Identification of the long, edited dsRNAome of LPS-stimulated immune cells

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Endogenous double-stranded RNA (dsRNA) must be intricately regulated in mammals to prevent aberrant activation of host inflammatory pathways by cytosolic dsRNA binding proteins. Here, we define the long, endogenous dsRNA repertoire in mammalian macrophages and monocytes during the inflammatory response to bacterial lipopolysaccharide. Hyperediting by adenosine deaminases that act on RNA (ADAR) enzymes was quantified over time using RNA-seq data from activated mouse macrophages to identify 342 Editing Enriched Regions (EERs), indicative of highly structured dsRNA. Analysis of publicly available data sets for samples of human peripheral blood monocytes resulted in discovery of 3438 EERs in the human transcriptome. Human EERs had predicted secondary structures that were significantly more stable than those of mouse EERs and were located primarily in introns, whereas nearly all mouse EERs were in 3′ UTRs. Seventy-four mouse EER-associated genes contained an EER in the orthologous human gene, although nucleotide sequence and position were only rarely conserved. Among these conserved EER-associated genes were several TNF alpha-signaling genes, including Sp112a and Tnfrsf1b, important for processing and recognition of TNF alpha, respectively. Using publicly available data and experimental validation, we found that a significant proportion of EERs accumulated in the nucleus, a strategy that may prevent aberrant activation of proinflammatory cascades in the cytoplasm. The observation of many ADAR-edited dsRNAs in mammalian immune cells, a subset of which are in orthologous genes of mouse and human, suggests a conserved role for these structured regions.

[Supplemental material is available for this article.]
In these cases, a few endogenous ligands are known, including U2 RNA, snoRNAs, and tRNAs (Bernard et al. 2012; Youssef et al. 2015), but the complete pool of long dsRNA remains elusive. In addition to acting as a substrate for dsRBP sensors, dsRNA structures also mediate gene regulation in some cases. During normal growth of mouse macrophages, an alternatively polyadenylated form of the Slc7a2 mRNA, termed CTN-RNA, is sequestered in nuclear paraspeckles by a structured, ADAR-edited 3′ UTR (Prasanth et al. 2005). Slc7a2 encodes a solute transporter essential for the nitric oxide response, and its rapid expression is required upon recognition of pathogen-associated molecular patterns like LPS. Addition of LPS to macrophages results in the endonucleolytic cleavage of the edited Slc7a2 3′ UTR and release of the mRNA into the cytosol for rapid translation. The Slc7a2 unprocessed transcript is quite long (~60 kb), and the authors hypothesize that nuclear retention of CTN-RNA allows for rapid cleavage and transport, decreasing the time from stress recognition to response by skipping a lengthy transcription step (Prasanth et al. 2005). Giving some credence to this theory, additional cleaved, structured 3′ UTRs have been predicted, in silico, for both mouse and human, suggesting that the phenotype of nuclear retention is more widespread than appreciated (Osenberg et al. 2009).

Here, we characterize the long, edited dsRNAomes of mouse bone marrow-derived macrophages (BMDMs) and human peripheral blood monocytes (PBMs). We find interesting differences and similarities, including dsRNA structures in orthologs of both mouse and human genes. Analysis of the nuclear-cytoplasmic distribution of EER-containing transcripts suggests that a subset, like Slc7a2, is sequestered in the nucleus.

Results

Identification of long dsRNA by RNA-seq of LPS-stimulated macrophages and monocytes

As a first step toward understanding how dsRNA is regulated during inflammation, we defined the repertoire of long dsRNA in mammalian immune cells during inflammation. BMDMs were stimulated with bacterial LPS for 0, 6, and 12 h, followed by RNA extraction. Total RNA was depleted of rRNA, and a fraction was enriched for dsRNA via dsRNA-immunoprecipitation (dsRIP) with the J2 dsRNA-specific antibody. Libraries were prepared from total RNA and dsRIP samples and sequenced using Illumina 101-bp paired-end sequencing (Fig. 1A). As a comparison, we also aligned raw sequencing reads from a recent study that sequenced RNA isolated from LPS-stimulated human peripheral blood monocytes derived from patients of different ages using Illumina 50-bp single-end sequencing (Fig. 1A; Lissner et al. 2015). The inclusion of the human data set allowed for comparison of the long dsRNA repertoire between mouse and human, in a cell type that responds similarly to LPS-stimulation (Hambleton et al. 1996). While the preparation of the libraries was not completely identical (see Methods), the additional data set allowed for comparison of key features between the mouse and human long dsRNAomes of activated immune cells.

Sequencing reads were aligned using an RNA editing-aware version of GNUMAP-bs that tolerates A-to-G mismatches during alignment (Supplemental Table S1; Hong et al. 2013). ADARs will only deaminate rod-like, unbranched dsRNA, and the longer the dsRNA, the more adenosines that will be edited (Bass 1997). Typically, dsRNA must be at least 30 bp to activate an immune...
response (Weber et al. 2006; Schlee 2013), but when viral dsRNA structures are investigated, they are usually at least 100 bp long (Ptáček et al. 2015). To specifically identify the long cellular dsRNA that might interact with an immune response, we searched for clusters of edited reads, observed as A-to-G mismatches in RNA-seq data (Editing Enriched Regions [EERs]), using protocols similar to those previously described (Fig. 1B; Supplemental Fig. S1; Whipple et al. 2015). EERs were first defined as 50-nt windows containing ≥3 editing sites. Editing sites were only counted when ≥1% of the reads were edited. Each editing site also required ≥5 reads to be included in the analysis. Overlapping 50-nt windows were combined, and EERs within 2500 nt were classified as one EER to allow distant binding partners to form native structures. Visual inspection of EERs indicated that a 2500-nt gap combined EERs within a transcript (intramolecular interactions) but did not create EERs spanning multiple transcripts. As a final filtering step, we required that each EER be comprised of at least two combined windows within 2500 nt, to increase the likelihood of finding long structured regions. This editing pipeline identified 342 mouse EERs (mEERs) and 3438 human EERs (hEERs) (Table 1; Supplemental Tables S2, S3).

