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The *Rps23rg* gene family originated through retroposition of the ribosomal protein s23 mRNA and encodes proteins that decrease Alzheimer's β-amyloid level and tau phosphorylation

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Retroposition is an important mechanism for gene origination. However, studies to elucidate the functions of new genes originated through retroposition, especially the functions related to diseases, are limited. We recently identified a mouse gene, *Rps23* retroposed gene 1 (*Rps23rg1*), that regulates β-amyloid (Aβ) level and tau phosphorylation, two major pathological hallmarks of Alzheimer's disease (AD), and found that *Rps23rg1* originated through retroposition of the mouse ribosomal protein S23 (*Rps23*) mRNA. Here we show that retroposition of *Rps23* mRNA occurred multiple times in different species but only generated another functionally expressed *Rps23rg1*-homologous gene, *Rps23rg2*, in mice, whereas humans may not possess functional *Rps23rg* homologs. Both *Rps23rg1* and *Rps23rg2* are reversely transcribed relative to the parental *Rps23* gene, expressed in various tissues and encode proteins that interact with adenylate cyclases. Similar to the RPS23RG1 protein, RPS23RG2 can upregulate protein kinase A activity to reduce the activity of glycogen synthase kinase-3, Aβ level and tau phosphorylation. However, the effects of RPS23RG2 are weaker than those of RPS23RG1 and such a difference could be attributed to the extra carboxyl-terminal region of RPS23RG2, which may have an inhibitory effect. In addition, we show that the transmembrane domain of RPS23RG1 is important for its function. Together, our results present a new gene family, whose products and associated signaling pathways might prevent mice from developing AD-like pathologies.

INTRODUCTION

Alzheimer's disease (AD), the most common neurodegenerative disease associated with aging in humans, is pathologically characterized by the abnormal accumulation of extracellular neuritic plaques and intracellular neurofibrillary tangles (NFTs) in vulnerable brain regions. Neuritic plaques consist of deposits of variously sized small β-amyloid (Aβ) peptides (1,2), which are derived from the precursor protein (APP) through sequential proteolytic processing by β-secretase and γ-secretase (3). Multiple lines of evidence suggest that overproduction/aggregation of Aβ in the brain is the primary cause of AD pathogenesis (4). NFTs are composed largely of hyperphosphorylated twisted filaments of a microtubule-associated protein, tau (5,6). Although tau hyperphosphorylation and aggregation is believed to be downstream of Aβ pathology in

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AD, tau mutations causing hyperphosphorylation have been associated with frontotemporal dementia with Parkinsonism-17 and tau pathology has been found in a number of neurodegenerative diseases collectively termed tauopathy (6,7).

Genetically engineered mice have become a useful tool for AD research. However, so far, none of these mouse models can faithfully recapitulate all of the pathological features of AD. Additionally, wild-type mice do not develop age-associated AD-like pathologies (8–10). Several hypotheses have been proposed to explain the resistance of mice to AD-like pathologies, including the sequence disparity between human and mouse Aβ (and possibly tau) that underlie different aggregation capabilities (8–10), the short lifespan of mice relative to humans (10,11) and the differences in processing of human and mouse APP by BACE1 (12). However, it is possible that other genetic novelties within the mice genome may also contribute to such a resistance. Therefore, identification of new genes/proteins involved in the modulation of Aβ generation and/or tau hyperphosphorylation and the comparative study of these genes/proteins between different species may not only be important for developing AD therapeutics but also provide clues for understanding the discrepancy between humans and mice, which will be useful in developing better AD animal models.

The origination of genes with new functions is an important mechanism for generating genetic novelties within a species during its evolution. New genes can originate through different mechanisms such as exon shuffling, gene duplication, retroposition, mobile element integration, lateral gene transfer and gene fusion/fission (13). Retroposition is a process where a parental mRNA is reverse-transcribed and inserted into the organism's genome, creating duplicate genes in new genomic positions. Although a retroposed gene copy usually does not contain the promoter region from the parental gene and will die out as a processed pseudogene in most cases, it can occasionally recruit new regulatory sequences near the incorporation site and become a functionally expressed gene (13). Nevertheless, studies to elucidate the functions of these newly originated genes, especially the functions related to diseases, are limited (14,15).

We recently identified a mouse gene *Rps23rg1* (*Rps23* retroposed gene 1. Gene ID 546049, *C330021F23Rik*. Referred to as *Rps23r1* in previous publication but changed here, by suggestion of the Mouse Genomic Nomenclature Committee) that originated through retroposition of the mouse ribosomal protein S23 (*Rps23*) mRNA (16). In the present study, we show that retroposition of *Rps23* mRNA occurred multiple times in different species but only generated two functionally expressed genes, *Rps23rg1* and *Rps23rg2*, both reversely transcribed relative to *Rps23*, in mice. In addition, we show that the RPS23RG protein family members function similarly to reduce AD-like pathologies (Aβ levels and tau phosphorylation) by interacting with adenylate cyclases to upregulate protein kinase A (PKA) activity and downregulate glycogen synthase kinase-3 (GSK-3) activity.

