Bioinformatics Data Mining Approach Suggests Coexpression of AGTPBP1 with an ALS-linked Gene C9orf72

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ABSTRACT

BACKGROUND: Expanded GGGGCC hexanucleotide repeats located in the noncoding region of the chromosome 9 open reading frame 72 (*C9orf72*) gene represent the most common genetic abnormality for familial and sporadic amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Formation of nuclear RNA foci, accumulation of repeat-associated non-ATG-translated dipeptide-repeat proteins, and haploinsufficiency of *C9orf72* are proposed for pathological mechanisms of C9ALS/FTD. However, at present, the physiological function of C9orf72 remains largely unknown.

METHODS: By searching on a bioinformatics database named COXPRESdb composed of the comprehensive gene coexpression data, we studied potential C9orf72 interactors.

RESULTS: We identified the ATP/GTP binding protein 1 (*AGTPBP1*) gene alternatively named *NNA1* encoding a cytosolic carboxypeptidase whose mutation is causative of the degeneration of Purkinje cells and motor neurons as the most significant gene coexpressed with C9orf72. We verified coexpression and interaction of AGTPBP1 and C9orf72 in transfected cells by immunoprecipitation and in neurons of the human brain by double-labeling immunohistochemistry. Furthermore, we found a positive correlation between AGTPBP1 and C9orf72 mRNA expression levels in the set of 21 human brains examined.

CONCLUSIONS: These results suggest that AGTPBP1 serves as a C9orf72 interacting partner that plays a role in the regulation of neuronal function in a coordinated manner within the central nervous system.

KEYWORDS: AGTPBP1, amyotrophic lateral sclerosis, bioinformatics, C9orf72, CCP1, coexpression, COXPRESdb, frontotemporal dementia, NNA1

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Introduction

Expanded GGGGCC hexanucleotide repeats located in the noncoding region of the chromosome 9 open reading frame 72 (*C9orf72*) gene represent the most common genetic abnormality for familial and sporadic amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), suggesting that two distinct disease entities ALS and FTD constitute an overlapping continuum affecting multiple systems in the central nervous system (CNS).[1,2] The patients with C9orf72 repeat expansions exhibit a clinical phenotype characterized by the earlier disease onset with bulbar, cognitive and behavioral impairment, psychosis, and parkinsonism.[3,4] The brains of C9ALS/FTD show not only the TAR DNA-binding protein-43 (TDP-43) pathology but also an accumulation of TDP-43-negative p62-positive neuronal cytoplasmic and nuclear inclusions prominent in the cerebellar granular cell layer and the hippocampal dentate gyrus.[5]

Recent studies have proposed three potential mechanisms for the pathogenesis of C9ALS/FTD.[6,7] First, mRNAs coding for expanded C9orf72 repeats transcribed from sense and antisense strands are concentrated on nuclear RNA foci that sequester vital RNA-binding proteins, leading to aberrant mRNA splicing and processing of the genes crucial for neuronal function.[8,9] Second, bidirectional transcripts of expanded C9orf72 repeats are translated into potentially neurotoxic dipeptide-repeat (DPR) proteins by the unconventional mechanism termed repeat-associated non-ATG (RAN) translation.[10,11] DPR proteins constitute a component of TDP-43-negative p62-positive inclusions in the brain of C9ALS/FTD. Third, repeat expansions cause transcriptional silencing of the *C9orf72* gene by epigenetic mechanisms that

involve hypermethylation of CpG islands and histone trimethylation, leading to haploinsufficiency responsible for the loss of normal function of C9orf72.[12,13] Expanded hexanucleotide repeats adopt a G-quadruplex conformation that directly interferes with transcription.[14] Notably, C9orf72 protein levels are reduced in the brain of C9ALS/FTD.[15] Furthermore, deletion of zebrafish or *Caenorhabditis elegans* C9orf72 orthologs causes degeneration of motor neurons, suggesting that the loss of normal function of C9orf72 is detrimental for motor neuron survival.[16,17] However, intraventricular injection in the mouse brain of an antisense oligonucleotide selectively targeting the sense strand of repeat-containing RNA reduces C9orf72 mRNA levels without any behavioral and pathological changes, contradicting the hypothetical view of hapoinsufficiency.[8]

C9orf72 is an evolutionarily conserved protein of unknown function, expressed most abundantly in neurons in the CNS under normal physiological conditions.[18] C9orf72 is distantly related to the differentially expressed in normal and neoplastic cells (DENN) family of GDP-GTP exchange factors (GEFs) that activate Rab GTPases.[19,20] Actually, C9orf72 regulates Rab GTPase-mediated endosome trafficking.[21] Previous studies identified ubiqulin-1 (UBQLN1), acting as an adaptor protein that mediates the translocation of polyubiquitinated proteins to the proteasome for degradation, as a direct interacting partner of C9orf72, suggesting that C9orf72 plays a regulatory role in the ubiquitin/proteasome system, a key machinery for cellular protein homeostasis.[21,22] However, at present, the precise physiological function of C9orf72 remains largely unknown.

In the present study, we attempted to discover novel C9orf72 interactors by searching coexpressed genes on a bioinformatics database named COXPRESdb composed of the comprehensive gene coexpression data of human and non-human species.[23] We identified the ATP/GTP binding protein 1 (*AGTPBP1*) gene encoding a cytosolic carboxypeptidase (CP), originally identified as "nervous system nuclear protein induced by axotomy 1" (NNA1), rapidly induced in motor neurons following sciatic nerve injury,[24] as the most significant gene coexpressed with C9orf72. Then, we verified coexpression and interaction of C9orf72 and AGTPBP1 in transfected cells by immunoprecipitation (IP) and in neurons of the human brain by double-labeling immunohistochemistry (IHC). These results suggest that AGTPBP1 serves as a C9orf72 interacting partner that plays a role in the regulation of neuronal function in a coordinated manner within the CNS.

Methods

Human brain tissues. For IHC, serial sections of the frontal cortex (FC) and the hippocampus were prepared from autopsied brains of 10 sporadic Alzheimer's disease (AD) patients, composed of five men and five women with the mean age of 70 ± 8 years, and 11 non-AD patients, composed of six men and five women with the mean age of 75 ± 8 years. The non-AD group includes four normal subjects died of non-neurological causes (NC), three patients with sporadic Parkinson's disease (PD), and four with sporadic ALS. All AD cases satisfied the Consortium to Establish a Registry for Alzheimer's Disease criteria for diagnosis of definite AD.[25] They were categorized into the stage C of amyloid deposition and the stage VI of neurofibrillary degeneration, following the Braak staging system.[26] Additional cases, for which frozen brain samples were available, were included for biochemical studies. The demographic profile of these samples was shown in Supplementary Table 1. Autopsies were performed at the National Center Hospital, National Center of Neurology and Psychiatry, Japan, or Kohnodai Hospital, National Center for Global Health and Medicine (NCGM), Japan. The comprehensive examination of autopsied brains by three established neuropathologists (TI, YS, and KA) validated the pathological diagnosis. In all cases, written informed consent was obtained. The Ethics Committee of the National Center of Neurology and Psychiatry for the Human Brain Research, the Ethics Committee of the National Center for Global Health and Medicine on the Research Use of Human Samples, and the Human Research Ethics Committee of the Meiji Pharmaceutical University approved the present study. Our research complied with the principles of the Declaration of Helsinki.

