Introduction

Adenosine to inosine deamination of RNAs is a conserved RNA-editing mechanism that is widespread in metazoa. The enzymatic reaction is performed by adenosine deaminases that act on RNA (ADARs) that recognize structured and double-stranded (ds) RNA. Inosines are recognized as guanosines by many cellular processes and, therefore, editing can alter the amino acid sequence of encoded proteins, change splice patterns, alter RNA-folding, or modify binding sites for associated proteins. In all metazoa tested so far, ADARs have been shown to be essential for development and normal life.

Mice have two active ADAR enzymes, ADAR1 and ADARB1 (generally referred to as ADAR2), which exhibit overlapping, yet distinct substrate specificity. So far, editing events that lead to an amino acid exchange in the encoded protein were primarily observed in RNAs transcribed in neuronal tissues that encode neuronal receptors and ion channels. In most cases, these editing events have significant impact on protein structure and function. The majority of editing events observed in neuronally expressed protein coding regions are mediated by ADAR2. Editing events in protein coding regions are less abundant outside the nervous system and may also be attributed to ADAR1 activity. Moreover, abundant editing is found in non-coding regions of mRNAs such as introns and UTRs where repetitive elements lead to the formation of extensive double-stranded regions. Editing in UTRs can be performed by both ADAR1 and ADAR2.

Consistently, lack of ADAR1 or ADAR2 leads to different phenotypes. ADAR1−/− mice die at embryonic stage (E12.5) due to liver disintegration and defects in hematopoiesis, whereas ADAR2−/− mice are prone to seizures and die shortly after birth. Altered editing patterns are also found in patients suffering from psychiatric disorders, neurodegenerative diseases, or malignant gliomas. Following editing patterns throughout development, it was also shown that editing frequencies in the nervous system increase, despite a constant expression of ADARs.

Comprehensive computational and sequence analysis has revealed novel editing events in the coding region of mRNAs outside neuronal tissues. Editing of Filamin A (FLNA) is most abundant in the gastrointestinal tract and only to a lesser extent in the nervous system. Using knockout mice, we show that ADARB1 (ADAR2) is responsible for the majority of FLNA editing, while ADAR1 can edit Filamin A mRNA in some tissues quite efficiently. Interestingly, editing levels of Filamin α and β do not follow the same trend across tissues, suggesting a substrate-specific regulation of editing.

Spatio-temporal profiling of Filamin A RNA-editing reveals ADAR preferences and high editing levels outside neuronal tissues

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RNA editing by ADARs can change the coding potential of protein-coding mRNAs. So far, this type of RNA editing has mainly been shown to affect RNAs expressed in the nervous system with much lower editing levels being observed in other tissues.

The actin crosslinking proteins filamin α and filamin β are widely expressed in most tissues. The mRNAs encoding either protein are edited at the same position leading to a conserved Q to R exchange in both proteins. Using bar-coded next generation sequencing, we show that editing of filamin α is most abundant in the gastrointestinal tract and only to a lesser extent in the nervous system. Using knockout mice, we show that ADARB1 (ADAR2) is responsible for the majority of FLNA editing, while ADAR1 can edit filamin α mRNA in some tissues quite efficiently. Interestingly, editing levels of filamin α and β do not follow the same trend across tissues, suggesting a substrate-specific regulation of editing.
membrane receptors to the actin cytoskeleton and regulate their precise location and transport. Moreover, Filamin A also acts as a co-localization factor for signaling pathways and a mechanical element for caveolae and membrane ruffle formation.

The Filamin A transcript is edited at a single position in exon 42, which leads to a Q/R substitution in Ig-repete 22 of the encoded protein. This site is located in the very interactive rod 2 domain, implying that it could affect the FLNA interactome. The editing site and sequence context have been found conserved from birds to mammals suggesting that FLNA editing must have functional consequences, which are currently not known. Moreover, the very same position is also edited in the highly conserved filamin B pre-mRNA, also giving rise to a Q/R substitution in repeat 22 of the encoded protein. To get some clues on the functional relevance of FLNA editing, to determine the extent of editing in different tissues and developmental stages, and to determine the enzyme responsible for editing we applied Illumina deep sequencing to FLNA amplicons of spliced mRNAs isolated from different tissues throughout development. Based on these data we generated the spatio-temporal profile of Filamin A editing in mature mRNAs.

