Deficiency of murine UFM1-specific E3 ligase causes microcephaly and inflammation

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Abstract

The UFM1 conjugation system is a Ubiquitin (Ub) -like modification system that is essential for animal development and normal physiology of multiple tissues and organs. It consists of UFM1, a Ub-like modifier, and the UFM1-specific enzymes (namely E1 enzyme UBA5, E2 enzyme UFC1 E2, and E3 ligases) that catalyze conjugation of UFM1 to its specific protein targets. Clinical studies have identified rare genetic variants in human UFM1, UBA5 and UFC1 genes that were linked to early-onset encephalopathy and defective brain development, strongly suggesting the critical role of the UFM1 system in the nervous system. Yet the physiological function of this system in adult brain remains not defined. In this study, we investigated the role of UFM1 E3 ligase in adult mouse and found that both UFL1 and UFBP1 proteins, two components of UFM1 E3 ligase, are essential for survival of mature neurons in adult mouse. Neuron-specific deletion of either UFL1 or UFBP1 led to significant neuronal loss and elevation of inflammatory response. Interestingly, loss of one allele of UFBP1 genes caused occurrence of seizure-like events. Our study has provided genetic evidence for the indispensable role of UFM1 E3 ligase in mature neurons and further demonstrated the importance of the UFM1 system in the nervous system.

Introduction:

Post-translational modification (PTM) of target proteins by Ubiquitin (Ub) and Ubiquitin-like (Ubl) modifiers plays pivotal roles in numerous cellular processes, ranging from cell cycle progression, DNA damage response, protein trafficking to autophagy. UFM1 (Ubiquitin-fold modifier 1) is a small Ubl modifier with 85 amino acid residues and highly conserved in multi-cellular organisms [1]. With a limited primary sequence identity with Ub, Ufm1 displays a solution structure of ubiquitin fold that is shared by other Ubls 12. However, unlike Ub and some Ubls with di-glycine (GG) residues at the carboxyl (C)-terminus of their active forms, the active Ufm1 contains Valine-Glycine (VG) residues at its C-terminus, and its Glycine residue is covalently conjugated to the lysine residues of target proteins via an iso-peptide bond. Ufm1 conjugation to its target proteins, a process termed as UFMylation, is accomplished in multi-step biochemical reactions that are catalyzed by a set of UFM1-specific enzymes, namely, UFM1-activating E1 enzyme UBA5, UFM1-conjugating E2 enzyme UFC1, and UFM1-specific E3 ligase that consists of UFL1 and its co-factor UFBP1 protein [2].

The physiological function of the UFM1 conjugation system has remained poorly defined until very recently. Multiple lines of genetic evidence have demonstrated its essential role in animal development. Germ-line deletion of individual UFMylation genes, including UFM1, UBA5, UFL1 and UFBP1, leads to defective erythropoiesis and embryonic lethality around E11.5-E13.5 days [3–5]. In adult animals, acute ablation of either UFL1 or UFBP1 caused pancytopenia and severe anemia, further demonstrating the indispensable role of UFMylation in definitive erythropoiesis days [3–5]. In addition to mouse genetics, clinical data have also shown that defective UFMylation contributes to the pathogenesis of disease pathogenesis, including hematopoietic diseases [6, 7], diabetes [8], ischemic heart injury [9], skeletal dysplasia [10], atherosclerosis [11], and cancer [12, 13]. Loss-of-function mutation in UFBP1 gene was found to be a causative factor in Shohat-type spondyloepimetaphyseal dysplasia (SEMD), a skeletal
dysplasia that affects cartilage development [14]. Mutations in UFM1-specific protease UFSP2 were found in the patients with Beukes hip dysplasia [10]. Intriguingly, a number of clinical studies have identified rare variants of human UFM1, UBA5 and UFC1 genes that were linked to early-onset encephalopathy and defective brain development [15–25]. Genetic study using Drosophila model revealed that knockdown of either UBA5, UFM1 or UFC1 induced locomotive defects and a shortened lifespan accompanied by aberrant neuromuscular junctions (NMJs) [16], while similar effect was reported in C. elegans and zebrafish models [15]. CNS-specific knockout UFM1 in mouse led to microcephaly and postnatal death within 1 day after birth [17]. In addition to the role in neurons, lower expression of UFM1 E3 ligase UFL1 (named as Maxer in the cited study) is implicated in functional deficiency of Bergmann glial cells that might contribute to the non-cell-autonomous pathology of spinocerebellar ataxia [26]. Collectively, these recent findings underscore the importance of the Ufm1 system in normal development and physiology of multiple systems, especially the central nervous system. In this report, we further investigate the role of Ufm1-specific E3 ligase in the murine adult brain.

