Molecular determinants and guided evolution of species-specific RNA editing

Robert A. Reenan

Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, Connecticut 06030, USA

Most RNA editing systems are mechanistically diverse, informationally restorative, and scattershot in eukaryotic lineages[1]. In contrast, genetic recoding by adenosine-to-inosine RNA editing seems common in animals; usually, altering highly conserved or invariant coding positions in proteins[2–4]. Here I report striking variation between species in the recoding of *synaptotagmin I* (*sytI*). Fruitflies, mosquitoes and butterflies possess shared and species-specific *sytI* editing sites, all within a single exon. Honeybees, beetles and roaches do not edit *sytI*. The editing machinery is usually directed to modify particular adenosines by information stored in intron-mediated RNA structures[5–7]. Combining comparative genomics of 34 species with mutational analysis reveals that complex, multi-domain, pre-mRNA structures solely determine species-appropriate RNA editing. One of these is a previously unreported long-range pseudoknot. I show that small changes to intronic sequences, far removed from an editing site, can transfer the species specificity of editing between RNA substrates. Taken together, these data support a phylogeny of *sytI* gene editing spanning more than 250 million years of hexapod evolution. The results also provide models for the genesis of RNA editing sites through the stepwise addition of structural domains, or by short walks through sequence space from ancestral structures.

RNA editing systems programmatically alter messenger RNA sequences after transcription from genomic templates and are found enigmatically scattered among phyla. One example, base modification through the hydrolytic deamination of adenosine to inosine (A-to-I) by ADARs (for 'adenosine deaminases acting on RNAs'), can result in informational recoding: the ribosome interprets inosine as guanosine[8]. Curiously, this recoding occurs almost exclusively in gene products whose primary function is fast neuronal signalling[3], in keeping with the observed neurological phenotypes of ADAR-deficient *Caenorhabditis elegans*, *Drosophila* and mice[9–11]. Further, human neurological disease has been associated with altered gene recoding[12,13]. The biological consequence of ADAR action at a particular site can vary markedly between genes and between species. Certain mammalian ionotropic glutamate receptor (iGluR) genes recode a functionally critical glutamine (Q) codon to that of arginine (R). Studies involving animals genetically altered in this recoding event are revealing. For instance, the GluR-2 (Q/R) site must be edited at nearly 100% or mice develop epilepsy and die postnatally[14], whereas lack of editing at the GluR-6 (Q/R) site (normally about 75%) is relatively benign[15], resulting in modest changes in synaptic plasticity and seizure vulnerability. *C. elegans* iGluR genes do not edit the same conserved amino acid; however, when an R-encoding version is introduced into worms, neurotoxicity and lethality ensue[16].

Previous reports indicate that A-to-I RNA editing varies between arthropod species[3,17,18]. Although the mechanism of gene recoding frequently involves imperfect base pairing of exonic and intronic sequences[5–7,19], the molecular basis of species-specific editing is unknown. *Drosophila synaptotagmin I* (*dsytI*), the Ca^{2+} sensor for synchronous neurotransmitter release[20], is a target of A-to-I RNA editing[3]. One exon, whose boundaries are conserved in all synaptotagmins[21], was shown to possess four editing sites (A to D) that recode highly conserved positions. To investigate whether editing varied between species, I performed reverse-transcriptase polymerase chain reaction (RT–PCR) to obtain *sytI* complementary DNAs from *Anopheles gambiae* (malaria mosquito), *Manduca sexta* (tobacco hawkmoth), *Apis mellifera* (honeybee), *Tribolium castaneum* (red flour beetle) and *Blattella germanica* (German cockroach). Direct sequence analysis of RT–PCR amplification products was performed to identify RNA editing sites, as described previously[3]. Editing was not detectable in *A. mellifera*, *T. castaneum* or *B. germanica sytI* genes. The remaining arthropods edited *sytI*, but no two species possessed the same set of editing sites (Fig. 1a, b). All