RESULTS

To identify RPS23RG1 homolog(s), we used the 141 amino acid-long mouse RPS23RG1 protein sequence to blast GenBank protein database and found another three mouse proteins with very high homology to RPS23RG1: EG381438 (Gene ID 381438, identities = 116/117, 99%), LOC100040998 (Gene ID 100040998, identities = 137/139, 98%) and LOC100039346 (Gene ID 100039346, identities = 126/134, 94%), of which the latter two are predicted by automated computational analysis. Hence, we designated their encoding genes as *Rps23rg2* (protein RPS23RG2 for EG381438), *Rps23rg3* (RPS23RG3 for LOC100040998) and *Rps23rg4* (RPS23RG4 for LOC100039346), respectively (Fig. 1A). RPS23RG1 has a predicted transmembrane domain (Fig. 1A) and previously we found that RPS23RG1 is a type Ib transmembrane protein that has a normal type I transmembrane protein orientation but no cleavable signal peptides. Here we found that RPS23RG2–RPS23RG4 also have the predicted transmembrane domain (Fig. 1A), suggesting that they are also type Ib transmembrane proteins. Blast with the mouse RPS23RG1 protein sequence also identified a predicted hypothetical protein LOC738579 (Gene ID 738579, identities = 50/74, 67%) with a relatively high homology, but lacking the predicted transmembrane domain from chimpanzees (*Pan troglodytes*) (Fig. 1A), which we designated *Rps23rg5* (and the protein as RPS23RG5). Moreover, the blast search showed that RPS23RG1 shares some homology with the CRA_d isoform of human ATG10 [ATG10 autophagy related 10 homolog (*S. cerevisiae*). Gene ID 83734, identities = 33/40, 82%]. However, further analyses revealed that the *ATG10* CRA_d isoform originated through a different mechanism from that of *Rps23rg1–Rps23rg5* (see below).

To study whether *Rps23rg* family genes are expressed in mice, we carried out reverse transcription–PCR (RT–PCR) and found that *Rps23rg1* was expressed in various mouse tissues (Fig. 1B). In addition, we found that *Rps23rg2* was also expressed (Fig. 1B). However, we did not detect the expression of *Rps23rg3* or *Rps23rg4* in any tissue by RT–PCR with several pairs of primers (data not shown), implying that the two genes predicted by automatic computational analysis are either not real functional genes (i.e. they are processed pseudogenes) or they are expressed at very low levels that cannot be detected by RT–PCR. For *Rps23rg5*, we did not study whether it is expressed in chimpanzees due to limited resources. However, since *Rps23rg5* was also predicted by automated computational analysis and we failed to detect its expression in humans by RT–PCR (data not shown), even though *Rps23rg5* originated before the divergence between humans and chimpanzees (see below), it is possible that *Rps23rg5* may also be a pseudogene without functional expression.

In our previous study, we found that the *Rps23rg1* gene originated through retroposition of the mouse *Rps23* mRNA (16). Therefore, we also compared gene sequences of other *Rps23rg* members to those of *Rps23*. Consistent with the results from the *Rps23rg1* comparison, sequence alignments and analyses showed that *Rps23rg2–Rps23rg4* and *Rps23rg5* gene sequences were highly homologous to the reverse and complementary sequences of mouse and chimpanzee *Rps23* mRNA, respectively, and were all intronless within the homologous region (Fig. 2A and B). These results clearly demonstrate that all of the *Rps23rg* gene family members originated

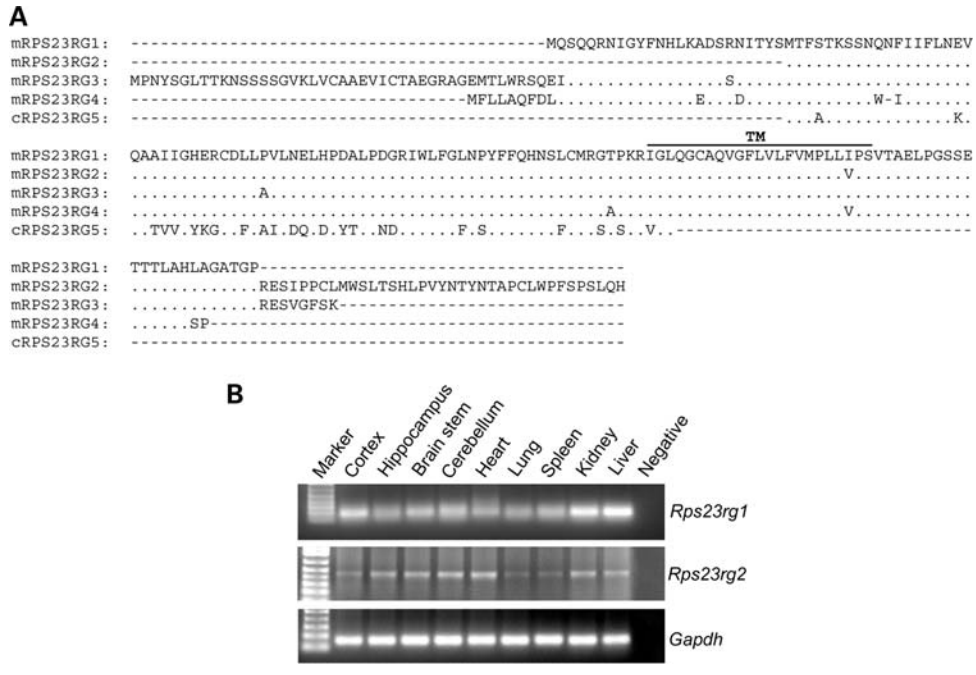

Figure 1. Identification of the RPS23RG family. (**A**) Sequence alignment of the five RPS23RG family proteins. RPS23RG1–RPS23RG4 were found in mice (m). RPS23RG5 was found in chimpanzees (c). '.', identical amino acids; '-', deletions; TM, predicted transmembrane domain. (**B**) *Rps23rg1* and *Rps23rg2* are expressed in multiple mouse tissues. RNAs from various tissues of a two-month-old C57BL6 mouse were extracted, reverse-transcribed and used as templates for RT–PCR with primers specific for *Rps23rg1* and *Rps23rg2*. A pair of primers targeting a conserved region of human and mouse *Gapdh* was used as control.

through retroposition of *Rps23* mRNA, but are reversely transcribed, relative to their parental genes.

