Title	The neural ELAVL protein HuB enhances endogenous proto-oncogene activation
Author(s)	Hatanaka, Tomoyuki; Higashino, Fumihiro; Tei, Kanchu; Yasuda, Motoaki
Citation	Biochemical and biophysical research communications, 517(2), 330-337 https://doi.org/10.1016/j.bbrc.2019.07.089
Issue Date	2019-09-17
Doc URL	http://hdl.handle.net/2115/79285
Rights	© <2019>. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	The neural ELAVL protein HuB enhances endogenous.pdf

[Instructions for use](#)

The neural ELAVL protein HuB enhances endogenous proto-oncogene activation

Tomoyuki Hatanaka^{1,3}, Fumihiro Higashino², Kanchu Tei³, Motoaki Yasuda^{1*}

¹ Department of Oral Molecular Microbiology, Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido University.

² Department of Oral Pathology and Biology, Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido University.

³ Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido University.

Corresponding author

Motoaki Yasuda: Department of Oral Molecular Microbiology, Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido University, N13 W7, Kita-ku, Sapporo 060-8586, Japan. Tel: +88(011)706-4241, Fax: +88(011)706-4901
moyasuda@den.hokudai.ac.jp

Abstract

The cytoplasmic distribution of the HuR/ELAVL1 (embryonic lethal abnormal vision 1) protein is recognized as an important prognostic factor of malignant tumors. However, the previous study suggests that exogenous over-expression of HuR is not sufficient for nuclear export. Conversely, the predominantly cytosolic distribution of neuron-specific human ELAV members, including HuB/ELAVL2, HuC/ELAVL3, and HuD/ELAVL4, has been reported. In the present study, we demonstrated the expression of HuB in several types of cancer cells, but expression of HuC and HuD was not observed. In addition, our results indicated that HuR and HuB formed a complex in the cytosolic fraction of cancer cells via the RRM3 region. Ectopic expression of HuB was capable of initiating the cytosolic translocation of HuR from the nucleus to the cytosol. Furthermore, HuB-transduced cancer cells displayed significant nuclear export of HuR, with quantitative PCR experiments revealing the simultaneous upregulation of HIF-1 α , c-Fos, c-MYC, and Ets2 basal mRNA expression. Phorbol 12-myristate 13-acetate (PMA)-stimulated HuB-transduced cells demonstrated significantly enhanced activation of endogenous c-Fos and CREB dependent cascades. Finally, co-transfection of HuB with the E1 region of type 5 human adenovirus significantly enhanced E1 transformation activities but that of HuR with the E1 region did not. Collectively, our findings suggest that the neural Hu family protein HuB plays a major role in the activation of

memory-related proto-oncogenes.

Key words: HuR, HuB, 3' UTR, proto-oncogene

Introduction

The messenger RNA of growth factors, cytokines, and proto-oncogenes contains an AU-rich element (ARE) in the 3' untranslated region (3' UTR). The half-life of ARE mRNA is typically extremely short. In this process, the nonamer UUAUUUAUU is considered a minimal functional consensus sequence of ARE, and ARE binding proteins such as AUF1, TTP, and TIA-1 bind to ARE mRNAs (e.g., P21, cyclin D1, c-fos, c-myc, TNF-alpha, GM-CSF, Cox-2, IL-2, and IL-3) at the consensus nonamer, thus promoting deadenylation and the targeting of the mRNA for decay[1].

The HuR/ELAVL1 protein is a member of the ELAV (embryonic lethal abnormal vision system) family and is expressed ubiquitously. As an RNA binding protein, HuR recognizes consensus ARE sequences and is known to stabilize the ARE mRNA of c-Fos, MyoD, p21, Cyclin A, Cyclin B1, Cyclin D1, NOS II/iNOS, GM-CSF, TNF-a, Cox-2, IL-3, vascular endothelial growth factor (VEGF), and Myogenin [1]. In normal cells, HuR displays nuclear localization; however, several reports considered the cytoplasmic distribution of HuR as an important prognostic factor for malignant tumors [2]. HuR can also be localized in the cytoplasm during senescence or under stress conditions, such as heat shock or infections [3] [4] [5]. However, our previous study demonstrated that the ectopic over-expression of HuR is not sufficient for stabilizing the expression of the *HIF1A* gene [6]. Therefore, it is likely

that other binding partners are required for the nuclear export of HuR in malignant tumors.