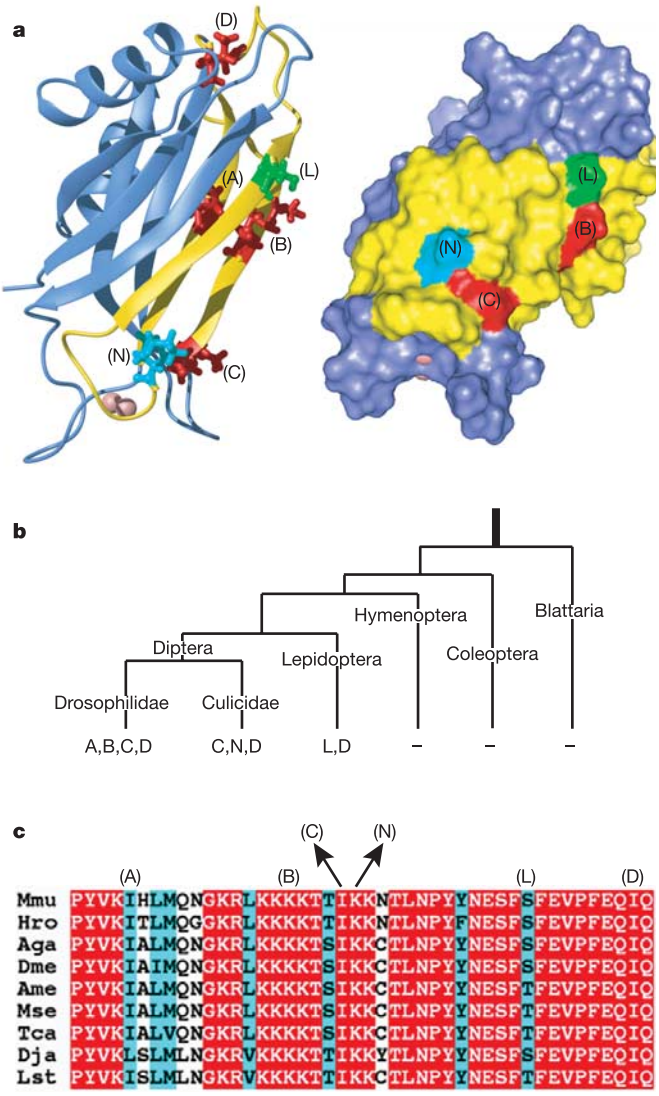

Figure 1 Species-specific genetic recoding of *synaptotagmin I*. **a**, Left: ribbon structure of the synaptotagmin I C_2B domain. Yellow corresponds to the conserved exon found in all *sytI* orthologues. Side chains of *D. melanogaster sytI* editing sites A–D (red), mosquito-specific site N (blue) and lepidopteran-specific site L (green) are indicated. Right: electrostatic surface potential showing surface-projecting residues of editing sites B, C, N and L (colours as in **a**). **b**, Cladogram of taxa in this study and editing sites present: Diptera (Drosophilidae (*Drosophila melanogaster*), Culicidae (*Anopheles gambiae*)), Lepidoptera (*Manduca sexta*), Hymenoptera (*Apis mellifera*), Coleoptera (*Tribolium castaneum*) and Blattaria (*Blattella germanica*). Dashes indicate no detectable editing. **c**, Protein sequence alignment of *synaptotagmin I* editing exon orthologues of the animal kingdom. Shaded invariant (red) and conserved (blue) amino acid residues are shown. Editing sites A–D, L and N result in the following recoding events (single-letter amino acid codes): site A, I → V; site B, K → R; site C, I → V; site D, I → M; site L, T → A; site N, K → R.

had a common editing site (D). In addition, *D. melanogaster* and *A. gambiae* shared a site (C). Finally, each species was found to possess species-specific editing sites (A, B, N and L). That species-specificity of RNA editing was a conserved feature among closely related species was confirmed by analysing *sytI* editing in additional representatives within each taxonomic group (Supplementary Table S1).

These editing sites in insects alter invariant or highly conserved residues within the SytI C2B domain (Fig. 1c), a Ca^{2+}-dependent phospholipid-binding machine essential for the rapid and synchronous release of neurotransmitter. Three species-specific editing sites (B, N and L) as well as shared editing site C recode amino acids positioned on an interaction surface that is crucial for proper SytI function[22–25] (Fig. 1a). *Aplysia californica* (sea hare) employs alternative splicing of the same exon, generating functionally distinct SytI isoforms[26]. A single amino acid difference accounted for differential function of these isoforms, corresponding to editing site L, and resulting in nearly the same amino acid change (Thr → Gly by alternative splicing, versus Thr → Ala by RNA editing).

Conservation of specific ADAR modification between related species can result in the simultaneous conservation of *cis* elements that direct RNA structure formation[17,27–29]. To test this rule for *sytI*, genomic sequences were next cloned and sequenced for the genomic region spanning the editing sites from ten members of the family Drosophilidae with estimated divergence times ranging from 15 million to 80 million years ago. Sequence alignment revealed two invariant intronic elements, E1 (33 nucleotides) and E2 (48 nucleotides) (Fig. 2a, Supplementary Fig. S1). The remaining intron sequences were highly divergent, although interelement spacing was a conserved feature.