The *Rps23* gene is highly conserved among species and belongs to the ribosomal protein family that is crucial for ribosome function (17). It has been reported that human ribosomal protein genes have generated a large number of processed pseudogenes through retroposition (18). Therefore, to further understand the origination of *Rps23rg* family genes, we screened for all *Rps23*-like sequences in human, mouse and rat genomes and constructed their phylogenic relationships. The results showed that retroposition of *Rps23* occurred in all three species and occurred more frequently in rodents than in humans (Fig. 3). The results also demonstrated that *Rps23rg1–Rps23rg4* originated in mice after the divergence between mice and rats, whereas *Rps23rg5* originated after the divergence between rodents and primates but before the divergence between humans and chimpanzees (Fig. 3). Furthermore, to identify potential functional *Rps23rg* homologs in humans, we selected fragments covering 100 Kb of the 5′ and the 3′ region adjacent to each of the identified *Rps23*-like sequences in the human genome for gene prediction and found no *Rps23rg*-like genes (data not shown). We also carried out RT–PCR with primers binding regions right next to these identified human *Rps23* retroposition sites and failed to obtain any positive amplification (data not shown).

We focused our functional studies on *Rps23rg1* and *Rps23rg2* since only these two genes were found to be expressed. RPS23RG2 is 24 amino acids shorter at the amino-terminus and 37 amino acids longer at the carboxyl-terminus than RPS23RG1. Otherwise, RPS23RG1 and RPS23RG2 have only one amino acid difference within the predicted transmembrane domain (Fig. 1A). Previously we showed that RPS23RG1 is a type Ib transmembrane protein and can be delivered to the cell surface (16). Here we found that RPS23RG2 and its truncated form lacking the 37 amino acids at the carboxyl-terminus (RPS23RG2CΔ37) can also be delivered to the cell surface (Fig. 4B), implying that these RPS23RG family members have same subcellular localizations. Since overexpressed RPS23RG1 has been found to interact with adenylate cyclases and upregulate the cAMP level, activating PKA activity and thereby inhibiting GSK-3 activity, tau phosphorylation and Aβ generation (16), we investigated whether RPS23RG2 has a similar function. We found that overexpressed RPS23RG2 also interacted with overexpressed adenylate cyclase 8 (Fig. 4A). In addition, overexpression of RPS23RG2 in human HeLa cells stably expressing human APP Swedish mutation upregulated PKA activity (with more CREB phosphorylation), decreased GSK-3α/β activities (with more GSK-3α/β phosphorylation), Aβ level and tau phosphorylation, and increased sAPPα release and APP carboxyl-terminal fragments (CTFs) levels (Fig. 4B). However, the effects of RPS23RG2 were much weaker than those of RPS23RG1 (Fig. 4B). In contrast, the truncated RPS23RG2CΔ37 form not only interacted with adenylate cyclase 8 (Fig. 4A), but also had effects on modulating PKA and GSK-3 phosphorylation/activity, APP processing/Aβ level and tau phosphorylation that were much stronger than those of RPS23RG2 and comparable to those of RPS23RG1 (Fig. 4B). These results imply that the extra carboxyl-terminus of RPS23RG2 may inhibit its effects on downstream signaling. On the other hand, the amino-terminal region of RPS23RG1 may not be functionally important since RPS23RG2CΔ37 lacks this region and has comparable effects to those of RPS23RG1.

