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Splice Variant of Mouse *Stam2* mRNA in Nervous and Muscle Tissue Contains Additional Exon with Stop Codon within Region Codings for VHS Domain

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Aim To analyze the alternatively spliced variant of *Stam2* mRNA and determine its distribution in mouse tissues.

Methods We identified a novel alternatively spliced exon by cloning and sequencing of *Stam2* cDNA obtained from tissue samples of 3-5 months old male C57Bl/6NCrl mice. The sequence of the alternatively spliced exon was analyzed by bioinformatic tools. The tissue distribution of different *Stam2* mRNA variants was determined by reverse transcription-polymerase chain reaction, and the consequences of the alternative splicing at the protein level were analyzed by western blot with the polyclonal anti-STAM2 antibody.

Results The novel alternatively spliced exon 1A of mouse *Stam2* gene was inserted within *Stam2* coding region and it contained a stop codon. The exon did not bear similarities to any other cDNA or protein sequence in the mouse, rat, or human databases. Both mRNA variants, with and without exon 1A, were present in the cortex, hippocampus, olfactory bulb, medulla oblongata, spinal cord, cerebellum, and the skeletal and heart muscle, while the other analyzed organs contained only the variant without the additional exon. The mRNA with the included exon did not give rise to a protein form detectable by western blotting with the polyclonal anti-STAM2 antibody.

Conclusion The alternatively spliced exon 1A was included in mRNA splice variant present in the nervous and muscle tissues. The alternative splicing event did not have major impact on STAM2 production and functionality. It seems that exon 1A is an evolutionary new exon created by exonization of an intronic sequence.

Membrane traffic is a dynamic process responsible for the maintenance of diverse cellular activities, including stress response, down-regulation of cell-surface molecules, and signal transduction. Molecules on the cell surface, together with those from outside the cell, could be internalized into the early endosome and subsequently either recycled back to the cell surface or degraded via multivesicular bodies (MVB) in the lysosome (1,2). The selection of the right cargo toward these two different pathways is not completely understood, but monoubiquitination and deubiquitination processes could play an important role (3).

Signal-transducing adaptor molecules 1 and 2 (STAM1 and STAM2) are proteins sharing 55% of protein sequence and considered to play a role in the endosomal sorting of the ubiquitinated cargo proteins (4,5). They form a ternary complex with hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and epidermal growth factor receptor pathway substrate clone No. 15 (EPS15) (6). The complex is located at the cytosolic side of the endosome and it sorts ubiquitinated membrane proteins into the clathrin-coated microdomains, thereby preventing their recycling to the cell surface (6,7). STAM proteins possess Vps-27/HRS/STAM (VHS) and ubiquitin-interacting motif (UIM) domains that are involved in ubiquitin binding (5). VHS domain is a conserved region, found exclusively at the N-terminus of proteins and therefore its topology is considered relevant for its function (8,9).

Alternative mRNA splicing is a regulated process of differential inclusion or exclusion of regions of the pre-mRNA and is a major source of protein diversity in higher eukaryotes (10). Alternative splicing is widespread in eukaryotic genomes and more than 60% of all human genes have alternatively spliced variants (11). It plays important roles not only in human physiology and disease, but also in evolution by increasing the rate of evolutionary alterations, such as exon creation (12).

Our aim was to analyze the newly identified alternatively spliced exon 1A included within VHS region of *Stam2* gene and determine its distribution, because it has been questioned if the alternatively spliced variant influences STAM2 protein domains, especially in regard to VHS domain.

Material and methods

Animals

We used the inbred mouse strain C57Bl/6NCrl and transgenic mouse line *Stam2*^{Gt1Gaj} in our investigation. All experiments except western blotting were performed on 3-5-month-old male C57Bl/6NCrl mice. Five mice were used to search for additional alternatively spliced exons of *Stam2* by reverse transcription-polymerase chain reaction (RT-PCR) and three mice were used to determine the tissue distribution of exon 1A and the presence of exon 1B. For the western blot, one male C57Bl/6NCrl mouse and one male *Stam2*^{Gt1Gaj} mouse were used. *Stam2*^{Gt1Gaj} mice were generated by gene trap method based on random insertion of nonhomologous DNA vector pKC199βgeo in the genome of embryonic stem cells at the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany (13). The inserted gene trap vector contained the splice acceptor sequence from mouse *Hoxc9* gene fused to promoterless *βgeo*, which is *lacZ-NeoR* fusion gene (14). The transgene insertion was in *Stam2* gene, within intron between exons 2 and 3. It interfered with the further *Stam2* mRNA transcription after the insertion site. The *Stam2*^{Gt1Gaj} mouse line was kept on C57Bl/6NCrl genetic background through 16 generations. Genotyping of transgenic animals was performed by PCR on tail DNA. Homozygous animals were obtained by intercrosses of heterozygotes.