The mouse dsRIP experiment identified additional mEERs relative to the total RNA samples, but no significant enrichment of expression was observed for specific EERs in the dsRIP samples compared to the total RNA samples (Supplemental Fig. S2). Possibly, mEERs lack structures typically bound by the J2 dsRNA antibody (≥40 bp of dsRNA). Alternatively, the J2 antibody may not bind highly edited RNA efficiently. As an additional control, we trimmed the mouse reads using the cutadapt application (http://cutadapt.readthedocs.org) to create a 50-bp single-end library more directly comparable to the human library. By this method, we identified fewer mEERs (156 mEERs vs. 342 from 101-bp paired-end reads) that still exhibited the same trends (Supplemental Fig. S2D) as the larger data set summarized in Figure 3 below. The trimmed data set agrees with our analysis determined from the full-length 101-bp paired-end reads, and as such, we included only the EERs determined from full-length mouse reads in the remainder of these analyses.

hEERs are longer and more structured than mEERs

hEERs were significantly longer than mEERs, with a median length of 845 nt relative to a median length of 546 nt for mEERs (Supplemental Fig. S3A). mEERs did not exhibit significantly more stable structures than length-matched controls that were selected with the same read-depth criteria as the EERs (Fig. 1C). In contrast, hEERs exhibited structures with significantly increased stability (decreased folding free energy) relative to length-matched controls (Fig. 1D). In addition, hEERs exhibited more stable folding free energy per nucleotide (ΔG/nt) than mEERs (Supplemental Fig. S3B). However, visual inspection of structures predicted with folding algorithms (RNAfold, Mfold) (Zuker 2003) revealed regions of double-stranded structure that correlated with regions of editing for both human and mouse EERs. Structures often showed regions of editing separated by large, unstructured loops (e.g., see Fig. 4E,F below). Examination of individual EERs, created without using a 2500-nt gap parameter to combine EERs, indicated that hEERs were composed of significantly longer, and a greater number of, individual regions of editing, in part explaining the increased stability observed in hEERs relative to mEERs (Supplemental Fig. S3C,D). Repetitive elements in the mouse genome are more divergent in sequence than those of the human genome, resulting in fewer pairing partners and decreased levels of dsRNA compared to the human transcriptome, which is composed primarily of Alu repeats (Neeman et al. 2006).

mEERs and hEERs exhibit features expected of double-stranded regions

For both mouse and human (Fig. 1E,F), editing sites in EERs showed nearest neighbor preferences strongly resembling those from the literature (ADAR1-5′ U > A > C > G; 3′ G > C = A > U; ADAR2-5′ U > A > C > G; 3′ G > C > U = A) (Eggington et al. 2011). These preferences remained stable throughout LPS-stimulation in both organisms (data not shown). In addition, we confirmed editing of EERs by Sanger sequencing of cDNA derived from mouse BMDMs, RAW264.7 cultured macrophages, and phorbol myristate acetate (PMA)-differentiated human THP-1 macrophages (Supplemental Fig. S4A,B). Finally, as additional support for the structure of hEERs, we intersected human EER-associated genes (hEAGs) with known DICER1 binding sites from the literature and found that many of the hEAGs are in fact bound by human DICER1 (observed 719/2792 hEAGs, expected 579/2792, P < 0.0001 by χ² test) (Rybak-Wolf et al. 2014).

mEERs and hEERs are spread throughout the genome

We mapped the chromosomal locations of EERs using the Iidiographica program (Kim and Ono 2007). EERs were spread across all mouse and human chromosomes, including the X and Y Chromosomes (Fig. 2A,B). Consistent with the fact that mice and humans do not have holocentric chromosomes, EERs did not cluster on chromosome arms as observed for Caenorhabditis elegans (Whipple et al. 2015), but we did observe an abundance of EERs on human Chromosome 19. Chromosome 19 is dense with genes and repeats (Grimwood et al. 2004), likely driving the higher abundance of EERs. We then intersected EERs with chromosomal locations of all genes generated from the UCSC Table Browser. The majority of EERs were in annotated genes, with a small subset in intergenic space, >1 kb from a gene (Fig. 3A–C). Interestingly, EERs were in genes that were longer than expected (Supplemental Fig. S4C). To determine the location of EERs within protein-coding genes, we annotated the location of each EER using gene features for that organism obtained from the UCSC Table Browser. Most mEERs were within 3′ UTRs, and hEERs were primarily in introns, consistent with previous studies (Fig. 3D; E-Lev-Maor et al. 2008; Hundley and Bass 2010; Gu et al. 2012; Liddicoat et al. 2015). A small fraction of mEERs were in introns in our analysis, but visual inspection indicated that most of these were 3′ UTR isoforms of splice variants, again suggesting that mouse dsRNA is predominantly located in 3′ UTRs. As observed for gene length, mouse and human EERs were in longer than expected introns and 3′ UTRs (Fig. 3F; G); however, EER length did not correlate with the length of the 3′ UTR or intron, indicating that increased length did not simply derive from EER length (Supplemental Figs. S5, S6). Gene ontology analyses comparing mouse EER-associated genes (mEAGs) with all expressed genes using GOrilla indicated no enrichment in mEAGs.

### Table 1. Identification of EERs

| Gap (nt) | Mouse | Human |
|---------|-------|-------|
| # Windows | >1 | 2500 | 2500 | 0 | 2500 | 2500 |
| # EERs | 1351 | 925 | 342 | 15,181 | 8332 | 3438 |
for any particular category, with only five enrichment categories meeting low levels of significance (Supplemental Table S4; Eden et al. 2007, 2009). A similar analysis performed for hEERs versus expressed genes indicated a slight enrichment in immune processes, not surprising given that monocytes are effectors of the immune response, and further, that LPS treatment increases the expression of many immune-related genes (Supplemental Table S5).