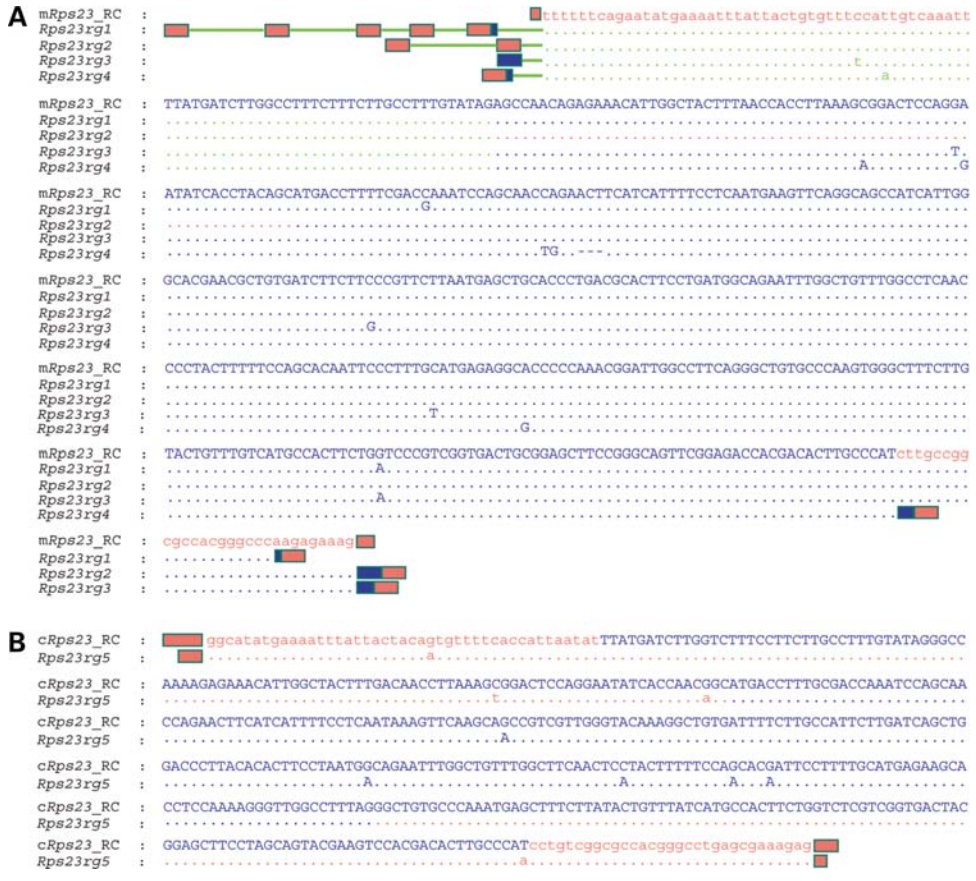

Figure 2. Origination of the *Rps23rg* gene family through retroposition of *Rps23* mRNA. The reverse and complementary (RC) sequences of mouse *Rps23* (m*Rps23*) and chimpanzee *Rps23* (c*Rps23*) cDNAs were aligned with mouse *Rps23rg1–Rps23rg4* (**A**) and chimpanzee *Rps23rg5* (**B**), respectively. Small letters indicate the intron sequence (in green) or untranslated region sequence of the exon (in red). Capital letters indicate protein-encoding sequence (in blue). Additional gene parts of *Rps23rg* family members recruited from incorporated chromosomal sites are indicated by lines (for introns) and boxes (for exons) (not drawn to proportion). The colors red, green and blue indicate untranslated exons, introns and protein-encoding regions, respectively. '.', identical nucleotides; '-', deletions.

Since RPS23RG1 is a type Ib transmembrane protein and interacts with transmembranous adenylate cyclases (16), we speculated that the transmembrane domain of the RPS23RG1 protein is important for its interaction with adenylate cyclases and for its activity. Therefore, we substituted the transmembrane domain of RPS23RG1 with that of APP or nicastrin, both of which are type I transmembrane proteins. As expected, co-immunoprecipitation studies showed that there is no interaction between adenylate cyclase 8 and the RPS23RG1 with an APP transmembrane domain substitution (Fig. 5A) or a nicastrin transmembrane domain substitution (data not shown). In addition, substitution of the RPS23RG1 transmembrane domain with that of APP or nicastrin abolished the effects of RPS23RG1 on upregulating phopshorylation of CREB and GSK-3α/β and reducing the level of Aβ (Fig. 5B).

Blast of the GenBank database with the RPS23RG1 protein sequence revealed that the amino-terminus of RPS23RG1 shares homology with the carboxyl-terminus of the rare isoform CRA_d of the human ATG10 protein, whereas RPS23RG1 does not share any homology with the common ATG10 protein (Fig. 6A). However, detailed sequence analyses revealed that the human *ATG10* CRA_d isoform did not originate through retroposition of the human *Rps23* mRNA but rather acquired the homology by 'hijacking' an exonal domain of the human Rps23 gene: the human *ATG10* gene is localized on chromosome 5 next to, but reversely transcribed relative to, the human *RPS23* gene (Fig. 6A). Therefore, a part of exon 4 of human *RPS23* is recruited by the *ATG10* CRA_d isoform during its transcription and translation, encoding the ATG10 CRA_d isoform's carboxyl-terminus. Since *Rps23* is highly conserved among different species and mouse *Rps23rg1* is reversely transcribed relative to mouse *Rps23*, the amino-terminus of RPS23RG1 becomes homologous to the carboxyl-terminus of ATG10 CRA_d isoform (Fig. 6A). The differences in origination suggest that the *ATG10* CRA_d isoform should not be considered a member of the *Rps23rg* family. Although ATG10 is thought to be an E2-like enzyme and involved in two ubiquitin-like modifications essential for autophagosome formation (19), the exact function of ATG10 CRA_d isoform remains unknown. However, it is unlikely that ATG10 CRA_d isoform functions similarly to RPS23RG1 through the homologous domain, since the amino-terminus of RPS23RG1 is dispensable for its function (Fig. 4). Indeed, overexpression of ATG10 CRA_d isoform, as well as ATG10, had no effects on promoting phosphorylation of CREB and GSK-3α/β or on reducing the level of Aβ as RPS23RG1 did (Fig. 6B).