Reverse transcriptase-polymerase chain reaction, cloning, and sequencing

Total RNA was isolated from organs of adult C57Bl/6NCrl mice. The animals were killed by cervical dislocation. Approximately 100 mg of 18 different tissues (cortex, hippocampus, olfactory bulb, medulla oblongata, spinal cord, cerebellum, heart, lungs, thymus, spleen, liver, pancreas, kidney, suprarenal gland, testis, muscle, bone and abdominal adipose tissue) of 3 C57Bl/6NCrl mice was isolated, frozen in liquid nitrogen and homogenized in 1 mL of cold TRI REAGENT solution (MRC Inc. Cincinnati, OH, USA) by tissue homogenizer (Ultra-turrax T25, Janke & Kunkel IKA Labortechnik, Staufen, Germany). The RNA was isolated according to the manufacturer's instructions and dissolved in diethylpyrocarbonate (DEPC)-treated water.

First-strand cDNA synthesis was performed from 1-2 μg total RNA with 200 U of reverse transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase RNase H^-, Promega, Madison, WI, USA), 15 U RNasin Plus RNase Inhibitor (Promega), 200 ng oligonucleotide primer specific for *Stam2* (5′ TAAACAGCACACCCACAAAG 3′), and 0.5 mmol/L deoxynucleotide triphosphates (Promega) in 25-μL reaction mix.

Polymerase chain reactions (50 μL volume) were carried out using 1 U of DNA polymerase (*Taq* DNA Polymerase; Promega), 0.2 mmol/L deoxynucleotide triphosphates, and 20 pmol of each primer in a PCR machine GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, USA). The amplified products were electrophoresed on a 1.8%-agarose gel and visualized by ethidium bromide, using 100-bp DNA ladder (Promega) to estimate the band sizes. Five brain cDNA samples from 5 C57Bl/6NCrl mice were used for 40 RT-PCR reactions with different primer combinations covering the complete coding sequence of *Stam2* (Figure 1A). The samples of the different tissues from 3 C57Bl/6NCrl mice were tested by RT-PCR to determine the presence of exons 1A and 1B. The primers were: A1 (5′ GCCAGAGCGTCGGGATTCAG 3′), A2 (5′ GAGTGCTTCCAACCCTGTCA 3′), A3 (5′ GAAAAGCGCTCTCCCACTCT 3′), A4 (5′ AAGCTCGTGTTTCCCTGAGA 3′) and A5 (5′ CCATGGAGGAGATTCATGCT 3′) (Figure 1A).

The selected products generated by PCRs were cloned into a plasmid (pGEM-T Vector System I; Promega), amplified and sequenced in the sequencing machine Dye Terminator Cycle Sequencing Ready Reaction with Amplitaq DNA Polymerase using ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Western blot analysis

Tissue homogenates were prepared from the brain, liver, and heart of a C57Bl/6NCrl mouse and a *Stam2*^{Gt1Gaj} mouse in the lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris · Cl, pH 8.0), 1% Igepal (Sigma, St. Louis, MO, USA), and 2x Protease Inhibitor Coctail Complete (Roche, Basel, Switzerland) by tissue homogenizer Ultra-turrax T25 (Janke & Kunkel IKA Labortechnik). The equivalent amounts of the protein from the tissue homogenates were run on 10%-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to nitrocellulose membranes (Protran BA 85, Schleicher & Schuell, Dassel, Germany). The membranes were blocked for 1 hour with blocking buffer (Tris buffer saline, 0.1% Tween 20, and 5% milk powder) and incubated for 2 hours with a polyclonal antibody against mouse STAM2 (1:500 dilution in blocking buffer) (15). Subsequently, blots were incubated with Antirabbit HRP (Promega) secondary antibody for 1 hour (1:2500 dilution). Bound antibodies were detected by TMB Stabilized Substrate (Promega) peroxidase substrate.