To determine whether the E1/E2-containing intron directs the RNA editing of *Drosophila sytI*, the sequences spanning the edited exon, the intron and the downstream exon were expressed as a minigene in *Drosophila* S2 cells together with *Drosophila* ADAR (dADAR). Efficient and specific editing was observed with the use of a restriction-enzyme assay for sites C and D (see Methods), demonstrating the sufficiency of these sequences to direct ADAR modification (Fig. 2c, WT). No editing was observed at inappropriate adenosines or in the absence of co-transfected dADAR (data not shown). RNA structural computations with MFOLD consistently predicted two mutually exclusive lowest-energy structures: one pairing E1 with the upstream exonic region of editing sites B and C (domain I), and one pairing the downstream distal E2 with the exonic region of editing site D (domain II). The assumption of a pseudoknot structure reconciled these two duplexes in one structure (Fig. 2b). To probe this hypothetical structure, potentially disruptive mutations were introduced into domains I and II (Fig. 2b, c). Mutations M1 and M2 singly abolished editing at site D, whereas editing at site C occurred normally. Likewise, mutations M3 and M4 singly abolished editing at site C, whereas editing at site D was unaffected. Structurally compensatory double mutations M12 and M34 each restored editing in their respective domains, thus validating numerous predicted base-pair interactions. Because

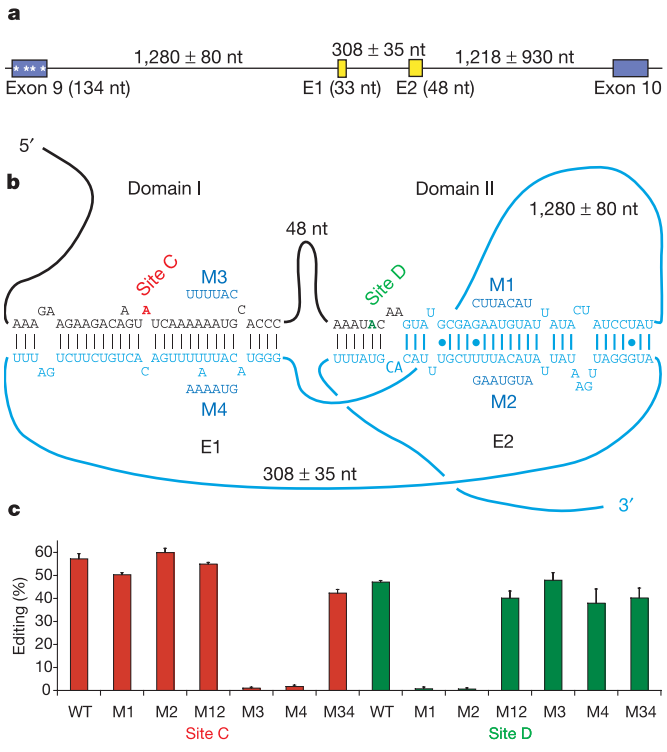

Figure 2 *Drosophila sytI* pre-mRNA forms a pseudoknot. **a**, Genomic organization of Drosophilid *sytI* gene editing sites. Exons (blue boxes), conserved elements E1 and E2 (yellow) and intron (line) shown with spacings as indicated (means ± s.d.). **b**, Predicted pseudoknot domain structure of *dsytI* pre-mRNA. Exon (black), intron (blue), and editing site C (red) and D (green) sequences are indicated. Mutations introduced into domains I and II are indicated above or below mutated sequences (M1–M4). **c**, Effects of mutations on the editing of sites C (red) and D (green) are indicated for disruptive single mutations (M1–M4) and compensatory double mutations (M12 and M34). WT, wild type. Values are means ± s.d.