Results:

The component of the Ufm1 conjugation system in adult mouse brain tissues

We first examined expression of the UFMylation components in adult mouse brain. Immunoblotting results has shown that UFMylation genes, including UFM1, UBA5, UFL1, UFBP1 and CDKRAP3, are ubiquitously expressed in multiple regions of mouse brain (Fig. 1a), while UFMylated RPL26, the principal UFM1 target, is enriched in the hippocampus (Fig. 1a, marked by arrow). Immunofluorescence staining of UBA5, UFL1 and UFBP1 in cortex and hippocampus showed that their expression were largely in the neurons (Fig. 1b and c). Our results suggest that the genes involved in the UFMylation pathway are broadly expressed in murine adult brain.

Neuron-specific deletion of UFL1 and UFBP1 causes microcephaly in adult mouse

Previous studies have shown that knockdown of the UFM1 system in invertebrate and zebrafish models causes defective neuromuscular junctions and decreased locomotor function ) [16, 15], yet these models may not fully recapitulate the clinical manifestation of neurological abnormality associated with the deficiency of the UFM1 system. In attempt to interrogate the role of the UFMylation pathway in adult central nervous system, we crossed UFL1 and UFBP1 floxed mice with Camka2-Cre transgenic mice to generate neuron-specific knockout mouse model (Fig. 2a and b). Camka2-Cre mice has been widely used to study gene function in postnatal brain [27]. Cre activity is mainly expressed but not restricted to pyramidal cell layers of cerebral cortex and hippocampus in postnatal mouse (http://www.informatics.jax.org/allele/ MGI:2177650?recomRibbon = open). Knockout of UFL1 and UFBP1 was confirmed by reduced protein level in brain lysates (Fig. 2c). Either UFL1^f/f;Camka2-Cre
(referred as to \textit{UFL1}^{Δ/ΔCamk2a} hereafter) or \textit{UFBP1}^{f/f};Camk2a-Cre (named as \textit{UFBP1}^{Δ/ΔCamk2a}) mice did not exhibit obvious developmental retardation. However, in comparison to wild-type mice, beginning around the age of 3–4 months, at least half of \textit{UFL1}^{Δ/ΔCamk2a} and \textit{UFBP1}^{Δ/ΔCamk2a} mice (both male and female) developed certain behavioral abnormalities, including mania, enhanced aggressiveness, remarkable jumping ability, and sometimes premature death without apparent cause (unpublished observation). Interestingly, both \textit{UFL1}^{Δ/ΔCamk2a} and \textit{UFBP1}^{Δ/ΔCamk2a} mice exhibited significant microcephaly (Fig. 2d and e), a phenotype that is reminiscent of the symptom of certain patients with the genetic variants of the UFM1 system [15, 25]. This result suggest that like other components of the UFM1 system, the UFM1 E3 ligase is also essential for the normal physiology and function of the central nervous system.

**UFL1 and UFBP1 deficiency causes significant neuronal loss and elevated cell death**

We further examined brain histology of \textit{UFL1} and \textit{UFBP1} conditional KO mice. In the hippocampus, \textit{UFBP1} ablation led to substantial loss of CA1 neurons while \textit{UFL1} deficiency resulted in nearly complete loss of CA1 region (Fig. 3a). In the cortex, in contrast to distinctive 6-layer structure in wild-type brain, either \textit{UFBP1} or \textit{UFL1} deficiency caused remarkable loss of neurons in all layers, which in turn resulted in decreased cortical thickness (Fig. 3b) and disorganized and unrecognizable cortical layers (Fig. 3a). Neuronal loss in both KO brains was further confirmed by staining of neuronal marker NeuN (Fig. 3c). Moreover, the CA1 neurons in \textit{UFBP1}^{Δ/ΔCamk2a} brain exhibited attenuated MAP2 staining, indicating significant loss of dendrites (Fig. 3d). In accordance with the phenotype of neuronal loss, either \textit{UFL1}^{Δ/ΔCamk2a} or \textit{UFBP1}^{Δ/ΔCamk2a} cortex contained more TUNEL-positive cells, indicating the increase of cell death (Fig. 3e and f). Together, these results strongly suggest that UFL1/UFBP1 is essential for survival of cortical and hippocampal neurons.