A subset of mEAGs have EERs in the human ortholog

Two hundred eighty-five of 342 mEERs intersected with an annotated RefSeq gene (Supplemental Table S2). Of these mEAGs, 74 of 285 (observed 74; expected 24 based on mouse/human expression patterns; \( P < 0.0001 \) by \( \chi^2 \) test) also showed an EER in the orthologous human gene (Fig. 4A). The EERs were not necessarily in the same location for each ortholog (3′ UTR or intron) (Fig. 4B), and only two EAGs had partial nucleotide sequence conservation (Cds2, 92% identity over 30 nt, 85% identity over 37 nt; Abhd2 85% identity over 109 nt). We focused on two orthologous pairs of EAGs that lacked nucleotide sequence conservation, Sppl2a/SPPL2A and Tnfrsf1b/TNFRSF1B (mouse/human ortholog) (Fig. 4C,D; Supplemental Fig. S7). Sppl2a encodes an intra-membrane aspartic protease important for cleavage of type II membrane signal peptides such as TNF alpha, while Tnfrsf1b encodes a TNF alpha receptor that directs auxiliary functions of TNF alpha signaling, including cellular apoptosis (Holtmann et al. 2002; Friedmann et al. 2006). Sppl2a contained an EER in an annotated 3′ UTR for mouse and a region that showed contiguous read coverage with the 3′ UTR of human SPPL2A, likely an unannotated 3′ UTR (Fig. 4C,D). Each EER folded into a predicted structure with significant stretches of dsRNA; however, consistent with previous analyses (Fig. 1C,D), the hEER appeared considerably more structured than the mEER (cf. Fig. 4E,F). Both of the predicted structures showed base-pairing between edited regions that are distantly located in the 3′ UTR, with intervening, unedited regions characterized by numerous loops and mismatches. In the mouse, the intervening unstructured region was a larger fraction of the EER than that of the human transcript, consistent with the lower stability predicted for mEERs compared to hEERs (see Supplemental Fig. S3B). Folding the entire 3′ UTR of both Sppl2a transcripts resulted in similarly complex structures with editing sites mapping to the predicted dsRNA regions, indicating that the entire 3′ UTR is likely involved in folding (data not shown). Tnfrsf1b showed similar EERs to Sppl2a, except the human transcript contained three separate EERs, within the 3′ UTR and two introns (Supplemental Fig. S7). Again, the predicted 3′ UTR-containing EER of human Tnfrsf1b showed more dsRNA regions than the mEER (Supplemental Fig. S7).

Several highly edited mEAGs are important for TNF alpha regulation

We focused on a list of the top 25 (”TOP25”) mEERs as ranked by best window of editing (highest number of editing sites in 50 nt) (Table 2). The highest ranking mEER by this metric was in the 3′ UTR of the Calcrl gene. The Calcrl EER showed 27 editing sites in a single 50-nt window. mEERs in the TOP25 list were all associated with protein-coding genes, and 24 of 25 were within an annotated 3′ UTR, unannotated 3′ UTR, or a 3′ UTR created by alternative splicing. Several TOP25 mEAGs encode proteins involved in TNFalpha signaling, including SPP2A, TNFRSF1B, the TNF alpha receptor TNFRSF14, and the TNF alpha-regulated protein GPNMB (Friedmann et al. 2006; Tomihari et al. 2009; Yang et al. 2012; Shui and Kronenberg 2014). Interestingly, we also observed the Slc7a2 gene in the
TOP25 list. As mentioned, an alternatively polyadenylated form of Slc7a2, known as CTN-RNA, contains a structured, edited 3′ UTR that contributes to its nuclear retention (Prasanth et al. 2005). During the LPS response, the structured 3′ UTR of CTN-RNA is cleaved by an unknown RNase, and the mRNA is released from the nucleus to mediate the nitric oxide response. Of possible relevance, the Tnfrsf1b transcript is cleaved in its 3′ UTR by ZC3H12A during the inflammatory response, again raising the possibility that 3′ UTR structure contributes to post-transcriptional regulation of inflammatory pathways (Uehata et al. 2013).

A subset of mEAGs is differentially edited and expressed

We considered the possibility that mEAGs might be regulated like Slc7a2 and performed several analyses to look for similarities. Slc7a2 regulation involves alternative polyadenylation, and we found that 73/342 mEERs contained an alternative polyA site (P < 0.0001 by χ² test) (Supplemental Table S2; alternative polyA sites from the GenXPro APADBv2 database). Two hundred fifty-five of 316 mEAGs contained alternative polyA sites, not necessarily overlapping the mEER (P < 0.0001 by χ² test) (Supplemental Table S2). Using RNA-seq data from our input samples, we confirmed that several TOP25 mEAGs were differentially expressed in response to LPS, similar to Slc7a2 (Supplemental Fig. S8A). Slc7a2 showed the highest fold induction at 6 h post-LPS treatment, and H2-T24, a mouse-specific histocompatibility locus, the highest up-regulation at 12 h post-LPS treatment. However, many TOP25 mEAGs were down-regulated at 6 and 12 h post-LPS induction, including Gm449 (2900026A02Rik), Rpa1, and Mad2l1. Editing was not correlated with altered expression, and an increase in expression led to either an increase or decrease in editing depending on the gene (Supplemental Fig. S8B).

A fraction of mEAGs is localized to the nucleus

Given the similarities to Slc7a2, we determined the localization of mEAGs within the cell. We utilized a published data set where BMDMs were activated, fractionated, and deep-sequenced (Bhatt et al. 2012). We aligned the raw reads (FASTQ files) using the GNUMAP-bs aligner and calculated FPKM scores for each transcript using the USeq application DefinedRegionDifferentialSeq (Nix et al. 2008). From this analysis, we determined a relative cytoplasmic/nuclear abundance for the TOP25 mEAGs in unstimulated cells. As a control, Actb and U2 RNA were shown to be enriched in the cytoplasm and nucleus, respectively (Fig. 5A). Fourteen of 24 (~58%) TOP25 mEER-associated transcripts that met read coverage requirements were skewed toward the nucleus relative to the cytoplasm, whereas random protein-coding genes were almost exclusively in the cytoplasm (Fig. 5B). These findings were validated by monitoring levels of Actb, Snord50a, Nudt21, Mad2l1, H2-T24, and Sppl2a transcripts by qRT-PCR of fractionated RAW264.7 macrophages (Fig. 5C). Snord50a, a C/D box class snoRNA, served as a more consistent marker than U2 of nuclear retention in our experiments in RAW264.7 macrophages. An analysis of all mEAGs indicated that 73 of 241 (30%) genes with sufficient read coverage to be included in the analysis were skewed toward nuclear localization (Fig. 5D). Orthologous hEAGs were significantly more numerous in nuclear-localized mEAGs relative to...
cytoplasmic mEAGs (Fig. 5E), despite no significant difference in folding free energy for the two populations of mEAGs (Fig. 5F). hEERs display similar characteristics to mEERs and Slc7a2.