The other three members of the human ELAV family (HuB/ELAVL2, HuC/ELAVL3, and HuD/ELAVL4) are expressed specifically in the nervous system and are referred to as neuron-specific ELAVs (nELAVs) [3]. While HuR/ELAV1 and nELAVs share 70–85% amino acid sequence homology (the most divergent region is the N-terminal end), nELAVs are primarily localized to the cytoplasm [7], whereas HuR/ELAVL1 is localized to the nucleus. Because of the specific distribution of nELAVs in neuronal tissues, these proteins were initially reported as autoantibody targets in patients with paraneoplastic neurological disorders [8]. In small-cell lung carcinoma cases, ELAVs on the surface of tumor cells have also been shown to elicit an autoimmune response [9]. However, the precise role of nELAVs in non-neural cancer cells remains unclear.

In the present study, we evaluated the divergent expression of HuB/ELAVL2 among different types of cancer cells. Those that endogenously expressed HuB formed a complex between the ubiquitously expressed cellular HuR and ARE mRNAs such as HIF-1 α . In addition, our results demonstrated that exogenous HuB can initiate the translocation of endogenous HuR from the nucleus to the cytoplasm. Moreover, our findings indicated that exogenous HuB enhanced adenovirus E1-dependent carcinogenesis.

Materials and Methods

Cells, plasmids, luciferase assays, and mammalian two-hybrid assays

All cell lines were purchased from the JCRB cell bank of the National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan). H1299-mock and H1299-HuB cells were established by retrovirus infection and puromycin (1 μ g/mL, Sigma-Aldrich, St. Louis, MO, USA) selection for two weeks. pAP1-LUC and pCRE-LUC plasmid were purchased from Agilent Technologies (Santa Clara, CA, USA). Full-length PTBP1 (GenBank accession no. NM 002819), HIF-1 α (GenBank accession no. NM 001530), HuC, and HuD were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). HuB and HuR cDNAs were amplified by PCR and cloned into the pcDNA3 (Thermo Fisher, Waltham, MA, USA), pEGFP (Takara, Otsu, Japan), pBIND (Promega, Madison, WI, USA), pACT (Promega, Madison, WI, USA), or pBabe puro vectors (Cell Biolabs, San Diego, CA, USA). The left 4021 bp of Ad 5 genomic DNA were cloned into a pCR- Blunt vector (Invitrogen, Carlsbad, CA). ARE sequences were cloned into pmir GLO (Promega, Madison, WI, USA). Luciferase sequence-containing plasmids were developed by lipofection (Lipofectamine2000, Promega, Madison, WI, USA), and luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Mammalian two-hybrid experiments were performed using the CheckMate Mammalian Two-Hybrid System

(Promega, Madison, WI, USA) according to the manufacturer's instructions.

To detect an activation of AP1 or CRE luciferase reporter, 1×10^5 of H1299 cells were stimulated for 12 h with PMA (200 nM) and ionomycin (1 µg/ml) in the absence of FBS.

Real-Time PCR

Cytoplasmic RNA was extracted using hypotonic buffer, containing 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 0.1 mM DTT, and TRIzol reagent (Thermo Fisher, Waltham, MA, USA). All cDNA samples were synthesized utilizing the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Quantitative PCR experiments were performed with the KAPA SYBR FAST qPCR kit (Kapa Biosystems, Boston, USA) and the ABI StepOne Real-Time PCR system (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. The primer pairs used for rat cDNA amplification were as follows: Forward primer for HIF-1α: 5'- CCTTCGATCAGTTGTCACCA-3' and reverse primer for HIF-1α: 5'- TGGGTAGGAGATGGAGATGC-3', forward primer for c-Fos: 5'- GGGAGGACCTTATCTGTGCG-3' and reverse primer for c-Fos: 5'- CACACTCCATGCGTTTTGCT-3', forward primer for c-MYC: 5'- GGACTTGTTGCGGAAACGAC-3' and reverse primer for c-MYC: 5'- CTCAGCCAAGGTTGTGAGGT-3', forward primer for Ets2: 5'- CAGGATGAATGATTTCGG-