**UFL1 and UFBP1 deficiency led to exacerbated inflammation**

To evaluate the effect of \textit{UFL1}/\textit{UFBP1} deficiency on gene expression, we performed RNA-seq analysis of cortical tissues of \textit{UFL1}^{Δ/ΔCamk2a} or \textit{UFBP1}^{Δ/ΔCamk2a} mice. With 1.3-fold cut-off (log KO/WT, \textit{Padj} < 0.05), there were 906 genes down-regulated and 715 genes up-regulated in the cortex of \textit{UFBP1}^{Δ/ΔCamk2a} mice (Fig. 4a and supplemental table 1). In comparison, fewer differential expressed genes (DEGs) has been identified in \textit{UFL1}^{Δ/ΔCamk2a} (Fig. 4d and supplemental table 2). Gene Ontology (GO) analysis showed that genes critical in synapse and dendrite development were down-regulated in both mouse models (Fig. 4b and e and supplemental tables 3 and 4), while genes responsible for innate and adaptive immunity were significantly increased in both KO cortex (Fig. 4c and f and supplemental tables 5 and 6). In \textit{UFBP1}^{Δ/ΔCamk2a} cortex, both glial and microglial markers were among the most up-regulated genes, including GFAP, IBA1 and CD68 (Fig. 4g and supplemental table 1), and their elevated expression was further confirmed by immunostaining of glial marker GFAP and microglial marker IBA1, respectively (Fig. 4h and
i). Consistently, the genes that are involved in inflammatory response, such as ITGAX, CD74, complement components C1q, C4b and C3, and chemokines Ccl3 and Ccl6, are highly up-regulated in KO cortex (Fig. 4j and supplemental tables 1 and 5). Interestingly, ER stress-induced genes including CHOP, ATF3, HMOX1 and pro-apoptotic gene BID were increased in UFBP1Δ/ΔCamk2a cortex (Fig. 4k and supplemental table 1). Taken together, gene profiling results have suggested that UFL1 and UFBP1 deficiency in the brain causes dramatic inflammatory response to brain injury.

Seizure-like events occurred in UFBP1 Heterozygous mice

Our previous study has shown that germ-line ablation of UFBP1 gene by gene trap led to embryonic lethality at E13.5 day due to defective erythroid development, but heterozygous mice survive and develop normally [5]. No histological abnormity was found in the brain of these heterozygous mice (unpublished observation). Interestingly, our research staff occasionally noticed occurrence of rare seizure events in UFBP1 heterozygous mice but in any wild-type mice. This surprising observation prompted us to further determine whether decreasing level of UFBP1 causes any neurological abnormality. Open field test failed to reveal any significant difference between wild-type and UFBP1 heterozygous mice (unpublished data). We further performed continuous electroencephalograph (EEG) recording in 4-month old wild-type and UFBP1 heterozygous mice. Intriguingly, all UFBP1 heterozygous mice tested (n = 3, male) exhibited abnormal EEG that indicates occurrence of spontaneous and recurrent seizure-like event (Fig. 5a). In contrast, no abnormal EEG event was recorded in the control mice (Fig. 5a). Frequency of seizure-like event varied in individual mouse, and overall frequency was higher than 15 events per 24 hours (Fig. 5b). This result suggests that loss of one UFBP1 allele may cause neurological abnormality and spontaneous seizure-like events.

Discussion:

Here we report that neuron-specific abolition of either UFL1 or UFBP1, two components of the UFM1-specific E3 ligase, led to significant loss of neurons in cortex and hippocampal CA1 regions and caused elevated inflammatory response. Furthermore, we found that heterozygous mice with loss of one UFBP1 wild-type allele exhibited abnormal EEGs that indicate occurrence of spontaneous seizure-like events. Our study have first demonstrated that the UFM1 E3 ligase is essential for normal function of murine central nervous system.