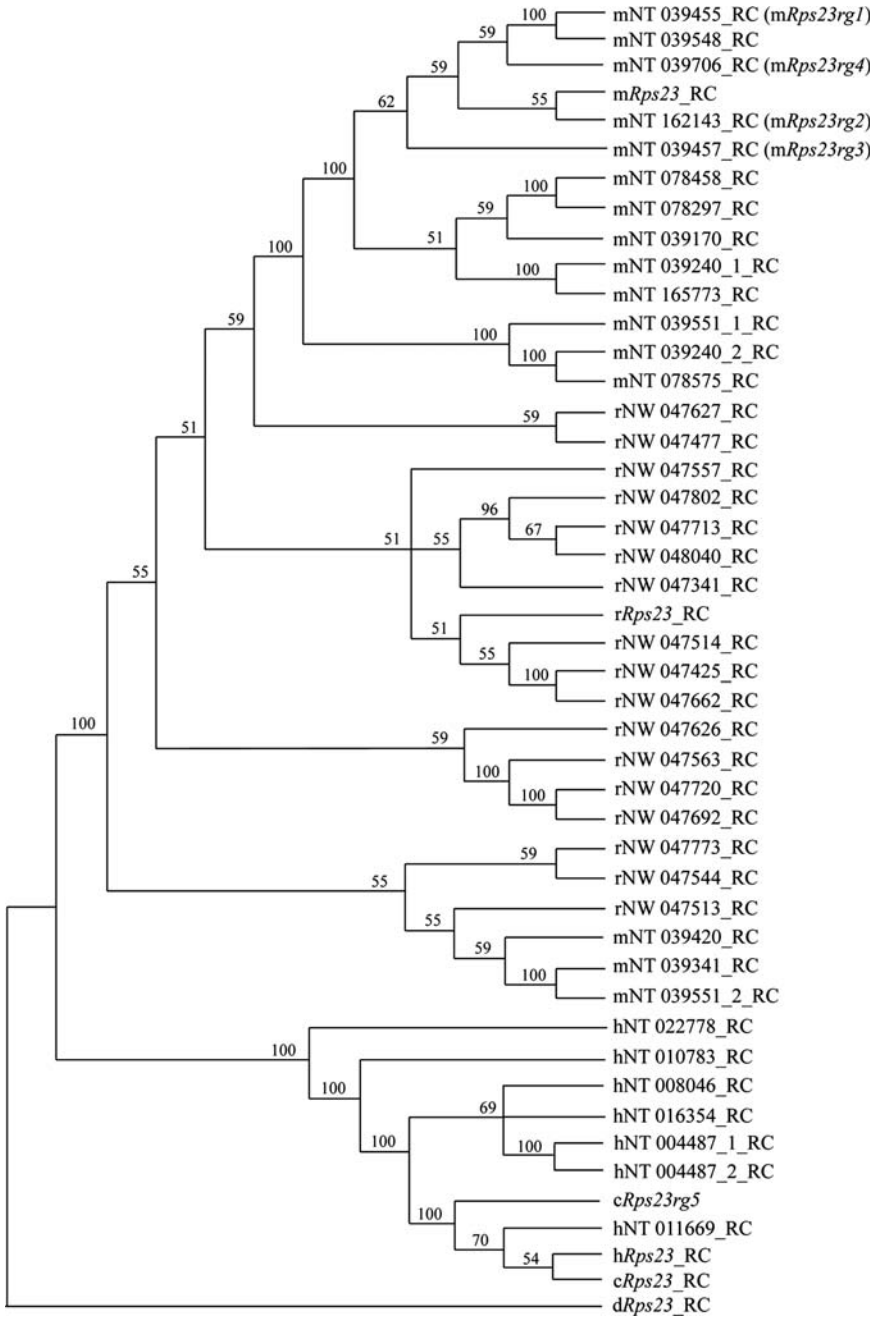

Figure 3. Phylogenic relationships of *Rps23rg1–Rps23rg5*, *Rps23* mRNAs and other *Rps23*-like sequences in humans, rats and mice. *Rps23*-like sequences in human (h), rat (r) and mouse (m) genomes were identified by a blast search of GenBank with respective *Rps23* mRNA sequences. Each identified sequence was indicated by its GenBank locus. Two *Rps23*-like sequences found in a same GenBank locus were separated by a suffix of 1 or 2. Reverse and complementary (RC) sequences of *Rps23* mRNA and *Rps23*-like sequences, including the chimpanzee (c) and dog (d) *Rps23* mRNAs, were aligned with mouse *Rps23rg1–Rps23rg4* and chimpanzee *Rps23rg5*. The consensus tree was generated using the maximum parsimony method and rooted with dog *Rps23*. Numbers on the branches indicate the consensus of a 1000 bootstrap analysis.

DISCUSSION

Here we have identified a new gene family that originated through retroposition of the *Rps23* mRNA. RPS23 belongs to the ribosomal protein family which is crucial for ribosome function (17). Mammals have 79 ribosomal proteins. Interestingly, it has been found that for most if not all human ribosomal proteins, the single, functional gene has generated a large number of processed pseudogenes (18), suggesting that transcripts of these ribosomal protein genes have a tendency to retropose. Indeed, we have found multiple *Rps23* retroposition events in humans and many more in mice and rats (Fig. 3). In addition, we found retroposition of *Rps23* in rhesus monkeys and dogs (data not shown). Although most of the retroposed gene copies probably have become processed pseudogenes, in the case of the *Rps23rg* gene family, at least for