3' and reverse primer for Ets2: 5'- CGCGATGAATGACTTGG-3', forward primer for VEGF: 5'-TCACCAAGGCCAGCACATAG-3' and reverse primer for VEGF: 5'-GAGGCTCCAGGGCATTAGAC-3', forward primer for PTBP1: 5'-CCAAGTTCGGCACAGTGTTG-3' and reverse primer for PTBP1: 5'-TATACCAGGTGCACCGAAGG-3', forward primer for rat HuR: 5'-GTCCAGAGGGGTTGCGTTTA-3' and reverse primer for HuR: 5'-CCAAACGGCCCAAACATCTG-3', forward primer for HuB: 5'-AGGTTTTCTCCAATGACCAT-3' and reverse primer for HuB: 5'-TTAGGCTTTGTGCGTTTTGT-3', forward primer for HuC: 5'- CTCATCGCCAGGTTCTCGC-3' and reverse primer for HuC: 5'- TCATGGTCACGAAGCCGAAA -3'., forward primer for HuD: 5'- AATCGGGGGTTTCAGCTCAC-3' and reverse primer for HuD: 5'-TCTGTTGTTGCTGGAGGGTC -3'. Real-time quantitative PCR was performed using the ABI PRISM7700 Sequence Detection System. All expression values were normalized against β -actin. All amplifications were performed thrice in triplicate.

Western blotting, fluorescent microscopy, and RIP assays

Cells were scraped and incubated with a hypotonic buffer containing 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 0.1 mM DTT and placed on ice for 10 min, followed

by centrifugation at 1,500 rpm for 5 min. Supernatants were saved as the cytosolic fraction. Both the supernatants and the precipitated nuclei were boiled with 2X SDS sample buffer for 2 min and followed by Western blotting. Anti-HuR mouse monoclonal antibodies (clone 3A2: Santa Cruz, Dallas, TX, USA), anti-HuB (MLB, Nagoya, Japan), anti-HuR (MLB, Nagoya, Japan), anti-HA (Santa Cruz, Dallas, TX, USA), and anti-GFP (MLB, Nagoya, Japan) rabbit polyclonal antibodies were used for Western blotting, immunoprecipitation, or RNA immunoprecipitation experiments. EGFP and DsRed tagged plasmids transfected cells were fixed with 4% paraformaldehyde and analyzed by FV10 confocal laser scanning microscope (Olympus, Tokyo, Japan). RNA precipitation was performed using a RIP assay kit (MLB, Nagoya, Japan). Precipitated RNA was then reverse-transcribed using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), followed by quantitative RT-PCR or RT-PCR.

Statistical analysis

Statistical differences were analyzed by the Student's *t*-test. P values < 0.05 were considered statistically significant.

Results

Cytosolic HuR expression is required for ARE mRNA stabilization

To investigate the biological function of ARE sequences for protein expression, we introduced four independent luciferase constructs tagged with ARE sequences in the 3' UTR (Fig. 1A) using several cell lines and compared their relative luciferase activities. As shown in Fig. 1B, the three cell lines transfected with ARE-tagged luciferase plasmids displayed diminished luciferase activities compared with those transfected with the control luciferase plasmid. In addition, ARE-dependent suppressive functions for luciferase expression were impaired in U87MG and HEK293 cells compared with H1299 cells (Fig. 1B). We also found that H1299 cells, which showed minimum luciferase expression with each ARE-tagged luciferase construct, expressed only a slight amount of HuR in the cytosolic fraction. In contrast, U87MG and HEK293 cells expressed a substantial amount of HuR protein in both the nuclear and cytoplasmic fractions (Fig. 1C).