We wanted to compare hEERs to mEERs for key features of Slc7a2 regulation. In our analysis, 473 of 3438 hEERs contained an alternative polyA site ($P < 0.0001$ by $\chi^2$ test) (Supplemental Table S3; GenXPro APADBv2 Database), a key feature of mouse Slc7a2 regulation. Of 2792 hEAGs, 2467 contained alternative polyA sites, not necessarily overlapping the EER sequence ($P < 0.0001$ by $\chi^2$ test) (Supplemental Table S3). We compared our hEERs to the in silico analysis of Osenberg et al. that identified putative human cleaved RNAs, similar to Slc7a2. We observed overlap with 28 of the 566 putatively cleaved segments (Osenberg et al. 2009). These findings suggest that a small subset of human mRNAs may undergo regulation similar to the mouse Slc7a2 transcript, as postulated (Osenberg et al. 2009). Recent work from the Mayr group shows that many human genes utilize multiple 3′ UTRs to control gene expression. Intersection of hEAGs with this data set indicated that 1025 of 2792 hEAGs are in transcripts that utilize alternative polyadenylation to produce multiple 3′ UTRs, again similar to the model for Slc7a2 ($P < 0.0001$ by $\chi^2$ test) (Supplemental Table S3; Lianoglou et al. 2013). In addition, many intriguing genes contained hEERs that are directly related to dsRNA biology, including MAVS, EIF2AK2, DDX58, IFNAR1, IFNAR2, IFNGR1, and IFNGR2 (Supplemental Table S3).

### Discussion

The mechanisms by which ADARs contribute to the inflammatory response are an area of intense study (Mannion et al. 2014; Liddicoat et al. 2015; Pestal et al. 2015), yet the endogenous targets of ADAR during normal growth and subsequent inflammation are unknown. In this study, we set out to define the endogenous pool of long, edited dsRNAs that serve as substrates for dsRBPs, including ADAR. Using both experimentally generated data and publicly available data sets, we identified 342 regions of predicted structure in the mouse and 3438 regions from human samples, during the inflammatory response to LPS (Table 1). The overabundance of EERs in human transcripts is in agreement with the large number of edited Alu elements in the human genome (Kim et al. 2004; Price et al. 2004). We observed that many editing sites within hEERs (53%) intersected Alu elements. However, in contrast to a recent report (Bahn et al. 2015), we did not see a difference in the number of hEER-editing sites intersecting Alus in introns versus 3′ UTRs (52% versus 58%). This difference could relate to many factors, including differences in analysis, cell type, and the dsRNA structures in question.

Mice have a more divergent repertoire of repetitive sequences, which includes an Alu-like element (B1 repeat) and several other types of repeats. This divergence drives a decrease in editing levels due to fewer potential pairing partners (~30- to 40-fold lower than in human) (Neeman et al. 2006). Thirty-six percent of mEER-associated editing sites intersected with repetitive elements as well, most commonly the B1 repeat. Although the difference in absolute
numbers for EERs between mouse and human was somewhat expected, the difference in genomic location was surprising. The majority of hEERs were within introns, whereas mEERs were almost exclusively in 3' UTRs, a feature also observed in a smaller mouse data set from the literature (Fig. 3D,E; Liddicoat et al. 2015). It is possible that deeper sequencing of the mouse transcriptome will reveal additional EERs. Previous work from our laboratory using the nematode C. elegans also revealed many EERs located within intronic sequences, suggesting that structured regions in the mouse may serve additional or different regulatory functions than those in the worm and human (Whipple et al. 2015). Although the human transcriptome contained a lower fraction of EERs within 3' UTRs relative to introns, the absolute number of EERs within 3' UTRs is higher in human than mouse (307 hEERs versus 109 mEERs).

Intriguingly, ~26% of mEAGs (74 EERs) had an EER in the orthologous human gene (Fig. 4A), a startling finding given that only 59 conserved editing sites are observed between mouse and human, and this conservation was limited to very short regions of the associated gene (Cds2, 92% identity over 30 nt, 85% identity over 37 nt; Abhd2 85% identity over 109 nt). The conservation of EERs in orthologous genes suggests a key role for these structures in regulation of gene function, and the lack of sequence conservation suggests it is the dsRNA structure that is most important.

As a first step toward determining the functional importance of EERs in gene regulation, we considered the example of Slc7a2, where a structured 3' UTR regulates nuclear localization of the transcript (Prasanth et al. 2005). We determined the localization of mEAGs using a published data set from the laboratories of Doug Black and Steve Smale (Bhatt et al. 2012). Realignment of these data using our pipeline indicated that 58% of the TOP25 mEERs, and a significant percentage (30%) of all mEAGs, are enriched in the nuclear fraction (Fig. 5D). Thirty-eight percent of these nuclear-retained RNAs have an EER in the orthologous human gene, again implying an evolutionarily conserved importance for nuclear retention of structured RNA (Fig. 5E). Since induction of interferon by viral dsRNA occurs in the cytoplasm, it is intriguing to consider that nuclear retention of endogenous

