Stress-induced Apoptosis Associated with Null Mutation of ADAR1 RNA Editing Deaminase Gene*

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Abstract

One type of RNA editing involves the conversion of adenosine residues into inosine in double-stranded RNA through the action of adenosine deaminases acting on RNA (ADAR). A-to-I RNA editing of the coding sequence could result in synthesis of proteins not directly encoded in the genome. ADAR edits also non-coding sequences of target RNAs, such as introns and 3′-untranslated regions, which may affect splicing, translation, and mRNA stability. Three mammalian ADAR gene family members (ADAR1–3) have been identified. Here we investigated phenotypes of mice homozygous for ADAR1 null mutation. Although live ADAR1−/− embryos with normal gross appearance could be recovered up to E11.5, widespread apoptosis was detected in many tissues. Fibroblasts derived from ADAR1−/− embryos were prone to apoptosis induced by serum deprivation. Our results demonstrate an essential requirement for ADAR1 in embryogenesis and suggest that it functions to promote survival of numerous tissues by editing one or more double-stranded RNAs required for protection against stress-induced apoptosis.

Members of the adenosine deaminases acting on RNA (ADAR)3 deaminate adenosine residues to inosine specifically in double-stranded RNA (dsRNA). The inosine residue converted from adenosine in RNA is detected as an A-to-G change of the cDNA sequence, and the translation machinery reads inosine as guanosine. Thus, A-to-I RNA editing could lead to alteration of codons and functions of the affected genes when it occurs within the coding sequences (1–3). For instance, RNA editing results in dramatic changes of glutamate receptor (GluR) ion channel properties such as Ca2+ permeability and kinetics of channel gating (4, 5), whereas substantial decrease of G-protein coupling functions is found with RNA editing isoforms of serotonin receptor subtype 2C (5-HT2C-R) (6–8). RNA editing also alters channel closure rate and tetramerization of squid voltage-gated K+ channels (9, 10). However, dsRNAs formed by inverted repeats of non-coding sequences such as introns and 3′-untranslated regions (UTRs) may be the most common substrates of this type of RNA editing (11). Here activation of the alternative splicing pathway or changes in splicing rate, translation efficiency, or mRNA stability may result from editing (11, 12). Curiously, repetitive elements such as Alu and LINE1 are commonly present in these intronic and UTR sequences subjected to A-to-I RNA editing, raising the possibility that A-to-I RNA editing may be involved in a mechanism regulating the repetitive sequences and transposons in mammalian genomes (11). Furthermore, dsRNA formed between sense mRNA and antisense transcripts (more frequently transcribed than previously thought), may be subjected to A-to-I RNA editing (13, 14). Control of cytoplasmic mRNA levels through antisense transcripts and A-to-I RNA editing has been proposed (13). Finally, ADAR appears to prevent silencing of transgenes in Caenorhabditis elegans regulated through their dsRNA transcripts and the RNA interference (RNAi) mechanism (15). The findings suggest that in certain circumstances RNAi and A-to-I RNA editing mechanisms crisscross for epigenetic control of gene expression.

The first member of the ADAR gene family, ADAR1, was cloned following the biochemical purification and microsequencing of bovine ADAR1 protein (16, 17). The search for ADAR1-like genes led to the cloning of ADAR2 (18–21) and ADAR3 (21, 22). Identification of fish ADAR1 to ADAR3 revealed conservation of the ADAR gene family members in vertebrates through evolution (23, 24). In invertebrates, a single Drosophila dADAR (25) and two less conserved C. elegans c.e.ADAR1 and c.e.ADAR2 (26) have been identified. Studies of in vitro RNA editing using purified recombinant ADAR proteins indicate that there are significant site-selectivity differences displayed by different ADAR gene family members (6, 18–21, 27). For instance, ADAR2 edits almost exclusively the D site of 5-HT2C-R and the Q/R site of GluR-B RNA, whereas ADAR1 selectively edits the A and B sites of 5-HT2C-R RNA and the intronic hot spot (+60 site) of GluR-B RNA. No in vitro RNA editing activity of ADAR3 has been demonstrated yet (21, 22). The editing site-selectivity of different ADARs, which is highly discriminating, indicates a significant difference in their interactions with substrate RNAs through their unique structural features such as N termini and numbers and sequences of dsRNA binding domains. In addition, both ADAR1 and ADAR2 form a homodimer complex, and functional interactions between the two monomer subunits appear to be essential for the site-selective editing mechanism (28). A wide range of phenotypic alterations following inactivation of ADAR gene family members has been reported. Lack of
Genotyping. By using one of these ES clones, we generated a germ-line allele (floxed ADAR1 gene) and three separate DNA probes specific for the 5′G418 and ganciclovir were screened by Southern hybridization using clones examined.