Immunohistochemistry. The brain tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Tissue sections were deparaffinized and heat-treated in 10 mM citrate sodium buffer, pH 6.0 by autoclaving them at 110 °C for 15 minutes in a temperature-controlled pressure chamber (Biocare Medical). They were incubated at room temperature (RT) for 15 minutes with 3% hydrogen peroxide-containing methanol to block the activity of endogenous peroxidase. They were incubated with phosphate-buffered saline (PBS) containing 10% normal goat serum at RT for 15 minutes to block nonspecific staining. Then, the tissue sections were incubated at 4 °C overnight with a rabbit polyclonal anti-AGTPBP1 antibody raised against a peptide spanning amino acid residues 837–1186 of the human AGTPBP1 protein (14067–1-AP; Proteintech), a rabbit polyclonal anti-human C9orf72 antibody raised against a peptide spanning amino acid residues 165–215 of the human C9orf72 protein (sc-138763; Santa Cruz Biotechnology), whose specificity was validated previously,[22] or a mouse monoclonal anti-AGTPBP1 antibody raised against a recombinant protein fragment spanning amino acid residues 368–753 of the human AGTPBP1 (9A3; LifeSpan Biosciences). The specificity of anti-AGTPBP1 antibodies was validated by western blot (WB) of the recombinant human AGTPBP1 protein tagged with hemagglutinin (HA) expressed in HEK293 cells (Supplementary Fig. 1). After washing with PBS, the tissue sections were labeled at RT for 30 minutes with a peroxidase-conjugated secondary antibody (Nichirei Corporation), followed by incubation with diaminobenzidine tetrahydrochloride substrate (Vector Laboratories).

Table 1. Top 20 significant genes coexpressed with C9orf72 on COXPRESdb.

RANK	ENTREZ GENE ID	GENE	GENE NAME	HSA1 MR	HSA2 MR	HSA3 MR
1	23287	*AGTPBP1*	ATP/GTP binding protein 1 (nervous system nuclear protein induced by axotomy 1)	3	2.5	1
2	153020	*RASGEF1B*	RasGEF domain family, member 1B	9.4	71.1	616.8
3	8915	*BCL10*	B-cell CLL/lymphoma 10	12.1	1088.1	2270.8
4	9262	*STK17B*	Serine/threonine kinase 17b	12.1	393.5	860.5
5	83478	*ARHGAP24*	Rho GTPase activating protein 24	16.9	3815.7	4213.3
6	80853	*JHDM1D*	Jumonji C domain containing histone demethylase 1 homolog D (S. cerevisiae)	19.6	81.2	262.4
7	23312	*DMXL2*	Dmx-like 2	20.5	133.7	351.3
8	79663	*HSPBAP1*	HSPB (heat shock 27kDa) associated protein 1	26.1	63.2	30.4
9	8676	*STX11*	Syntaxin 11	32.9	1342	2474.8
10	100113407	*TMEM170B*	Transmembrane protein 170B	34.4	NA	595.1
11	1540	*CYLD*	Cylindromatosis (turban tumor syndrome)	36.9	338.3	17.6
12	10135	*NAMPT*	Nicotinamide phosphoribosyltransferase	37.6	114.5	300.6
13	9841	*ZBTB24*	Zinc finger and BTB domain containing 24	45.4	2530.5	3076.2
14	51132	*RLIM*	Ring finger protein, LIM domain interacting	47	103.8	2330.5
15	7456	*WIPF1*	WAS/WASL interacting protein family, member 1	49.2	4282.7	5392.5
16	81553	*FAM49A*	Family with sequence similarity 49, member A	49.6	255.2	393.1
17	85464	*SSH2*	Slingshot homolog 2 (Drosophila)	54	999	818.5
18	4783	*NFIL3*	Nuclear factor, interleukin 3 regulated	59.7	553.5	1550
19	55454	*CSGALNACT2*	Chondroitin sulfate N-acetylgalactosaminyltransferase 2	60.5	87.9	139.4
20	5277	*PIGA*	Phosphatidylinositol glycan anchor biosynthesis, class A	60.8	47	71.6

Notes: The set of genes coexpressed with C9orf72 are identified by database search on COXPRESdb ver 6.0. The correlation coefficient is transferred to mutual rank (MR) on Human Gene-U133 Plus 2 Array (Hsa1), Human Gene 1.0 ST array (Hsa2), and RNAseq-based coexpression data (Hsa3). The smaller value of MR indicates higher significance.
Abbreviation: NA, not available.

They were processed for a counterstain with hematoxylin. For negative controls, the primary antibodies were omitted from the reaction.

For double labeling, tissue sections were stained with a mixture of sc-138763 and 9A3 antibodies. They were washed with PBS, followed by incubation with a mixture of Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen) and Alexa Fluor 568-conjugated anti-mouse IgG (Invitrogen). The nuclei of the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI).

RT-PCR analysis. Total cellular RNA was extracted from human neural cell lines and tissues by using TRIZOL (Invitrogen). RNA treated with DNase I was processed for cDNA synthesis using oligo$(dT)_{20}$ primers and SuperScript II reverse transcriptase (Invitrogen). cDNA was amplified by PCR using HotStarTaq Plus DNA polymerase (Qiagen Inc.) on a thermal cycler at 94 °C for 30 seconds for denaturation, at 62 °C for 30 seconds for annealing, and 72 °C for one minute for extension. PCR cocktails were prepared individually by mixing with sense and antisense primer sets following: 5′cagcacgtgttgtagtttggaggg3′ and 5′agtcaagcaggctggaaggcagat3′ for a 213 bp product of the human *AGTPBP1* gene (NCBI Reference Sequence Number NM_015239); 5′ccttgatttaacagcagagggcga3′ and 5′tttccccacaccactgagctactt3′ for a 210 bp product specific for the *C9orf72* isoform a gene (NM_018325); and 5′ccatgttcgtcatgggtgtgaacca3′ and 5′gccagtagaggcaggatgatgttc3′ for a 251 bp product of the glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene (NM_002046), serving as an internal control. cDNA was amplified by PCR for 30 cycles except for G3PDH that was amplified for 26 cycles.

For quantitative RT-PCR (qPCR), cDNA prepared from frozen human brain tissues and a reference RNA of the human FC (AM6810; Ambion–Invitrogen) was amplified by PCR on LightCycler 96 (Roche Diagnostics) using SYBR Green I and the primer sets described above. The expression levels of target genes were standardized against the levels of G3PDH detected in the corresponding cDNA samples. All the assays were performed in triplicate.

Coexpression database search. The COXPRESdb version 6.0 (coxpresdb.jp) is a freely accessible bioinformatics database, composed of the comprehensive information on gene coexpression collected from large sets of public microarray data to predict gene functions of 11 species, including *Homo sapiens, Mus musculus, Rattus norvegicus, Drosophila melanogaster,* and *C. elegans*.[23] For human genes, COXPRESdb includes coexpression data derived from microarrays of 85,723 samples,

whose raw data are extracted from ArrayExpress and normalized by the robust multiarray average method. Additionally, COXPRESdb includes RNA-Seq-based coexpression data of 5,636 samples. COXPRESdb enables us to identify coexpression relationships for a given gene with the reliability score and to illustrate the interactive network of the genes surrounding a focused gene on Cytoscape web. By importing "C9orf72" as a query into COXPRESdb, we identified the set of genes coexpressed with C9orf72, which potentially contain candidates for C9orf72 interactors.