| mEER ID | BW #Obs | EER length (nt) | Associated gene | Gene length (nt) | Location | Function of gene product |
|---------|---------|----------------|----------------|-----------------|----------|------------------------|
| mEER1   | 27      | 404            | Calcrl         | 94,752          | 3' UTR   | Adrenomedullin receptor; GPCR |
| mEER2   | 26      | 75             | Ppp6r1         | 27,408          | 3' UTR   | Protein phosphatase regulatory subunit in NF-κB regulation |
| mEER3   | 19      | 1453           | Mod21l         | 5616            | 3' UTR   | Component of mitotic spindle checkpoint |
| mEER4   | 19      | 610            | Gpnmb          | 34,368          | Intron (3' UTR isomor) | Feedback regulation of proinflammatory responses to LPS |
| mEER5   | 17      | 2749           | H2-T24         | 14,832          | 3' UTR   | Major histocompatibility gene |
| mEER6   | 16      | 564            | Rnf168         | 23,952          | 3' UTR   | Contributes to class switch recombination in immune system |
| mEER7   | 15      | 1136           | Slfn5          | 11,568          | 3' UTR   | Hematopoietic cell differentiation |
| mEER8   | 14      | 84             | Prkcb          | 345,648         | Intron (3' UTR isomor) | Regulation of B-cell receptor signalosome and oxidative stress |
| mEER9   | 14      | 296            | Gm449          | 67,992          | 3' UTR   | Signal peptidase cleaving TNF alpha during immune response |
| mEER10  | 14      | 1806           | Sppl2a         | 42,816          | 3' UTR   | DNA replication, recombination, and repair |
| mEER11  | 13      | 497            | Rpa1           | 50,112          | 3' UTR   | Biosynthesis of pyroglutamyl peptides |
| mEER12  | 13      | 668            | Qpctl          | 8976            | 3' UTR   | Permease for arginine, lysine, and ornithine during immunity |
| mEER13  | 12      | 2861           | Slc7a2         | 59,904          | 3' UTR   | Contributes to activation of antiviral immunity |
| mEER14  | 12      | 3619           | Tnfsf14        | 6336            | 3' UTR   | Galactosidase important for RBC recycling |
| mEER15  | 12      | 856            | Gla            | 12,960          | (Unannotated) | Choline transporter |
| mEER16  | 12      | 1747           | Scl2a2         | 182,064         | (Unannotated) | Modulation of protein synthesis |
| mEER17  | 12      | 781            | Dnajc1         | 197,136         | (Unannotated) | Galactosidase important for RBC recycling |
| mEER18  | 12      | 1295           | Nudt21         | 17,616          | (Unannotated) | 3' RNA cleavage and polyadenylation processing |
| mEER19  | 12      | 2008           | Tnfsf1b        | 33,408          | (Unannotated) | Recruitment of anti-apoptotic proteins |
| mEER20  | 11      | 213            | Dnase2a        | 14,352          | (Unannotated) | Hydrolysis of dsDNA under acidic conditions |
| mEER21  | 11      | 750            | Tmem69         | 4272            | (Unannotated) | – |
| mEER22  | 11      | 302            | Noll10         | 82,080          | 3' UTR   | Possible substrate recognition component of E3 ubiquitin ligase |
| mEER23  | 11      | 557            | Tulip4         | 144,672         | 3' UTR   | Paralog of primate Trim5a antiviral factor |
| mEER24  | 11      | 277            | Trim12c        | 14,592          | 3' UTR   | Converts phosphatidic acid to CDP-diacylglycerol |
| mEER25  | 10      | 1776           | Cds2           | 48,864          | 3' UTR   | – |

BW #Obs = # observed editing sites in best 50-nt window. (mEER) mouse editing enriched region, (GPCR) G-protein coupled receptor, (RBC) red blood cell.
dsRNA serves to prevent aberrant activation of host inflammatory cascades; however, further experiments will be necessary to definitively test this hypothesis. Many EERs also contained, or were in proximity to, alternative polyA sites in both mouse and human, supporting the idea that some EERs undergo regulation similar to Slc7a2.

In addition, intersection of our data with that from the Mayr group indicated that many hEERs are in genes that utilize alternative 3′ UTRs (Lianoglou et al. 2013), another feature of the Slc7a2 mechanism of regulation. We were particularly interested in immune-relevant EAGs, such as Sppl2a and Tnfrsf1b. We determined that these transcripts, important for TNF alpha signaling, contained EERs in their human orthologs as well. It is also possible that EERs influence other processes within the cell. For example, circularization of exons to create circular RNAs (circRNAs) is promoted by complementary Alu repeats in flanking introns (Dubin et al. 1995; Liang and Witusz 2014; Zhang et al. 2014; Ivanov et al. 2015). Not surprisingly, these Alu repeats are hyperedited, and knockdown of ADAR1 increases the levels of a subset of circRNAs (Ivanov et al. 2015). In fact, an intersection of hEERs with circRNAs derived from circBase (circBase.org) revealed that 1424 of 3438 hEERs intersect at least one circRNA (observed 1424; expected 1244; P < 0.0001 by χ² test), whereas no mEERs intersected circRNAs. Since production of circRNAs often requires sequences in introns, the latter is consistent with our observation that very few mEERs are in introns.

As expected for targets of ADAR editing, hEERs were predicted to fold into structures that were significantly more stable than length-matched controls; surprisingly, this difference was not observed for mEERs, and mEERs and length-matched controls showed similar folding free energies (Fig. 1C,D). Slight differences in human and mouse ADAR protein sequences could explain this difference, but no overt differences were observed in the catalytic or dsRNA binding domains (data not shown), and ADAR nearest neighbor preferences were also similar (Fig. 1E,F). We did observe that hEERs were comprised of longer, more numerous individual EERs (Supplemental Fig. S3C,D). Additionally, more distant pairing partners for mEERs correlated with intervening regions comprised of long unstructured regions that might lower stability and increase predicted folding free energies, as noted for Sppl2a and Tnfrsf1b (Fig. 4E,F; Supplemental Fig. S7).

We identified 3438 hEERs that were primarily located in introns of protein-coding genes (Fig. 3E). GO analysis of these genes showed a plethora of immune-relevant genes, as expected given the importance of monocytes in the immune response. hEERs are in a variety of dsRBPs and signaling molecules, including EIF2AK2, DDX58, and MAVS. An intriguing possibility is that molecules important for sensing dsRNA are also regulated by dsRNA content in the cell and within their own message. In addition to dsRBPs, many important immune factors, including IFNAR1/2 receptors, IFNGR1/2, multiple STAT proteins, MAPK2K1/2, and multiple IRAK1/2/3/4 proteins have hEERs within their sequences (Supplemental Table S3). The editing observed in immune transcripts is interesting for several reasons, including the intimate relationship between dsRNA and innate immunity and the known intersection of hADAR1 with immunity and disease. With the identification of the mouse and human long dsRNAomes, the stage is now set to define the targets of ADAR, and other dsRBPs, that are dysregulated during disease to produce an inflammatory response.

Methods

Reagents
All chemical reagents were purchased from Sigma-Aldrich unless noted.
Cell culture
All mice utilized in this study were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Utah (Protocol number 12-11008). These protocols follow US federal guidelines described by the Office of Laboratory Animal Welfare (OLAW) as described in the Guide for the Care and Use of Laboratory Animals, 8th Edition. 

