ARTICLE

Received 13 Feb 2013 | Accepted 2 Jul 2013 | Published 1 Aug 2013

DOI: 10.1038/ncomms3232

Tertiary structural elements determine the extent and specificity of messenger RNA editing

Leila E. Rieder¹, Cynthia J. Staber², Barry Hoopengardner³ & Robert A. Reenan¹

The specificity and extent of RNA editing by ADAR enzymes is determined largely by local primary sequence and secondary structural imperfections in duplex RNA. Here we surgically alter conserved *cis* elements associated with a cluster of ADAR modification sites within the endogenous *Drosophila paralytic* transcript. In addition to the local requirement for a central imperfect RNA duplex containing the modified adenosines, we demonstrate that a secondary RNA duplex containing splicing signals strongly modulates RNA editing. A subtle non-coding mutation, extending base pairing of this accessory helix, confers significant phenotypic consequences via effects on splicing. Through mutation/counter-mutation, we also uncover and functionally replace a highly conserved intronic long-range tertiary pseudoknot that is absolutely required for deamination of one particular adenosine in the central duplex. Our results demonstrate that complex RNA tertiary structures, which may be difficult to predict computationally, form *in vivo* and can regulate RNA-editing events.

¹ Department of Molecular Biology, Cellular Biology, and Biochemistry, Brown University, Providence, Rhode Island 02912, USA. ² Stowers Institute for Medical Research, Kansas City, Missouri 64110, USA. ³ Department of Biomolecular Sciences, Central Connecticut State University, New Britain, Connecticut 06050, USA. Correspondence and requests for materials should be addressed to R.R. (email: robert_reenan@brown.edu).

The spatial folding of RNA *in vivo* poses an experimentally difficult but vital issue for key biological processes. Recognition of double-stranded RNA substrates by proteins depends largely on target structure¹. Such proteins bind through structural motifs seeking generic dsRNA features rather than primary nucleotide sequence. Although recent evidence suggests that dsRNA-binding domains (RBDs) are capable of primary sequence recognition via the shallow minor groove of duplex RNA², three-dimensional RNA structure has a major role in highly specific protein-RNA recognition, supporting diverse cellular functions such as splicing, RNA interference and translation. ADAR (adenosine deaminases that act on RNA) family members possess dsRBDs, recognize diverse RNA substrates and catalyse the deamination of adenosines into inosines in a post-transcriptional process known as A-to-I RNA editing³. As the chemical properties of inosine mimic those of guanine, cellular machines, including the ribosome, interpret inosine as guanine. Therefore, RNA editing effectively causes an adenosine to guanosine change in the primary sequence of mRNAs, potentially recoding the resulting peptides.

A-to-I editing is thought to exist as a mechanism to expand protein repertoires within the nervous system^{4–6}. ADARs are strikingly conserved among metazoa^{3,7} and engineered model organisms lacking ADAR activity display severe neurological phenotypes, including seizures and neurodegeneration^{8–11}. Furthermore, ADARs are localized to the neuronal nucleus¹²

and nucleolus¹³, supporting the role of editing in nascent, immature RNA molecules. One exemplar *Drosophila* neuronal editing substrate is *paralytic* (*para*), an essential action potential sodium channel gene in the fly genome¹⁴. In contrast, nine voltage-gated sodium channel genes ($Na_v1.1$ – $Na_v1.9$) are encoded in the mammalian genome¹⁵. Yet *para* transcripts are extensively alternatively spliced and edited (Fig. 1a), post-transcriptional modifications that result in over 2 million potential sodium channel isoforms¹⁶.

ADAR proteins contain at least one dsRBD, congruent with edited adenosines residing within dsRNA structures¹⁷. Nevertheless, very few ADAR targets have been probed to reveal the extent to which three-dimensional properties contribute to editing specificity. Complicating this picture is the observation that the isolated ADAR catalytic domain, lacking dsRBDs, edits certain target dsRNAs¹⁸, and that mutations in the catalytic domain of human ADAR2 cause altered editing site specificity¹⁹. At the RNA level, ADAR substrates range from perfectly complementary long RNA hairpins (HPs), which are promiscuously hyper-edited at up to 40% of adenosines²⁰, to shorter (~25 bp) imperfectly paired substrates, which are specifically edited at one or a few adenosines²¹.

The menagerie of specifically edited substrates includes simple short HPs, such as the 71-nt stem-loop directing editing at the mammalian GluR-2 R/G site²², as well as comparatively enormous pseudoknotted structures, such as that found in the

Figure 1 | Organization of *para* exon 19 editing sites and ADAR dependence. (a) The *para* locus contains 26 constitutive exons (blue), 13 alternative exons (yellow) and 11 sites of RNA editing (red). The boxed region in (a) is enlarged in (b), showing the three nearby editing sites in constitutive exon 19. (c) Sequence conservation of the region in (b) from 13 species of *Drosophilidae* found in the UCSC Genome Browser. Three regions of intronic homology, the ECS ('editing site complementary sequence'), DCS ('donor-site complementary sequence'), and HP are putative *cis* sequences. (d) The indicated region of the *para* transcript from exon 19 (blue) and the downstream intron (black), folded in Sfold, show the conserved *cis* sequences denoted in (c) involved in double-stranded structures. Edited adenosines are shown in red. (e) Wild-type (Canton-S) editing at the three sites in exon 19 is apparent as mixed A/G peaks in electropherograms from reverse-transcribed *para* mRNA. (f) Editing at these sites is sensitive to dADAR levels, as editing in a hypomorphic *dADAR* mutant (*dADAR*^{hyp}) are greatly reduced. (g) Editing is completely abolished in the *dADAR*^{5g1} null mutant.

Drosophila synaptotagmin-1 transcript, whose elements span thousands of nucleotides²³. Most RNA structures that recruit ADAR proteins involve exonic editing sites that interact via base pairing with complementary conserved intronic *cis* sequences. The diversity of ADAR targets provides a unique source for studying RNA–protein and RNA–RNA interactions.