Vector construction, transfection, and IP. The expression vector, composed of the HA-tagged full-length open reading frame (ORF) of the human *AGTPBP1* gene, was obtained from Applied Biological Materials. The full-length ORF of the human *C9orf72* gene and the gene encoding the CP domain spanning amino acid residues 819–1096 of AGTPBP1 (NM_015239.2) (Supplementary Fig. 2) were individually amplified by PCR using PfuTurbo DNA polymerase (Agilent Technologies) with appropriate primers. Subsequently, PCR products were cloned in the expression vector named p3XFLAG-CMV7.1 (Sigma), pCMV-Myc (Clontech Laboratories, Inc.), pEGFP-C1 (Clontech), or pRFP-C1 (homemade) to express a fusion protein N-terminally tagged with Flag, Myc, EGFP, or RFP, respectively. The vectors were transfected in HEK293 cells or NSC-34 motor neurons[27] (Cosmo Bio Co., Ltd.) by using lipofectamine (LPF) 2000 reagent (Invitrogen) for transient expression. After cotransfection of the vectors, the protein extract was processed for IP with mouse monoclonal anti-Flag M2 affinity gel (A2220; Sigma), mouse monoclonal anti-HA agarose (A2095; Sigma), or rabbit polyclonal anti-Myc-conjugated agarose (A7470; Sigma), followed by WB with a mouse monoclonal anti-FLAG M2 antibody (F1804; Sigma), a rabbit polyclonal anti-HA antibody (H6908 Sigma), a rabbit polyclonal anti-C9orf72 antibody (sc-138763), or a rabbit polyclonal anti-GFP antibody (sc-8334; Santa Cruz Biotechnology). In some experiments, protein G agarose (Roche Diagnostics) was utilized for IP.

SiRNA-mediated knockdown of AGTPBP1. The siRNA product directed to the human AGTPBP1 mRNA named Hs_AGTPBP1_7371 and a negative control RNA named Mission_SIC-001 were obtained from Sigma. They were introduced in SK-N-SH neuroblastoma cells at a concentration of 100 nM by using LPF RNAiMax reagent (Invitrogen), followed by processing for qPCR and WB analysis.

Overexpression of POU2F1. The full-length ORF of the human POU class 2 homeobox 1 (POU2F1) gene was amplified by PCR with appropriate primers. The PCR product was cloned in the expression vector named pcDNA3.1/V5-His TOPO (Invitrogen) without the inclusion of a V5-His tag. The POU2F1 expression vector or the control vector named pcDNA3.1/V5-His-TOPO/lacZ was transfected in SK-N-SH cells by using LPF 2000 reagent for transient expression, followed by processing for qPCR and WB analysis.

WB analysis. To prepare total protein extract, brain tissues and cultured cells were homogenized in RIPA buffer (Sigma) supplemented with a cocktail of protease inhibitors (Sigma), and followed by centrifugation at 12,000 rpm for 10 minutes at RT to harvest the supernatant. The protein separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was transferred onto nitrocellulose membranes, which were incubated at RT overnight with anti-AGTPBP1 antibody, anti-C9orf72 antibody, or anti-POU2F1 (OCT1) antibody (GTX105202; GeneTex, Inc.). Then, the membrane was incubated at RT for 30 minutes with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology). The specific reaction was visualized by exposing membranes to a chemiluminescent substrate (Thermo Scientific). After the antibody was stripped by incubating membranes at 50 °C for 30 minutes in the stripping buffer, composed of 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol, it was processed for relabeling with a mouse monoclonal anti-actin, beta (ACTB) antibody (AC-15; Sigma) or a goat polyclonal anti-HSP60 antibody (sc-1052; Santa Cruz Biotechnology), serving as an internal control of protein loading. The signal intensity of the major band was quantified by using ImageJ (National Institute of Health). The expression levels of AGTPBP1 and C9orf72 were standardized by the corresponding signal intensity of ACTB.

Statistical analysis. In qPCR and WB analysis, the statistical significant difference between AD ($n = 7$) and non-AD ($n = 14$) groups was evaluated by Student's t-test. The P-value smaller than 0.05 by a two-tailed test was considered as significant. The statistical significance among the three groups was evaluated by one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. The correlation between the two groups was evaluated by Pearson's correlation coefficient test. The correlation coefficient (r) larger than 0.4 was considered as positive correlation.

Results

COXPRESdb search indicates coexpression of AGTPBP1 with C9orf72. By searching on COXPRESdb,[23] we identified the *AGTPBP1* gene as the most significant gene coexpressed with C9orf72 (Table 1). The correlation coefficient (r) for gene expression levels between C9orf72 and AGTPBP1 was 0.406, indicating a discernible positive correlation between both (Fig. 1A). The molecular network analysis of the top 20 coexpressed genes showed that C9orf72 directly interacts with AGTPBP1, RASGEF1B ($r = 0.268$), ARHGAP24 ($r = 0.171$), HSPBAP1 ($r = 0.312$), and PCIF1 ($r = 0.327$) (Fig. 1B). AGTPBP1 is originally identified as NNA1 that is rapidly induced in motor neurons following sciatic nerve injury in response to regeneration of motor neurons.[24] Owing to the highest rank of significance for coexpression and the pathological relevance to ALS, thereafter, we concentrated on AGTPBP1 as the most promising candidate for further investigation.

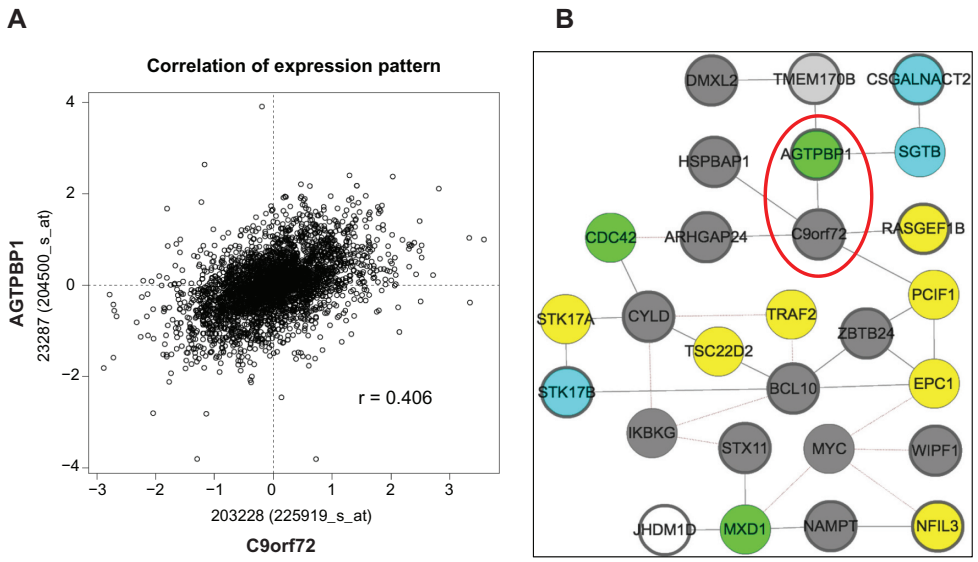

Figure 1. COXPRESdb search indicates coexpression of AGTPBP1 with C9orf72. The genes coexpressed with C9orf72 were identified by database search on COXPRESdb, as listed in Table 1. Interactive network of top 20 genes coexpressed with C9orf72 was visualized on Cytoscape web. (**A**) Correlation profile of gene expression between C9orf72 (*x* axis: probe ID 225919_s_at) and AGTPBP1 (*y* axis: probe ID 204500_s_at). (**B**) Molecular network of top 20 genes coexpressed with C9orf72. The color of nodes indicates yellow (nuclear), green (cytosol), blue (mitochondria), white (plasma membrane), and grey (ambiguous), determined by WoLF localization prediction program. The interaction between C9orf72 and AGTPBP1 is highlighted by red ellipse.