Laboratory studies of various mouse genetic models have demonstrated the indispensable role of the UFM1 system in embryogenesis, development and normal physiology of multiple tissues and organs [2]. Its importance is further supported by identification of multiple UFMylation gene variants in genetic diseases such as skeletal dysplasia and neurological disorders. Recent human genetic studies indicate that the neural system appears to be more susceptible to perturbation of the UFMylation pathway than other systems. Most disease-causing mutations of human UFM1, UBA5 (E1) and UFC1 (E2) genes were found in pediatric patients with neurological anomalies, ranging from dystonia, ataxia, abnormal EEGs, epileptic seizure to microcephaly and global developmental delay [15–25]. These abnormalities were
manifested in various syndromes, such as hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC), severe early-onset encephalopathy with progressive microcephaly, severe infantile-onset encephalopathy, and autosomal recessive cerebellar ataxia (ARCA) [15–25]. Biochemically, mutations identified in UBA5 and UFC1 genes compromise their enzymatic activity, thereby resulting in attenuation of the UFMylation pathway [15, 24]. Interestingly, a pathogenic variant of UFSP2, a tentative de-UFMylase, was recently identified in pediatric patient with a severe syndrome of neurodevelopmental disability and epilepsy [28]. Although the impact of the mutations in these genes on UFMylation of specific targets remains to be further evaluated, it is reasonable to postulate that a well-balanced UFMylation activity is essential for normal development and function of neurons, and disruption of this balance (either insufficient or over-accumulated UFMylated products) may lead to neurological disorders with varying degree of severity.

Although the critical role of UBA5 (UFM1-specific E1) and UFC1 (UFM1-specific E2) in the nervous system has been strongly supported by both clinical data and animal studies, the involvement of UFM1-specific E3 ligase in brain development remains largely unknown. Using neuron-specific knockout mouse models in the current study, we have demonstrated that UFL1/UFBP1 complex, the only known UFM1-specific E3 ligase identified so far, is indeed important for normal function of murine brain. As originally reported, the Cre recombinase activity in Camk2a-Cre line T29-1 is predominantly expressed in the CA1 pyramidal cell layer in the hippocampus during the third and fourth postnatal week, and then spreads to the forebrain and other regions in later postnatal stages ([27] and https://www.jax.org/strain/005359). Consistent with the distribution of Cre activity in adult brain, Cre-mediated knockout of either UFL1 or UFBP1 mainly affects the neurons in the cortex and CA1 region of hippocampus. More TUNEL positive cells are present in KO mice indicating the essential role of UFL1 and UFBP1 in neuronal survival (Fig. 3). The underlying cause of neuronal death remains to be further elucidated. The UFMylation pathway has been implicated in cell processes such as autophagy, protein translation, ribosome-associated protein quality control (RQC) and Unfolded Protein Response (UPR) [2]. Impairment of one or multiple of these pathways may lead to disruption of neuronal proteostasis, thereby resulting in elevated cell death. Further mechanistic investigation is needed to identify key UFM1 substrates and elucidate functional impact of UFMylation of these targets in neuronal survival and cellular processes.

In addition to neuronal survival, UFL1 or UFBP1 may be also involved in normal neuronal function. This notion is supported by our finding of seizure-like phenotype manifested by UFBP1 heterozygous mice. Although we cannot exclude the contribution of non-neuronal effect of UFBP1 deletion to this phenotype at this stage, this finding raises an intriguing possibility that partial loss of UFMylation function may play a broader role in the pathophysiology of neurological disorders than our current view, which is mainly focused on early-onset pediatric encephalopathy. Interestingly, GWAS study has identified UFBP1 (also known as DDRGK1) as a new risk locus in Parkinson’s disease [29], while UFL1 expression is decreased in schizophrenia patients [30]. Thus, further investigation of the UFM1 system in the neural system may unravel a novel insight into the pathogenic mechanism of neurological disorders as well as new therapeutic targets for these diseases.
Materials And Methods:

Generation of neuron-specific knockout mice of UFBP1 and UFL1

To generate neuron-specific knockout mouse models, UFL1 and UFBP1 floxed mice (described in [4, 5]) were crossed with Camk2a-Cre transgenic line T29-1 (stock #005359, The Jackson Laboratory). Genotyping of UFL1 and UFBP1 floxed allele was described previously [4, 5], and Camk2a-Cre allele was genotyped according to the standard protocol of The Jackson Laboratory. Mice were housed in the animal facility of Augusta University. All animal procedures were approved by AU and Vanderbilt University IACUCs.