Precocious phenotypes of ADAR1 null mutation

In this study, we investigated the role played by ADAR1 during development by using a mouse line harboring a new ADAR1 null mutation allele established via the Cre-LoxP system. Analysis of staged embryos revealed that ADAR1−/− homozygous embryos die at E11.0 to E12.5. Most significantly, widespread apoptosis was detected in many tissues of ADAR1−/− embryos collected live at E10.5 to E11.5, despite their normal gross appearance. Elevated apoptosis was detected also in ADAR1−/− primary embryonic fibroblasts subjected to serum deprivation. Our results suggest that A-to-I editing at a single unknown dsRNA(s) by ADAR1 is required for cell survival and embryo development by protecting against stress-induced apoptosis.

MATERIALS AND METHODS

Mice—FVB/N mice were purchased from Taconic Farms, and Ella-Cre and Alb-Cre mice were from The Jackson Laboratory. BALB/c SCID mice were obtained from the Wistar Animal Facility.

Oligonucleotides—The following oligonucleotides used for PCR genotyping and FISH oligonucleotide primer extension analysis were synthesized at the University of Pennsylvania, Cancer Center Nucleic Acid Facility: PR-1, 5′-GGGATCCCCAGGGTGGAGATG-3′; PR-2, 5′-GGCTCTAGAAAAGGAGCCACACCGCACAGCAA-3′; PR-3, 5′-GGGATCCTCTTGTTGAGCTTTG-3′; PR-4, 5′-GGCTCTAGAAATCAAACATGACATGAGG-3′; 5′-TGGGATCCTCTTGTTGAGCTTTG-3′; PPR-1, 5′-GGCCAGCTACAATGTTCTCAATGTG-3′; PKRE2S, 5′-GCTCTAGAGAATCAAACCCACAAGAGGCCAG-3′; PKRE2A, 5′-GCTCTAGAGAATCAAACCCACAAGAGGCCAG-3′; Neo3, 5′-GCTCTAGAGAATCAAACCCACAAGAGGCCAG-3′; and Neo3, 5′-GCTCTAGAGGAGGGCACAGCCACAGCA-3′. PCR Genotyping—Genotypes carrying the ADAR1wt/flox allele were identified by PCR genotyping. MEF cells were prepared from wild-type, ADAR1 wt/flox (35) and ADAR1−/− EIIa-Cre (34) mice with the primers PKRE2S, PKRE2A, and Neo3. Four PCR cycles were performed using the primer set PKRE2S and PKRE2A. The presence of the Cre transgene was determined by including the primer set PRCre-1 and PRCre-2 during PCR. Genotyping for PKR (dsRNA-activated serine/threonine kinase) alleles was done using the primer set PKRE2S, PKRE2A, and Neo3. S. PCR cycle conditions used are as follows: 94 °C, 2 min for 1 cycle, 94 °C, 30 s; 71 °C, 30 s; 72 °C, 30 s for 30 cycles, and then 72 °C, 5 min for 1 cycle.

Mice for in vivo and in vitro experiments were housed in wire-bottomed, ventilated stainless-steel cages and maintained on a 12-h light/dark cycle. Mice were fed standard food and had free access to water.

Apoptosis-prone Phenotypes of ADAR1 Null Mutation

Western Immunoblot Analysis—Serum samples collected from 6-week-old

Phosphor Imaging System (Amersham Biosciences). Immunohistochemistry—Mouse embryos were fixed in 4% paraformaldehyde and embedded in paraffin. MEF cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Nonidet P-40. Mouse monoclonal antibody (mAb) 15.8.6 (Molecular Probes, Eugene, OR) was used to detect ADAR1 expression. Paraffin-embedded whole embryo or adult mouse liver sections were subjected to TUNEL staining using an In Situ Cell Death Detection Kit (Roche Applied Science). After labeling apoptotic cells with anti-fluorescein antibody-conjugated with alkaline phosphatase, the sections were counterstained with methyl green. Direct fluorescent labeling during the TUNEL reaction was used for detection of apoptotic MEF cells subjected to serum deprivation.

RNA Editing Assay—Quantification of editing efficiency at selected sites of Glur and H1-R RNAs extracted from teratomas was carried out by dideoxynucleotide/primer extension assay as described previously (29). The primer sets used for the dideoxynucleotide primer extension assay were PKRE2S (H1-R site), EXMQR5 (GluR-5, Q/R site), and EXMQR6 (GluR-6, Q/R site). The ratio of the edited and unedited RNAs was estimated by quantifying the radioactivity of the primer-extended products with a Phosphor Imaging System (Amersham Biosciences).