Containing 11 highly specific editing sites (Fig. 1a), *paralytic* mRNA provides an exceptional opportunity to study enzyme–substrate interactions directing RNA editing. However, previous *in vivo* studies of *paralytic* have only demonstrated an implied necessity for secondary structure²⁴. Here we describe the *in vivo* molecular consequences of RNA structural perturbations, introduced *via* knock-in into the endogenous *para* locus, using homologous recombination (HR)²⁵. These mutations probe conserved *cis* sequences in predicted RNA structures and establish the necessity of imperfect double-strandedness around editing sites. We also reveal the presence of an accessory duplex formed around the splicing-donor site, which may mediate an interaction between RNA splicing and the extent of editing. Finally, our data demonstrate that a highly conserved tertiary pseudoknot (PK) is indispensable in directing ADAR to only one of three nearby adenosines. Replacing the PK with a functional analogue indicates the generality of the mechanism. Our data demonstrate that proximity of editing sites within a central duplex does not imply functional coupling. We show that extent and selectivity of editing can be determined by structural components assembled from distant sequence elements.

Results

RNA structural predictions for RNA-editing sites. The *Drosophila paralytic* transcript possesses three nearby edited adenosines (1–3) in exon 19 (Fig. 1b), apparent as mixed A/G peaks in wild-type electropherograms of RT–PCR products (Fig. 1e), and is predicted to form a dsRNA secondary structure (Fig. 1d). Editing is sensitive to levels of *Drosophila* ADAR (dADAR), as flies carrying a hypomorphic dADAR allele (~80% dADAR reduction)²⁶ show reduced editing levels (Fig. 1f) and dADAR null males⁹ do not edit these sites (Fig. 1g). Editing at these three adenosines is conserved across *Drosophila* species²⁴ (Supplementary Fig. S1).

Previous study of these sites predicted and experimentally validated the minimal sequences necessary for RNA editing²⁴. However, only two species were compared when making these predictions. A more comprehensive study could reveal additional conserved elements. Therefore, we obtained sequences from 37 species of the genus *Drosophilidae* (Supplementary Fig. S2). Sequence alignments revealed several areas of extreme intron conservation (Fig. 1c, Supplementary Fig. S2). The RNA folding algorithm Sfold²⁷ predicted structural features of the *paralytic* transcript in this region, including three striking features corresponding to regions of high sequence conservation (Fig. 1c,d).

The expected central duplex places the three editing sites within a 77 bp-long imperfect duplex formed between the exon and downstream intron possessing homology to the edited region ('editing site complementary sequence,' ECS; Supplementary Fig. S3a). In addition, a highly invariant area of the intron, the 'donor-site complementary sequence' (DCS; Supplementary Fig. S3b), is predicted to form a secondary RNA duplex encompassing the 5'-splice donor site. Finally, a predicted intronic local HP of unknown function is highly conserved in all species surveyed (Supplementary Fig. S3c).

A central RNA duplex is required for RNA editing *in vivo*. Traditional experimental manipulation of RNA-editing structures are usually conducted *in vitro*²⁸ or with mini-gene reporters in

culture^{29,30}. Many of these studies generally recapitulate editing and delineate important *cis* elements. We chose to use ends-out HR to study the *in vivo* details of RNA editing, because precise mutation of genes preserves endogenous gene regulation.³¹

Briefly, the process of HR introduces mutations into the genome, as well as a 76-bp LoxP remnant directed to a region of the intron with low-sequence conservation³¹ (Fig. 2a). The targeting construct encompasses a *mini-white* marker gene flanked by LoxP sites and two homology arms into which the desired mutations are engineered (Fig. 2b). After targeting, Cre-recombinase removes the *mini-white* gene, leaving a single LoxP remnant. A wild-type construct containing no mutations in the *para* locus provides the appropriate control for all other mutants (Figs 2c, 3a and 5a).

We first assessed the role of the predicted ECS by deleting the 39 intronic nucleotides predicted to pair with the edited region. As expected, when the *para* ECS is deleted, editing is completely abolished (Fig. 2d) compared with the LoxP control (Fig. 2c). Although all three edited adenosines require the ECS-directed central duplex, editing at only the first and third sites alter amino acids (Glu to Arg, Asn to Asp, respectively). We next mutated the first and third edited adenosines to guanosines, mimicking complete ADAR modification at these sites (Fig. 2e). This mutation ('GAG') restores predicted base pairing at site 3 (versus an A–C mismatch, Fig. 2c), resulting in a dramatic 2.5-fold increase in editing at synonymous site 2 (Fig. 2f). These data are consistent with previous observations²⁴, which suggest that editing of site 2 is coupled to editing at site 3. In addition, ADAR enzymes prefer editing adenosines neighbored by a 3'-guanosine and a 5'-adenosine³². Adenosines mismatched to a cytosine are also favored³³, suggesting that editing at site 3, an 'ideal' dADAR target, precedes editing at site 2, which may occur as a sequential byproduct under little or no selective pressure.

A duplex containing splicing signals modulates RNA editing.

The highly conserved DCS sequence is predicted to generate a dsRNA structure encompassing the 5'-splice donor (Fig. 3a). We therefore used HR to precisely delete the DCS (24 nt) (Fig. 3b). In 'DCS delete' alleles of *para*, editing is reduced uniformly at all three sites compared with the LoxP control (Fig. 3d, Supplementary Fig. S4a,b). We considered the possibility that the wild-type DCS duplex retards splicing, directly or indirectly, thereby enhancing editing. As the ECS is within the intron, more efficient splicing in DCS delete may disfavour editing by removal of the intron.