Next, we introduced a HuR expression plasmid into the H1299 cells together with an ARE-tagged luciferase construct, in order to examine whether over-expressed HuR could improve luciferase expression levels. However, as indicated in Figure 1D, no obvious improvement in luciferase expression was detected in HuR over-expressed H1299 cells. Therefore, we considered that another molecule may be involved in the cytosolic localization of HuR in

HEK293 and U87MG cells. To further investigate the endogenous expression of other Hu family genes, we performed Real-Time PCR experiments. Fig. 1E indicates that HuC and HuD were almost undetectable; however, a significant amount of HuB mRNA expression was confirmed in U87MG and HEK293 cells but not in H1299 cells.

Ectopic HuB expression trans-locates HuR from the nucleus to the cytoplasm and stabilized ARE mRNAs

Next, we introduced all four HA-tagged Hu family genes, together with the ARE-tagged luciferase plasmids, into the H1299 cells to confirm whether exogenously expressed HuB could improve luciferase expression. There are two splicing variants of human HuB, according to the GeneBank database. We designated the shorter splicing variant as HuB-S and the longer splicing variant as HuB-L (Fig. 2A). Only a 13 amino acid sequence, located in the hinge region, differs between HuB-S and HuB-L. A BLAST search revealed high homology between HuR and HuB, with approximately 80% amino acid sequence homology. However, as indicated in Fig. 2B, only HuB-S-, HuB-L-, and HuD-introduced cells showed a gain in relative luciferase activity. Further immunoprecipitation experiments revealed that a HuR/HuB complex was formed in HEK293 cells, predominantly in the cytosolic fraction, but no evidence of a HuR/HuB complex was found in either the nuclear or cytosolic fractions of

H1299 cell lysates (Fig. 2C).

Next, to investigate whether exogenous HuB can translocate “endogenous” HuR from the nucleus to the cytosol, we established a stable high HuB-expressing cell line (H1299-HuB) using a retrovirus system (the mock infectant was designated as H1299-mock). As indicated in Fig. 2D, the cytoplasmic translocation of endogenous HuR was observed in H1299-HuB cells.

The C-terminal of the RRM3 domain is responsible for HuB/HuR complex formation

Next, to confirm the binding activity between HuB and HuR, we performed a mammalian two-hybrid experiment using HuR bait and HuB wild-type or mutant fishes (Fig. 3A represents a schematic illustration of the HuR and HuB mutants). Surprisingly, the affinity between HuB and HuR was found to be stronger than that between HuR and HuR (Fig. 3B). The results also demonstrated that both N-terminal and C-terminal sequences were involved in complex formation.

Next, we performed immunoprecipitation experiments using HA-tagged HuR, the HA-tagged HuB mutants, and EGFP-tagged HuR. As indicated in Fig. 3C, the C-terminal region of the RRM3 domain was found to be responsible for the complex formation between HuB and HuR.

To further investigate the translocation of the HuB/HuR complex, we transfected DsRed-

tagged HuR together with EGFP-tagged HuR or HuB (HuB-S, HuB-L, and deletion mutants) into H1299 cells. As shown in Fig. 3D, EGFP-HuB (both HuB-S and HuB-L) was able to translocate the DsRed-HuR signals from the nucleus to the cytosol, whereas EGFP-HuR was not. The results also demonstrated that the N-terminal 38 amino acids of the HuB protein was responsible for the cytosolic distribution of HuB. Therefore, it is likely that the N-terminal and C-terminal domains of HuB play different roles independently.

Exogenous HuB expression and tumor progression and carcinogenesis

Next, to investigate the role of the HuB/HuR complex in cancer cells, we performed RNA immunoprecipitation (RIP) experiments. H1299-mock cells and H1299-HuB cells were lysed and immunoprecipitated with anti-HuR or anti-HuB antibodies, followed by reverse transcription reactions and Real-Time PCR for HIF-1 α . Only a background level of HIF-1 α mRNA was immunoprecipitated with anti-HuB antibodies in H1299-mock, whereas significant amounts of HIF-1 α mRNA were detected in H1299-HuB lysates (Fig. 4A). It is intriguing that significantly increased amounts of HIF-1 α mRNA were precipitated with the anti-HuR antibody in H1299-HuB lysates. Indeed, these results suggest that cytosolic HuR/HuB or HuB/HuB complexes may have a higher affinity for HIF-1 α mRNA compared with the HuR/HuR complex.