Interconverting exons 2–7 of the ADAR1 protein (38) was used for immunohistochemical analysis. Paraspecific mouse tissue staining was blocked using the M.O.M. Kit (Vector, Burlingame, CA). Primary antibody was detected using the horseradish peroxidase-labeled Biotin-X-Fluorescein conjugate (Jackson Laboratory, Bar Harbor, ME) for immunofluorescence and the horseradish peroxidase-labeled Biotin-Streptavidin (Roche Applied Science) for indirect immunohistochemistry. After labeling with mAb 15.8.6 and the Renaissance (Molecular Probes, Eugene, OR), sections were counterstained with methyl green.
RESULTS

Generation of Floxed ADAR1 and ADAR1+/− Heterozygous Mouse Lines—To investigate ADAR1 functions during development, a new floxed ADAR1 allele (ADAR1–2loxP or ADAR1flo) was created in mice using the LoxP/Cre system (Fig. 1A). We first established a floxed ADAR1–3loxP allele in R1 ES cells (Fig. 1B). Because the presence of the Neo cassette might interfere with the promoter activity and/or mRNA processing of the ADAR1 gene, the Neo cassette was deleted by in vitro transient transfection with a Cre expression vector, resulting in an ADAR1–2loxP allele (ADAR1flo). Germ line chimeras and mice heterozygous for the ADAR1flo allele were obtained (Fig. 1C, lane 3). Heterozygous ADAR1flo mice were bred inter se, generating fertile and healthy ADAR1flo/flo (floxed ADAR1+/−) homozygote mice (Fig. 1C, lane 4). These mice harboring the ADAR1flo allele were crossed with a universal Cre deleter line EIIa-Cre (34). We expected that this cross would result in ADAR1+/− progeny bearing an embryonic lethal phenotype, considering our previous observation with high level chimeric embryos prepared with ADAR1+/− donor ES cells that died before E14 with anemia and liver degeneration (37). However, to our surprise healthy, live born ADAR1+/−/del (ADAR1+/−) heterozygous mice were generated at normal Mendelian ratios after in vivo deletion of the ADAR1 C terminus by Cre recombinase (Fig. 1C, lane 5) (discussed below).

Embryonic Lethal ADAR1+/− Mice—The intercrossing of ADAR1+/− F1 mice generated approximately a 2:1 ratio of live...
**Table I**

| Age  | Wild-type (+/+), live | Heterozygous (+/-), live | Homozygous (−/−), Live | Homozygous (−/−), Dead |
|------|----------------------|--------------------------|-------------------------|------------------------|
| >1 week | 31                   | 61                       |                         |                        |
| E12.5 | 13                   | 16                       | 4                       |                        |
| E11.5 | 15                   | 44                       | 8                       | 12                     |
| E11.0 | 20                   | 44                       | 12                      | 5                      |
| E10.5 | 10                   | 25                       | 14                      |                        |
| E9.5  | 6                    | 12                       | 8                       |                        |

ADAR1+/− and wild-type mice (Table I). No live ADAR1−/− homozygotes, however, resulted from this intercross (Table I). Therefore, we analyzed staged embryo and were able to collect live wild type and ADAR1+/− (E9.5−12.5) and ADAR1−/− (E9.5−11.5) embryos. The expected ratio of live wild-type, het erozygous, and homozygous embryos was recovered at E10.5 (Table I). However, a large proportion of ADAR1−/− embryos died around E11.0−12.0. These embryos, which presumably died, were grossly normal except for occasional pallor and reduced size. Surviving ADAR1−/− embryos appeared to be normal, although some embryo proper and yolk sac membranes were pale (Fig. 2A). At this embryonic stage (E11−12), the majority of circulating erythrocytes are yolk sac-derived (termed primitive). Examination of peripheral blood collected from E11.0 ADAR1−/− embryos revealed no morphological abnormalities of primitive erythrocytes indicating that their differentiation is qualitatively normal (Fig. 2B). Thus, the pallor observed in these embryos could be due to either hemorrhage or quantitative defects in primitive erythropoiesis. Hemorrhage was indeed detected in the abdominal cavity area of some ADAR1−/− embryos. We previously observed defects in definitive-type (fetal liver-derived) erythropoiesis associated with ADAR1 deficiency (37). Early embryonic death precluded direct examination of fetal liver hematopoiesis in our ADAR1−/− embryos. However, definitive hematopoietic progenitors are also present in embryonic yolk sac (38, 40). Therefore, colony-forming progenitor assays were carried out with E9.5 yolk sacs. No significant differences in the numbers of definitive erythropoiesis (fetal liver and yolk sac) progenitors were found among ADAR1+/−, ADAR1−/−, and wild-type embryos (Fig. 2C), suggesting that definitive hematopoiesis is intrinsically normal in the absence of ADAR1. Thus, the nearly complete block to definitive erythropoiesis that we observed previously in ADAR1-deficient embryos could be due to defects in the fetal liver microenvironment (37).

