HuR and the decreased expression of *Sirt1* in TO cells could be accounted for by the decreased binding of ELAVL1/HuR to the *Sirt1* mRNA, and not only due to its localization. However, this mechanism may have a limited influence in TO and OT cells, since SAM and AdoX had no significant effect on the binding of ELAVL1/HuR to *Sirt1* mRNA.

The non-adapted response to ER stress produced by ELAVL1/HuR nuclear retention and the related impaired export of mRNAs may be part of the puzzle explaining the wide spectrum of clinical neurological manifestations of nutritional and inherited causes of Cbl deficiency. The transcriptomic changes in the cell model reported here are consistent with most of the clinical neurological manifestations of Cbl deficiency, with some of them related to brain development, affecting genes controlling cell cycle, apoptosis and differentiation and some to late stage neurological manifestations including genes involved in ER stress, neuroplasticity and myelin synthesis (8,13,14). The association of Cbl with abnormal brain ageing and neurodegeneration is consistent with the results of transcriptome indicat-





**Figure 9.** Influence of ELAVL1/HuR methylation and phosphorylation on its subcellular localization in fibroblasts from patients with CblC and CblG inherited defects. (A) Duolink Proximity Ligation Assay showing the subcellular mislocalization of methyl-ELAVL1/HuR in cells from three CblC patients (C1, C2, C3) and four CblG patients (G1, G2, G\*1, G\*2) stained with anti-methyl arginine and with anti-ELAVL1/HuR antibodies. Histograms show the mean number of red dots corresponding to the number of interactions between Me-Arg and ELAVL1/HuR per cell in each cell line. (B) Western blot of ELAVL1/HuR following immunoprecipitation against methyl-arginine (Me-Arg) or phospho-serine (P-Ser) in CblC and CblG fibroblasts. Cells were either treated by cobalamin (Cbl), okadaic acid or AdoX.

ing changes in gene functions like APP/presenilin pathways of Alzheimer disease (37).

The importance of ELAVL1/HuR mislocalisation in the transcriptomic changes observed in diseases with impaired Cbl metabolism may be underscored by the observation that a large proportion of its gene targets (82%) are distributed in the K-means clusters identified from the transcriptomic data. Its prominent role in the disrupted export of AU-rich element (ARE)-containing mRNA targets is also illustrated by the absence of mislocalisation of two other brain specific ELAVL1 member proteins, HUC and HUD (Supplementary Figure S9). Given the fact that it acts as a controller for neuronal stress to confer protection from neurodegeneration, and is also involved in brain develop-

ment and aging through controlling cell cycle, inflammation, genotoxic stress and cell death (38–42), mislocalized ELAVL1 targets can potentially contribute considerably to the neurological phenotypes of the Cbl metabolic errors. The recent large number of reports linking dysregulated RNA metabolism to both inherited and complex neurological disorders indeed support this notion (17,18,38). Interestingly, ELAVL1/HuR is the only ubiquitous ELAVL protein whose expression is not limited to the neuronal tissues (43). This highlights its potential involvement via dysregulation of RNA processing in both hematological and visceral manifestations of Cbl deficiency.

In conclusion, we show here that Cbl deficiency can disrupt RBP-dependent nucleocytoplasmic shuttling of mR-

NAs, most prominently via ELAVL1/HuR. This new insights shall help in the near future the reexamination of the pathological consequences on developmental and ageing processes caused by Cbl deficiency and Cbl inborn error and in the search for innovative therapies for inherited disorders of Cbl metabolism.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## FUNDING

Institutional grants were received from: the i-SITE Lorraine University of Excellence (LUE); the French National Agency for Research (ANR); Region of Lorraine (France); 'Etude des mécanismes moléculaires à l'origine des pathologies associées à un dysfonctionnement du métabolisme des monocarbones' that is co-funded by the European Union within the framework 'FEDER-ESF Lorraine et Massif des Vosges 2014–2020'. Funding for open access charge: Inserm and University of Lorraine.

*Conflict of interest statement.* None declared.

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