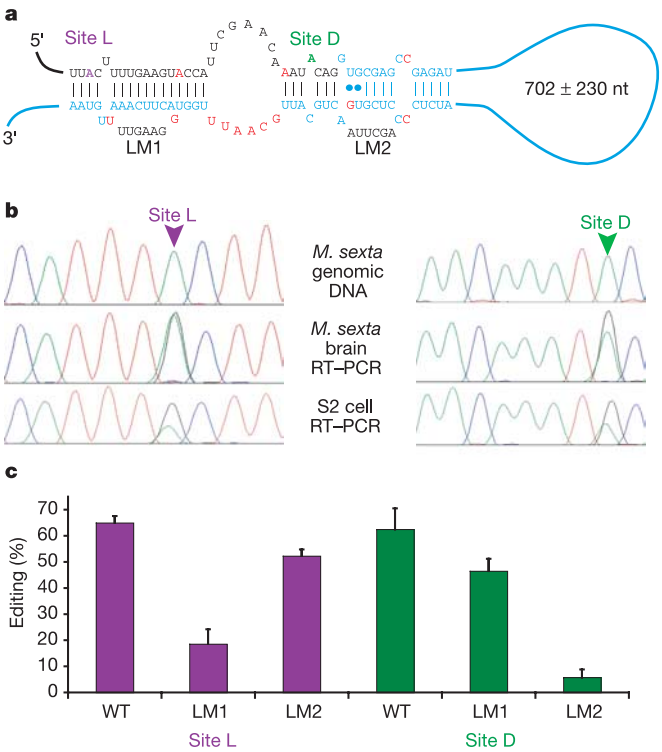

Figure 3 Structure of lepidopteran *sytI* editing site and heterologous editing by dADAR. **a**, Predicted structure of lepidopteran *sytI* pre-RNA. Exon (black), intron (blue) and variant positions (red) are indicated (see Supplementary Fig. S3). Variation in loop sequence length is indicated (mean ± s.d). Intronic mutations are indicated (LM1 and LM2). **b**, Electropherograms of *M. sexta sytI* sites L (left) and D (right). Sequences were generated from genomic DNA PCR products (top), RT–PCR products from *M. sexta* brain RNA (middle) and RT–PCR products from *M. sexta* minigene expressed in *Drosophila* S2 cells with dADAR (bottom). **c**, Mutations LM1 and LM2 (as in **a**) were introduced into the *M. sexta* minigene and expressed in S2 cells with dADAR. Values are means ± s.d.

editing in each domain of the *dsytI* structure can be disrupted independently, it seems unlikely that *dsytI* editing proceeds through a stepwise mechanism invoking sequential editing. Rather, both duplex regions probably exist in the pre-mRNA, forming a long-range pseudoknot containing two domains of ADAR action.

Lepidopterans modify *sytI* at site D, as well as a Lepidoptera-specific site (L). Genomic DNA sequences spanning the same region studied in *Drosophila* species were obtained for ten species of moths and butterflies. Comparison revealed highly conserved intronic sequences downstream of the editing sites, comprising a single extended region with limited sequence variation (Supplementary Fig. S2). To test whether these sequences direct editing, *M. sexta sytI* genomic sequences encompassing the edited exon, downstream intron and downstream exon were expressed as a minigene in *Drosophila* S2 cells along with dADAR. Efficient and specific editing was observed at *M. sexta* sites D and L (Fig. 3b). The *M. sexta* intron is clearly sufficient to direct species-specific editing with a heterologous editing enzyme. Thus, little of the species-specificity of *syt I* editing must be due to differences in ADAR enzymes between species.

Structural predictions for the *M. sexta sytI* pre-mRNA consistently paired conserved intronic sequences with the region of editing sites D and L (Fig. 3a). Although the intronic conserved elements varied in sequence between moth and butterfly species, none of the differences altered the predicted secondary structure of the RNA (Fig. 3, Supplementary Fig. S3). Like *dsytI*, the lepidopteran substrate contains two duplex domains, each with a site of adenosine modification. Because dADAR efficiently and accurately edits the *M. sexta* minigene in *Drosophila* cells, the predicted structure was tested by mutation (Fig. 3a, c). Two mutations were engineered into intronic conserved sequences (LM1 and LM2). The LM1 mutation decreased editing at site L but editing at site D was relatively unaffected. Mutation LM2 disrupted editing at nearby site D but editing at site L occurred at nearly wild-type levels (Fig. 3c). Thus, *sytI* RNA editing in Lepidoptera occurs through two mutationally separable domains of ADAR action whose overall structural arrangement is substantially different from that seen in Drosophilidae.