RNA-seq, differential and enrichment analysis

After perfusion, cortical tissues of 3 wild-type and 3 KO mice were dissected and homogenized in TRIzol (ThermoFisher Scientific). Total RNAs were isolated with Zymo Direct-zol RNA Miniprep Plus kit (R2072, Zymo Research, Irvine, CA). RNA-seq and analyses were conducted by Novogene (Beijing, China). After data filtering, the genes were mapped to the genome with STAR software, and quantification was determined by HTSeq software. DESeq2 was used for differential analysis, and ClusterProfiler was used for enrichment analysis (GO, KEGG and Reactome).

Histology, immunofluorescent staining and immunoblotting

Hematoxylin and eosin (H & E) staining and Nissl staining was performed by the Histology core of Augusta University. Immunofluorescent staining and immunoblotting were performed as described previously [5]. Bright field and Epiuorescence images were obtained using Zeiss Observer D1 with AxioVision 4.8 software (Carl Zeiss Microscopy GmbH, Jena, Germany) and Keyence BZ-X700 fluorescent microscope with its corresponding software (Keyence America, Itasca, IL, USA). All histopathological analysis and quantification were performed blindly by experienced lab personnel who had no prior knowledge on the genotypes of animals and tissues.

The antibodies used in this study included: Uba5 (Li Lab), Ufl1 (Li lab), Cdk5rap3 (Li lab), Ufbp1 (21445-1-AP, Proteintech, Rosemont, IL), Ufm1 (Ab109305, Abcam, Waltham, MA), RPL26 (#5400, Cell Signaling, Danvers, MA), NeuN (MAB377, MilliporeSigma, Burlington, MA), MAP2 (#8707, Cell Signaling), GFAP (#3670, Cell Signaling), IBA1/AlF-1 (#17198, Cell Signaling), and β-Actin (#3700, Cell Signaling). All affinity-purified and species-specific HRP- and fluorophore-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Video-EEG monitoring.

Mice were anesthetized with 2–3% inhaled isoflurane and mounted on a stereotaxic apparatus (Stoelting, Wood Dale, IL). A prefabricated mouse headmount (Pinnacle Technologies) was fastened onto the skull with 4 electrodes contacting the dura, two placed 1 mm anterior to bregma and two placed 7 mm anterior
to bregma, each being 1.5 mm lateral to the central sulcus. Mice were returned to the home cage for at least 7 days for recovery then placed in recording chambers for continuous 24-h simultaneous video and EEG recording. The EEG recordings were performed at Vanderbilt mouse neurobehavioral lab core.

**TUNEL staining**

TUNEL staining was performed using *in situ* cell death detection kit (TMR Red, Roche, Basel, Switzerland) according to the manufacturer's instruction.

**Statistical analysis**

All statistical analyses were performed using Graph Prism 9 software. *P* values were determined by unpaired *t*-tests between two set of data. A *p* value less than 0.05 was considered to be significant.

**Declarations:**

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**Competing Interest:**

The authors have no relevant financial or non-financial interests to disclose

**Author contribution:**

JZ, HZ, SL, MQ, TZ, YH contribute to material preparation, data collection and analysis. JZ, HZ, YH, YC and HL contribute to experimental design and data analysis. JZ, HZ and HL contribute to drafting the manuscript, and all authors contribute to commenting and revising the manuscript.

**Data availability**

All original RNA-seq datasets are available as requested.

**Ethics approval:**

The authors state that all animal research complied with USDA Animal Welfare Act and Regulations, and all experimental procedures were approved by Augusta University and Vanderbilt University IACUCs.

**Consent to participate**

Not applicable
Consent to publish

Not applicable

Acknowledgements

Not applicable

References:


Figures

Figure 1

Expression of UFMylation genes in adult mouse brain. **a.** Protein levels of UFMylation genes in the subregion of the adult mouse brain. Potential UFMylated RPL26 is indicated by arrow.