Bioinformatics

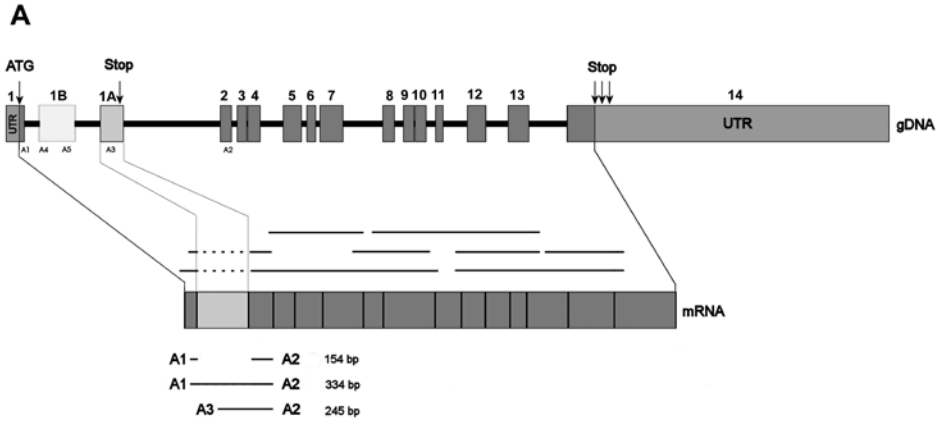
The alternatively spliced exon nucleotide sequence was compared with nucleotide sequence

databases Homo sapiens LATESTGP database, Mus musculus LATESTGP database and Rattus norvegicus LATESTGP database by Ensembl Genome Browser (*www.ensembl.org)* and to nonredundant nucleotide sequence database, GenBank+EMBL+DDBJ mouse, human, non-mouse and non-human EST databases and PDB nucleotide database by BLASTN program (*www.ncbi.nlm.nih.gov/BLAST/*). The results were confirmed by UCSC Genome Browser and BLAT Search Genome (*http://genome.ucsc.edu/*). Additional alternatively spliced exons in *Stam2* were also searched by UCSC Genome Browser (*http://genome.ucsc.edu/*). The nucleotide sequences were translated to protein sequences by ExPASy Translate tool (*www.expasy.ch/tools)* and compared with non-redundant GeneBank CDS, non-redundant SwissProt sequences, and PDB protein database by BLASTP program (*www.ncbi.nlm.nih.gov/BLAST/*).

Results

Exon 1A is a novel alternatively spliced exon of Stam2 gene

Among RT-PCRs done on cDNA obtained from the mouse brain with primers specific for *Stam2*, different primer pairs resulted in the product of expected size and the additional 200-base pairs longer product (Figure 1A). The same difference in size obtained by different prim-



B

TCGCCCAGCTGGGGCAGTCGGAGTCCCGCCAGAGCGTCGGGATTCAGCTCGGGTTGCCGG
CGTGATGCCTCTGTTCACTGCCAACCCGTTCGAGCAAGATGTGGTCTCTCATGTGTCAGAA
AAGCGCTCTCCCACTCTGCTACACCCCTGTTACATCCCTGCTACATCCCACTCTGCTACATC
TTTCCTCGGTTGCCTGCTGTCCCTAACTCTGACATCTGCTTAAAGACATGGAGTCTGTCTCT
CTTCCAAAACTCCTGAGTTCAAGGGATCCTCCTGCCTCACCCTCCCAGAAAAAGCCACAAA
TGAGTACAACACCACAGAAGACTGGAGTCTGATCATGGACATCTGTGACAGGGTTGGAAG
CACTCCCAGTGG

Figure 1. Diagram of genomic *Stam2* DNA and the coding region of *Stam2* mRNA with alternatively spliced exon 1A and hypothetical exon 1B between the first two exons. **A.** The start and stop codons are indicated on the genomic DNA. The exons' numbers are above them. The lines above the mRNA represent the products of reverse transcriptase-polymerase chain reaction (RT-PCR), checked for alternatively spliced exons. Of a total of 40 reactions, 8 are depicted. Lines containing a portion of discontinued line corresponding to exon 1A represent two products: with and without exon 1A included. The lines below the mRNA show the position of primers (A1, A2, and A3) and fragments amplified by RT-PCR used for determination of the alternatively spliced mRNA tissue distribution. Primers A4 and A5 specific for the hypothetical exon 1B are also depicted. **B.** Nucleotide sequence of exons 1, 1A, and 2. Exon 1A is located between exons 1 and 2 and its sequence is underlined. The start codon ATG in exon 1 and the stop codon TAA in exon 1A are marked.

er pairs indicated that the longer product could represent an alternative splicing event. The PCR products were cloned and sequenced, and the sequence obtained corresponded to those of *Stam2* cDNA including the additional novel fragment of 189 nucleotides (Figure 1B; GenBank accession number: DQ141211). The fragment matched a part of the intron sequence between *Stam2* exons 1 and 2 from the bases 52668294 to 52668482 of the mouse chromosome 2. Therefore, it was referred to as *Stam2* exon 1A.