Coexpression of AGTPBP1 and C9orf72 mRNA in human neural cells. Next, we studied mRNA expression of AGTPBP1 and C9orf72 in human neural cell lines and tissues by RT-PCR and qPCR. By RT-PCR, the human cerebrum (CBR), astrocytes, neuronal progenitor (NP) cells, NTera2 teratocarcinoma-derived neurons, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, U-373MG glioblastoma, T98 glioblastoma, and HMO6 immortalized microglia constitutively expressed both AGTPBP1 and C9orf72 transcripts, along with G3PDH (Fig. 2, panels A–C, lanes 1, 3–10). In contrast, no products were amplified, when the reverse transcription step was omitted (Fig. 2, panels A–C, lane 2). By qPCR, we found positive correlation between AGTPBP1 and C9orf72 mRNA levels in the set of eight human neural cells in culture, as indicated by $r = 0.874$ and $P = 0.005$ in Pearson's correlation (Fig. 2, panels D–F). Thus, we found that AGTPBP1 and C9orf72 transcripts are coexpressed widely in various human neural cells in culture.

Molecular interaction of AGTPBP1 and C9orf72 in cultured cells. By co-IP experiments, we verified an interaction between the full-length AGTPBP1 protein tagged with HA and the full-length C9orf72 tagged with Flag, when both were transiently expressed in HEK293 cells (Fig. 3, panels A and B). Next, we studied the molecular interaction of C9orf72 with the CP domain of the AGTPBP1 protein. The amino acid sequence of this domain is highly conserved through evolution (Supplementary Fig. 2). The CP domain tagged with Flag interacted with the full-length C9orf72 protein tagged with Myc or EGFP in HEK293 cells (Fig. 3, panels C and D). Furthermore, we identified the molecular interaction of endogenous AGTPBP1 and C9orf72 proteins in SK-N-SH cells by co-IP (Fig. 3, panel E). We also found colocalization of EGFP-tagged AGTPBP1 and RFP-tagged C9orf72, expressed chiefly in the cytoplasm of NSC-34 motor neurons (Fig. 4, panels A–F). Thus, we verified that AGTPBP1 is coexpressed and interacts with C9orf72 at protein levels in cultured cells.

Coexpression of AGTPBP1 and C9orf72 mRNA and protein in human brains. To study the pathological relevance of coexpression of AGTPBP1 and C9orf72 to human neurodegenerative diseases, we performed IHC, qPCR, and WB analysis of human brains. By single-labeling IHC, we identified intense immunoreactivity for AGTPBP1 or C9orf72, both of which were expressed chiefly in cell bodies and axonal and dendritic processes of various neurons, along with variable immunoreactivities in the neuropil, in the frontal cortex (FC), and the hippocampus of brains of normal control (NC) subjects, AD, PD, and ALS (Fig. 5, panels A–D). By double-labeling IHC, we identified coexpression of AGTPBP1 and C9orf72 located in the cytoplasm of hippocampal CA1 neurons in NC brains (Fig. 4, panels G–I). Although the number of AGTPBP1-immunoreactive neurons was reduced in AD brains, senile plaques did not exhibit an accumulation of AGTPBP1 or C9orf72 immunoreactivity (data not shown).

Next, we studied AGTPBP1 and C9orf72 mRNA and protein expression levels in frozen tissues of the FC, derived from four NC, six ALS, four PD, and seven AD cases. By qPCR, AGTPBP1 mRNA levels were not significantly different between AD and non-AD cases ($P = 0.900$) (Fig. 6A

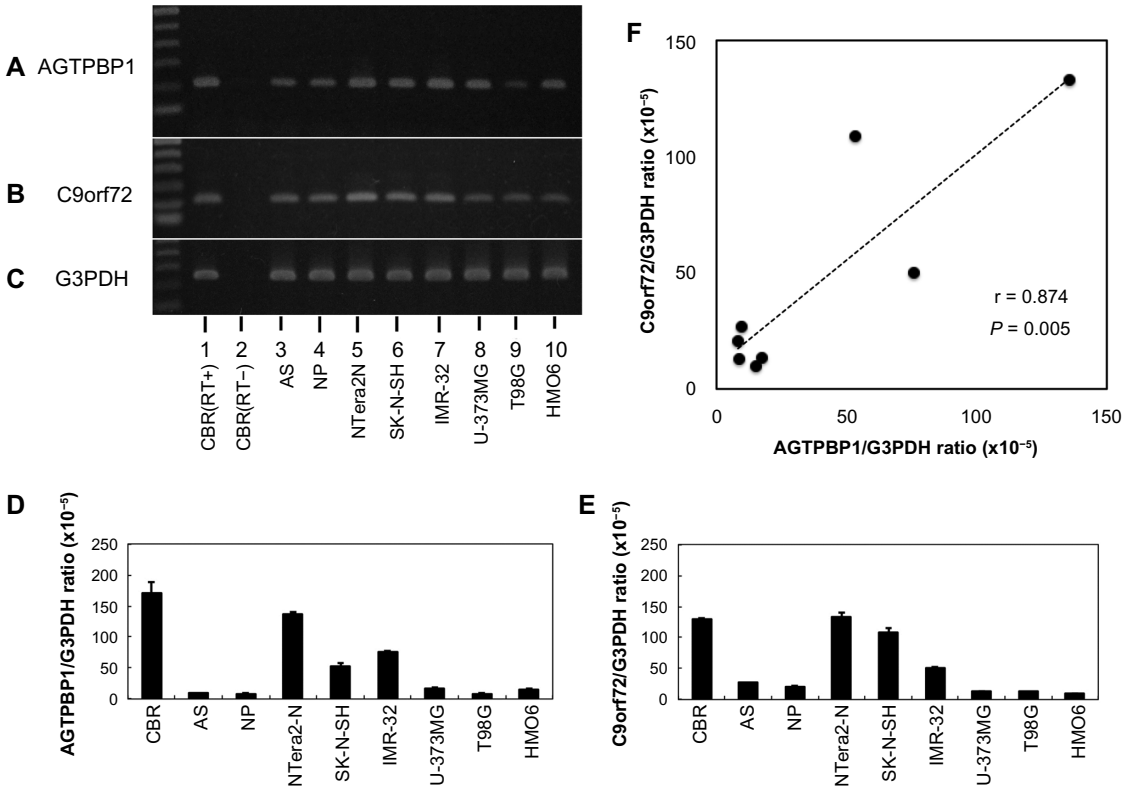

Figure 2. Coexpression of AGTPBP1 and C9orf72 mRNA in human neural cells. The mRNA expression was studied by RT-PCR and qPCR in human neural tissues and cultured cells. The panels (**A**–**C**) represent RT-PCR of (**A**) AGTPBP1, (**B**) C9orf72, and (**C**) G3PDH. The lanes (1–10) indicate (1) the FC of the human CBR with inclusion of the reverse transcription (RT) step, (2) CBR without inclusion of the RT step, (3) astrocytes, (4) NP cells, (5) NTera2 teratocarcinoma-derived neurons (NTera2 N), (6) SK-N-SH neuroblastoma, (7) IMR-32 neuroblastoma, (8) U-373MG glioblastoma, (9) T98G glioblastoma, and (10) HMO6 immortalized microglia. The panels (**D, E**) represent qPCR of (**D**) AGTPBP1 and (**E**) C9orf72, standardized against the levels of G3PDH, serving as an internal control. The panel (**F**) indicates Pearson's correlation between AGTPBP1 and C9orf72 mRNA expression levels in the set of eight human neural cells.

and C), although C9orf72 mRNA levels were modestly reduced in AD cases, when compared with the levels in non-AD cases ($P = 0.047$) (Fig. 6B and C). Importantly, we found a clear positive correlation between AGTPBP1 and C9orf72 mRNA expression levels in the set of 21 human brains examined ($r = 0.614$ and $P = 0.003$ in Pearson's correlation) (Fig. 6D). By WB, AGTPBP1 protein levels were not significantly different between AD and non-AD cases ($P = 0.403$) (Fig. 7A and B). The levels of C9orf72 protein were also not significantly different between AD and non-AD cases ($P = 0.137$) (Fig. 7A and B). No obvious correlation was found between AGTPBP1 and C9orf72 protein expression levels in the set of 21 human brains examined ($r = 0.290$ and $P = 0.203$ in Pearson's correlation) (Fig. 7C).