As a constant for transient transfection experiments (Fig. 2B), Real-Time PCR results demonstrated that the mRNA expressions of HIF-1 α , c-Fos, c-Myc, and Ets2 were higher in H1299-HuB cells compared with H1299-mock cells (Fig. 4B). We also detected the increased expression of other ARE mRNAs, including VEGF and PTBP1 (Fig. 4B). Next, we performed RNA immunoprecipitation (RIP) assays using HEK293 cells, to confirm the binding between “endogenous” HuB and ARE mRNAs. As shown in Fig. 4C, endogenous HuB proteins were found to be coprecipitated with tumor angiogenesis related ARE mRNAs, including PTBP1, HIF-1 α , and VEGF.

To further investigate the phenotypes of HuB-infectant cells (H1299-HuB), AP1 or cAMP-response element (CRE) luciferase reporter transfected H1299-mock and H1299-HuB cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Our results demonstrated significantly enhanced activation of AP1 promoter and CRE promoter in H1299-HuB (Fig. 4D).

Next, we performed transformation assays with rat cells to investigate the carcinogenic activity of exogenous HuB. Normal rat fibroblasts (3Y1) were transfected with the adenovirus E1 region, which contains E1A and E1B. As shown in Fig. 4E, the number of foci significantly increased with the combination of E1 and HuB-S or HuB-L but not HuR.

Discussion

The ELAVL/Hu family of proteins is highly conserved, and their binding affinity for ARE mRNA is rather indiscriminative. Therefore, the stabilization of ARE mRNA may primarily be achieved through the expression level or subcellular localization of Hu family proteins. Indeed, high expression of cytoplasmic HuR was implicated in ARE mRNA stabilization and the aggressiveness of malignant tumors. However, HuR over-expression does not appear to be sufficient for the stabilization of HIF-1 α mRNA. In this study, we demonstrated attenuated ARE mRNA decay among some cell lines that express high levels of HuB. In such cells, HuB was shown to form a cytosolic complex with HuR. RNA immunoprecipitation (RIP) assays further revealed a direct association between the HuB/HuR complex and ARE mRNAs, including HIF-1 α , PTBP1, and VEGF. We also described that over-expressed EGFP-tagged HuB, but not that of HuR, was sufficient for the translocation of over-expressed DsRed-tagged HuR from the nucleus to the cytosol. In addition, mammalian two-hybrid and immunoprecipitation experiments revealed that the C-terminal half of RMM3 was responsible for the association between HuB and HuR. The N-terminal region of HuB was shown to be required for the cytosolic localization of the HuB/HuR complex. Collectively, these results suggested that HuB plays a major role in the cytosolic translocation of HuR, as well as the stabilization of ARE mRNA.

Tumor angiogenesis is a distinct step of cancer progression and metastasis [10]. Indeed, a large body of evidence indicates that the enhanced vessel permeability of tumor vascular networks contributes to both tumor growth and cancer cell dissemination. Among the numerous molecular pathways involved in this process, VEGF has been shown to be a critical molecule that regulates all aspects of tumor-induced angiogenesis, with cancer cells themselves serving as the main source of VEGF within the tumor mass. When perfusion of the tumor mass environment is impaired by tumor dilatation, the resulting reduction in oxygen tension serves to activate HIF-1 α , the main transcription factor of VEGF. In our previous report, we demonstrated that polypyrimidine tract-binding protein 1 (PTBP1) is a translational enhancer of HIF-1 α [6]. As shown in Fig. 4B, the three ARE mRNAs we studied were shown to be stabilized in HuB-expressing H1299 cells compared with H1299-mock cells. In addition, our RIP assay results suggested the co-precipitation of the mRNAs with endogenous HuB in HEK293 cells (Fig. 4C). Moreover, ectopically expressed HuB in H1299 cells (H1299-HuB cells) was capable of initiating both the translocation of nuclear HuR to the cytoplasm and the stabilization of PTBP1, HIF-1 α , and VEGF-A mRNA. Thus, it is likely that this mRNA stabilization, which is dependent on the HuB/HuR complex, may be involved in tumor progression.