BMDMs were isolated and differentiated from femurs and tibias of 7- to 8-wk-old male C57BL/6 mice (Jackson Laboratory), as described (Lochhead et al. 2012). Macrophages were differentiated for 7 d in differentiation media (RPMI 1640, Life Technologies; 20% horse serum, Life Technologies; 2 mM L-glutamine, Life Technologies; 20 µg/mL gentamicin; Blango and Bass, 2013). Macrophages were differentiated into macrophages using 5 ng/mL PMA treatment for 48 h.

RNA isolation and immunoprecipitation of dsRNA
RNA was extracted from 10-cm plates of stimulated mouse macrophages using TRIzol Reagent (Life Technologies). Samples were then treated with TURBO DNase (Life Technologies) and ethanol-precipitated. RNA was resuspended in water and stored at −80°C. Purified RNA was immunoprecipitated using the J2 dsRNA-specific antibody (English and Scientific Consulting; Lot #J2-1104). In brief, the product of 9 µg of RiboZero-treated RNA was incubated in binding buffer (150 mM NaCl, 50 mM TRIS pH 8.0, 1 mM EDTA, 1% NP-40) with 5 µg of J2 antibody and RNaseOUT (Life Technologies) rotating overnight at 4°C. J2-bound dsRNA was incubated in binding buffer with 25 µl of prewashed protein-A/G agarose beads for 4 h at 4°C, followed by 5x washes in cold binding buffer. RNA was then extracted with TRIzol Reagent as above.

Library preparation and high-throughput sequencing
After RNA fragmentation, cDNA libraries were generated from total RNA using the Illumina TrueSeq Stranded Total RNA Library Prep Kit according to the manufacturer’s specifications (Illumina). DMSO (2.5%) was added to the cDNA synthesis reaction to promote read-through of structured RNA. Libraries were evaluated for the appropriate size distribution using a Bioanalyzer DNA 1000, followed by 101-cycle paired-end sequencing using the Illumina HiSeq 2000 platform at the Huntsman Cancer Center High Throughput Genomics Core. hEERs were evaluated for the appropriate size distribution using a Bioanalyzer DNA 1000, followed by 101-cycle paired-end sequencing using the Illumina HiSeq 2000 machine, with single-end sequencing reads of 50 bp in length (Lissner et al. 2015).

Sequence alignment with GNUMAP-bs
Alignments were performed as described (Whipple et al. 2015), with slight adaptations for mouse and human genomes. Paired-end sequencing reads were aligned using the RNA editing-aware version of GNUMAP-bs to the Mus musculus GRCm38/mm10 genome and human GRCh37/hg19 (GNUMAP-bs only) (Hong et al. 2013). GNUMAP-bs was run as previously described with arguments “--lib_type wt1 --read_type ma --num_threads 12 --mpi 0 -nt_conv a2i -top_k 10 hash 10 --map_quality sensitive.” Alignments were filtered using the SamTranscriptomeParser application of the Useq package (http://useq.sourceforge.net) with options to remove excessive mismatches (“-a 10000”), reverse strands of paired reads (“-r”), and merge paired reads (“-p”). Analyses were completed with (“-n 1000000”) and without (“-n 1”) repetitive sequences, with no major differences. As such, data for replicate mapping reads were included in all analyses to take into account the repetitive nature of mammalian genomes (Price et al. 2004; Neeman et al. 2006). AlignmentEndTrimmer, a Useq application (Whipple et al. 2015), was used to trim low quality bases at ends of reads and eliminate all reads with >1 non-A-to-G mismatch. The error rates after trimming were comparable to the expected Illumina HiSeq 2000 error rate of 0.001 (average rate for mouse, 0.0011; average rate for human, 0.0004; Illumina.com).

Detection of EERs through RNA editing
The RNA editing pipeline was performed as described (Whipple et al. 2015). In brief, BAM files produced by SamTranscriptomeParser were fed into samtools mpileup (http://www.htslib.org/) (Li et al. 2009) to create pileup files containing genome position information. In addition to disabling base alignment quality (BAQ) computation, anomalous read pairs were used to generate pileup files. Next, the Useq application RNAEditingPileupParser was utilized to parse pileup files for A-to-G transitions with ≥5 overlapping reads on the same strand. RNAEditingScanSeqs, an additional Useq application, was then used to scan the parsed pileup files for 50-nt windows containing ≥3 editing sites where ≥1% of the reads at that site were edited. Overlapping editing-enriched windows were combined. EERs within 2.5 kb were merged using the EnrichedRegionMaker Useq tool. The distance of 2.5 kb was chosen by manually curating output and identifying a distance that would combine EERs within a single transcript but not combine EERs from separate transcripts. The final output was adapted to create a six column BED file containing all identified EERs.

Bioinformatics analysis of EERs
For annotation of EERs, BED files of Ensembl genome annotations (GRCm38/mm10 or GRCh37/hg19) were obtained from the UCSC Genome Browser and intersected with EERs using the annotateBed tool of the BEDTools suite (bedtools.readthedocs.org/) (Quinlan and Hall 2010; Quinlan 2014). The percentage of EER bases overlapping an annotation is reported as overlap. Output was manually curated to prevent overlap of features; precedence was given to noncoding RNA > coding exons > 3' UTR > 5' UTR > introns. In all cases, features and EERs were required to be on the same strand to be considered intersected. If an EER did not intersect any known region, it was considered intergenic.

The intersection of EER lists with published data sets of interest was performed using the Useq applications FileMatcher or IntersectRegions as appropriate. BEDTools intersectBed, annotateBed, and genomeCoverageBed tools were used where appropriate to gather additional information as noted in text.
Validation of EERs

Total RNA was isolated by TRIzol extraction of activated BMDMs, RAW264.7 macrophages, or PMA-differentiated THP-1 macrophages and treated with TURBO DNase (Life Technologies) as described above. cDNA was generated using SuperScript III (Life Technologies) and amplified by PCR. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and submitted for Sanger sequencing at the University of Utah DNA Sequencing Core (cores.utah.edu) after confirming the presence of only one PCR product. All primers are listed in Supplemental Table S6.