Next, we mutationally extended the DCS duplex by 9 bp ('DCS zip'), hypothetically stabilizing the DCS duplex. The DCS zip mutation (Fig. 3c) resulted in substantially increased editing at all three sites (Fig. 3d, Supplementary Fig. S4c). Our data support *in vitro* experiments documenting the interplay between editing and splicing³⁴. When the splicing machinery recognizes the donor site, the intron is removed and further processing, maturation and export should decrease the likelihood that dADAR recognizes the transcript due to loss of double-strandedness. Further, *para* transcripts with a wild-type intron or deleted for the DCS were properly spliced, whereas those from the DCS zip allele exhibited improper splicing (Fig. 3e). Sequencing of aberrant products in DCS zip animals revealed cryptic splice donor-site activation within constitutive exon 19 and upstream alternative exon h (Fig. 3f). All of these variants result in frameshift mutations and, if translated, substantially truncated channel proteins. Our observations strongly suggest that the DCS is involved in a structure that influences splicing, indirectly modulating editing levels in the central RNA duplex by influencing intron removal.

Figure 2 | Editing mutations introduced by HR demonstrate necessity of central duplex. (a) The region of the *para* locus targeted by HR encompasses exon 19 and the putative *cis* elements (black). (b) The targeting construct for HR involves two ‘arms’ (white), homologous to the endogenous *para* locus and bordered by unique restriction sites. A *mini-white* reporter gene flanked by LoxP sites (red) is situated between the arms and targeted to a region in the intron of low sequence conservation. (c) As a proper control, a construct containing wild-type arms is targeted to create the LoxP control line, which is wild-type except for the LoxP remnant in the intron. Editing at the three sites occurs in this control. (d) When the 39 nt ECS intronic *cis* sequence is deleted through HR (‘ECS delete’), editing is completely abolished at all three adenosines. (e) The ‘GAG’ mutation replaces the non-silent first (Glu > Arg) and third (Asn > Asp) adenosines with guanosines (green carrots), resulting in sodium channel peptides representing only the completely edited form. (f) The GAG mutation (red) causes editing at the silent site 2 to increase compared with the LoxP control (grey). Bars represent 95% confidence intervals from three biological replicates (*** $P < 0.0001$, one-way ANOVAs with Dunnett *post-hoc* tests ($\alpha = 0.05$), please see Supplementary Fig. S10a for raw data and specific *P*-values).

We performed quantitative RT-PCR on RNA extracted from DCS zip and LoxP control animals (Supplementary Fig. S5a). This analysis revealed a significant decrease in full-length spliceoform in the DCS zip mutants compared with the LoxP control, while the presence of several aberrant spliceoforms was significantly increased in the mutant animals (Supplementary Fig. S5b). In addition, the DCS zip allele also gives rise to significantly more total *para* transcript than control, suggesting that transcription from the *para* locus is upregulated in DCS zip animals, perhaps to compensate for low levels of properly spliced *para* transcript and Para sodium channel protein.

The *para* locus encodes the only action potential-generating sodium channel gene in *Drosophila*¹⁴, and excitability of the fly nervous system is highly sensitive to *para* dosage³⁵. Animals hemi- or homozygous for temperature-sensitive (ts) *para* alleles, which reduce *para* expression, display ts-paralytic phenotypes,

while null alleles of *para* result in unconditional lethality. Therefore, we assessed the phenotypic consequences of altered *para* splicing in our DCS zip mutant. *Drosophila* hemi- or homozygous for DCS zip show a significant ts-paralytic phenotype (Fig. 4a, Supplementary Fig. S5c), similar to other *para*^{ts} alleles. To investigate the dosage of full-length functional *para* transcript originating from the DCS zip allele (Fig. 3c), we crossed females homozygous for the DCS zip allele to males null for endogenous *para* on the X chromosome, rescued by a duplication of *para* translocated to chromosome four. The lethality of female progeny from this cross demonstrates that the DCS zip allele is haploinsufficient. However, female siblings carrying the wild-type *para* duplication on chromosome four were rescued for viability (Fig. 4b). Interestingly, although males contain only one X chromosome, males hemizygous for the DCS zip allele are viable, likely due to the upregulation of genes on the

Figure 3 | Mutations in the donor-site complementary sequence (DCS) affect editing and splicing. (a) In the LoxP control, the wild-type DCS intronic sequence is predicted to involve the donor site in secondary structure. (b) The ‘DCS delete’ mutation excises the conserved 24 nt DCS *cis* sequence from the intron. (c) Seven intronic nucleotides (green) are added to the wild-type DCS (‘DCS zip’), and are predicted to result in a more stable secondary structure around the splice donor. (d) Editing at all three sites significantly decreases in the ‘DCS delete’ *Drosophila* lines compared with the LoxP control (grey), whereas editing significantly increases in animals homozygous for the ‘DCS zip’ allele. Bars represent 95% confidence intervals from three biological replicates ($***P < 0.0001$, one-way ANOVAs with Dunnett *post-hoc* tests ($\alpha = 0.05$), please see Supplementary Fig. S10a for raw data and specific *P*-values). (e) PCR was performed on cDNA reverse-transcribed from *para* mRNA. Not all *para* transcripts from the ‘DCS zip’ allele are full-length (FL). Lane 1, 100 bp ladder; lane 2, LoxP control; lane 3, DCS delete; lane 4, DCS zip. Sizes of bands are indicated on the 1.5% agarose gel. (f) Aberrant bands from (e) were isolated and sequenced to determine the activated cryptic donor sites within constitutive exon 19 (blue) and alternative exon h (yellow). Primers (green) in exons h and 20 produce the full-length PCR product (a) from the wild-type splice donor. Aberrant shorter products (b–d) are formed owing to activation of cryptic alternative donor sites in exons h and 19.

X chromosome through the process of dosage compensation³⁶. In summary, the extent of the DCS/donor-site duplex appears to modulate the degree of RNA editing. The level of editing in the central duplex can be ‘tuned’ up or down, and we propose that this is an effect modulated directly or indirectly through effects on splicing. Subtle changes predicted to strengthen the DCS duplex enhance editing at the molecular level, but do so at the cost of a substantial deleterious phenotypic consequence at the organismal level.