In normal cells, HuR proteins are predominantly expressed in the nucleus, and they are

exported to the cytoplasm in a CRM1-dependent manner under certain limited conditions [11]. Conversely, the ubiquitous cytoplasmic distribution of HuR in several types of cancer cells has been reported. The idea that the cytoplasmic distribution of the HuR complex is CRM1-independent in some cases is intriguing. Human adenovirus E4 orf6 is one of the binding partner proteins in the CRM1-independent nuclear export of the HuR complex [4]. Several reports demonstrated that E4 orf6 enhances the oncogenic effect of adenovirus E1A and E1B [12] [13]. As shown in Figure 4E, the numbers of transformed colonies that were transformed by E1 and HuB were significantly higher compared with E1-HuR co-transformed or E1-transformed colonies. Our results demonstrated that the over-expression of HuR alone was not capable of enhancing the transformation activities of the adenovirus E1 genes; it is likely that other protein binding partners of HuR are required for enhanced transformation activity.

The *c-fos*, *ets*, and *c-myc* genes are endogenous counterparts of the viral oncogenes *v-fos*, *v-ets*, and *v-myc*. They are also transcription factors, and their activities are regulated by strict control systems, including an ARE-dependent mRNA destabilization system, since each gene exhibits a strong onco-genetic nature [14] [15]. Therefore, the HuB-dependent prolonged expression of these transcription factors is considered a risk factor for carcinogenesis. However, neural cells do not have the ability to divide, which is required for

oncogenesis. Therefore, it seems likely that HuB expression is not a risk for oncogenesis in neural cells. On the contrary, the prolonged expression of *c-fos* may contribute to long-term neuronal plasticity, including learning and memory [16]. Kaczmarek et al discovered that *c-fos* mRNA can accumulate in different structures of the brain after various behavioral training regimens [17] [18] [19]. It has been also reported that histon acetyltransferase (HAT) activity of CREBBP (CREB-binding protein), another ARE mRNA, is involved in memory consolidation [20]. Berto et al also reported that HuB regulation of transcript expression is critical for neural functions and clinically relevant to autism spectrum disorder [21]. Thus, the stabilization of these proto-oncogene mRNAs may be beneficial for neuronal cell function.

It is intriguing that five of the six so-called “Yamanaka Thomson factors,” oct3/4, sox2, klf4, c-myc, lin28A, and nanog, contain ARE sequences in their 3' UTR (by search of the ARE data base [22]). Lin28B shares more than 70% of amino acids and contains an ARE sequence in the 3' UTR. Researchers also reported that the Lin28B promoter sequence contains c-Myc binding sites [23]. In addition, HuB is the only Hu member that is expressed in the ovary among the three neuronal Hu proteins [24]. Thus, it is plausible that the HuB/HuR complex may be involved in cell reprogramming and differentiation.

Collectively, our findings indicate that HuB functions as an enhancer for multiple proto-oncogenes, which are required for certain neural functions, including long-term neuronal

plasticity and learning and memory. However, these functions are also known to be involved in a positive loop for carcinogenesis in dividing cells and tumor progression.

Acknowledgement

This work was supported by Grants-in-Aid for Science Research B-24390285, B-19390477, and C-18591994 provided by the Japan Society for the Promotion of Science.