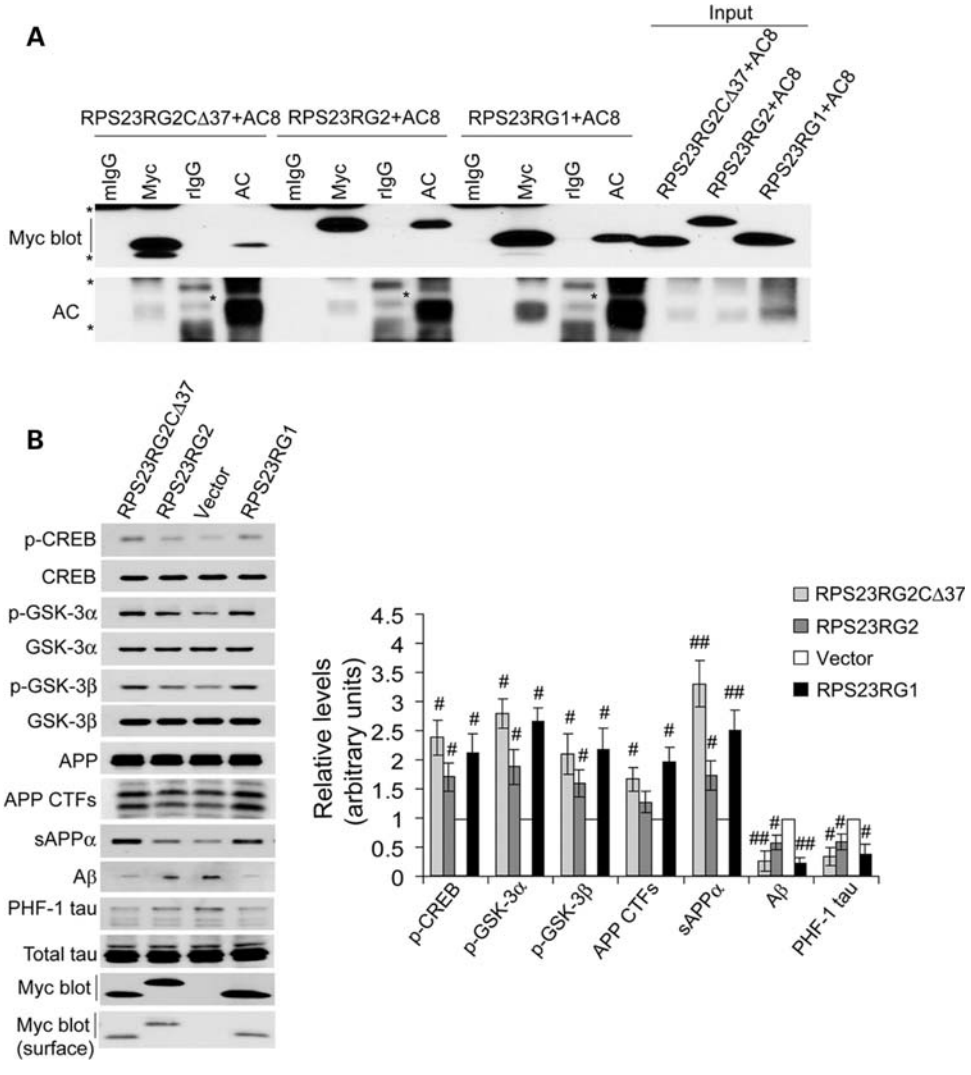

Figure 4. RPS23RG family proteins interact with adenylate cyclases, activate PKA and reduce GSK-3 activity, Aβ level and tau phosphorylation. (**A**) RPS23RG family proteins interact with adenylate cyclase 8 (AC8). N2a cells were transfected with RPS23RG1, RPS23RG2 or RPS23RG2CΔ37, all of which are Myc-tagged, together with AC8. Co-immunoprecipitation assays in these cells showed that an adenylate cyclase (AC) antibody pulled down RPS23RG1, RPS23RG2 and RPS23RG2CΔ37 as the Myc antibody 9E10 did (upper panel), and that the Myc antibody pulled down AC as the AC antibody did (lower panel). However, mouse IgG (mIgG) and rabbit IgG (rIgG) did not pull down RPS23RG family proteins or AC. *Non-specific bands. Note that rIgG pulled down a non-specific band that is localized slightly higher than the AC band. (**B**) RPS23RG family proteins modulate the activities of PKA and GSK-3, APP processing/Aβ level and tau phosphorylation. Overexpression of RPS23RG1, RPS23RG2 and RPS23RG2CΔ37 in human HeLa cells stably expressing human APP Swedish mutation increased the levels of phosphorylated CREB indicative of upregulated PKA activity, phosphorylated GSK-3α/β indicative of reduced GSK-3 activity, sAPPα and APP CTFs, and decreased Aβ level and tau phosphorylation. p: phosphorylated forms. In some experiments, transfected cells were subjected to biotinylation and affinity precipitation to detect cell surface levels of transfected proteins. In some experiments, protein levels were quantified by densitometry and normalized to those of controls for comparison (set as one arbitrary unit). Error bars indicate SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. *P*-values were calculated using two-tailed Student's *t*-test ($n = 3$).

Rps23rg1 and *Rps23rg2*, the retroposed gene copies have recruited host chromosome sequences adjacent to the incorporation sites as new regulatory elements and/or exon/intron units to form functional genes. However, the *Rps23rg* gene family members are reversely transcribed relative to the parental *Rps23* mRNA, thereby encoding proteins with no sequence homology to those of RPS23 proteins.

Since RPS23RG family members show beneficial functions for inhibiting AD-like pathologies (Aβ production and tau phosphorylation), one priority is to identify any human homologs for potential therapeutic targeting. However, our database search found that only the human ATG10 CRA_d isoform shares homology to a dispensable domain of RPS23RG1. In addition, ATG10 CRA_d isoform did not have the same effect as RPS23RG1 and *ATG10* CRA_d isoform did not originate in the same way as the *Rps23rg* family members (Fig. 6). Furthermore, although we found that the predicted *Rps23rg5* gene originated before the divergence between humans and chimpanzees, our RT–PCR amplification with primers specific to the predicted *Rps23rg5* cDNA failed to generate any positive result from various human tissue/cell samples (data not shown), suggesting that there is no functionally expressed *Rps23rg5* in