**b.** Immunostaining of UBA5, UFBP1 and UFL1 in the cortex. **c.** Immunostaining of UBA5, UFBP1 and UFL1 in the CA1 region of the hippocampus.
Figure 2

Neuron-specific deletion of UFL1 and UFBP1 causes microcephaly in adult mouse. **a.** Generation of neuron-specific UFL1 knockout mice UFL1$^{D/D\text{Camk2a}}$. **b.** Generation of neuron-specific UFBP1 knockout mice UFBP1$^{D/D\text{Camk2a}}$. **c.** Protein level of UFL1 and UFBP1 in knockout mice. The level was normalized to actin control. **d.** Representative adult brains from wild-type, UFL1$^{D/D\text{Camk2a}}$, and UFBP1$^{D/D\text{Camk2a}}$ mice (male, 4-month old). **e.** The ratio of brain/body weight of wild-type, UFL1$^{D/D\text{Camk2a}}$, and UFBP1$^{D/D\text{Camk2a}}$ mice. **p < 0.01** (n=5, 4-month old male). Similar result was obtained with females mice (data not shown).
Figure 3

*UFL1* and *UFBP1* deficiency causes significant neuronal loss and elevated cell death. 

**a.** Nissl and H & E staining of wild-type, *UFL1<sup>D/DCamk2a</sup>* and *UFBP1<sup>D/DCamk2a</sup>* brain sections. All mice were 4-month old. 

**b.** Thickness of cortex of wild-type, *UFL1<sup>D/DCamk2a</sup>* and *UFBP1<sup>D/DCamk2a</sup>* brain. *** p < 0.001, **** p < 0.0001 (n=4 mice per genotype). 

**c.** NeuN staining of cortical and hippocampal CA1 regions of wild-type, *UFL1<sup>D/DCamk2a</sup>* and *UFBP1<sup>D/DCamk2a</sup>* brain. 

**d.** MAP2 staining of cortical and hippocampal CA1 regions of wild-type, *UFL1<sup>D/DCamk2a</sup>* and *UFBP1<sup>D/DCamk2a</sup>* brain. 

**e.** TUNEL-staining of cortex of wild-type, *UFL1<sup>D/DCamk2a</sup>* and *UFBP1<sup>D/DCamk2a</sup>* brain sections. 

**f.** Quantitation of TUNEL-positive cells. The number of TUNEL-positive cells was scored in a double-blinded manner. **** p < 0.0001 (n=4 mice per genotype).
Figure 4

UFL1 and UFBP1 knockout leads to exacerbated inflammation. a. The number of genes whose expression in the cortex was affected by UFBP1 deletion. b. GO enrichment analysis of up-regulated DEGs in UFBP1<sup>D/DCamk2a</sup>. c. GO enrichment analysis of down-regulated DEGs in UFBP1<sup>D/DCamk2a</sup>. d. The number of genes whose expression in the cortex was affected by UFL1 deletion. e. GO enrichment analysis of up-regulated DEGs in UFL1<sup>D/DCamk2a</sup>. f. GO enrichment analysis of down-regulated DEGs in UFL1<sup>D/DCamk2a</sup>.
Expression of glial and microglial cell markers GFAP, IBA1 and CD68 in UFL1<sup>D/DCamk2a</sup> cortex. * p < 0.05, *** p < 0.001 (n=3). h. GFAP and i. IBA1 staining of wild-type, UFL1<sup>D/DCamk2a</sup> and UFBP1<sup>D/DCamk2a</sup> brains. j. Expression of inflammatory genes in UFBP1<sup>D/DCamk2a</sup> cortex. ** p < 0.01, *** p < 0.001 (n=3). k. Expression of ER stress-induced genes and proapoptotic BID in UFBP1<sup>D/DCamk2a</sup> cortex. ** p < 0.01, *** p < 0.001 (n=3). All data are mean ± SEM

**Figure 5**

Occurrence of seizure-like events in UFBP1 Heterozygous mice. a. Representative examples of EEG recording of wild-type and three male UFBP1 Heterozygous mice (4-month old). b. Quantitation of the parameters of seizure-like events. The data were scored by analysis of full EEG recordings.

**Supplementary Files**

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