References

- [1] C. Barreau, L. Paillard, H.B. Osborne, AU-rich elements and associated factors: are there unifying principles?, *Nucleic acids research*, 33 (2005) 7138-7150.
- [2] I. Kotta-Loizou, C. Giaginis, S. Theocharis, Clinical significance of HuR expression in human malignancy, *Medical oncology*, 31 (2014) 161.
- [3] P.J. Good, The role of elav-like genes, a conserved family encoding RNA-binding proteins, in growth and development, *Seminars in cell & developmental biology*, 8 (1997) 577-584.
- [4] F. Higashino, M. Aoyagi, A. Takahashi, M. Ishino, M. Taoka, T. Isobe, M. Kobayashi, Y. Totsuka, T. Kohgo, M. Shindoh, Adenovirus E4orf6 targets pp32/LANP to control the fate of ARE-containing mRNAs by perturbing the CRM1-dependent mechanism, *The Journal of cell biology*, 170 (2005) 15-20.
- [5] J.P. Jehung, T. Kitamura, A. Yanagawa-Matsuda, T. Kuroshima, A. Towfik, M. Yasuda, H. Sano, Y. Kitagawa, K. Minowa, M. Shindoh, F. Higashino, Adenovirus infection induces HuR relocalization to facilitate virus replication, *Biochemical and biophysical research communications*, 495 (2018) 1795-1800.
- [6] M. Yasuda, T. Hatanaka, H. Shirato, T. Nishioka, Cell type-specific reciprocal regulation of HIF1A gene expression is dependent on 5'- and 3'-UTRs, *Biochemical and biophysical research communications*, 447 (2014) 638-643.

- [7] H.J. Okano, R.B. Darnell, A hierarchy of Hu RNA binding proteins in developing and adult neurons, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 17 (1997) 3024-3037.
- [8] A. Szabo, J. Dalmau, G. Manley, M. Rosenfeld, E. Wong, J. Henson, J.B. Posner, H.M. Furneaux, HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal, *Cell*, 67 (1991) 325-333.
- [9] J. Dalmau, H.M. Furneaux, C. Cordon-Cardo, J.B. Posner, The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues, *The American journal of pathology*, 141 (1992) 881-886.
- [10] D.R. Bielenberg, B.R. Zetter, The Contribution of Angiogenesis to the Process of Metastasis, *Cancer J*, 21 (2015) 267-273.
- [11] I.E. Gallouzi, C.M. Brennan, J.A. Steitz, Protein ligands mediate the CRM1-dependent export of HuR in response to heat shock, *RNA*, 7 (2001) 1348-1361.
- [12] M. Moore, N. Horikoshi, T. Shenk, Oncogenic potential of the adenovirus E4orf6 protein, *Proceedings of the National Academy of Sciences of the United States of America*, 93 (1996) 11295-11301.
- [13] M. Dobbelstein, J. Roth, W.T. Kimberly, A.J. Levine, T. Shenk, Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence, *EMBO*

J, 16 (1997) 4276-4284.

[14] J.L. Veyrune, G.P. Campbell, J. Wiseman, J.M. Blanchard, J.E. Hesketh, A localisation signal in the 3' untranslated region of c-myc mRNA targets c-myc mRNA and beta-globin reporter sequences to the perinuclear cytoplasm and cytoskeletal-bound polysomes, J Cell Sci, 109 (Pt 6) (1996) 1185-1194.

[15] Y. You, C.Y. Chen, A.B. Shyu, U-rich sequence-binding proteins (URBPs) interacting with a 20-nucleotide U-rich sequence in the 3' untranslated region of c-fos mRNA may be involved in the first step of c-fos mRNA degradation, Mol Cell Biol, 12 (1992) 2931-2940.

[16] S. Tonegawa, M. Pignatelli, D.S. Roy, T.J. Ryan, Memory engram storage and retrieval, Curr Opin Neurobiol, 35 (2015) 101-109.

[17] L. Kaczmarek, J.A. Siedlecki, W. Danysz, Proto-oncogene c-fos induction in rat hippocampus, Brain Res, 427 (1988) 183-186.

[18] L. Kaczmarek, E. Nikolajew, c-fos protooncogene expression and neuronal plasticity, Acta Neurobiol Exp (Wars), 50 (1990) 173-179.

[19] J. Jaworski, K. Kalita, E. Knapska, c-Fos and neuronal plasticity: the aftermath of Kaczmarek's theory, Acta Neurobiol Exp (Wars), 78 (2018) 287-296.

[20] E. Korzus, M.G. Rosenfeld, M. Mayford, CBP histone acetyltransferase activity is a critical component of memory consolidation, Neuron, 42 (2004) 961-972.

[21] S. Berto, N. Usui, G. Konopka, B.L. Fogel, ELAVL2-regulated transcriptional and splicing networks in human neurons link neurodevelopment and autism, *Hum Mol Genet*, 25 (2016) 2451-2464.