Knockdown of AGTPBP1 does not reduce C9orf72 expression levels in SK-N-SH cells. Next, to investigate a regulatory role of AGTPBP1 in the *C9orf72* gene expression, we performed experiments on siRNA-mediated knockdown of AGTPBP1 in SK-N-SH cells. The introduction of the siRNA product directed to AGTPBP1 but not of a negative control RNA reduced the levels of AGTPBP1 mRNA by 63.1% (Fig. 8, panel A) and protein by 81.8% (Fig. 8, panel C, lane 3). In contrast, the siRNA treatment did not reduce the levels of C9orf72 mRNA (Fig. 8, panel B) and protein (Fig. 8, panel D, lane 3). These observations did not support a direct involvement of AGTPBP1 in the regulation of *C9orf72* gene expression.

Overexpression of POU2F1 does not elevate AGTPBP1 expression levels in SK-N-SH cells. To identify a common transcriptional regulator for coexpression of AGTPBP1 and C9orf72, we determined the promoter region spanning –1,000 to +200 bp from the transcriptional start sites, by searching on the DBTSS database (dbtss.hgc.jp) combined with the transcription factor-binding site prediction program named Match (www.gene-regulation.com/cgi-bin/pub/programs/match). As a result, we identified six distinct binding sites for POU2F1, a transcription factor of the POU transcription factor family, in both AGTPBP1 and C9orf72 promoters. Importantly, POU2F1 is known to regulate neuronal differentiation.[28] To investigate a role of POU2F1 in coexpression of AGTPBP1 and C9orf72, we performed experiments on transient overexpression of POU2F1 in SK-N-SH cells (Fig. 9, panel C, lane 2). However, forced expression of exogenous POU2F1 did not alter the levels of AGTPBP1 mRNA expression (Fig. 9, panel A), although it elevated C9orf72 mRNA levels by 1.86-fold in SK-N-SH cells (Fig. 9, panel B). These results did not support the view that

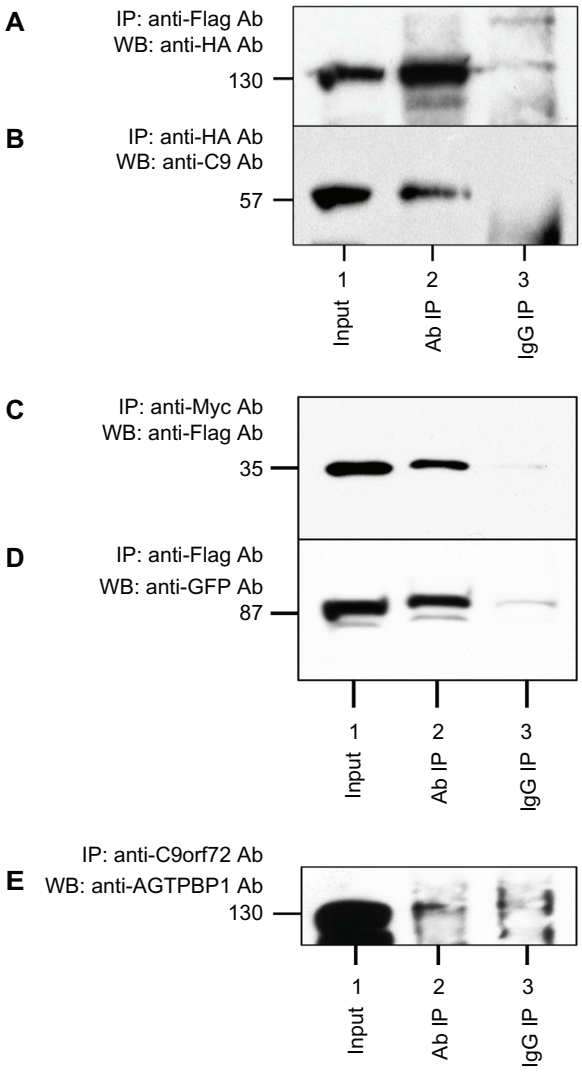

Figure 3. Molecular interaction of AGTPBP1 and C9orf72 in cultured cells. The full-length AGTPBP1 protein tagged with HA and the full-length C9orf72 protein tagged with Flag were coexpressed in HEK293 cells. The protein extract was processed for (**A**) IP with anti-Flag antibody followed by WB with anti-HA antibody or (**B**) IP with anti-HA antibody followed by WB with anti-C9orf72 antibody. The CP domain of AGTPBP1 tagged with Flag and the full-length C9orf72 protein tagged with Myc or EGFP were coexpressed in HEK293 cells. The protein extract was processed for (**C**) IP with anti-Myc antibody followed by WB with anti-Flag antibody or (**D**) IP with anti-Flag antibody followed by WB with anti-GFP antibody. The protein extract of SK-N-SH cells was processed for (**E**) IP with anti-C9orf72 antibody followed by WB with anti-AGTPBP1 antibody. The lanes represent (1) input control, (2) IP with the specific antibody, and (3) IP with normal IgG.

POU2F1 acts as a coregulatory transcription factor for AGTPBP1 and C9orf72.

Discussion

In the present study, we identified AGTPBP1 as the most significant gene coexpressed with C9orf72 by searching on COXPRESdb. The AGTPBP1 gene encodes cytosolic carboxypeptidase 1 (CCP1), alternatively named NNA1 characterized by rapid induction in motor neurons following sciatic nerve injury.[24] We verified coexpression and interaction of AGTPBP1 and C9orf72 in cultured cells and in neurons of the human brain by IP and IHC. Importantly, we found a clear positive correlation between AGTPBP1 and C9orf72 mRNA expression levels in the set of eight human neural cell lines and in the set of 21 human brains examined. In contrast, we could not find a statistical significant correlation between AGTPBP1 and C9orf72 protein expression levels in the set of 21 human brains. This discrepancy is in part attributable to rapid postmortem degradation of brain proteins. These observations suggest that AGTPBP1 serves as a C9orf72 interacting partner that plays a role in neuronal function in a coordinated manner within the CNS.

By database search on the Human Protein Atlas (www.proteinatlas.org), we found that both AGTPBP1 and C9orf72 are broadly expressed outside the CNS at moderate levels in the gallbladder, stomach, small intestine, colon, kidney, urinary bladder, testis, prostate, uterus, placenta, skeletal muscle, cardiac muscle, lung, tonsil, thyroid gland, and adrenal gland, suggesting that they play some physiological roles in non-neural cells. We recently studied an exon array transcriptome dataset of laser microdissection-purified cervical cord motor neurons derived from C9ALS patients and NC subjects.[29] We found that the expression levels of AGTPBP1 and C9orf72 exons are reduced concurrently in C9ALS by –2.5-fold and –7.1-fold, respectively, supporting the present observations.