To confirm that exon 1A represents an alternatively spliced exon in the mouse *Stam2* gene, PCRs with the genomic DNA and the brain cDNA were performed. Primer pairs spanning exons 1 and 2, exons 1 and 1A, and exons 1A and 2 never yielded any product in the reactions with the genomic DNA, whereas the expected product sizes were obtained with the cDNA samples.

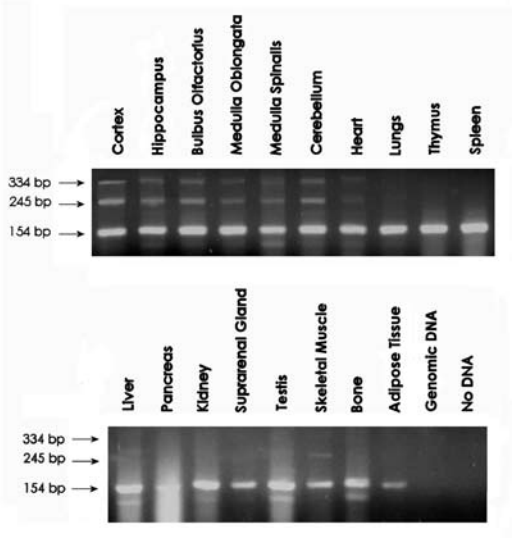


Figure 2. Distribution of the alternatively spliced *Stam2* mRNA with exon 1A in mouse tissues determined by reverse transcriptase-polymerase chain reaction (RT-PCR). A 155-bp band (from primer A1 to A2) represents the mRNA form without exon 1A (constitutive form), and 245-bp (from primer A3 to A2) and 334-bp (from primer A1 to A2) fragments represent the mRNA form with exon 1A included (alternatively spliced form). The alternatively spliced form was present in nervous and muscle tissues. The bands of 245 bp and 334 bp in the heart and skeletal muscle are shown very weakly. The bands representing the constitutive *Stam2* mRNA are stronger than bands representing alternatively spliced transcript. The band of approximately 100 bp visible in some samples represents an unspecific PCR product. Primers are shown in Figure 1A.

This proved that newly identified exon 1A corresponded to the alternatively spliced *Stam2* exon.

There were no matches of exon 1A to any sequence in mouse cDNA databases, except a single match corresponding to an intron sequence from genomic DNA (ie, part of *Stam2* intron), assumed to be assigned by mistake as a cDNA (GenBank accession number: CA569372). In addition, a search of mouse, rat and human cDNA and protein databases showed that there was no other sequence similar to *Stam2* exon 1A in mouse, rat and human cDNA, suggesting that the alternatively spliced exon 1A is unique for mouse, ie, it is not evolutionary conserved. The comparison of mouse *Stam2* exon 1A sequence to genomic DNA of mouse, rat and human revealed 88.6% identity between *Stam2* exon 1A and a fragment of the rat chromosome 3 from base 34149796 to 34149973 residing in the intron between the first two exons of the predicted rat *Stam2* gene (GenBank accession number: NM001012085.1). Nevertheless, this similarity was comparable to that of the whole corresponding *Stam2* intron (88.3%), implying that it only reflected the general similarity of the rat and mouse genomes. No similarity with human genomic DNA was found.