**Widespread Apoptosis in ADAR1−/− Embryos**—Histological examination of ADAR1−/− embryos revealed aberrant features noted previously in the fetal livers of ADAR1+/− chimeras (e.g. decreased density of hepatocytes) (37). Many of these liver cells possessed pyknotic nuclei, a key hallmark of cells that die from apoptosis (Fig. 3B). Subsequent immunohistochemical examination using the TUNEL assay revealed that many tissues including vertebra and heart had undergone apoptosis (Fig. 3F). The highest levels of apoptosis were detected within the liver for both hepatocytes and hematopoietic cells, possibly contributing to the anemic appearance of these embryos (Fig. 3H). Apoptosis was detected even within the primordial livers of some ADAR1−/− embryos collected at E10.5, although the gross outward appearance of these embryos was indistinguishable from that of wild-type embryos (data not shown).

In order to demonstrate that the elevated apoptosis was caused by ADAR1 deficiency and not due to aberrant ADAR1 products generated by the gene targeting maneuver, we then conducted real time reverse transcriptase-PCR experiments using total RNA samples extracted from E11.0 embryos, in an attempt to detect aberrant ADAR1 transcripts derived from the ADAR1 null allele. Two sets of PCR primers were used: one set consisted of a sense primer located in exon 5 and an antisense primer located in exon 10, and another set consisted of a sense primer located in exon 11 and an antisense primer located in the 3′-UTR. We detected PCR products corresponding to spliced mRNAs encompassing exons 5−10 in wild-type embryos as expected. PCR products corresponding to aberrant ADAR1 transcripts containing sequences encompassing spliced exons 5−11, intron 11, and the 3′-UTR, anticipated to be synthesized from the ADAR1−/− allele, were also detected at a level equal to PCR products derived from normal mRNAs in ADAR1+/− embryos (data not shown). By using an ADAR1-specific mAb, we next conducted immunostaining and Western blot analysis to see whether their translation products were detectable. The mAb used for these experiments was raised against the region corresponding to E2 to E7, and thus it should recognize both normal ADAR1 and truncated ADAR1 proteins that contain dsRNA binding domains (E2 to E7), if they are stable enough to be present at detectable levels. However, no truncated ADAR1 proteins were detected in ADAR1−/− embryos by immunostaining (Fig. 3D) and by Western blot analysis (Fig. 3, right panel), perhaps due to poor translation efficiency of the aberrant transcripts or unstable nature of the translation products. Moreover, trace amounts of truncated ADAR1, if generated from the ADAR1 null allele, do not appear to act as a trans-dominant factor, because our ADAR1−/− heterozygote embryos, potentially capable of producing such aberrant ADAR1 proteins, are normal and display no detectable levels of apoptosis. Thus, truncated protein products are very unlikely to have their own deleterious gain-of-function that would underlie the elevated apoptosis detected with ADAR1−/− homozygote embryos. Most importantly, immunostaining analysis revealed also that regions exhibiting excessive apoptosis in ADAR1−/− embryos corresponded to tissues expressing the highest levels of ADAR1 in wild-type embryos (Fig. 3, C and F).

**Phenotypic Analysis of Mice with Liver-specific ADAR1 Ablation**—To decipher the mechanism underlying the elevated apoptosis in ADAR1−/− mice, we established also a liver-specific ADAR1 null allele (ADAR1floxtfloxAbl-Cre+) by crossing ADAR1floxtflox and Abl-Cre deleter mice. Cre-recombinase expression under the control of the albumin gene promoter is inactive during development; thus, it is unlikely to affect the erythropoiesis occurring within the fetal liver (35). The homozygous mice (ADAR1floxtfloxAbl-Cre+) are viable despite their much smaller size (about 50−70% of wild-type mice). Analysis of chromosomal DNA extracted from the liver of these mice indicated an average 40−60% removal of the floxed region of the ADAR1 gene by Cre recombinase (data not shown). Blood tests carried out with 6-week-old homozygous mice revealed significant abnormalities as follows: up to 4-fold decrease in the levels of glucose and elevated levels (up to 10-fold) of liver enzymes such as alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and alkaline phosphatase, indicative of hepatocellular damage. Histological analysis of liver sections from 6-week-old homozygous mice revealed disorganized liver architecture, as well as many aberrant hepatocytes with enlarged nuclei (Fig. 4B). Most significantly, TUNEL assays once again detected many hepatoeytes undergoing apoptosis in these ADAR1 ablated mice (Fig. 4D), similar to what we observed in ADAR1−/− embryos. No hematopoietic abnormalities were observed in late stage ADAR1floxtfloxAbl-Cre− embryos (E16−19) or newborn.
mice, an expected finding because the recombinase is activated postnatally.