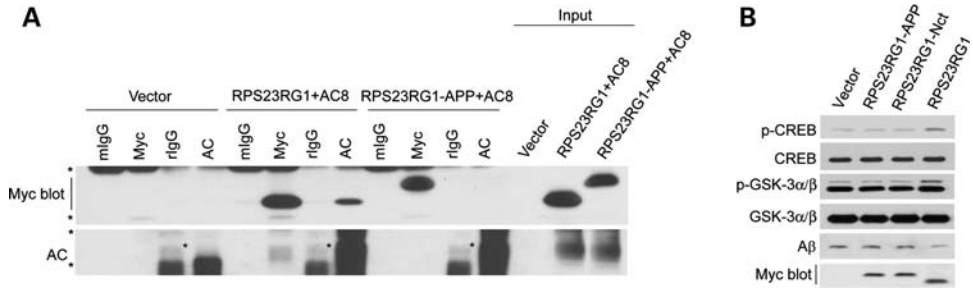

Figure 5. The transmembrane domain is crucial for RPS23RG1's function. (**A**) The transmembrane domain is required for its interaction with AC. N2a cells were transfected with Myc-tagged RPS23RG1 or Myc-tagged RPS23RG1 carrying the APP transmembrane domain, together with AC8. Co-immunoprecipitation assays in these cells showed that the AC antibody pulled down RPS23RG1 but not RPS23RG1 carrying the APP transmembrane domain (upper panel), and that the Myc antibody pulled down AC only in cells transfected with RPS23RG1 but not in cells transfected with RPS23RG1 carrying the APP transmembrane domain (lower panel). *Non-specific bands. (**B**) Ablation of RPS23RG1 transmembrane domain abolishes its activity. RPS23RG1, RPS23RG1 carrying the APP transmembrane domain (RPS23RG1-APP) or the nicastrin transmembrane domain (RPS23RG1-Nct) and a blank vector were transfected into N2a cells stably expressing human APP Swedish mutation or HeLa cells stably expressing human APP Swedish mutation. Overexpression of RPS23RG1-APP and RPS23RG1-Nct failed to increase the levels of phosphorylated CREB and phosphorylated GSK-3α/β or decrease the level of Aβ as RPS23RG1 did.

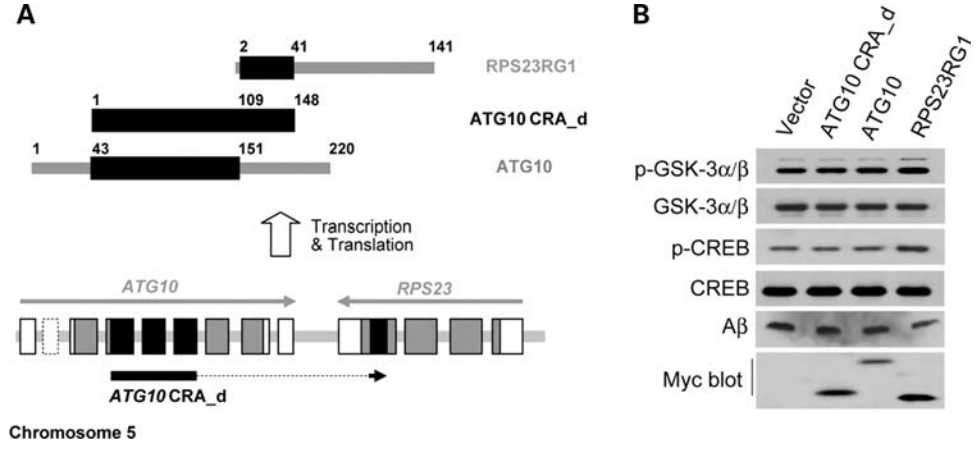

Figure 6. ATG10 CRA_d isoform originated differently from RPS23RG family members and does not have the same effect. (**A**) The origin of the *ATG10* CRA_d isoform is different from that of *Rps23rg* family members. The human *ATG10* gene (gray boxes, CDS, white boxes, UTRs; dashed white box, alternative ATG10 isoform UTR) is located next to, but in an opposite direction relative to that of *RPS23* (gray boxes, CDS; white boxes, UTRs) on chromosome 5 (not drawn in proportion). The regions of the *ATG10* and the *RPS23* genes responsible for encoding the ATG10 CRA_d isoform were indicated with black boxes. The carboxyl-terminal region (amino acids 109–148) of ATG10 CRA_d isoform is homologous to the amino-terminal region (amino acids 2–41) of RPS23RG1. The amino-terminal region (amino acids 1–109) of ATG10 CRA_d isoform is identical to that of the middle region (amino acids 43–151) of the major ATG10 isoform. (**B**) The ATG10 CRA_d isoform does not have the same effect as RPS23RG1. N2a cells stably expressing human APP Swedish mutation or HeLa cells stably expressing human APP Swedish mutation were transfected with ATG10 CRA_d isoform, ATG10, RPS23RG1 or blank vector. Overexpression of the ATG10 CRA_d isoform and ATG10 failed to increase the levels of phosphorylated CREB and phosphorylated GSK-3α/β or decrease the level of Aβ as RPS23RG1 did.

humans. On the other hand, we have demonstrated that the transmembrane domain of RPS23RG1 is important for its function (Fig. 5), whereas the predicted RPS23RG5 lacks this transmembrane domain (Fig. 1A). Therefore, even if RPS23RG5 is present in humans, it might not have the same function as that of RPS23RG1. Moreover, we predicted genes within the regions covering all the identified *Rps23*-like sequences in the human genome and found no *Rps23rg*-like genes. RT–PCR with primers binding regions right next to these human *Rps23* retroposition sites also failed to obtain positive amplification (data not shown). Hence, although we cannot completely exclude the possibility of existence of functional *Rps23rg* homologous gene in humans, we believe that the chance will be low.

During aging, humans are susceptible to the pathogenesis of AD which is typically characterized by Aβ overproduction/aggregation and tau hyperphosphorylation. In contrast, wild-type mice rarely develop AD-like pathologies (8–10). The differences in AD susceptibility between humans and mice have been attributed to the sequence disparity between human and mouse Aβ (and possibly tau) that underlie different aggregation capabilities (8–10), the short lifespan of mice relative to humans (10,11) and the differences in processing of human and mouse APP by BACE1 (12). The presence of the functional *Rps23rg* family members in mice may provide additional explanation for why mice are resistant to AD pathogenesis. On the other hand, although it is unlikely that humans possess *Rps23rg* family homologs, RPS23RG family members can reduce Aβ level and tau phosphorylation not only in mouse cells (data not shown), but also in human cells (Fig. 4B), suggesting that RPS23RG protein family-mediated signaling

pathways are active in humans and these pathways may be potential AD therapeutic targets.