Stam2 mRNA splice variant with exon 1A included is present in the nervous and muscle tissue

To determine the tissue distribution of exon 1A in mouse, the RT-PCRs were performed on RNA samples from 18 different mouse tissues and organs. Three primers, A1, A2, and A3, specific for exons 1, 2, and 1A, respectively, were used in the same reaction set (Figures 1A and 2). Three products were expected as follows: the shorter product resulting from the primers in exons 1 and 2 (A1/A2) corresponding to exon 1A exclusion, the longer product resulting from the same primers corresponding to exon 1A inclusion, and the product of the intermediate size, resulting from the primers in exons 1A and 2 (A2/

A3), specific for the mRNA with exon 1A (Figures 1A and 2). All three products were present in the samples from the nervous system (cortex, hippocampus, olfactory bulb, medulla oblongata, spinal cord, and cerebellum) and those from the skeletal and heart muscle, showing that both splice variants were present in these samples. Only a single product corresponding to the exclusion of exon 1A was present in the lungs, thymus, spleen, liver, pancreas, kidney, suprarenal gland, testis, bone and abdominal adipose tissue (Figure 2). Although RT-PCR is not a quantitative method, the intensities of the obtained bands indicated that the mRNA without exon 1A was more abundant than the mRNA with exon 1A included.

Exon 1A is a unique alternatively spliced exon in Stam2 gene

To test if there were additional alternatively spliced exons of *Stam2*, 40 different RT-PCRs with primers covering the complete coding sequence of *Stam2* were performed on mouse brain cDNA (Figure 1A). The RT-PCR demonstrated that there was no other alternatively spliced exon, except exon 1A, in the coding region of *Stam2* cDNA obtained from the mouse brain. It was also shown that a transcript containing exon 1A contained all 14 *Stam2* exons.

The USCS Genome Browser revealed many cDNA clones from different mouse tissues or cell cultures matching *Stam2* only intron sequence. However, only a single clone (GenBank accession number: AW647621), beside matching the intron sequence, included the relevant neighboring exon sequence, indicating that it could represent an alternatively spliced *Stam2* cDNA. The AW647621 clone was generated from the differentiated murine adipose 3T3 cell line. The hypothetical additional exon was referred to as *Stam2* exon 1B. Its sequence was located in the same intron as exon 1A, between *Stam2* exons 1 and 2, which is the longest *Stam2* intron (Figure 1A).

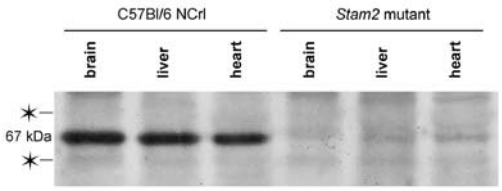


Figure 3. Western blot analysis showing a single form of STAM2 protein in the brain, liver, and heart of the wild type mouse. Asterixes indicate the expected positions of the presumed products of the alternatively spliced *Stam2* mRNA. The predicted molecular weight of the alternative splice forms of STAM2 is either 5 kDa higher (with stop codon readtrough), or 6 kDa lower than the major form of STAM2 (without readtrough, but with translation start from the first following ATG signal). Protein samples from the brain, liver, and heart tissue of *Stam2*^{Gt1Gaj} homozygous mutant mouse with impaired STAM2 protein production served as a negative control.

To check if exon 1B was present in the cDNA of different mouse tissues, we performed RT-PCRs with primers spanning *Stam2* exons 1 and 2 (primers A1/A2), exons 1 and 1B (primers A1/A4), and exons 1B and 2 (primers A5/A2) (Figure 1A). Exon 1B was not detected in any tissue tested, including the abdominal adipose tissue. These results indicated that exon 1A was a unique alternatively spliced exon in *Stam2* gene.

Only STAM2 protein corresponding to the mRNA without exon 1A was detected by western blot

The translation of exon 1A nucleotide sequence into the protein sequence revealed stop codons in all three reading frames, particularly the UAA stop codon in the open reading frame of *Stam2* (Figure 1B). Therefore, the coding region of the transcript containing alternative exon 1A was only 162 nucleotides long and corresponded to 53 aminoacids. This short protein would share with STAM2 only 13 aminoacids at its N-terminus, hence the transcript containing exon 1A would not contribute to a protein with STAM2 function.

To test if the alternatively spliced mRNA variant gave rise to a protein sharing, the functional domains with STAM2 protein despite the presence of the stop codon, or if the hypothetical 53 aminoacids protein interfered with the

normal STAM2 production, western blot analysis with polyclonal anti-STAM2 antibody was performed on samples from the brain and heart, expressing the both mRNA variants, and the liver, expressing only the mRNA variant without exon 1A (Figure 3). To distinguish specific from unspecific binding of the used polyclonal antibody against STAM2, the wild type mice were compared to the mutant *Stam2*^{Gt1Gaj} mice, where *Stam2* was modified by gene trap (13). Due to the vector insertion, the production of STAM2 protein in the homozygous *Stam2*^{Gt1Gaj} mutants was strongly impaired. Western blot analysis showed a single specific band of 67 kDa present in all samples of the wild type mouse, corresponding to the STAM2 protein without exon 1A inclusion. No specific bands corresponding to the presumed products of the alternatively spliced mRNA or to their degradation products were detected. In addition, there was no difference in the intensities of the other weaker bands observed when the samples from wild type and mutant mouse were compared, showing that the other bands were unspecific (Figure 3). These results indicated that the inclusion of exon 1A did not result in a protein sharing the functional domains with STAM2, neither did it interfere with STAM2 protein production.