Results and Discussion

Previous studies have shown that A to I editing levels for protein-coding targets expressed in the mammalian brain increase gradually during development, from embryo to adulthood. To test whether this trend would also apply to Filamin A and whether editing can also be found in other organs of non-neuronal origin we determined FLNA editing levels using Illumina deep sequencing. Using bar-coded amplicons of FLNA cDNA we compared editing levels in various tissues from developmental stages E15, P0, P20, and adult (> 4 mo) mice. Depending on the sample, read depths varied from a minimum of 450 reads to 961 000 reads maximum with an average of 161 500 reads per sample. As the overall mistakes in base calling were around 0.3%, A to G transitions that were lower than 0.5% were not considered for further analyses.

Due to the small size of E15 embryos we separated head, brain, whole thorax and abdomen, and abdomen only in these early stages. In contrast to the majority of mammalian protein coding targets of A to I editing, filamin A mRNA showed highest editing in the abdomen, reaching up to 12%, while editing levels in the brain were around 2%, similar to the editing levels previously observed for the R/G site in Gria2 (Fig. 1A; Table 1).

Filamin A editing levels increased with age and, hence, higher editing levels were observed in young and adult mice. Most interestingly, the overall frequency distribution of editing events varied dramatically from previous studies on other substrates. Our analysis revealed that editing levels of Filamin A mRNA are highest in 4-mo-old mice with stomach showing 95% A–G transitions, large intestine (86% A–G transitions), and lung (68% A–G transitions). Nevertheless, editing levels were also high in different parts of the brain (30–40% of A–G transitions), as well as in heart (56% A–G transitions), and bladder (55% A-G transitions) (Fig. 2A; Table 2). To verify the high editing levels observed in non-neuronal tissues and to compensate for any inter-individual differences, we have tested editing levels in the gastro-intestinal tract by Sanger sequencing on three additional mice. These data independently confirmed the very high editing levels in stomach (~85–90%), large intestine (~80%), and heart (~60%) (data not shown). Thus, while direct Sanger sequencing generally detected slightly less editing events the general observation of very high editing levels in the gastrointestinal tract could be verified. This also demonstrates that inter-individual differences are low, as already discussed elsewhere.

FLNA editing levels had also been determined for a few human embryonic and adult tissues. The limited number of human tissues investigated makes a direct comparison of human and mouse editing levels difficult. However, in humans—like in the mouse—embryonic editing levels were very low. In adult human brain, liver, kidney, and spleen editing reached about 25%. In the mouse, in contrast, editing levels of up to 40% were observed in the brain, while editing levels in kidney, liver and spleen were in a similar range between 15–25%. Whether humans also show high editing levels in the gastrointestinal tract remains to be determined.

ADAR1 and ADAR2 have different, yet overlapping target specificities. Moreover, ADAR1 and ADAR2 show different but partially overlapping expression patterns. We therefore wanted to determine which enzyme would be largely responsible for the observed editing events. In a previous study, we had already shown that editing of FLNA in post-natal brain can be achieved by both ADAR1 and ADAR2. We therefore performed deep sequencing of organ and development-specific cDNA samples derived from ADAR2/− mice and embryos. In the absence of ADAR2, editing levels were strongly reduced, indicating that ADAR2 is the main enzyme responsible for editing of FLNA RNA. In embryos lacking ADAR2, the rate of A to G transitions dropped below.
the background level in head and brain (Fig. 1B; Table 3). The only significant editing levels of 1.2% were detectable in samples derived from thorax and abdomen. In postnatal tissues only few organs showed editing levels well above 1% (Fig. 2B; Table 4). The highest editing levels were detected in bone, pancreas, spleen, and liver. In the spleen of P0 animals editing reached 26%, followed by 17% editing detected in adult bone. Editing in liver varied from 8% to 1% while editing in pancreas varied from 7 to 5% between P0 and P120, respectively (Fig. 2B; Table 4). Again, editing in neuronal tissues was lower than in other organs but reached at least 4% in adult cerebellum.