Anopheles gambiae and *Aedes aegypti sytI* share editing sites with *Drosophila* species (C and D), whereas mosquito-specific site N occurs between sites C and D (Fig. 1c). Because the editing of *Drosophila sytI* at sites C and D is directed by a conserved pseudoknot structure, a comparative sequence analysis of five mosquito species was performed to identify conserved intronic sequences (Supplementary Fig. S4). Like *Drosophila*, mosquitoes possess distinct E1-like and E2-like elements that are predicted to form a homologous pseudoknot structure, with some alterations. Many of these differences are structurally silent; however, key changes are observed in mosquito domain I in the vicinity of editing site C, including the extensive disruption of base pairing near editing site B, which is not edited in mosquitoes (Fig. 4a and Supplementary Fig. S5). Expression of the *Anopheles gambiae sytI*

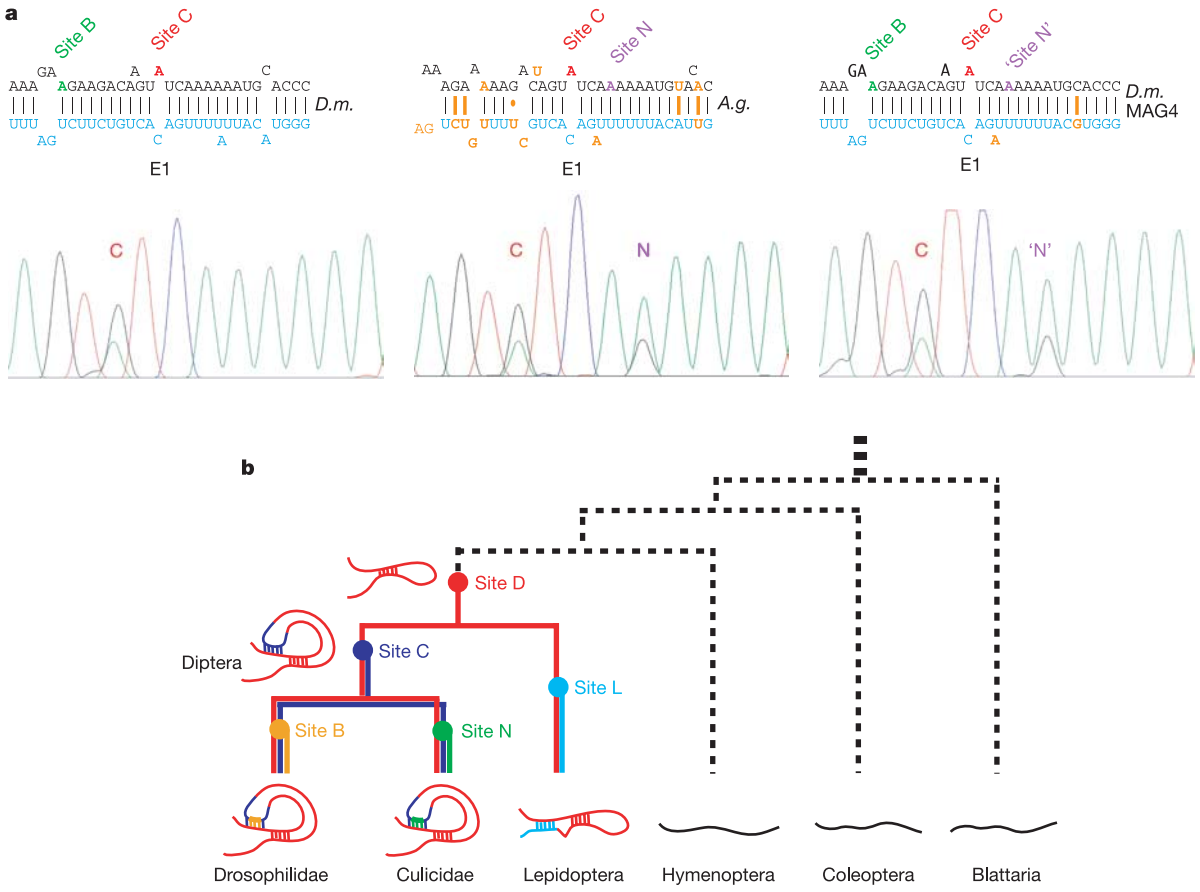

Figure 4 Guided evolution of species-specific RNA editing. **a**, Structure of pseudoknot domain I showing exon (black) and intron (blue) sequences for *Drosophila* (*D.m.*, left), *Anopheles* (*A.g.*, middle) and MAG4 mutation (*D.m.* MAG4, right). Differences of *Anopheles* domain I from *Drosophila* are indicated in orange (middle). The MAG4 mutations (right) are also indicated in orange. Editing status is shown in electropherograms below structures. **b**, Phylogeny of *sytI* RNA editing. Extant *synaptotagmin I* pre-mRNA structures and proposed ancestor molecules are shown associated with a cladogram of ordinal relationships of taxa in this study[30]. Nodes denoting ancestral origins of particular editing events are indicated by circles. Unedited *sytI* mRNAs are depicted as unstructured (black).