The HP structure is required for editing site selection. The highly conserved intronic HP structure (Fig. 5a) has no obvious relationship to either the ECS or DCS duplexes, and is without precedence in other known ADAR targets. Surprisingly, deletion of the HP preferentially abolished editing at site 1 (Fig. 5g, Supplementary Fig. S4d). Although the nucleotide sequence of the HP stem differs between species, comparative sequence analyses revealed extensive sequence co-variation, maintaining base pairing (Supplementary Fig. S6a,b), suggesting that the structure (Fig. 5b) rather than primary sequence is necessary for editing. In particular, two unpaired bulged regions of the HP stem are almost invariably pyrimidines, and the HP stem therefore resembles a

‘barbell.’ We introduced the ‘HP zip’ mutation, which base pairs most of the bulged pyrimidines, converting the HP stem into a more perfect duplex (Fig. 5c). The HP zip mutation preferentially reduced editing at site 1, indicating the modulatory nature of the unpaired residues in the HP (Fig. 5g, Supplementary Fig. S4e).

The ‘barbell’ structure of the HP is preserved in most species of *Drosophilidae* (Supplementary Fig. S6a,b). However, *D. willistoni* subgroup members lack the HP barbell structure (Supplementary Fig. S6c). We wanted to assess whether this natural variation of the HP, resembling the HP zip synthetic allele, alters editing (Fig. 5c). We compared editing at site 1 to editing at site 3 in species across *Drosophilidae* (Supplementary Fig. S1). Editing at site 1 was significantly decreased in two closely related species, *D. willistoni* and *D. equinoxialis*, which lack the canonical barbell shape (Supplementary Fig. S6c,d). These data further suggest that the barbell shape serves to modulate editing at site 1.

To test whether the HP stem sequence is necessary for editing at site 1, we introduced helix-disrupting mutations in the 5′ or 3′ side of the stem (Figs. 5d,e). The ‘HP stem 1’ and ‘HP stem 2’ mutations both selectively abolish editing at site 1, while combining these two compensatory mutations (‘HP stem double,’ Fig. 5f) rescues editing at the first adenosine (Fig. 5g, Supplementary Fig. S4f–h). Thus, editing at site 1 requires both

Figure 4 | Phenotypic consequences of aberrant splicing from the ‘DCS zip’ allele. (a) Male flies of the indicated genotypes were placed in room temperature glass vials and then submerged in a circulating 39 °C water bath. Time to first paralysis event was recorded over 5 min. Flies hemizygous for the ‘DCS zip’ allele paralyse significantly faster than those with the LoxP control allele (log-rank test; $P < 0.0001$). $N = 105$ flies per genotype (biological replicates). Ribbons represent s.d. (b) Virgin females homozygous for the LoxP control or ‘DCS zip’ alleles were crossed to males with a *para* deletion on the X chromosome (*para*^{lk5}) rescued by a wild-type chromosomal duplication on the fourth chromosome (*para* dup). Allele contributions from each parent are shaded. No female progeny with only one copy of the ‘DCS zip’ *para* allele were found (red shaded box), although this lethality is rescued by the wild-type *para* duplication on the fourth chromosome. Lethality is not observed in male progeny hemizygous for the ‘DCS zip’ allele, a phenomenon that is likely due to dosage compensation of the male X chromosome.

Figure 5 | Editing at site 1 within the central duplex requires the HP element. (a) The highly conserved predicted wild-type HP element. (b) Conservation of the HP across *Drosophilidae*. Black regions of the structure are conserved, while red are variable. Blue represents conserved pyrimidine bulges. Numbers refer to the nucleotides in the loops or base pairs in the stem. (c) The ‘HP zip’ mutation pairs some of the bulged, conserved pyrimidines (green) into the HP stem. (d) The ‘HP stem 1’ mutation introduces a destabilizing *PacI* site into one side of the stem, while (e) the ‘HP stem 2’ mutation introduces the *PacI* mutation from the opposite side. (f) Combining the HP stem 1 and 2 mutations (‘HP stem double’) restores predicted base pairing in the HP stem. (g) Deleting the HP (black) results in a selective loss of editing at site 1 compared to the LoxP control (grey), while pairing the pyrimidine bulges (‘HP zip’; yellow) results in a reduction of editing at site 1 compared with the LoxP control. Both the ‘HP stem 1’ and ‘2’ mutations (orange and red, respectively) selectively abolish editing at site 1, while the restorative double mutation (‘HP stem double’; brown) rescues editing at site 1. Bars represent 95% confidence intervals from three biological replicates (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, one-way ANOVAs with Dunnett *post-hoc* tests ($\alpha = 0.05$), please see Supplementary Fig. S10a for raw data and specific P -values).

the presence of the HP as well as the specific paired and unpaired bases within that structure, while two other adenosines only 10 nucleotides away are unaffected by the presence and integrity of the HP.

A long-range tertiary PK is mediated by the HP. A closer inspection of both the HP primary sequence as well as its potential to contribute to other structural elements was revealing. First, although the HP stem covaries between *Drosophila* species, the HP loop is completely invariant (Fig. 5b, Supplementary Fig. S3c). Second, a short sequence just 3' to the ECS is also completely conserved, is not predicted to base pair and creates a docking site for the HP loop sequence—a potential tertiary PK interaction (Fig. 6a). Such interactions are very difficult to predict

with current RNA folding software programs. Furthermore, there is no precedence for the requirement of a long distance tertiary structural element directing specific RNA editing in a different dsRNA helix.