AGTPBP1 belongs to the evolutionarily conserved M14 metallocarboxypeptidase family that catalyzes the hydrolysis of C-terminal amino acids from their substrates.[30] AGTPBP1 is widely expressed in differentiating neurons, including motor neurons, in the developing CNS and the adult CNS.[24] Loss-of-function mutations in the *AGTPBP1* gene cause Purkinje cell degeneration (*pcd*) and retinal photoreceptor degeneration, followed by accumulation of DNA damage.[31–33] Furthermore, a missense mutation that reduces the catalytic activity of AGTPBP1 causes lower motor neuron disease in sheep.[34] These observations, taken together, suggest that AGTPBP1 plays a pivotal role in differentiation and survival of cerebellar, retinal, and motor neurons. Importantly, the CP domain of AGTPBP1 is indispensable for the rescue of the *pcd* phenotype in mutant mice.[35]

Increasing evidence shows that AGTPBP1 functions as a deglutamylating enzyme that catalyzes the hydrolysis of polyglutamate chains expressed on tubulins.[36,37] The *pcd* mice lacking functional AGTPBP1 exhibit microtubule hyperglutamylation linked with neurodegeneration.[38] Furthermore, mutant AGTPBP1 promotes aberrant location of lysyl oxidase propeptide in the nuclei of Purkinje cells, followed by interfering with NF-κB RelA signaling and by reducing stability of microtubule-associated proteins.[39] AGTPBP1 has a nuclear export signal capable of binding directly to the export receptor exportin 1, indicating that subcellular location of AGTPBP1 is affected by the mechanism involved in

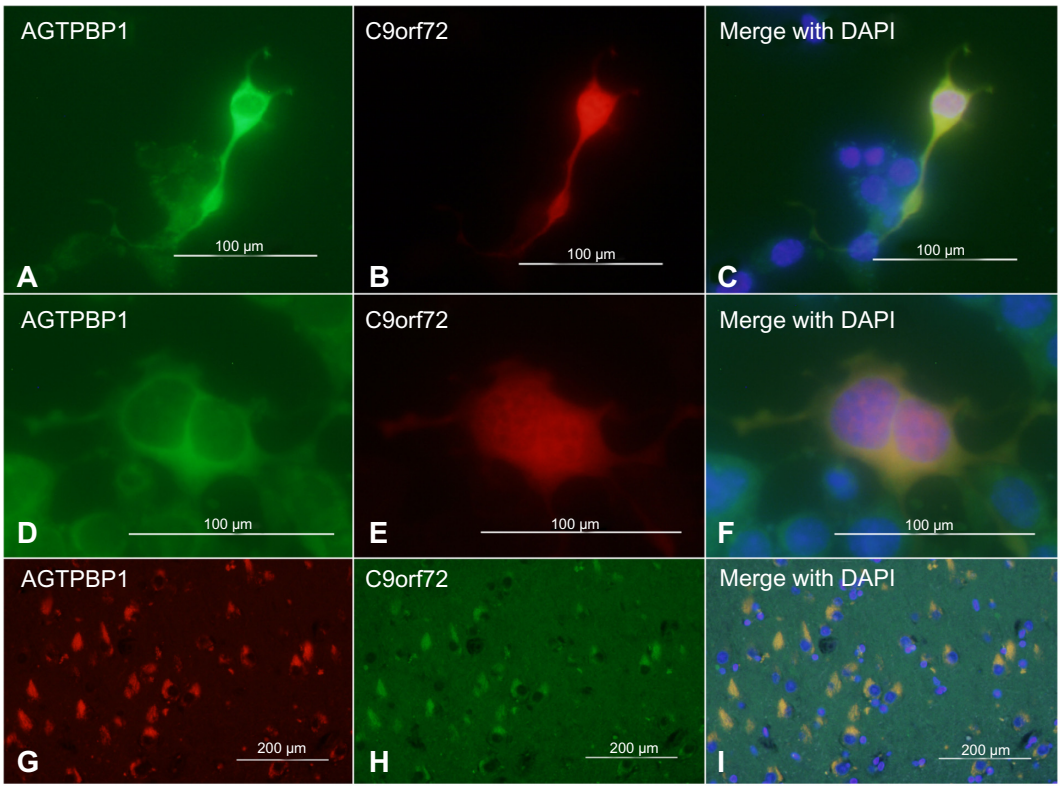

Figure 4. Coexpression of AGTPBP1 and C9orf72 in cultured cells and human brains. The vectors of EGFP-tagged AGTPBP1 and RFP-tagged C9orf72 were cotransfected in NSC-34 motor neurons. Coexpression of AGTPBP1 and C9orf72 was studied in the CA1 hippocampal region of human brains derived from normal control (NC) subjects by double-labeling IHC. The panels (**A**–**I**) represent (**A, D**) **(A, D)** NSC-34, AGTPBP1 (green), (**B, E**) NSC-34, C9orf72 (red), (**C, F**) merge of AGTPBP1 and C9orf72 labeled with DAPI, (**G**) CA1, AGTPBP1 (red), (**H**) CA1, C9orf72 (green), and (**I**) merge of AGTPBP1 and C9orf72 labeled with DAPI.

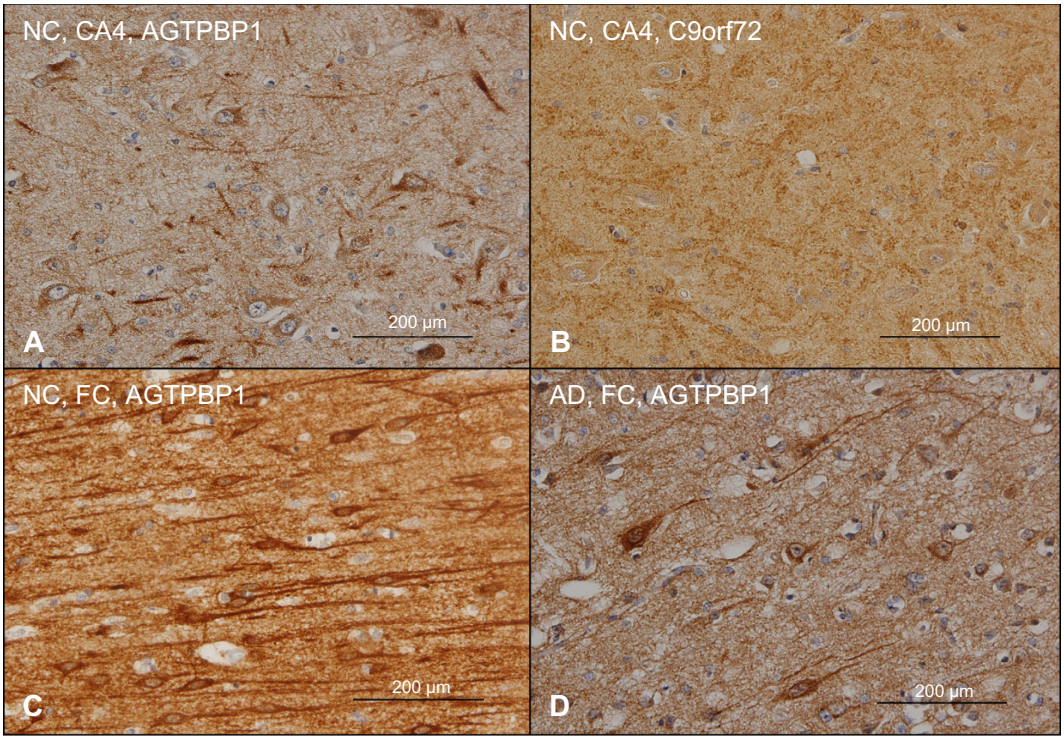

Figure 5. IHC of AGTPBP1 and C9orf72 expression in human brains. The immunoreactivity for AGTPBP1 or C9orf72 was studied in the hippocampus CA4 region and the frontal cortex (FC) of NC and AD brains by IHC. The panels **(A–D)** indicate **(A)** NC, CA4, AGTPBP1, **(B)** NC, CA4, C9orf72, **(C)** NC, FC, AGTPBP1, and **(D)** AD, FC, AGTPBP1. Scale bar = 200 μm.