Discussion

A *Stam2* transcript containing a newly identified exon 1A was sequenced and characterized by use of bioinformatics, RT-PCR and western blot. This transcript was present in the nervous and muscle tissue together with the previously known transcript without exon 1A, and it was absent from all other tested tissues. Moreover, no other additional *Stam2* transcripts were detected, which indicated that *Stam2* transcription resulted in a single constitutive mRNA form present in all adult tissues and another, less abundant, alternatively spliced form, which includes exon 1A present only in the subset of the tissues.

The alternatively spliced exon 1A identified in *Stam2* gene corresponded to a mouse specific intron sequence that was spliced as an exon. It seems that the appearance of the alternatively spliced transcript is a recent evolutionary event since exon 1A is not evolutionary conserved and is present only in the alternatively spliced transcript form (16,17). Likewise, the exons included only in the alternative splice forms (as opposed to the constitutive or major transcript form) are usually not conserved (12,18). The lack of similar sequence in the mouse genome and the presence of the similar sequence in the corresponding intron of the hypothetical rat *Stam2* implies that exon 1A appeared by direct recruitment of the intron sequence but not by insertion of a sequence originating from a different part of the genome (19,20). The recruitment of intronic sequences as new alternatively spliced exons was proposed to have an important role in evolutionary emergence of new protein sequences in eukaryotes (12,16,19,21).

It was questionable if the appearance of the new exon in the alternatively spliced transcript resulted in a new function. We speculated that it may have resulted in a modified STAM2 protein or influenced the production of STAM2 protein in the nervous and muscle tissues. The insertion of exon 1A near the STAM2 N-terminus, within the region coding for the VHS domain, would certainly influence this domain. Moreover, as exon 1A contained the stop codon, the presumed result of the translation was a short 53-aminoacids long protein lacking all STAM2 functional domains.

Splicing of transcripts into unproductive mRNAs, which could be targeted for degradation, represents a possible mechanism to regulate gene expression (22). For example, a point mutation in Alu element residing in the third intron of ornithine aminotransferase gene activates a cryptic splice site and consequently leads to the introduction of an incomplete Alu element into an open reading frame (23). The in-frame stop

codon carried by Alu element results in a truncated protein and ornithine aminotransferase deficiency. In the same way, the production of the described splice variant of *Stam2* could represent an option to be used by splicing machinery to regulate the expression of *Stam2* depending on the ratio of the exon inclusion.

There was also a possibility that a changed, but functional, STAM2 protein form was produced from the alternatively spliced mRNA containing the UAA codon by a phenomenon called readthrough, although a readtrough at UAA codon has shown to be less effective than at UGA and UAG codons (24). In case that the stop codon would not be recognized, the resulting protein would have VHS domain disturbed, but other protein domains would be preserved. The same would be achieved if the cryptic ATG translation start after the stop codon would be used. Nevertheless, western blot analysis with polyclonal anti-STAM2 antibody showed no other protein form except regular STAM2 protein. Therefore, it seemed that the alternative splicing did not have an impact on the STAM2 production or function. Since the evolution of new coding sequences from noncoding ones is an active ongoing process in eukaryotes, it is possible that creation of exon 1A is just a recent attempt of evolution to regulate *Stam2* function (19). The considered possibilities highlight the versatility of the splicing mechanisms in achieving various tasks, which can either occur spontaneously and be selected by evolution, or be artificially designed and used for experimental and therapeutic purposes.

In conclusion, alternatively spliced exon 1A was included in mRNA splice variant present in the nervous and the muscle tissues, but the alternative splicing did not have a major impact on STAM2 production and functionality. The facts that exon 1A represented a mouse intron sequence containing the stop codon, which was unique in mouse genome and not evolutionary conserved, and which was present only in the alternatively spliced transcript form in a subset of mouse tissues, implied that exon 1A is evolutionary new exon created by exonization of intronic sequence.

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