We were struck by the similarity of the predicted *para* PK to a structural element of the group II self-splicing intron from an extremophilic bacterium³⁷. In this autocatalytic intron, the α/α' kissing-loop interaction scaffolds the formation of a five-way helical junction necessary for self-catalysis (Supplementary Fig. S7)³⁸. The primary sequence of the α/α' kissing-loop interaction differed from that of the *para* HP loop/dock interaction at only three positions. We mutationally converted the HP loop sequence to the α sequence from the kissing-loop interaction (Fig. 6b) and the docking site into that of the α' (Fig. 6c). We found that these mutations, designed to disrupt the putative tertiary PK, independently, selectively and completely abolish editing at *para* site 1 (Fig. 6e, Supplementary Fig. S4i,j). Combining the mutations in the double mutant, recapitulating the naturally occurring α/α' kissing loop within the *paralytic* intron (Fig. 6d), restores editing (Fig. 6e, Supplementary Fig. S4k). Thus, our data demonstrate that the tertiary PK exists *in vivo*, uses a structure similar to a known example, and selectively directs the RNA-editing enzyme to deaminate only one of the three nearby edited adenosines in a separate duplex.

Discussion

The RNA informational complement of only four nucleotides, amplified by simple base-pairing rules, nevertheless allows for an infinite variety of secondary and tertiary structures, surfaces, and chemistries. *In silico* approaches, such as comparative genomics and structural predictions, are useful in identifying base pairing interactions. However, higher order RNA structures may be difficult to predict. Most structural studies of ADAR targets focus on the necessity of a central RNA duplex containing the adenosines destined for modification. In contrast, our study revealed distinct structural elements that have roles in both modulating and specifying sites of RNA editing *in vivo*.

Many A-to-I editing sites are found near intron–exon boundaries, where edited adenosine(s) and splicing signals lie in the same dsRNA region³⁴. The highly conserved DCS/donor-site RNA duplex suggests a balance between splicing and editing; postponing splicing by occlusion of splicing signals would extend time for RNA folding, ADAR binding or both, resulting in increased editing. The deletion of the DCS decreases editing overall and appears to have no deleterious effect on splicing. Thus, the DCS may serve as an editing modulator. Interestingly, the predicted DCS of *Musca domestica* is more extensive and a fourth adenosine is edited within this structure (Supplementary Fig. S8a,b,e) supporting the notion that DCS has a role in splicing and editing. It is tempting to propose that deleting the DCS exerts effects by improving the rate with which splicing signals are accessed by the spliceosome, and thus the rapidity of intron removal. However, further experiments would be necessary to determine the exact role of the DCS.

We previously reported that the ‘no action potential temperature-sensitive’ (*mle^{napts}*) mutation in the maleless dsRNA helicase results in a ‘splicing catastrophe’²⁴, causing exon skipping due to failure to utilize the 5'-donor site of exon 19, and a ts-paralytic phenotype due to lack of sufficient full-length *para* transcripts. The present study further shows that aberrant splicing of *para* and ts-paralysis can be caused by increasing base pairing of the structure encompassing the same 5'-donor site. The DCS zip mutation adds 7 intronic nucleotides 271 nucleotides from the splice donor (Fig. 3c), extending the DCS/donor-site helix. Even this modest non-coding change results in a dramatic increase in

Figure 6 | The HP is involved in a tertiary PK that directs selective editing in the central duplex and can be functionally replaced. (a) The predicted intronic tertiary PK is composed of seven base pairs. Exon, blue; intron, black; editing sites, red. (b) The ‘Loop > α ’ mutation alters three nucleotides in the HP loop (green) so that the sequence recapitulates the α sequence from the group II self-splicing intron. (c) The ‘Dock > α' ’ mutation introduces three nucleotide changes (green) in the docking site of the predicted *para* PK, altering the sequence into that of the self-splicing intron α' sequence. (d) The double ‘Loop/dock > α/α' ’ mutation restores the predicted PK interaction using the α and α' sequences (green) from the group II self-splicing intron kissing-loop interaction. (e) Both the ‘Loop > α ’ and the ‘Dock > α' ’ mutation (light and dark purple, respectively) abolish editing at site 1, while the double mutation, ‘Loop/dock > α/α' ’ (blue) rescues editing at this site compared to the LoxP control (grey). Bars represent 95% confidence intervals from three biological replicates (***) $P < 0.0001$, one-way ANOVAs with Dunnett *post-hoc* tests ($\alpha = 0.05$), please see Supplementary Fig. S10a for raw data and specific *P*-values).

RNA editing at the cost of cryptic donor-site activation (Fig. 3f). Owing to this aberrant splicing, we hypothesize that fewer functional sodium channel transcripts are produced (Supplementary Fig. S5b), decreasing channel expression.

One interesting difference between this phenomenon and the effect of the *mle^{napt}* mutation is that correctly spliced *para* transcripts in *mle^{napt}* show decreased levels of RNA editing. We do not see this as being inconsistent with a splicing interaction, as the mutant Mle protein produced from *mle^{napt}* was proposed to 'stall' on the dsRNA structure. The stalled helicase could potentially sterically interfere with ADAR binding, which would not be the case in the DCS zip allele, in which increased editing is seen.

An intronic duplex distant from splicing signals in the mammalian Gabra-3 transcript was recently reported to stimulate specific editing²⁸. The authors hypothesized that the duplex serves to generically increase ADAR concentration locally, differing from our proposed mechanism.

Sodium channel abundance affects *Drosophila* behaviour, development, reproduction and aging³⁹. Flies hemi- or homozygous for the DCS zip allele are ts-paralytic (Fig. 4a, Supplementary Fig. S5c), consistent with *para* alleles that confer phenotypes due to loss of *para* activity¹⁴. The substantial increase in editing in the DCS zip mutant comes at the expense of splicing defects, explaining why the DCS is not larger in size than observed. Splicing and editing occur co-transcriptionally⁴⁰, and recent evidence points toward a role for RNA structure in splicing^{41,42}.