Stress-induced Apoptosis in ADAR1/H11002/H11002 MEF Cells—To obtain insight into the protective function of ADAR1 against apoptosis, we prepared MEF from wild-type, ADAR1/H11001/H11002, and ADAR1/H11002/H11002 E9.5 embryos (Fig. 5A). Although ADAR1/H11002/H11002 MEF cells appear to grow normally under enriched culture conditions, they are prone to apoptosis when subjected to serum deprivation, whereas apoptosis was detected only at a minimum level in wild-type (Fig. 5B) and ADAR1/H11001/H11002 heterozygote MEF cells (data not shown). The following two forms of ADAR1 protein exist: a full-length 150-kDa form (p150) and a shorter 110-kDa form (p110). The ADAR1 mRNA transcribed from the interferon-inducible promoter (41) directs translation of p150, whereas two other ADAR1 mRNAs transcribed from constitutive promoters direct p110. The p150 protein is detected mainly in the cytoplasm, whereas the p110 protein is present exclusively in the nucleus (42). By using the ADAR1-specific mAb, Western blot analysis was performed with protein samples extracted from wild-type MEF cells subjected to serum deprivation as well as ADAR1/H11002/H11002 MEF cells. In ADAR1/H11002/H11002 MEF cells neither the p150 nor the p110 isoform of ADAR1 was detectable, whereas the p110 isoform was almost exclusively found in wild-type MEF cells grown under enriched culture conditions (Fig. 5C). Interestingly, however, the expression of ADAR1p150 increased significantly in wild-type MEF cells during serum deprivation, perhaps indicating a special requirement of this full-length ADAR1 isoform for cell survival (Fig. 5C).

Apoptosis Induced in ADAR1/H11002/H11002 Embryos Is Independent of PKR and RNase L Pathways—Among the many established death-signaling responses of cells (43–45), two pathways acti-
Fig. 3. Widespread apoptosis detected in ADAR1
tembryos. Hematoxylin and eosin staining (A and B), immunohistochemical analysis for expression of ADAR1 by using an ADAR1-specific mAb (C and D) and for TUNEL assays (E–H) were conducted with serial sections of wild-type and ADAR1 null embryos recovered live at E11.0. For immunostaining analysis of ADAR1
tembryos, the final color development step was carried out for twice as long (10 min) as in the control experiment (C) done for ADAR1
tembryos so that at least the outline of the null embryos can be seen. Otherwise, immunostaining of ADAR1
tembryos was in practice blank. By using the same ADAR1-specific mAb, Western blot analysis was carried out for protein samples extracted from whole embryo bodies (E11.0), demonstrating the absence of both normal and aberrant ADAR1 protein products in ADAR1
embryos (right panel). TUNEL assays carried out on whole embryos (E and F) and liver sections (G and H) reveal widespread apoptosis in tissues stained dark silver gray. The highest levels of apoptosis were detected in heart (ht), liver (lv), and vertebra (vt). Examples of apoptotic hepatocytes (black arrowheads) and hematopoietic cells (red arrowheads) are indicated (H).

Fig. 4. Increased apoptosis in liver of ADAR1flox/flox/Alb-Cre
mice. Liver sections from 6-week-old wild-type (A and C) and ADAR1flox/flox/Alb-Cre
mice (B and D) were either stained with hematoxylin and eosin (magnification ×200) (A and B) or analyzed with the TUNEL assay (C and D). Many cells with pyknotic nuclei are detected in ADAR1flox/flox/Alb-Cre
mice (B). Examples of apoptotic hepatocytes (black arrowheads) are indicated.

vated by dsRNA are known to lead to induction of apoptosis for cells under stress and/or infected with virus (46, 47). One pathway is mediated by dsRNA-activated serine/threonine kinase PKR (46, 47). The activated PKR phosphorylates the translation initiation factor eIF-2α, resulting in the shut down of the protein translation system and induction of apoptosis. ADAR1 inactivation may alter the processing of dsRNA(s), which may in turn activate PKR and consequently trigger apoptosis. If this were the case, then loss of PKR could suppress embryonic lethality caused by loss of ADAR1. To test this possibility, we attempted to generate ADAR1+/−/PKR−/−
mice, presuming that in the absence of PKR ADAR1 ablation would not activate the pathway leading to apoptosis. Mice homozygous for the PKR null mutation are viable unless infected with certain viruses (47, 48). We found no ADAR1+/−/PKR−/− mice among 140 progeny derived from the inter se cross of ADAR1+/−/PKR−/− mice, whereas ADAR1+/−/PKR+/− and ADAR1+/−/PKR+/− mice were recovered in approximately the expected ratio of 1:2 (Table II). Therefore, apoptosis and embryonic death caused by loss of ADAR1 does not appear to be mediated by activation of PKR.