MATERIALS AND METHODS

Sequence analyses

We blasted the GenBank database with the RPS23RG1 protein sequence to identify its homologs. *Rps23* cDNA sequences of humans, mice and rats were used to screen for *Rps23*-like sequences in the respective species' genome. Homologous sequences were aligned manually. For most identified sequences, we selected their homologous regions covering the complete RPS23 protein-encoding sequence to construct their phylogenic relationships, using the maximum parsimony method embedded in MEGA (20) and the dog *Rps23* sequence as a root. We generated a consensus tree with a 50% cut-off of a 1000 bootstrap analysis. For other sequences without a homologous region covering the complete RPS23 protein-encoding sequence, we used whatever homologous region they share with other sequences to construct consensus trees. All generated consensus trees were finally combined into one consensus tree. The potential transmembrane region in the RPS23RG1 protein was predicted using PredictProtein (21). For identified *Rps23*-like sequences in the human genome, we selected the fragment containing 100 Kb each of the 5′ and the 3′ region adjacent to these sequences for gene prediction, using Genscan (22,23).

Cell cultures

Maintenance of mouse neuroblastoma N2a cells, N2a cells stably expressing human APP Swedish mutation and human HeLa cells stably expressing human APP Swedish mutation has been previously described (16).

Reverse transcription–PCR

Total RNAs were extracted from tissues of a 2-month-old C57BL6 mouse using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After treatment with RNase-free DNase, equal amounts of RNAs were reverse-transcribed into first-strand cDNA using SuperScript First-Strand kit (Invitrogen). Synthesized cDNAs were used as templates for PCR with pairs of primers amplifying the *Rps23rg1* cDNA (5′-primer: TGGGTGAGGGCGTGCAGCTGT; 3′-primer: ACCTTGCC CACTTGTTTGCTTTTC), the *Rps23rg2* cDNA (5′-primer: GGCCCAAGAGAAAGCATTC; 3′-primer: TTGCACATT TAGCCTTAGAAC) and a conserved region of the human and the mouse *Gapdh* cDNAs (5′-primer: CCCTTCATT GACCTCAACTA; 3′-primer: CCTTCTCCATGGTGGT GAA).

Vectors, transfection, immunoblot and quantification

Vectors expressing RPS23RG1, RPS23RG2 or RPS23RG2CΔ37 (RPS23RG2 lacking 37 amino acids at its carboxyl-terminus) were constructed in the pCMV-Myc vector (Clontech, Mountain View, CA, USA) with a Myc tag at the amino-terminus and a His_6 tag (for RPS23RG1) or a HA tag (for RPS23RG2 and RPS23RG2CΔ37) at the carboxyl-terminus. Vectors expressing ATG10 and ATG10 CRA_d were constructed in pcDNA3.1 with a Myc-His tag at the carboxyl-terminus. A vector expressing RPS23RG1 was also constructed using the same strategy as a control. For transmembrane domain replacement studies, the transmembrane domain of RPS23RG1 (amino acids 96–118) in the pcDNA3.1 vector was substituted with that of APP (amino acids 625–648, based on APP695) or with that of nicastrin (amino acids 670–690). Cells were transiently transfected with these vectors using Lipofectmine reagent (Invitrogen). Aβ in conditioned media was either precipitated with trichloroacetic acid or immunoprecipitated with the antibody 6E10 (Covance, Emeryville, CA, USA) and then immunoblotted with 6E10. Soluble APPα (sAPPα) in conditioned media was also immunoblotted with 6E10. Cell lysates were subjected to SDS–PAGE and immunoblot with antibodies against phosphorylated CREB (Ser133) indicative of PKA activity, total CREB, phosphorylated GSK-3α/β (Ser21/9, inactive form) and total GSK-3α/β (Cell Signaling, Danvers, MA, USA), the 369 antibody recognizing APP/APP CTFs (24) and the Myc antibody 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In some experiments, cells were first transfected with a human tau vector (25) and cell lysates were additionally immunoblotted with antibodies against total tau (Abcam, Cambridge, MA, USA) and hyperphosphorylated PHF-1 tau (kindly provided by P. Davies at Albert Einstein College of Medicine). Protein levels were quantified by densitometry and normalized to those of controls for comparison (set as one arbitrary unit), using two-tailed Student's *t*-test. In some experiments, transfected cells were subjected to biotinylation and affinity precipitation with streptavidin beads (26) before SDS–PAGE to detect cell surface levels of RPS23RG proteins.

Co-immunoprecipitation

N2a cells were transfected with RPS23RG1, RPS23RG2, RPS23RG2CΔ37 or RPS23RG1 with an APP transmembrane domain or nicastrin transmembrane domain substitution, together with adenylate cyclase 8 (AC8) plasmid (kindly provided by D. R. Storm and G. Chan at University of Washington). Cells were then lysed in either CHAPSO buffer (1% CHAPSO, 25 mM HEPES, pH 7.4, 150 mM NaCl and 2 mM EDTA supplemented with protease inhibitors) or in NP40 buffer (1% NP40 in phosphate buffered saline, supplemented with protease inhibitors). Lysates were immunoprecipitated using mouse IgG, rabbit IgG and antibodies against Myc or adenylate cyclases (Santa Cruz Biotechnology) and Trueblot™ IP beads (eBioscience, San Diego, CA, USA), followed by immunoblot with the Myc antibody.

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