Our data suggest that an RNA duplex sequesters splicing signals and finely tunes RNA editing levels by directly or indirectly influencing splicing (Fig. 7a). A slow polymerase could also indirectly influence intron removal. Interestingly, editing levels increase in *Drosophila* carrying a mutant 'slow' RNA polymerase II compared with Canton-S wild-type animals

(Fig. 7b–c). The C4 point mutation confers a slower elongation rate on RNA polymerase II, and affects alternative splicing of endogenous genes in both human cells and *Drosophila*⁴³. We speculate that slower constitutive splicing (due to slowed polymerase) results in an increased level of editing.

The physiological importance of RNA editing on Para protein function is unknown, although editing occurs near sites implicated in human SCN1A-based epilepsy. Nevertheless, we assayed behavioural phenotypes in mutants that encode the extremes of editing: the GAG mutant (fully edited), resulting in Para channels with Arg and Asp residues inserted, and the ECS delete mutant (fully unedited), resulting in genomically encoded Glu and Asn residues. These two mutations conferred no detectable locomotor, diurnal or mating behavioural phenotypes (Supplementary Fig. S9). We conclude that RNA editing at these sites results in subtle alterations in channel function and behaviour that are too subtle for our methods of detection.

Complex tertiary interactions that extend base pairing to extrahelical and non-canonical base pairs in dsRNA structures are frequently essential to function, and are exemplified by the PK. For example, protein synthesis relies on a central PK in 30S ribosomal RNA to scaffold three major structural domains⁴⁴. Likewise, telomerase depends on a core PK within its RNA component⁴⁵. PKs also figure prominently in viral mRNAs during the process of ribosomal frameshifting⁴⁶. The PK interaction we report here is unique in directing specific RNA editing. Length and helix discontinuities (loops and bulges) within central RNA duplexes are usually thought to limit ADAR activity to specific adenosines⁴⁷.

We propose that the exon/ECS duplex is inconsistent in either size or structure with a standard binding of dADAR to the duplex for editing at site 1, and necessitated the evolution of a more complex structure to orchestrate binding of dADAR for site 1 editing (Fig. 7d,e). *M. domestica* and *Sarcophaga bullata* show

Figure 7 | A model for paralytic pre-mRNA editing and splicing. (a) Editing (red) and splicing (blue) are inversely correlated. When splicing is very slow or inefficient, editing is high, for example, in the *para* 'DCS zip' allele. If splicing is rapid or highly efficient, the paralytic structure is quickly resolved, resulting in lower levels of editing, for example, transcripts from the *para* 'DCS delete' allele. (b) Editing in the 'slow' RNA polymerase II mutant fly is higher at all three sites than in Canton-S (c). (d) dADAR dsRNA-binding domains (orange) bind to only the ECS to edit sites 2 and 3 into inosine (red). This occurs before the splicing machinery (blue) recognizes the splice donor. (e) dADAR binds to the ECS and HP duplexes to correctly position the catalytic domain (red) to edit *para* site 1.

RNA editing at sites 1–3 (Supplementary Fig. S8a,d), and comparative genomics reveal a conserved HP, located between DCS and ECS elements, with an invariant eight-nucleotide loop (Supplementary Fig. S8a–c). Curiously, the loop sequence differs from that seen in *Drosophilidae*, and no obvious conserved docking site was detected.

The *paralytic* PK interaction reported herein encompasses seven Watson–Crick base-pairing interactions. Although the HP loop and docking site are absolutely conserved in all studied *Drosophilidae* species (Fig. 5b, Supplementary Fig. S3c), replacing PK sequences with a naturally occurring motif supported editing (Fig. 6e). The kissing-loop interaction in the group II intron and the *para* HP loop/docking site interaction share 5/8-nucleotide identity, raising the question of whether there may be a constraint on this tertiary structural motif. Although the *Drosophila para* PK sequences are invariant, the α/α' sequences from group II introns do co-vary⁴⁸. Indeed, seven base pairs is thought to be a minimum required for rapid nucleotide annealing⁴⁹, although certain kissing loops comprising as little as two base pairs can have surprising stability⁵⁰.

Another persistent question is whether this novel structural arrangement is subject to novel regulatory inputs, especially from the environment. For example, might this type of site be sensitive to temperature, cellular stress or even function to sense metabolites in a manner similar to a riboswitch?

Recent effort in the editing field has focused on identification of new editing sites via RNAseq, thereby compiling comprehensive ‘inosinomes’⁵¹. Yet these studies are often controversial⁵², and pose substantial analytical challenges due to false positives⁵³. Editing is implicated in an expanding list of diseases, including breast cancer⁵⁴, suicidal depression⁵⁵ and amyotrophic lateral sclerosis (ALS). Substantial evidence points toward ADAR2 misregulation in motor neurons of ALS patients⁵⁶, and recent identification of a hexanucleotide repeat associated with ALS⁵⁷ has strengthened the hypothesis that aberrant RNA processing is involved in ALS, possibly through the sequestration of RNA-binding proteins, such as ADAR, with irregular transcripts in inclusion bodies⁵⁸.

Broadly, our observations suggest that proximity of editing sites does not imply functional coupling. Future efforts to find polymorphisms affecting editing levels need not assume that canonical ECS elements are sufficient to determine editing extent and specificity.

Lastly, our data on complex tertiary interactions could assist in the design of artificial editing substrates, enabling the co-option of endogenous ADAR enzymes as tools in specific RNA therapies. For example, a 937-bp antisense sequence designed to trigger RNA editing and degradation of the HIV *env* transcript, is already in clinical trials⁵⁹. Further, the adenosine preference of the human ADAR protein is mutable¹⁹, and the enzyme itself therefore presents a possible target for genetic disease therapy.

Methods

Sequence alignments. Sequence alignments were performed using the ClustalW multiple sequence alignment package included with MacVectortm 7.2 (Accelrys Inc.). Open and extend gap penalties were set to 1.0. The 37 species alignment was then used for the cladogram in the Supplementary material. The cladogram was generated using the neighbor-joining method, bootstrap (1,000 reps).