substrate in *Drosophila* cells along with dADAR resulted in the *Anopheles* pattern of editing, indicating that dADAR recognized the mosquito-specific editing site N (data not shown). To determine whether the editing of site N results from structural differences in domain I, two nucleotide changes were made in the *Drosophila sytI* expression construct in element E1, guided by the *A. gambiae* domain I structure (MAG4; Fig. 4a). The intronic MAG4 mutations are located more than 1,200 nucleotides from editing site C. Whereas no editing of site N occurs in the wild-type *dsytI* construct, the MAG4 mutations confer efficient modification of the mosquito site N adenosine on the *Drosophila* minigene RNA. Thus, small non-coding changes, far removed from a potential target adenosine, are capable of inducing sufficient structural change to directly evolve a site of gene recoding by ADAR modification.

The editing sites described here recode invariant or nearly invariant positions, a phenomenon seen in other targets of A-to-I editing in arthropods, molluscs and vertebrates. Together, these data imply a selective advantage for RNA editing by allowing protein sequences access to a mutational forbidden zone wherein historically invariant amino acids can be altered by degrees, in mRNA, but not through discrete genetic change in coding sequence. Access to this normally unattainable realm of protein space is mediated through complex RNA secondary structures under intense purifying selection. I suggest that the data presented here comprise a credible phylogeny of RNA editing for a gene, graphically illustrating descent with modification (Fig. 4b). RNA editing in insect *sytI* first seems to have evolved in the common ancestor of dipteran and lepidopteran lineages, beginning with site D. Beetles, roaches and reported vertebrate *sytI* genes are genomically incapable of evolving Ile → Met editing at the same location, for lack of a third-position adenosine in their isoleucine codons (ATT or ATC). The ancestral editing site D structure probably generated new editing sites through two methods. One, global intronic variation, led to the synthesis of entirely new, add-on oligonucleotide domains, such as those that direct editing sites C and L in dipterans and lepidopterans, respectively (Figs 2 and 3). Alternatively, sites wended their way through sequence space, generating nascent editing sites by means of a small number of changes to ancestral structures, as shown by the nature of sites A, B and N and the ability to transfer editing from the mosquito to fly pre-mRNA substrate readily by simple mutation. Of course, species-specific *sytI* RNA editing sites, directed by different structures from those presented here, might exist in other animals.

The evolutionary methods of creating editing sites proposed here have probably shaped most targets of ADAR-mediated recoding. Further challenges posed by this study lie in determining what advantage particular editing sites confer on species, the extent of standing variation of RNA editing, and the role, if any, of RNA recoding in the process of speciation. □

Methods

Multiple sequence alignment and structural predictions

Sequences were aligned with the GCG software package. Protein alignment shown in Fig. 1c was performed with the Pileup program using default settings. Sequences are abbreviated as follows: Mmu (*Mus musculus*), Hro (*Halocynthia roretzi*), Aga (*Anopheles gambiae*), Dme (*Drosophila melanogaster*), Ame (*Apis mellifera*), Mse (*Manduca sexta*), Tca (*Tribolium castaneum*), Dja (*Dugesia japonica*) and Lst (*Lymnaea stagnalis*). For *Drosophila* genomic DNA sequence alignments (Supplementary Fig. S1, Supplementary Table S1), alignments were performed on sequences spanning the editing exon through to element E2 because of large discrepancies in intron size downstream of E2. No significant conserved sequences were obtained in alignments downstream of E2 (data not shown). All DNA sequence alignments were performed with the gap creation and gap extension penalties set to a value of 1.

Structural predictions were performed using the MFOLD program of the Macfarlane Burnet Centre MFOLD server (http://mfold.burnet.edu.au/), setting the folding temperature to 25 °C. For *Drosophila* substrates, only the sequences from the editing exon as far as 100 nucleotides downstream of E2 were used.

RNA editing analysis of endogenous synaptotagmins

For flies, mosquitoes and beetles, whole-organism RNAs were isolated with TRI Reagent (Molecular Research Center). For moths, butterflies, bees and roaches, whole-head RNA was isolated. Species-specific primers were used for first-strand cDNA synthesis. PCR was then performed with species-specific *sytI* primers. Amplification products of the correct size were gel-purified and sequenced. The presence or absence of editing was assessed by the presence of mixed A/G signals in the electropherograms and singlet A signal from PCR products from genomic DNA for each species.