In addition to PKR, RNase L is also known to be activated by dsRNA during viral infection or stress response of cells (46). Following activation by binding of 2′-5′-oligoadenylate molecules, which are synthesized by 2′-5′-oligoadenylate synthetase, RNase L degrades nonspecific cellular RNA, leading cells to apoptosis. However, the stability of general rRNA and mRNA is not affected in ADAR1−/− embryos and MEF cells cultured in the absence of serum (data not shown). Thus, dsRNA activated oligoadenylate synthetase-RNase L pathway is also unlikely to underlie apoptosis detected in ADAR1−/−
and MEF cells subjected to serum deprivation.

Identification of in Vivo Editing Sites for ADAR1—Enzymatically active ADAR1 and ADAR2 proteins display distinctive in vitro editing-site selectivity with known RNA substrates (6, 18–21, 27). However, the involvement of ADAR1 in site-selective A-to-I RNA editing has never been demonstrated in vivo conclusively. This prompted us to develop a teratoma system that would enable us to assess the editing-site selectivity of ADAR1 in vivo. We established ES cell lines from ADAR1−/−, ADAR1+/−, and control embryos, and we used these to generate teratomas, in which a large fraction of ES cells differentiate into neuron-like cells (37). Total RNA extracted from teratomas derived from wild-type, ADAR1+/−, or ADAR1−/− ES cells were tested for several known A-to-I editing sites of GluR-B, GluR5, GluR6, and 5-HT2C R RNAs (Table III). An almost complete lack of editing at the A and B sites of 5-HT2C R RNA was
Fig. 5. Stress-induced apoptosis in ADAR1<sup>−/−</sup> MEF cells. A, ADAR1<sup>−/−</sup> MEF cells express no ADAR1. Fluorescent immunostaining localized ADAR1 to the nucleoplasm, nucleolus, and cytoplasm of wild-type but not ADAR1<sup>−/−</sup> MEF cells using primary 15.8.6 mAb specific to ADAR1 and secondary Alexa Fluor<sup>™</sup> 488-conjugated mAb against mouse IgG. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). B, serum deprivation-induced apoptosis in ADAR1<sup>−/−</sup> MEF cells. Cells were stained with propidium iodide (PI) for RNA, and analyzed with the
ADAR1 embryos die at the midgestation stage (37). However, true establishment of these sites as ADAR1 editing sites (31) and indicates the percentage of editing at the different editing sites examined.

| GluR-B | GluR | 5-HT2CR | PKR-independent embryonic lethal phenotypes of ADAR1 null mutant mice |
|--------|------|---------|---------------------------------------------------------------|
| ADAR1<sup>+/+</sup> | 11 | 23 | 0 | 48 |
| ADAR1<sup>-/-</sup> | 24 | 38 | 0 | 62 |
| ADAR1<sup>-/-</sup> | 19 | 31 | 0 | 44 |
| Total | 48 | 92 | 0 | 140 |

**DISCUSSION**

Here we demonstrate that ADAR1 is essential for life, acting to maintain the survival of numerous embryonic tissues. In addition, studies using MEF cells from gene-targeted mouse embryos indicate an essential role for ADAR1 in stress response to nutrient deprivation.

**ADAR1 Null Mutation Resulting in Embryonic Lethality—**

Our previous studies indicated that most ADAR1<sup>-/-</sup> chimeric embryos die at the midgestation stage (37). However, true ADAR1<sup>-/-</sup> heterozygous mice generated in the current study exhibit no obvious abnormalities. The mechanism underlying this apparent paradox is unclear. ADAR1<sup>-/-</sup> embryos and the ADAR1<sup>-/-</sup> chimeric embryos share several common features including anemia and degenerating fetal liver, suggesting that the phenotype of the latter resulted from ADAR1 deficiency. The ADAR1 targeting construct used for our previous chimera studies and the new floxed construct were both designed to inactivate the C-terminal deaminase domain (16, 49). The original construct, however, retained a PGK-neo cassette in the antisense orientation, whereas the new construct did not. Therefore, it is possible that readthrough antisense effects originating from the PGK promoter in the targeting vector could antagonize endogenous ADAR1 expression to account for the severe embryonic lethal phenotypes of ADAR1<sup>-/-</sup> chimeric embryos. Because homodimerization of ADAR1 is required for its enzymatic activity (28), the ADAR1 activity may be particularly sensitive to the decrease in the concentrations. In support of this possibility, ADAR1 mRNA levels in chimeric embryos were less than half-normal, 35–45% of wild type (37). Alternatively, the difference in phenotype penetrance observed between ADAR1<sup>-/-</sup> heterozygote mice and ADAR1<sup>-/-</sup> chimeric embryos may be due to strain and genetic background variation, which affects the phenotypes of numerous gene disruptions (50). The ADAR1<sup>-/-</sup> ES cell lines used for preparation of chimeric embryos were derived from R1 cells and thus in the SV129 strain background. However, ADAR1<sup>-/-</sup> heterozygote mouse lines had a 1:1 ratio of SV129 and FVB/N strain background at the F1 generation. Recently, the association of heterozygosity for the functional ADAR1 null mutation and dyschomatosis symmetrica hereditaria, a human pigmentary genodermatosis of autosomal dominant inheritance, have been reported (51). At this time, we do not know if this ADAR1 haploinsufficiency-related dyschomatosis symmetrica hereditaria known only for human so far will develop in ADAR1<sup>-/-</sup> mice upon repeated exposure to UV light (51).