RNA structural predictions. Structural predictions for regions encompassing all elements and the HP element alone were performed using the SFOLD web-based algorithm (<http://sfold.wadsworth.org/cgi-bin/index.pl>) using the SRNA package to generate general features and output for statistical RNA folding.

Drosophila stocks. Stocks were maintained at 25 °C under 12-h light/dark cycles on standard cornmeal molasses food. The ‘slow’ RNA polymerase mutant *Drosophila* line ‘RpII215^{C4}’ was obtained from Bloomington Stock Center (no. 3663).

Y.A. Savva and J.E.C. Jepson provided the *dADAR^{hyp}* and *dADAR^{5G1}* *Drosophila* lines B. Ganetzky supplied the *para^{lks}*; *para dup*/Ci stock.

Ends-out HR of the *paralytic* locus. We performed ends-out HR after Staber *et al.*³¹. This technique involves two 2.5 Kb arms homologous to the *para* locus and flanked by recognition sites for the Flp and I-Sce I endonucleases. We cloned and sequenced two homology arms in pTOPO (Life Technologies) and then ligated them into a p[w25.2] targeting vector. Primers used for Arm 1 were: PTMARM1-F, 5'-TCGTACGCTGTGCGGAGTAGTGAATCATCTTAG-3'; PTMARM1-R, 5'-TGGCGCGCCAGAGCGGAGCAAGAAATTCATCGG-3'.

Primers used for Arm 2 were: PTMARM2-F, 5'-TGGTACCGAGAAGATACTATGTATTTGGTAGC-3'; PTMARM2-R, 5'-TGCGGCCGCGACCAATCGTGTGTCATGTATGGTTCC-3'.

We achieved mutations in the desired region using the Quik Change II XL Site-directed Mutagenesis Kit (Agilent Technologies). The mini-gene *white⁺*, situated between the arms and flanked by LoxP sites, acts as a selectable eye colour marker and is removed later with Cre-recombinase. The vector was introduced into the *Drosophila* genome by injection (Genetic Services, Inc.).

We targeted the endogenous *para* locus so as to generate multiple independent events. We removed the *white⁺* marker from each line by crossing in Cre-recombinase and isolating individuals carrying targeted alleles with a single LoxP remnant. We then validated targeted alleles via Sanger sequencing (University of Wisconsin Biotechnology Center).

RNA-editing analyses. For each mutation analysed we performed six analyses, two PCRs each from three independent *Drosophila* HR lines. Species-specific editing data were obtained from three PCR replicates derived from a single RNA sample. For each sample, we extracted RNA from male heads ($N = 15–20$) using Tri Reagent (Molecular Research Center, Inc.). We amplified cDNAs via RT-PCR using a *para*-specific primer (Supplementary Table S1), performed PCR and electrophoresed samples on agarose gels. Species-specific editing data were obtained by designing PCR and RT primers to regions of homologous sequence (Supplementary Table S1). We cleaned the products using Wizard Gel and PCR Cleanup Kits (Promega) and detected editing by Sanger sequencing (University of Wisconsin Biotechnology Center) using a site-specific primer (Supplementary Table S1). We determined editing ratios by measuring the area under select A and G nucleotide traces in Adobe Photoshop.

Identification of cryptic splice site activation. We cleaned PCR products, as above, and sequenced individual products.

Temperature-sensitivity paralysis assay. We raised *Drosophila* at room temperature (18 °C) and tested all animals within 24 h after eclosion. We mouth pipetted single flies into room temperature glass vials, which we then submerged in a circulating 39 °C water bath. We measured time from submersion until first detectable paralytic event, defined as 5 s lying on the back or (rarely) side. We tested 105 flies per genotype (35 each from three independent HR lines) per sex.

Quantitative RT-PCR assay. We isolated mRNA from 40 male *Drosophila* heads per biological replicate using oligo d(T)₂₅ magnetic beads (New England Biolabs). We normalized and reverse-transcribed the mRNA using the iScript cDNA Synthesis Kit (Bio-Rad). We then performed quantitative RT-PCR using the SYBR Green PCR Master Mix (Applied Biosystems) and ran plates on the 7500 Fast Real-Time PCR System machine (Applied Biosystems). We performed three technical replicates per biological replicate, and three biological replicates for both the LoxP control and DCS zip lines. We used primers specific to the mature GAPDH transcript to normalize the data from replicates (Supplementary Table S1). To detect total *para* transcript, we designed primers specific to the 3' most exon–exon junction of the mature *para* mRNA. To detect spliceforms, we designed primer pairs within exons 19 and 20 (see Supplementary Fig. S5a, Supplementary Table S1). We performed melting curves on each PCR product to ensure primer specificity.

Activity analysis. Multiple lines of HR-constructed flies were first backcrossed to Canton-S for five generations. Three lines of each genotype were used in subsequent behavioural assays. Total activity was measured using individual activity monitors (TriKinetics). Each monitor consists of a horizontal glass capillary bisected by an infrared beam. Activity is quantified as the total number of beam breaks in 30-min bins over a 24-h period. Flies of the indicated genotypes were raised in 12-h/12-h light/dark conditions. Individual male flies (3–5 days post-eclosion) were placed in monitors and allowed to acclimate for 12 h before data collection. Data were collected and averaged over 3 days.

Mating analysis. Mating analyses were conducted between 0700 and 0900 hours, by a single observer blind to male genotype. Virgin males of the indicated genotypes and Canton-S virgin females were isolated in un-yeasted vials and aged 4 days. During each assay, the male was acclimated to the mating chamber for 5 min

before the female was added. All assays were conducted for 10 min or until successful copulation. Latency is defined as time before male orientation. Time spent courting is the proportion of the total assay time that the male spent following, singing, dancing, tapping and licking the female. Courting time before copulation is defined as the total time from the start of the assay until copulation by successful males.