Schneider cell RNA-editing system and substrate mutagenesis

RNA-editing reporter minigene constructs were generated by cloning the *sytI* editing exon, downstream intron and downstream exon into the pMT-V5/His vector (Invitrogen). dADAR expression construct was generated by cloning the +1, −3a alternative splice form of dADAR into pMT-V5/His. Schneider S2 cells were transfected with various editing reporter constructs (about 200 ng) with or without 2 μg of dADAR expression construct. Cultures were transfected with DNA using GeneJuice (Novagen) transfection reagent and induced with copper sulphate 5 h after transfection. Cell were harvested 3 days after transfection and total RNA was prepared with TRI Reagent. In all cases the RNA samples were treated with DNase (DNA-*free*; Ambion) to remove contaminating input DNA.

SytI minigene transcripts were amplified by RT–PCR from S2-cell total mRNA with gene-specific and vector-specific primers. RNA editing was quantified as follows. RNA editing of *dsytI* creates a *PshAI* restriction enzyme cutting site at site C or a *HpyCH4V* cutting site at site D. *M. sexta* RNA editing creates a *BcgI* cleavage site at editing site L and a *HpyCH4V* cutting site at site D. RNA samples were prepared from two to four independent transfections for each construct. For each RNA sample quantified, three independent RT–PCRs were performed with different primer sets; the resulting products were digested with either *PshAI*, *BcgI* or *HpyCH4V* and subjected to electrophoresis on an agarose gel. The intensities of bands corresponding to edited and unedited products were quantified; band intensities were corrected for band size. Editing frequencies reported are means ± s.d. All images were obtained on a Kodak Gel Logic 100 system and were taken under subsaturation conditions. Data were quantified with 1D v.3.6 image analysis software (Kodak).

Mutations indicated in Figs 2–4 were introduced with PAGE-purified mutagenic primers (about 50 nucleotides in length) (IDT) using the QuikChange II XL Site-directed Mutagenesis Kit (Stratagene). Mutations were induced on the full-length editing constructs in pMT-V5/His. All mutagenized templates were subject to 12–14 rounds of amplification, and the resultant transformed mutants were subjected to sequence analysis of the entire insert to confirm the mutations and lack of secondary mutations.

Received 25 October 2004; accepted 14 January 2005; doi:10.1038/nature03364.

1. Bass, B. L. (ed.) *RNA Editing* (Oxford University Press, Oxford, 2001).
2. Seeburg, P. & Hartner, J. Regulation of ion channel/neurotransmitter receptor function by RNA editing. *Curr. Opin. Neurobiol.* **13,** 279–283 (2003).
3. Hoopengardner, B., Bhalla, T., Staber, C. & Reenan, R. Nervous system targets of RNA editing identified by comparative genomics. *Science* **301,** 832–836 (2003).
4. Bhalla, T., Rosenthal, J. J., Holmgren, M. & Reenan, R. Control of human potassium channel inactivation by editing of a small mRNA hairpin. *Nature Struct. Mol. Biol.* **11,** 950–956 (2004).
5. Herb, A., Higuchi, M., Sprengel, R. & Seeburg, P. Q/R site editing in kainate receptor GluR5 and GluR6 pre-mRNAs requires distant intronic sequences. *Proc. Natl Acad. Sci. USA* **93,** 1875–1880 (1996).
6. Dawson, T., Sansam, C. & Emeson, R. Structure and sequence determinants required for the RNA editing of ADAR2 substrates. *J. Biol. Chem.* **279,** 4941–4951 (2004).
7. Higuchi, M. *et al.* RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. *Cell* **75,** 1361–1370 (1993).
8. Bass, B. L. RNA editing by adenosine deaminases that act on RNA. *Annu. Rev. Biochem.* **71,** 817–846 (2002).
9. Tonkin, L. A. *et al.* RNA editing by ADARs is important for normal behavior in *Caenorhabditis elegans*. *EMBO J.* **21,** 6025–6035 (2002).
10. Palladino, M. J., Keegan, L. P., O'Connell, M. A. & Reenan, R. A. A-to-I pre-mRNA editing in *Drosophila* is primarily involved in adult nervous system function and integrity. *Cell* **102,** 437–449 (2000).
11. Higuchi, M. *et al.* Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature* **406,** 78–81 (2000).
12. Gurevich, I. *et al.* Altered editing of serotonin 2C receptor pre-mRNA in the prefrontal cortex of depressed suicide victims. *Neuron* **34,** 349–356 (2002).
13. Kawahara, Y. *et al.* Glutamate receptors: RNA editing and death of motor neurons. *Nature* **427,** 801 (2004).
14. Brusa, R. *et al.* Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. *Science* **270,** 1677–1680 (1995).
15. Vissel, B. *et al.* The role of RNA editing of kainate receptors in synaptic plasticity and seizures. *Neuron* **29,** 217–227 (2001).
16. Aronoff, R., Mellem, J. E., Maricq, A. V., Sprengel, R. & Seeburg, P. H. Neuronal toxicity in *Caenorhabditis elegans* from an editing site mutant in glutamate receptor channels. *J. Neurosci.* **24,** 8135–8140 (2004).
17. Hanrahan, C. J., Palladino, M. J., Ganetzky, B. & Reenan, R. A. RNA editing of the *Drosophila* para Na^+ channel transcript. Evolutionary conservation and developmental regulation. *Genetics* **155,** 1149–1160 (2000).
18. Grauso, M., Reenan, R. A., Culetto, E. & Sattelle, D. B. Novel putative nicotinic acetylcholine receptor subunit genes, Dα5, Dα6 and Dα7, in *Drosophila melanogaster* identify a new and highly conserved target of adenosine deaminase acting on RNA-mediated A-to-I pre-mRNA editing. *Genetics* **160,** 1519–1533 (2002).
19. Lehmann, K. A. & Bass, B. L. The importance of internal loops within RNA substrates of ADAR1. *J. Mol. Biol.* **291,** 1–13 (1999).
20. Yoshihara, M. & Littleton, J. T. Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. *Neuron* **36,** 897–908 (2002).