Taken together, our data indicates that ADAR2 is the primary enzyme responsible for editing of FLNA mRNA. Nonetheless, in some tissues and some developmental stages editing by ADAR1 still reaches significant levels, even in the absence of ADAR2. In spleen, for instance, editing levels peak at 14% at p120 in the wild type, but can increase to 26% at P0 in ADAR2-/- knockout animals. Also, in bone, editing levels reach 30% in the wild type vs. 16% in the ADAR2-/- knockout.

We have obviously considered the possibility of barcoding or basecalling problems. We have therefore confirmed the most important samples derived from ADAR2-/- samples by conventional Sanger sequencing (data not shown). Increased editing in the absence of one ADAR enzyme suggests competitive binding of different ADARs to one and the same site. Lack of one ADAR would then facilitate access to an editing site for another ADAR enzyme that might act more efficiently at this site. Similar phenomena had been described before.4

Both, in the presence and absence of ADAR2, editing levels in most tissues increase with age. However, in some non-neuronal tissues like large intestine, cartilage, skin, liver, or spleen, editing levels decreased from P0 to P120; sometimes even below the levels of basecalling errors, which we defined around 0.3% (compare Fig. 2A and B).

Recently, it was noted that the mRNA encoding the paralogous protein filamin B is edited at exactly the same position as filamin A, also leading to a Q to R exchange in Ig repeat 22.27 We therefore wanted to test whether editing levels in FLNB mRNA would follow the same trend as editing levels in FLNA. We therefore amplified and sequenced the FLNB mRNA from the same cDNAs used for the analysis of FLNA editing. cDNAs from stomach, large intestine, heart, bladder, and lung were analyzed using Sanger sequencing. Interestingly, while FLNA editing levels were highest in stomach, pancreas, liver, and bone, reaching at least 25% in the spleen.
Table 5. As the very same cDNA samples to determine editing in filamin A and filamin B mRNAs were used, the observed differences in editing levels cannot reflect differences in expression of ADARs in these samples. We therefore compared the ratios of FLNA and ADAR2 expression in a few isolated tissues (Fig. 3B). However, no clear trend between editing levels and FLNA/ADAR2 ratios could be observed. Stomach, showing very high editing levels had a very high FLNA to ADAR2 ratio while cerebellum with also high editing levels had a low FLNA to ADAR2 ratio. It therefore appears more likely that other factors that can enhance or repress editing in a tissue- and substrate-specific manner will show different expression levels in those tissues that have a different trend for FLNA and FLNB editing. In fact, we have recently shown that some RNA-binding proteins can selectively modulate editing of some substrates, most likely by competitive binding to editing sites.\(^{48,49}\) Therefore, if some RNA-binding proteins can specifically interact with the editing sites in either FLNA or FLNB pre-mRNAs, editing in either one of these substrates might be specifically repressed.

In summary, our comprehensive analysis of editing in the filamin A encoding mRNA shows very high editing levels outside the nervous system. Our study shows further that FLNA editing is mainly achieved by ADAR2 but that in some cases ADAR1 can efficiently compensate for ADAR2. High editing levels in some tissues in the absence of ADAR2 might possibly result from facilitating access of ADAR1.\(^4\) However, clarification of this point will require further experimentation. Most interestingly, our finding that editing levels in FLNA are highest outside neuronal tissues suggests further novel and unexplored roles for RNA editing. Given the many functions of filamin A, ranging from actin crosslinking to receptor interaction it may be too premature to speculate about the function of FLNA editing. However, editing may well alter the interaction repertoire of FLNA. With editing levels as high as 90% in the gastrointestinal tract of adult mice dropping to a mere 4–5% of editing in ADAR2\(^{-/-}\) mice and editing levels of more than 12% in the abdomen of wild-type embryos it is unlikely that the high editing levels in the gastrointestinal tract represent an ADAR1-mediated anti-inflammatory response. Instead, a constitutive function for the edited version of FLNA seems most likely.