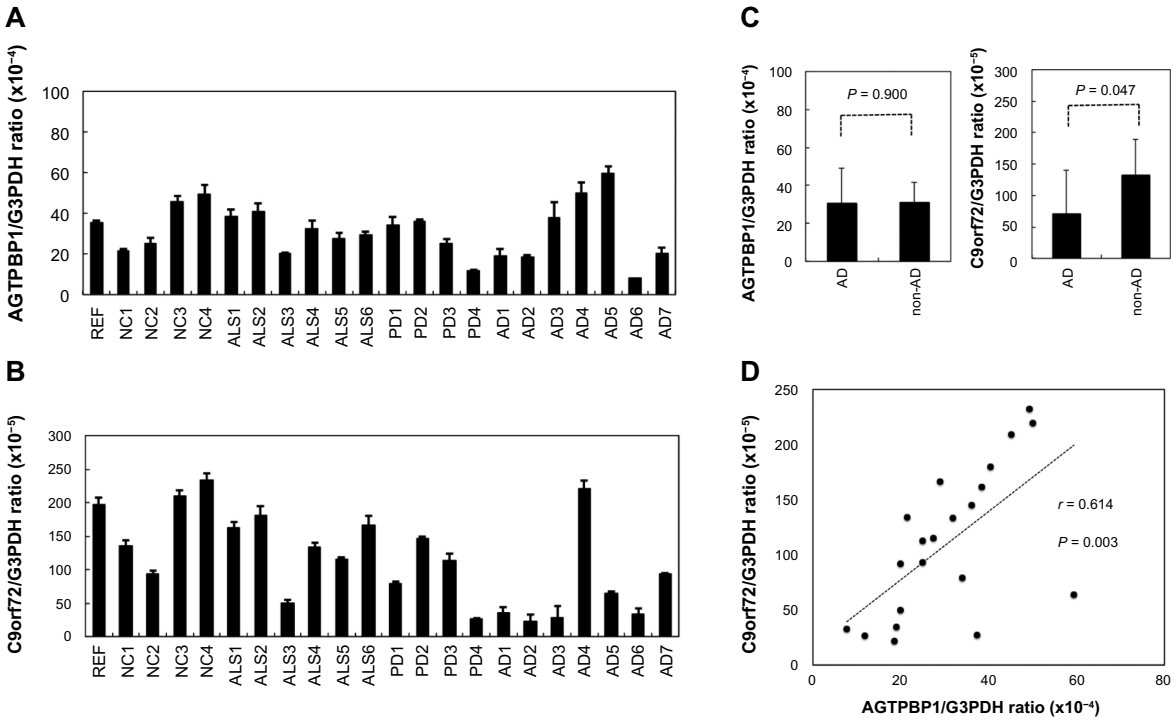

Figure 6. AGTPBP1 and C9orf72 mRNA expression in human brains. The mRNA expression levels were studied by qPCR in human brain tissues derived from a reference of the human FC (REF), four NC, six ALS, four PD, and seven AD cases listed in Supplementary Table 1. The expression levels were standardized against those of G3PDH, serving as an internal control: (**A**) AGTPBP1, (**B**) C9orf72, and (**C**) comparison between AD and non-AD groups. The panels indicate (left) AGTPBP1 and (right) C9orf72. (**D**) Pearson's correlation between AGTPBP1 and C9orf72 mRNA expression levels.

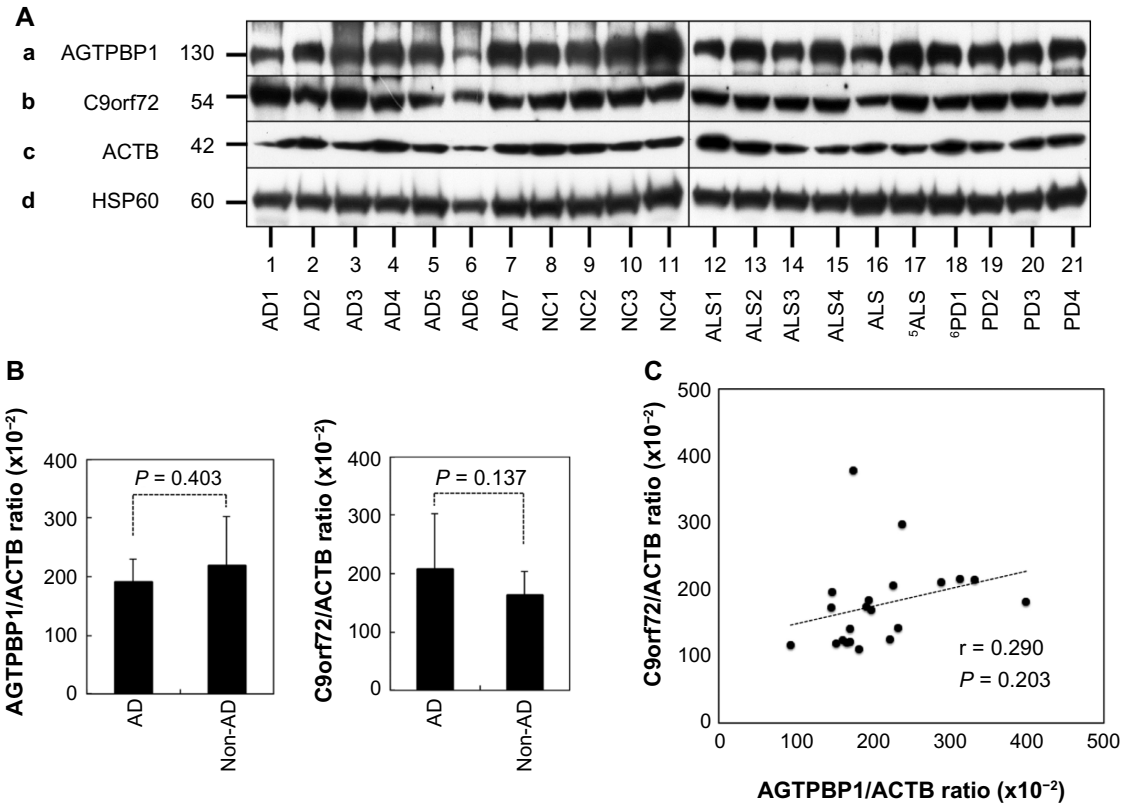

Figure 7. AGTPBP1 and C9orf72 protein expression in human brains. The protein expression levels were studied by WB in human brain tissues derived from four NC, six ALS, four PD, and seven AD cases listed in Supplementary Table 1. The expression levels were standardized against those of ACTB, serving as an internal control. (**A**) WB of (a) AGTPBP1, (b) C9orf72, (c) ACTB, and (d) HSP60. (**B**) Comparison between AD and non-AD groups. The panels indicate (left) AGTPBP1 and (right) C9orf72. (**C**) Pearson's correlation between AGTPBP1 and C9orf72 protein expression levels.