21. Craxton, M. Genomic analysis of synaptotagmin genes. *Genomics* **77,** 43–49 (2001).
22. Mackler, J. M. & Reist, N. E. Mutations in the second C2 domain of synaptotagmin disrupt synaptic transmission at *Drosophila* neuromuscular junctions. *J. Comp. Neurol.* **436,** 4–16 (2001).
23. Chapman, E. R., Desai, R. C., Davis, A. F. & Tornehl, C. K. Delineation of the oligomerization, AP-2 binding, and synprint binding region of the C2B domain of synaptotagmin. *J. Biol. Chem.* **273,** 32966–32972 (1998).
24. Rickman, C. *et al.* Synaptotagmin interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate. *J. Biol. Chem.* **279,** 12574–12579 (2004).
25. Grass, I., Thiel, S., Honing, S. & Haucke, V. Recognition of a basic AP-2 binding motif within the C2B domain of synaptotagmin is dependent on multimerization. *J. Biol. Chem.* **279,** 54872–54880 (2004).
26. Nakhost, A., Houeland, G., Blandford, V. E., Castellucci, V. F. & Sossin, W. S. Identification and characterization of a novel C2B splice variant of synaptotagmin I. *J. Neurochem.* **89,** 354–363 (2004).
27. Reenan, R., Hanrahan, C. & Ganetzky, B. The mle(napts) RNA helicase mutation in *Drosophila* results in a splicing catastrophe of the *para* Na^+ channel transcript in a region of RNA editing. *Neuron* **25,** 139–149 (2000).
28. Aruscavage, P. & Bass, B. A phylogenetic analysis reveals an unusual sequence conservation within introns involved in RNA editing. *RNA* **6,** 257–269 (2000).
29. Kung, S. S., Chen, Y. C., Lin, W. H., Chen, C. C. & Chow, W. Y. Q/R RNA editing of the AMPA receptor subunit 2 (GRIA2) transcript evolves no later than the appearance of cartilaginous fishes. *FEBS Lett.* **509,** 277–281 (2001).
30. Wheeler, W. C., Whiting, M., Wheeler, Q. D. & Carpernter, J. M. The phylogeny of the extant hexapod orders. *Cladistics* **17,** 113–169 (2001).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements I thank L. Reenan for discussions; B. Hoopengardner, T. Bhalla and A. Das for comments on the manuscript; B. Hoopengardner for sharing certain genomic DNA templates and for assistance with S2 cell culture; UCHC Molecular Core Facility staff for diligent sequencing efforts; and M. Lalande for his encouragement. This work was supported by grants from the National Science Foundation and National Institutes of Health (R.A.R.).

Competing interests statement The author declares that he has no competing financial interests.

Correspondence and requests for materials should be addressed to R.A.R. (rreenan@neuron.uchc.edu).

erratum

Pleistocene to Holocene extinction dynamics in giant deer and woolly mammoth

A. J. Stuart, P. A. Kosintsev, T. F. G. Higham & A. M. Lister

Nature **431,** 684–689 (2004).

In Fig. 4 of this Letter, some of the data were not properly aligned with their location labels. The corrected figure is shown here. □