[22] T. Bakheet, E. Hitti, K.S.A. Khabar, ARED-Plus: an updated and expanded database of AU-rich element-containing mRNAs and pre-mRNAs, *Nucleic acids research*, 46 (2018) D218-D220.

[23] T.C. Chang, L.R. Zeitels, H.W. Hwang, R.R. Chivukula, E.A. Wentzel, M. Dews, J. Jung, P. Gao, C.V. Dang, M.A. Beer, A. Thomas-Tikhonenko, J.T. Mendell, Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation, *Proceedings of the National Academy of Sciences of the United States of America*, 106 (2009) 3384-3389.

[24] P.J. Good, A conserved family of elav-like genes in vertebrates, *Proceedings of the National Academy of Sciences of the United States of America*, 92 (1995) 4557-4561.

Figure legends

Figure 1

A: Schematic illustration of the Luciferase constructs.

B: Results of reporter assays among the three cell lines. Error bars indicate the standard deviation of triplicate experiments. P values were analyzed by the Student's *t*-test.

C: Immunoblot results for the indicated cell lines. N indicates the nuclear fraction, and C indicates the cytosolic fraction.

D: Relative amount of luciferase activities of transiently transfected H1299 cells. The combinations of plasmids are indicated at the bottom. Error bars indicate the standard deviation of triplicate experiments.

E: Relative amount of HuB, HuC, and HuD in several cell lines. Each amount of reverse transcribed cDNAs were evaluated with standard curves.

Figure 2

A: Immunoblot results for the indicated plasmids and the schematic illustration of HuB.

B: Relative amount of luciferase activities of transiently transfected H1299 cells. The combinations of plasmids are indicated at the bottom. Error bars indicate the standard deviation of triplicate experiments.

C: Co-precipitation results of the indicated cells. Each fractionated lysate was immunoprecipitated with normal serum (N.S.) or anti-HuB antibody and blotted with the anti-HuR antibody. N indicates the nuclear fraction and C indicates the cytosolic fraction.

D: Immunoblot results for the nuclear and cytosolic lysates of the indicated cell lines. N indicates the nuclear fraction, and C indicates the cytosolic fraction.

Figure. 3

A: A schematic illustration of the HuB mutants.

B: Results of mammalian two-hybrid experiment. Combinations of plasmids are indicated at the bottom. pACT empty vector or pACT-Hu series, pBIND HuR, and Gal4 luciferase reporter plasmids (pG5) were co-transfected into H1299 cells.

C: Results of co-immunoprecipitation of the indicated transiently transfected H1299. Cells were co-transfected with EGFP-HuR and HA-tagged Hu series plasmids. Lysates were precipitated with the anti-HA antibody and blotted with the indicated antibodies (anti-GFP or anti HA antibody).

D: Fluorescent images of transiently transfected H1299 cells. H1299 cells were transfected with a combination of Ds-Red-tagged HuR and EGFP-tagged Hu plasmids. A clear translocation of DsRed-tagged HuR from the nucleus to the cytosol was demonstrated in

EGFP-HuB-S and EGFP-HuB-L co-transfected cells.

Figure. 4

A: Immune-precipitated HIF-1 α mRNA is indicated. A significant difference in the immunoprecipitated mRNA amounts was detected in the H1299-HuB cells compared with H1299-mock cells.

B: Relative amounts of mRNA as determined by Real-Time PCR are indicated.

C: Results of the RNA immunoprecipitation of HEK293 cells. Obvious amplifications of the indicated cDNAs were demonstrated.

D: Results of AP1 and CRE luciferase assays. Stimulated H1299-HuB cells (PMA: 200 nM, Ionomycine: 1ug/ml for 12 hr) showed enhanced luciferase activities.

E: Results of the transformation assays. 3Y1, which are normal rat fibroblasts, were transfected with the indicated combinations of plasmids and cultured for 4 weeks. Significant differences between the foci were detected among the transfectants.

Fig. 1

Fig. 2

Fig. 3

A

B

C

D

Fig. 4