Subcellular fractionation and nuclear localization

FASTQ files from Bhatt et al. (SRP008831) (Bhatt et al. 2012) were aligned using GNUMAP-bs, and FPKM values were determined for cytoplasmic and nuclear fractions of mEAGs using the USEq program DefinedRegionDifferentialSeq. A ratio of cytoplasmic to nuclear FPKM was then calculated to represent the relative localization of genes of interest. To verify findings, cytoplasmic and nuclear fractions from RAW264.7 macrophages were collected as described (Bhatt et al. 2012). Briefly, monolayers of cells in 10-cm plates were washed twice with cold PBS + 1 mM EDTA, followed by gentle scraping of cells into 1 mL of PBS + 1 mM EDTA. Plasma membranes were incubated in 300 µL of lysis buffer (10 mM Tris-HCl [pH 7.5], 0.15% NP40, and 150 mM NaCl) for 5 min. Lysates were layered over a chilled 24% sucrose cushion made in lysis buffer without detergent and centrifuged for 10 min at 4°C at 14,000 rpm. Four hundred microliters of supernatant were collected and served as the cytoplasmic fraction. The nuclei pellet was rinsed with ice-cold PBS + 1 mM EDTA and resuspended in 50% glycerol. An equal volume (200 µL) of cold nuclei lysis buffer (10 mM Tris-HCl [pH 7.5], 0.15% NP40, and 150 mM NaCl) for 5 min at 4°C at 14,000 rpm. Four hundred microliters of supernatant were made in lysis buffer without detergent and centrifuged for 10 min at 10,000 rpm.

Statistical analysis

Statistical analyses were performed using Prism 6.04 unless indicated (GraphPad Software). Student’s t-tests were performed with Welch’s correction for unequal variances when appropriate. Data distribution normality (Gaussian) was not assumed, such that nonparametric tests were also used where appropriate. P values of less than 0.05 were deemed significant for all experiments. χ² tests were used where appropriate to determine observed versus expected significance. A Z test for two population proportions was utilized to compare abundance of samples in two populations where appropriate.

Data access

High-throughput sequencing data and processed data files from this study have been submitted to the NCBI Gene Expression Omnibus ( GEO; http://www.ncbi.nlm.nih.gov/geo/) (Edgar et al. 2002) under SuperSeries accession number GSE75155.

Acknowledgments

We thank David Nix, Tim Mosbruger, and Darren Ames for thoughtful conversations and bioinformatics consultation throughout this project. We thank Joseph Whipple, Daniel Reich, Sarah Altschuler, and Osama Youssuf for thoughtful conversations on data and techniques. We thank the laboratories of Ryan O’Connell and Matthew Mulvey for providing cell culture lines and protocols. B.L.B. and M.G.B. were supported by grants from the National Institutes of Health (NIH): National Institute of Aging grant 5DP1AG044162 and National Institute of General Medical Sciences grant 5R01GM044073. In addition, M.G.B. was supported by an NIH microbial pathogenesis training grant T32AI054534. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Bahn JH, Ahn J, Lin X, Zhang G, Lee JH, Civelek M, Xiao X. 2015. Genomic analysis of ADAR1 binding and its involvement in multiple RNA processing pathways. Nat Commun 6: 6355.

Bass BL. 1997. RNA editing and hypermutation by adenosine deamination. Trends Biochem Sci 22: 157–162.

Bernard JJ, Cowling-Zitron C, Nakatsuki T, Muehleisen B, Muto J, Borkowski AW, Martinez L, Greidinger EL, Yu BJ, Gallo RL. 2012. Ultraviolet irradiation damages self noncoding RNA and is detected by TLR3. Nat Med 18: 1286–1290.

Bhatt DM, Pandya-Jones A, Tong AJ, Barozzi I, Lissner MM, Natoli G, Black DL, Smale ST. 2012. Transcript dynamics of proinflammatory genes revealed by sequence analysis of subcellular RNA fractions. Cell 150: 279–290.

Blow M, Futreal PA, Wooster R, Stratton MR. 2004. A survey of RNA editing in human brain. Genome Res 14: 2379–2387.

Cavassani KA, Ishii M, Wen H, Schaller MA, Lincoln PM, Lukacs NW, Hogaboam CM, Kunkel SL. 2008. TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. J Exp Med 205: 2699–2621.

de Faria IJ, Olmo RP, Silva EG, Marques JT. 2013. dsRNA sensing during viral infection: lessons from plants, worms, insects, and mammals. J Interferon Cytokine Res 33: 239–253.

Dubin RA, Kazemi MA, Oster H. 1995. Inverted repeats are necessary for circularization of the mouse testis Sry transcript. Gene 167: 245–248.

Eeden E, Lipson D, Yoge S, Yakhini Z. 2007. Discovering motifs in ranked lists of DNA sequences. PLoS Comput Biol 3:e39.

Eeden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. 2009. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics 10: 48.

Edgar R, Domrachev M, Lash AE. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30: 207–210.

Eggington JM, Greene T, Bass BL. 2011. Predicting sites of ADAR editing in double-stranded RNA. Nat Commun 2: 319.

Friedman E, Hauben E, Maylandt K, Schleger S, Vreugs S, Lichtenhaler SF, Kuhn PH, Stauffer D, Rovelli G, Martoglio B. 2006. SPPL2a and SPPL2b promote intramembrane proteolysis of TNF in activated dendritic cells to trigger IL-12 production. Nat Cell Biol 8: 843–848.

Green NM, Moody KS, Debatis M, Marshall-Broadley A. 2012. Activation of autoreactive B cells by endogenous TLR7 and TLR3 RNA ligands. J Biol Chem 287: 39789–39799.

Grinnwood J, Gordon LA, Olsen A, Terry A, Schmutz J, Lamerini J, Hellsten U, Goodstein D, Cournane O, Tran-Gyamfi M, et al. 2004. The DNA sequence and biology of human chromosome 19. Nature 428: 529–535.

Gu T, Baas FW, Simons AK, Acket-Bicknell CL, Braun RE, Hiibs MA. 2012. Canonical A-to-I and C-to-U RNA editing is enriched at 3’UTRs and microRNA target sites in multiple mouse tissues. PLoS One 7: e53720.
Liang D, Wilusz JE. 2014. Short intronic repeat sequences facilitate circular RNA biogenesis in animals. 

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Lev-Maor G, Ram O, Kim E, Sela N, Goren A, Levanon EY, Ast G. 2008. The Sequence Alignment/Map format and SAMtools. Bioinformatics 24: 2079–2086.

Kim DD, Kim TT, Walsh T, Kobayashi Y, Matise TC, Buyske S, Gabriel A. 2014. Complementary sequence-mediated exon circularization. J Immunol 189: 2488–2501.