Erythropoiesis normally begins in the yolk sac at about E7.5 where mainly primitive-type erythrocytes are generated until about E11. Around day 11–12, production shifts to the fetal liver where definitive (adult-type) erythrocytes and also myeloid lineages are produced. Definitive hematopoietic precursors are believed to initiate within the yolk sac and/or the aorta-gonadal-mesonephros region in the embryo proper and then migrate to the fetal liver (39, 40). The pallor observed in some ADAR1<sup>-/-</sup> embryos at E11.0–11.5 could be caused by either reduced production of yolk sac-derived primitive erythrocytes or hemorrhage, perhaps due to loss of vascular integrity associated with widespread apoptosis in the fetal liver and/or other tissues. Circulating primitive erythrocytes exhibited normal morphology, although we cannot rule out quantitative defects in yolk sac erythropoiesis. ADAR1 also appears to be important for definitive hematopoiesis as chimeric embryos generated with ADAR1<sup>-/-</sup> ES cells exhibited severe anemia associated with lack of definitive erythrocytes (37). In the current study, erythroid and myeloid progenitors were present in normal numbers in ADAR1<sup>-/-</sup> yolk sacs of E9.5 embryos and appeared to mature normally in vitro, as judged by morphology. These findings indicate that definitive hematopoietic progenitors capable of normal differentiation are present in ADAR1<sup>-/-</sup> embryos but fail to further develop in fetal livers. This is consistent with aberrant features of fetal livers noted previously with ADAR1<sup>-/-</sup> chimeric embryos (37) and ADAR1<sup>-/-</sup> embryos examined in the present study. Fetal livers deteriorating due to massive apoptosis are unlikely to provide a suitable microenvironment for hematopoietic maturation.

**Protective Role of ADAR1 for Cell Survival against Stress-induced Apoptosis—** Apoptosis is detected widely in many tissue...
Apoptosis-prone Phenotypes of ADAR1 Null Mutation

The specific identity of dsRNA(s) targeted by ADAR1, of which RNA processing/editing is critical for cell survival in mice, is currently unknown. Interestingly, absence of GluR-B RNA editing at the so-called Q/R site leads to a 10-fold reduction of the Bcl-xL expression levels in E11.0 ADAR1flox/flox mice, a separate, liver-specific ADAR1 null mutant mouse line. Finally, ADAR1−/− MEF cells are also prone to apoptosis when subjected to serum deprivation. These results suggest a generalized ADAR1 function in many tissues and MEF cells for survival against stress-induced apoptosis.