**Materials and Methods**

**Tissue isolation**

\(^{1}\)\(^{a}\) (ADAR2)-knockout mice were a kind gift of Peter Seeburg (MPI Heidelberg).\(^{12}\) Tissues expressing both active versions of ADAR were obtained from c57bl/6 wt mouse. Mice were sacrificed and organs were dissected and kept in liquid nitrogen. The tissues were macerated using 10% SDS and 0.5M EDTA before RNA isolation. For deep sequencing, tissues from single mice were analyzed per time point. For some tissues (stomach, intestine, heart), sequences from three additional mice were determined by Sanger sequencing.

**RNA isolation, reverse transcription (RT), and Filamin amplification**

Total RNA was isolated from homogenized organs by using PēqGOLD TRIzol reagent (PEQLAB Biotechnologie GmbH) using manufacturers protocols. Aliquots of isolated RNAs were

Table 1. Editing levels at E 15.5 in wild-type mice

| Region            | Editing in % |
|-------------------|--------------|
| head              | 2.5 (145k)   |
| brain             | 2.3 (385k)   |
| thorax-abdomen    | 6.2 (73k)    |
| abdomen           | 12.3 (9k)    |

E 15.5 embryos were dissected into the regions indicated and cDNAs of the FLNA mRNA were amplified, bar coded, and sequenced on an Illumina GA\(\text{II}\) platform. Editing levels are indicated in %. Read depth is indicated in 1000 (k) in brackets.
treated with DNase I (Fermentas 1 U/μL, 10U) at 37 °C for 1 h and kept in 96% ethanol prior to reverse transcription. cDNAs were synthesized using M-MLV Reverse Transcriptase kit (Invitrogen) and random hexamer primers in a total volume of 20 μl. Filamin A fragments were amplified in a 15 cycle PCR reactions using Phusion High Fidelity Polymerase (Finnzymes). Forward primers were designed to bind Filamin A exon 42 and contained the sequencing primer sequence and a 4-nucleotide barcode. The forward primer sequence was ACA CGACGCT CTTCCGATCT NNNN CC GCCTT ACTGTTTCTA GTCT. The sequencing primer part is in italics while NNNN is the position of the barcode. The FLNA hybridizing part is underlined.

The reverse primer was designed to bind Filamin A exon 43 and had the sequence GCTGGTTGAC CTTTAACCCT G. PCR products (50 nt long, spanning spliced exons 42–43) were purified from polyacrylamide gels by elution with 2.5M NH4OAc o/n at +4 °C, followed by ethanol precipitation. The eluted samples were used for second round of PCR with Phusion High Fidelity Polymerase (Finnzymes) and Illumina sequencing adaptor primers (15 cycles). Forward primer sequence AAT GATACGG CGACCACCGA GATCTA CACT CTTTCCCTAC ACGACGCTC T TCCGATCT; reverse primer sequence: CAAGCAGAAG ACGGCATACG AGCTCTTCCG ATCT GCTGGTTGACCTTTAA CCCTG. PCR fragments were again purified by PAGE and resuspended in water.

The same cDNAs were used to amplify filamin B with the forward primer in exon 41: CAGCCCTTAC CTGGTGCCCG T and the reverse primer in exon 42: CTTTGCATCA ATCTTCCCTT TCG.

Deep sequencing
Cluster generation and DNA sequencing was performed using the Illumina Genome analyzer (GA) Ix system. After sequencing, the adaptor and barcode sequences were removed using Cutadapt. Sequences were aligned to the predicted spliced product. Sequences not fitting the predicted spliced product except for the edited nucleotide were discarded.