Mannion NM, Greenwood SM, Young R, Cox S, Brindle J, Read D, Nellaker C, Vesely C, Ponting CP, McLaughlin PJ, et al. 2014. The RNA-editing enzyme ADAR1 controls innate immune responses to RNA. Cell Rep 9: 1482–1494.

Meltzer M, Long K, Nie Y, Gupta M, Yang J, Montano M. 2010. The RNA editing enzyme ADAR1 is induced in myoblasts by inflammatory ligands and buffers stress response. Clin Transl Sci 3: 73–80.

Neuman Y, Levanon EY, Jantch MF, Eisenberg E. 2006. RNA editing level in the mouse is determined by the genomic repeat repertoire. RNA 12: 1802–1809.

Nix DA, Coudry SJ, Boucher KM. 2008. Empirical methods for controlling false positives and estimating confidence in CisPosseq peaks. BMC Bioinformatics 9: 523.

Osenberg S, Dominissini D, Rechavi G, Eisenberg E. 2009. Widespread cleavage of A-to-I hyperediting substrates. RNA 15: 1632–1639.

Otsuka K, Jin G, Fang H, Angel P, Nowicki J, Kang Y, Jiang Z, Du X, Cook R, et al. 2007. Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. Immunity 27: 123–134.

Pan J, Fisher C, Liu J, Jiang Y, Huang S, Chen L. 2011. Bacterial LPS triggered TLR3 expression is critical for antiviral response in human monocytes: evidence for negative regulation by CYLD. J Immunol 23: 357–364.

Pestal K, Funk CC, Snyder JM, Price ND, Treuting PM, Stetson DB. 2015. Isoforms of RNA-editing enzyme ADAR1 independently control nucleic acid sensing. J Virol 89: 7735–7747.

Pflauffer CK, Mastorakos GM, Matchett WE, Ma X, Samuel CE, Cattaneo R. 2015. Measles virus defective interfering RNAs are generated frequently and early in the absence of C protein and can be destabilized by adenosine deaminase acting on RNA-1-like hypermutations. J Virol 89: 7735–7747.

Pinto Y, Cohen HY, Levanon EY. 2014. Mammalian conserved ADAR targets comprise only a small fragment of the human genome. Genome Biol 15: R5.

Prasanth KV, Prasanth SG, Xuan Z, Hearn S, Freier SM, Bennett CF, Zhang MQ, Spector DL. 2005. Regulating gene expression through RNA nucleic acid retention. Cell 123: 249–263.

Price AL, Eskin E, Pevzner PA. 2004. Whole-genome analysis of Alu repeat elements reveals complex evolutionary history. Genome Res 14: 2245–2252.

Quinan AR, 2014. BEDTools: the Swiss-Army tool for genome feature analysis. Curr Protoc Bioinformatics 47: 11.12.1–14.

Quinan AR, Hall IM. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26: 841–842.

Rabinovic R, Kabir K, Chen M, Yu Z, Zhang D, Luo X, Yang JH. 2001. ADAR1 is involved in the development of microvascular lung injury. Circ Res 88: 1066–1071.

Rybak-Wolf A, Jens M, Murakawa Y, Herzog M, Landthaler M, Rajewsky N. 2014. A variety of dicer substrates in human and e. elegans. Cell 159: 1153–1167.

Savva YA, Rieder LE, Reenan RA. 2012. The ADAR protein family. Genome Biol 13: 252.

Scadden AD. 2007. Inosine-containing dsRNA binds a stress-granule-like complex and downregulates gene expression in trans. Mol Cell 26: 491–500.

Schleier M. 2013. Master sensors of pathogenic RNA-RIG-I like receptors. Immunobiology 218: 1322–1335.

Shui JW, Kronenberg M. 2014. HVEM is a TNF receptor with multiple regulatory roles in the mucosal immune system. Immune Netw 14: 67–72.

Thomsen MC, Nielsen M. 2012. Seq2Log: a method for construction and visualization of amino acid binding motifs and sequence profiles including sequence weighting, pseudo counts and two-sided representation of amino acid enrichment and depletion. Nucleic Acids Res 40: W281–W287.

Tian B, Bevilaqua PC, Diegelman-Parente A, Mathews MB. 2004. The double-stranded-RNA-binding motif: interference and much more. Nat Rev Mol Cell Biol 5: 1013–1023.

Tomihata M, Hwang SH, Chung JS, Cruz PD Jr, Ariizumi K. 2009. Gpnmb is a TNF receptor with multiple regulatory roles in the mouse is determined by the genomic repeat repertoire. Traffic 14: 1719–1728.

Wang Q, Khillan J, Gadue P, Nishikura K. 2000. Requirement of the RNA editing enzyme ADAR1. J Biol Chem 275: 1725–1731.

Whipple JM, Youssef OA, Aruscavage PJ, Nix DA, Hong C, Johnson WE, Bass BL. 2015. Genome-wide profiling of the RNA-editing enzyme ADAR1 gene in embryonic erythropoiesis. Science 290: 1763–1768.

Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. 2006. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol 80: 5059–5064.

Whipple JM, Youssaf OA, Aruscavage PJ, Nix DA, Hong C, Johnson WE, Bass BL. 2015. Genome-wide profiling of the C. elegans dsRNAome. RNA 21: 786–800.

Wu Y, Wang H, Zhang J, Ma X, Meng J, Li Y, Hou Z, Luo X. 2009. Adenosine deaminase that acts on RNA-1 p150 in alveolar macrophage is involved in LPS-induced lung injury. Shock 31: 410–415.

Yang Y, Wang X, Moore DR, Lightfoot SA, Huycke MM. 2012. TNF-a mediated macrophage-induced bystander effects through Nefin-I. Cancer Res 72: 5219–5229.

Youssaf OA, Safran SA, Nakamura T, Nix DA, Hotamisligil GS, Bass BL. 2015. Potential role for snoRNAs in PKR activation during metabolic stress. Proc Natl Acad Sci USA 112: 5025–5029.

Zhang XO, Wang HB, Zhang Y, Lu X, Chen LL, Yang L. 2014. Complementary sequence-mediated exon circularization. Cell 159: 134–147.

Zucker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31: 3406–3415.

Received January 1, 2016; accepted in revised form April 18, 2016.