Supplemental Data

Tissue-specific expression and subcellular localization of ALADIN, the absence of which causes human triple A syndrome

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Results and Discussion

Cloning of a full-length *AAAS* cDNA

We identified a 1.4 kb insert clone, 282D10, from the normalized infant brain cDNA library (Soares et al.,1994) containing the EST 1190E. Because the mRNA transcripts for the EST 1190E were longer than 1.4 kb, the full-length cDNA was cloned by performing 5' RACE experiments using human liver total RNA as a template. The analysis of the full-length cDNA sequence revealed that it was identical to that of the AAAS gene (NM_015665).

Tissue distribution of *AAAS* mRNA in human

We performed Northern blot analysis to determine the tissue specificity of *AAAS* expression and the size of the transcript. The C-terminal probe spanning exons 4-16 recognized two transcripts, 2.1 and 2.7 kb in sizes (Figure 1). All tissues expressed both transcripts at various expression levels. A 85 bp DNA fragment representing a part of the first exon specifically recognized the 2.1 kb mRNA only. This exon contains the start codon of the *AAAS* gene, thus indicating that the 2.1 kb mRNA is the transcript encoding ALADIN. Currently, we know neither the nature of the 5' end of the 2.7 kb transcript nor the protein this transcript might encode and can not rule out the possibility that two transcripts are produced by alternative splicing in *AAAS* gene. The 2.1 kb mRNA was widely expressed in human tissues with strong expression in testis, pancreas, kidney and placenta (Figure 1). The relative tissue expression pattern of *AAAS* mRNA reported previously comprises data from tissues expressing both 2.1 and 2.7 kb transcripts, as the pattern was obtained by dot blot analysis using a probe spanning exons 7-14 (Tullio *et al*., 2000). In fact, the MTN blots probed with the DNA fragment corresponding to exons 7-14 displayed a tissue expression pattern identical to that seen with the probe encompassing exons 4-6 (Supplemental Data Figure S1 and Figure 1). Recently, it has been reported that the splice variant of human *AAAS*, *AAAS*-v2, which contains three

WD40 domains without exon 6 (Li *et al*., 2005). We do not know yet whether this variant is identical to our splice variant since we did not characterize the 2.7 kb transcript. But we think that *AAAS*-v2 is a different transcript from 2.1 and 2.7 kb transcripts in our report because these were expressed in heart, placenta, spleen, prostate, testis, colon and peripheral blood leukocyte, while *AAAS*-v2 was not expressed in these tissues and had a 1.7 kb fulllength cDNA.

Expression of ALADIN in human tissues

The expression of the *AAAS* gene product, ALADIN, was investigated by western blot analysis using antibodies raised against two separate peptides (Figure 2A-C). While anti-FLAG antibodies detected only fusion proteins, antibodies CNE19 and CVL16 detected an additional protein of 60 kDa from HeLa cell lysates (Figure 2D and E), thus indicating that both antibodies were able to detect not only exogenous ALADIN but also endogenous ALADIN of molecular weight 60 kDa. To investigate the expression of ALADIN in human tissues, western blot analysis was performed using antibody CNE19. As tissue availability was limited, we analysed ALADIN in human adrenal gland, pancreas, pituitary, kidney, skeletal muscle and placenta samples. Although several non-spe cific signals were present, ALADIN expression was evident in pancreas, adrenal and pituitary gland (Figure 3A). When western blot analysis was carried out with ALADIN-adsorbed CNE19 antiserum, the ALADIN signal was specifically abolished (Figure 3B), while non-specific signals were unaffected. This result clearly indicated that ALADIN was expressed in the tissues mentioned. Tissue-specific expression of ALADIN raises an interesting point with respect to ALADIN function in cells and focuses attention on tissue-specific symptoms of AS. Our results may suggest that tissuespecific expression of ALADIN in pancreas, adrenal and pituitary gland may reflect the disease-specific phenotype in AS. Interestingly, ALADIN was not detected in kidney, skeletal muscle and placenta, although *AAAS* mRNA expression was present. Further analysis is required to elucidate the tissuespecific symptoms of Triple A syndrome.