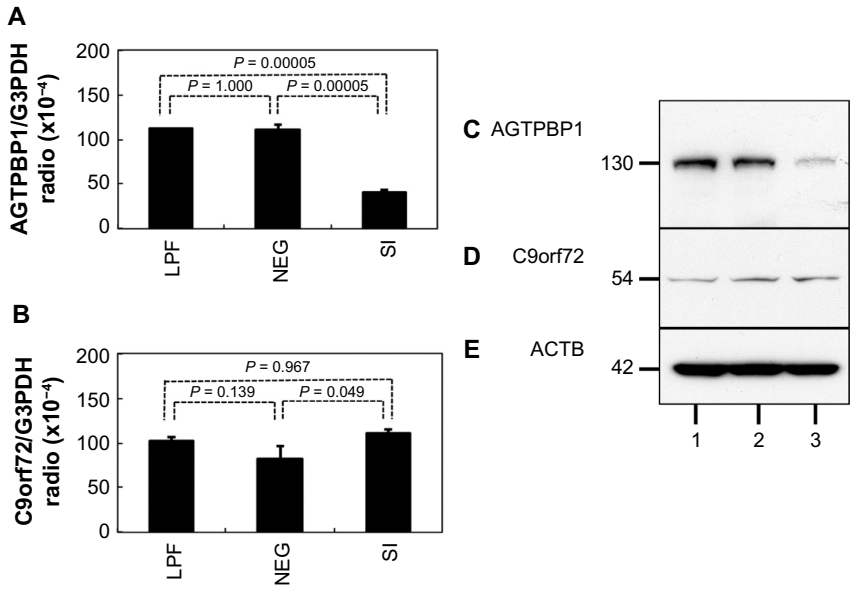

Figure 8. Knockdown of AGTPBP1 in SK-N-SH cells. The siRNA product directed to AGTPBP1 (SI) or a negative control RNA (NEG) was introduced in SK-N-SH cells by using Lipofectamine (LPF) RNAiMax reagent, followed by processing for qPCR and WB analysis. The panels (**A**, **B**) represent qPCR of (**A**) AGTPBP1 and (**B**) C9orf72 standardized against the levels of G3PDH mRNA, while the panels (**C**–**E**) indicate WB of (**C**) AGTPBP1, (**D**) C9orf72, and (**E**) ACTB, serving as an internal control. The lanes (1–3) represent the protein extract isolated from the cells treated with (1) LPF alone, (2) NEG, or (3) SI.

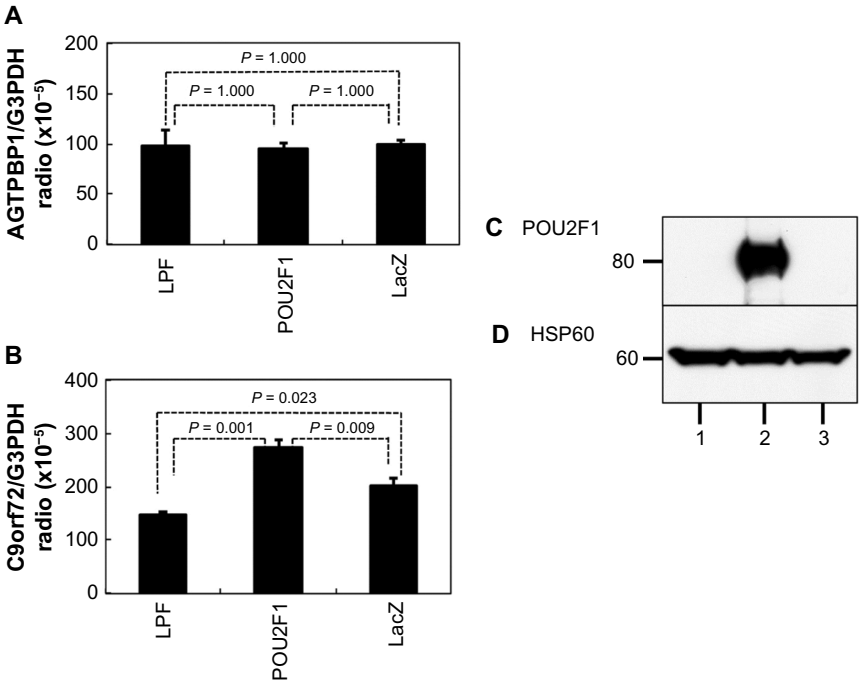

Figure 9. Overexpression of POU2F1 in SK-N-SH cells. The POU2F1 expression vector or the control vector expressing LacZ was transfected in SK-N-SH cells by using Lipofectamine (LPF) 2000 reagent, followed by processing for qPCR and WB analysis. The panels (**A, B**) represent qPCR of (**A**) AGTPBP1 and (**B**) C9orf72 standardized against the levels of G3PDH mRNA, while the panels (**C, D**) indicate WB of (**C**) POU2F1 and (**D**) HSP60, serving as an internal control. The lanes (1–3) represent the protein extract isolated from the cells treated with (1) LPF alone, or the cells transfected with the expression vector of (2) POU2F1 or (3) LacZ.

the nucleocytoplasmic transport.[40] Because AGTPBP1 acts as an aminopeptidase that regulates protein turnover by cleaving proteasome-generated peptides into amino acids, numerous intracellular peptides are accumulated in the brains of the *pcd* mice defective in functional AGTPBP1.[41] Interestingly, a recent study showed that transgenic mice with motor neuron-specific knockout of proteasome but not of autophagy showed progressive motor neuron degeneration, suggesting

that reduced protein turnover due to compromised proteasome function primarily contribute to ALS pathogenesis.[42] Importantly, we found that the mRNA expression levels of AGTPBP1 and C9orf72, along with SQSTM1 (p62), are concurrently elevated in SK-N-SH cells following treatment with MG-132, a cell-permeable proteasome inhibitor (unpublished data). These observations suggest a coordinated role of AGTPBP1 and C9orf72 in protection against an overload in the ubiquitin/proteasome system during the process of neurodegeneration.

Conclusions

By searching on COXPRESdb, we identified AGTPBP1 as the most significant gene coexpressed with C9orf72. We verified coexpression and interaction of AGTPBP1 and C9orf72 in cultured cells and in neurons of the human brain. We found a clear positive correlation between AGTPBP1 and C9orf72 mRNA expression levels in the set of 21 human brains examined. These results suggest that AGTPBP1 serves as a C9orf72 interacting partner that plays a role in the regulation of neuronal function in a coordinated manner within the CNS.

Acknowledgments

All autopsied brain samples were obtained from Research Resource Network (RRN), Japan.

Author Contributions

Designed the study, performed immunohistochemical analysis, and drafted the manuscript: JS. Performed biochemical analysis: SK, YK, YY, MT, JM. Validated the pathological diagnosis of autopsied brains: TI, YS, KA. All the authors have read and approved the final manuscript.

Supplementary Materials

Supplementary Figure 1. Characterization of anti-AGTPBP1 antibodies utilized in the present study. The full-length ORF of human AGTPBP1 tagged with HA was transiently expressed in HEK293 cells. The protein extract was processed for WB. The panels (A–C) indicate WB of (A) AGTPBP1, (left) 14067–1-AP antibody or (right) 9A3 antibody, (B) HA, and (C) HSP60, serving as an internal control. The lanes (1–3) represent the protein extract of (1) non-transfected cells, (2) the cells expressing HA-tagged AGTPBP1, and (3) the human FC. We found that HEK293 cells express an endogenous AGTPBP1 protein (panel A, lane 1).

Supplementary Figure 2. Multiple alignment of AGTPBP1 amino acid sequences. Multiple alignment analysis of amino acid sequences was performed by using CLC Sequence Viewer 7 (CLC bio-Qiagen). We imported amino acid sequences of the human AGTPBP1 protein (NP_056054.2) and its orthologs derived from *Pan troglodytes* (XP_001136417.1), *M. musculus* (NP_075817.2), *R norvegicus* (NP_001099570.1), *Gallus gallus* (XP_001233247.3), *Xenopus tropicalis (*NP_001107308.1), *and Danio rerio* (NP_001019616.1). The thick red line covers the CP domain of AGTPBP1 that is processed for cloning in the present study.

Supplementary Table 1. Demographic profile of human brain samples examined in the present study.

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