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REFERENCES

1. Bass, B. L. (2002) Annu. Rev. Biochem. 71, 817–846
2. Maas, S., Rich, A., and Nishikura, K. (2003) J. Biol. Chem. 278, 1391–1394
3. Seeburg, P. H., and Hartner, J. (2003) Curr. Opin. Neurobiol. 13, 279–283
4. Hohs, M., Single, F. N., Sommer, B., Sprengel, R., and Seeburg, P. H. (1993) Cell 75, 1361–1370
5. Lemel, H., Mombacher, J., Melcher, T., Höger, T., Geiger, J. R., Kuner, T., Monyer, H., Higuchi, M., Buch, A., and Seeburg, P. H. (1994) Science 262, 1709–1713
6. Burns, C. M., Chu, H., Rueret, S. M., Hutchinson, L. K., Canton, H., Sanders-Bush, E., and Emeson, R. B. (1999) Nature 397, 303–308
7. Niswender, C. M., Capeland, S. C., Herrick-Davis, K., Emeson, R. B., and Sanders-Bush, E. (1999) J. Biol. Chem. 274, 9472–9478
8. Wang, Q., O’Brien, P. J., Chen, C.-X., Cho, D.-S. C., Murray, J. M., and Nishikura, K. (2000) J. Neurochem. 74, 1280–1300
9. Paton, D. E., Silva, T., and Bezanilla, F. (1997) Neuron 19, 1120–1127
10. Rosenthal, J. C., and Bezanilla, F. (2002) Neuron 34, 745–757
11. Metz, D. P., Arusavech, P. J., and Bass, B. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7906–7911
12. Rueret, S. M., Dawson, T. R., and Emeson, R. B. (1999) Nature 339, 75–80
13. Cormeau, C. G. G. (2003) Nat. Biotechnol. 21, 371–372
14. Yoon, K., Dahary, D., Serek, R., levans, E. Y., Goldstein, O., Shoshan, A., Diber, A., Biton, S., Tamir, Y., Khosravi, R., Nemzer, S., Pinner, E., Balash, S., Bernstein, J., Savitsky, K., and Rotman, G. (2003) Nat. Biotechnol. 21, 379–386
15. Knight, S. W., and Bass, B. L. (2002) Mol. Cell 10, 809–817
16. Kim, U., Wang, Y., Sanford, T., Zeng, Y., and Nishikura, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11455–11460
17. O’Connell, M. A., Krause, S., Higuchi, M., Hsuan, J. J., Totty, N., Jenny, A., and Keller, W. (1995) Mol. Cell. Biol. 15, 1389–1397
18. Gerber, A. P., O’Connell, M. A., and Keller, W. (1997) RNA (New York) 3, 633–643
19. Lai, C. H., Chen, C.-X., Carter, K. C., and Nishikura, K. (1997) Mol. Cell. Biol. 17, 2413–2424
20. Metzler, T., Maas, S., Herb, A., Sprengel, R., Seeburg, P. H., and Higuchi, M. (1996) Nature 379, 460–464
21. Chen, C.-X., Cho, D.-S. C., Wang, Q., Lai, C. H., Carter, K. C., and Nishikura, K. (2000) RNA (New York) 6, 1391–1397
22. Hough, R. P., Lingma, A. T., and Bass, B. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 463–468
23. Slavov, D., Clark, M., and Gardiner, K. (2006) Gene (Amst.) 352, 41–51
24. Slavov, D., Cremonese-Jurcovic, T., Clark, M., and Gardiner, K. (2005) Genes (Amst.) 250, 53–60
25. Palladino, M. J., Keegan, L. P., O’Connell, M. A., and Reenan, R. A. (2000) Mol. Cell. Biol. 20, 6766–6774
26. Houlé, N. J., and Bass, B. L. (1999) Nature 397, 649–655
27. Lakos, M., Pichl, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W., and Westphal, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5860–5865
28. De La Paz, E., and Bass, B. L. (2002) ENDWORX 29, 93–97
29. Uchida, T., and Bass, B. L. (2002) ENDWORX 30, 135–138
30. Nakamura, S., Hashimoto, N., and Bass, B. L. (2002) ENDWORX 31, 93–97
31. Kawakubo, K., and Samuel, C. E. (2000) Cell 100, 463–477
32. Saito, K., and Bass, B. L. (2002) ENDWORX 33, 193–196
33. Kasai, A., and Bass, B. L. (2002) ENDWORX 34, 197–199
34. Wu, Y., and Bass, B. L. (2002) ENDWORX 35, 201–204
35. Toyooka, S., and Bass, B. L. (2002) ENDWORX 36, 205–208
36. Chen, C.-X., and Nishikura, K. (1997) Cell 88, 355–365
37. Tsujimoto, J. (2003) J. Cell. Biol. 158, 155–167
38. Samuel, C. E. (2001) Clin. Microbiol. Rev. 14, 778–809

# Q. Wang and K. Nishikura, unpublished results.
References

47. Williams, B. R. G. (1999) *Oncogene* 18, 6112–6120
48. Yi-Li, Y., Reis, L. F. L., Pavlovic, J., Aguzzi, A., Schäfer, R., Kumar, A., Williams, B. R. G., Aguët, M., and Weissmann, C. (1995) *EMBO J.* 14, 6095–6106
49. Lai, F., Drakas, R., and Nishikura, K. (1995) *J. Biol. Chem.* 270, 17098–17105
50. Ihle, J. N. (2000) *Cell* 102, 131–134
51. Miyamura, Y., Suzuki, T., Kono, M., Inagaki, K., Ro, S., Suzuki, N., and Tomita, Y. (2003) *Am. J. Hum. Genet.* 73, 693–699
52. Reenan, R. A., Hanrahan, C. J., and Ganetzky, B. (2000) *Neuron* 25, 139–149
53. Brennecke, J., Hipfner, D. R., Stark, A., Russel, R. B., and Cohen, S. M. (2003) *Cell* 113, 25–36
54. Xu, P., Vermeire, S. Y., Guo, M., and Hay, B. A. (2003) *Curr. Biol.* 13, 780–795
55. Lee, Y., Jeon, K., Lee, J.-T., Kim, S., and Kim, V. N. (2002) *EMBO J.* 21, 4663–4670
56. Lagos-Quintana, M., Rauhut, R., Meyer, J., Berkhards, A., and Tuschl, T. (2003) *RNA (New York)* 9, 175–179
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