Basecalling tables were used to define the level of noise at 0.3% for all possible transitions over the entire sequence length. A to G transitions above 0.5% at position 3 of the actual sequence, representing the editing site, were considered as true editing events.

Real-time PCR
To quantify the expression of filamin A, ADAR2, and actin in selected tissues, RNAs prepared for the deep sequencing was DNase I digested (Fermentas 1 U/μl, 10 U) and reverse transcribed using M-MLV Reverse Transcripase kit (Invitrogen) and random hexamer primers in a total volume of 20 μl. Real-time PCR was performed on an Eppendorf realplex 2 mastercycler, whereas using a GoTaq qPCR master mix (Promega) and following primers and reaction conditions: Filamin A sense 5’-GGTGACGCC CGCCGCCTTAC- 3’, anti- sense 5’-AAGATGCTGGC TGGTGGAC-3’, ADARb1 sense 5’-TCCTGCACTG ACAAGATAGC-3’,

| Organ          | P0      | P20     | P120    |
|----------------|---------|---------|---------|
| lung           | 48.6 (961k) | 57.8 (331k) | 66.5 (176k) |
| aortic arch    | 53.3 (550k) | nd      | 54.9 (550k) |
| heart          | 29.6 (595k) | 34.7 (16k) | 26.9 (134k) |
| muscle         | 28.9 (213k) | 26.6 (13k) | 33.7 (1k) |
| cartilage      | 18.5 (420k) | nd      | 29.0 (27k) |
| bone           | 22.6 (102k) | 18.7 (354k) | 30.5 (95k) |
| skin           | 23.1 (142k) | 16.3 (140k) | 26.3 (9k) |
| stomach        | 30.7 (163k) | 32.2 (135k) | 93.4 (146k) |
| small intestine| nd      | 2.3 (127k) | 15.9 (74k) |
| large intestine| nd      | 11.5 (24k) | 84.5 (138k) |
| pancreas       | 9.7 (414k) | 28.6 (558k) | 35.6 (166k) |
| spleen         | 11.7 (488k) | 11.6 (48k) | 13.9 (19k) |
| Liver          | 13.3 (55k)  | 14.9 (2k)  | 24.0 (0.5k) |
| kidney         | 8.8 (76k) | 11.5 (300k) | 22.8 (6k) |
| bladder        | 45.6 (392k) | nd      | 54.2 (114k) |
| brown fat      | 7.1 (228k) | nd      | nd |
| eyes           | 11.1 (431k) | nd      | 51.3 (355k) |
| olfactory bulb | 19.8 (288k) | 6.3 (29k) | 35.3 (28k) |
| cortex         | 10.3 (143k) | 9.0 (10k) | 38.0 (127k) |
| hippocampus    | nd      | 32.6 (230k) | 38.8 (41k) |
| thalamus/hypot.| nd      | 33.4 (119k) | 32.0 (12k) |
| cerebellum     | 33.7 (5k)  | 24.5 (28k) | 38.0 (111k) |
| spinal cord    | nd      | 21.2 (6k)  | 27.0 (5k) |

E 15.5 embryos were dissected into the regions indicated and cDNAs of the FLNA mRNA were amplified, bar-coded, and sequenced on an Illumina GA II platform. Editing levels were calculated for each tissue and are listed in %. Nd, not determined. Read depth is indicated in 1000 (k) in brackets.

| Region             | Editing in % |
|--------------------|--------------|
| head               | 0.3 (614k)   |
| brain              | 0.2 (305k)   |
| thorax+abdomen     | 1.3 (243k)   |
| abdomen            | 0.2 (675k)   |