Subcellular localization of endogenous ALADIN

Previous studies have shown that ALADIN localizes to the NPC using GFP-tagged fusion protein. Therefore, we analysed the subcellular location of endogenous ALADIN using the CNE19 antibody. We observed that ALADIN was present mainly in the nuclear fraction (Figure 4A) as reported previously (Cronshaw and Matunis, 2003). Moreover, ALADIN co-purified with the nuclear membrane when HeLa cells were fractionated into cytoplasm, nucleus, nucleoplasm and nuclear membrane (Figure 4B). Therefore, our data using ALADIN antibody show that endogenous ALADIN is localized in the nuclear membrane, presumably to NPC.

Subcellular mislocalization of ALADIN mutants

The C-terminal end of ALADIN contains Ser-His-Leu (Handschug *et al*., 2001), a putative peroxisomal targeting signal 1 (PTS1) (Gould *et al*., 1988). Therefore, we examined whether the SHL residues affected correct NPC targeting of ALADIN using artificial mutant constructs S544G and S544X. In contrast to the wt GFP-ALADIN which was localized to the nuclear membrane (Supplemental Data Figure S3), the S544G and S544X mutants were mislocalized to the cytoplasm and formed aggregates (Figure 5A). Cellular fractionation confirmed the cytoplasmic localization of mutants (Figure 5B). These results clearly indicate that the SHL is important for proper NPC targeting. There are several possible causes of mistargeting of these mutants. One possibility is the loss of NPC signal by the partial degradation of the mutant ALADIN. But it does not seem to be the case, since we can detect the same molecular weight mutant GFP-ALADINs as the wt GFP-ALADIN (Supplemental Data Figure S2). Another possibility is that C-terminal region around SHL is involved in ALADIN's targeting by undefined mechanisms. Those mechanisms may include binding with interacting proteins or its proper folding. Further study is needed to test this possibility. Based on the current understanding of NPC targeting signal of ALADIN, the serine residue at 544 plays a role in targeting of the nascent ALADIN.

Fig S1.

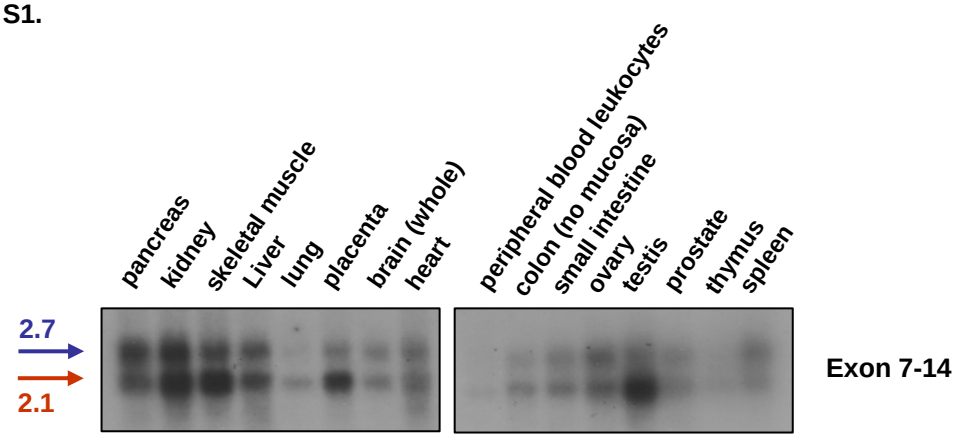

Fig S2.

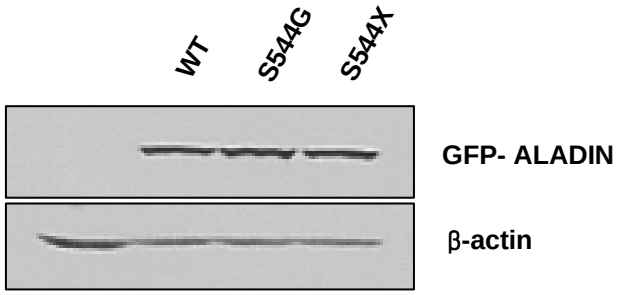

Fig S3.