Table 2. Editing levels in filamin A pre-mRNA during post-natal development

| Organ     | P0     | P20    | P120    |
|-----------|--------|--------|---------|
| lung      | 48.6   | 57.8   | 66.5    |
| aortic arch | 53.3   | nd     | 54.9    |
| heart     | 29.6   | 34.7   | 26.9    |
| muscle    | 28.9   | 26.6   | 33.7    |
| cartilage | 18.5   | nd     | 29.0    |
| bone      | 22.6   | 18.7   | 30.5    |
| skin      | 23.1   | 16.3   | 26.3    |
| stomach  | 30.7   | 32.2   | 93.4    |
| small intestine | nd   | 2.3    | 15.9    |
| large intestine | nd | 11.5   | 84.5    |
| pancreas  | 9.7    | 28.6   | 35.6    |
| spleen    | 11.7   | 11.6   | 13.9    |
| Liver     | 13.3   | 14.9   | 24.0    |
| kidney    | 8.8    | 11.5   | 22.8    |
| bladder  | 45.6   | nd     | 54.2    |
| brown fat | 7.1    | nd     | nd      |
| eyes      | 11.1   | nd     | 51.3    |
| olfactory bulb | 19.8 | 6.3    | 35.3    |
| cortex    | 10.3   | 9.0    | 38.0    |
| hippocampus | nd | 32.6   | 38.8    |
| thalamus/hypot.| nd | 33.4   | 32.0    |
| cerebellum | 33.7  | 24.5   | 38.0    |
| spinal cord | nd   | 21.2   | 27.0    |
anti-sense
5'-GGTTCACGAAAATGCTGAG 3'
Actin
β
sense
5'-CTTGGACGCTCTCTCGTTGC-3',
antisense
5'-ACGATGGAGGGAATACGC-3'
40 cycles of 95 °C/15 s, 57.5 °C/30 s, 72 °C/1 min, preceded by denaturation at 95 °C/3 min.
Relative difference in expression of Filamin A and ADARβ1 was calculated by the ΔCt method using actin β as a reference gene.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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CDNAs prepared from the organs indicated were amplified, bar-coded, and sequenced on an illumina GAII platform. Editing levels were calculated for each tissue and are listed in % Nd. Nd, not determined.

Table 4. Editing levels in filamin A pre-mRNA during post-natal development in ADAR2−/− mice

| Organ        | P0   | P20  | P120 |
|--------------|------|------|------|
| lung         | 0.2  | 0.2  | 0.2  |
| heart        | 0.4  | 0.4  | 0.4  |
| muscle       | 1.2  | 0.9  | 1.2  |
| cartilage    | 3.0  | 0.8  | nd   |
| bone         | 0.9  | 1.0  | 1.0  |
| Skin         | 2.3  | 1.3  | 1.5  |
| stomach      | nd   | 0.2  | 1.5  |
| small intestine | nd  | 3.0  | 3.3  |
| large intestine | 2.0 | 0.2  | 0.5  |
| pancreas     | 6.8  | 5.3  | 5.0  |
| spleen       | 26.0 | 3.6  | 1.2  |
| liver        | 8.0  | 2.0  | 0.9  |
| kidney       | 0.2  | 0.5  | 0.8  |
| bladder      | 0.1  | 0.1  | 0.4  |
| brown fat    | nd   | nd   | 1.4  |
| eyes         | 0.2  | 0.4  | 0.3  |
| olfactory bulb | nd  | 0.6  | 1.9  |
| cortex       | 0.2  | 0.2  | 1.1  |
| hippocampus  | nd   | 0.8  | 1.2  |
| thalamus/hypot. | nd | 0.8  | 6.1  |
| cerebellum   | 0.3  | 0.6  | 4.0  |
| spinal cord  | nd   | 0.3  | 1.1  |

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| Organ            | Editing level in % |
|------------------|--------------------|
| stomach          | 44                 |
| large intestine  |                    |
| heart            | 62                 |
| lung             | 28                 |
| bladder          | 11.50              |

CDNAs from the organs indicated were amplified and sequenced by Sanger sequencing. Peak heights were measured at the editing site and the percentage of editing was calculated by dividing the G peak height by the sum of the A and G peak heights.

Table 5. Editing levels in FLNB at P120 in wild-type mice

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