Statistical analyses. For editing analyses, we performed one-way ANOVAs ($\alpha = 0.05$) followed by Dunnett *post-hoc* tests. $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$. All experimental lines were compared to LoxP control lines. For specific *P*-values please see Supplementary Fig. S9. For paralysis analyses, we performed log-rank tests on the raw data. We performed one-way ANOVAs on qRT-PCR data after Rieu and Powers⁶⁰.

References

- Steffl, R., Skrisovska, L. & Allain, F. H. RNA sequence- and shape-dependent recognition by proteins in the ribonucleoprotein particle. *EMBO Rep.* **6**, 33–38 (2005).
- Masliyah, G., Barraud, P. & Allain, F. H. RNA recognition by double-stranded RNA binding domains: a matter of shape and sequence. *Cell. Mol. Life. Sci.* **70**, 1875–1895 (2012).
- Savva, Y. A., Rieder, L. E. & Reenan, R. A. The ADAR protein family. *Genome Biol.* **13**, 252 (2012).
- Stapleton, M., Carlson, J. W. & Celniker, S. E. RNA editing in *Drosophila melanogaster*: new targets and functional consequences. *RNA* **12**, 1922–1932 (2006).
- Hoopengardner, B., Bhalla, T., Staber, C. & Reenan, R. Nervous system targets of RNA editing identified by comparative genomics. *Science* **301**, 832–836 (2003).
- Rosenthal, J. J. & Seeburg, P. H. A-to-I RNA editing: effects on proteins key to neural excitability. *Neuron* **74**, 432–439 (2012).
- Nishikura, K. Functions and regulation of RNA editing by ADAR deaminases. *Annu. Rev. Biochem.* **79**, 321–349 (2010).
- 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).
- 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).
- Tonkin, L. A. & Bass, B. L. Mutations in RNAi rescue aberrant chemotaxis of ADAR mutants. *Science* **302**, 1725 (2003).
- Wang, Q., Khillan, J., Gadue, P. & Nishikura, K. Requirement of the RNA editing deaminase *ADAR1* gene for embryonic erythropoiesis. *Science* **290**, 1765–1768 (2000).
- Savva, Y. A. *et al.* Auto-regulatory RNA editing fine-tunes mRNA re-coding and complex behaviour in *Drosophila*. *Nat. Commun.* **3**, 790 (2012).
- Desterro, J. M. *et al.* Dynamic association of RNA-editing enzymes with the nucleolus. *J. Cell. Sci.* **116**, 1805–1818 (2003).
- Ganetzky, B. Genetic studies of membrane excitability in *Drosophila*: lethal interaction between two temperature-sensitive paralytic mutations. *Genetics* **108**, 897–911 (1984).
- Meisler, M. H. & Kearney, J. A. Sodium channel mutations in epilepsy and other neurological disorders. *J. Clin. Invest.* **115**, 2010–2017 (2005).
- 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).
- Bass, B. L. & Weintraub, H. A developmentally regulated activity that unwinds RNA duplexes. *Cell* **48**, 607–613 (1987).
- Eggington, J. M., Greene, T. & Bass, B. L. Predicting sites of ADAR editing in double-stranded RNA. *Nat. Commun.* **2**, 319 (2011).
- Kuttan, A. & Bass, B. L. Mechanistic insights into editing-site specificity of ADARs. *Proc. Natl Acad. Sci. USA* **109**, E3295–E3304 (2012).
- Wahlstedt, H. & Ohman, M. Site-selective versus promiscuous A-to-I editing. *Wiley Interdiscip. Rev. RNA* **2**, 761–771 (2011).
- Nishikura, K. *et al.* Substrate specificity of the dsRNA unwinding/modifying activity. *EMBO J.* **10**, 3523–3532 (1991).
- Aruscavage, P. J. & Bass, B. L. A phylogenetic analysis reveals an unusual sequence conservation within introns involved in RNA editing. *RNA* **6**, 257–269 (2000).
- Reenan, R. A. Molecular determinants and guided evolution of species-specific RNA editing. *Nature* **434**, 409–413 (2005).
- Reenan, R. A., Hanrahan, C. J. & 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).
- Gong, W. J. & Golic, K. G. Ends-out, or replacement, gene targeting in *Drosophila*. *Proc. Natl Acad. Sci. USA* **100**, 2556–2561 (2003).
- Jepson, J. E. *et al.* Engineered alterations in RNA editing modulate complex behavior in *Drosophila*: regulatory diversity of adenosine deaminase acting on RNA (ADAR) targets. *J. Biol. Chem.* **286**, 8325–8337 (2011).
- Ding, Y., Chan, C. Y. & Lawrence, C. E. Sfold web server for statistical folding and rational design of nucleic acids. *Nucleic Acids Res.* **32**, W135–W141 (2004).
- Daniel, C., Veno, M. T., Ekdahl, Y., Kjems, J. & Ohman, M. A distant cis acting intronic element induces site-selective RNA editing. *Nucleic Acids Res.* **40**, 9876–9886 (2012).
- Tian, N. *et al.* A structural determinant required for RNA editing. *Nucleic Acids Res.* **39**, 5669–5681 (2011).
- Bhalla, T., Rosenthal, J. J., Holmgren, M. & Reenan, R. Control of human potassium channel inactivation by editing of a small mRNA hairpin. *Nat. Struct. Mol. Biol.* **11**, 950–956 (2004).
- Staber, C. J., Gell, S., Jepson, J. E. & Reenan, R. A. Perturbing A-to-I RNA editing using genetics and homologous recombination. *Methods Mol. Biol.* **718**, 41–73 (2011).
- Lehmann, K. A. & Bass, B. L. Double-stranded RNA adenosine deaminases ADAR1 and ADAR2 have overlapping specificities. *Biochemistry* **39**, 12875–12884 (2000).
- Wong, S. K., Sato, S. & Lazinski, D. W. Substrate recognition by ADAR1 and ADAR2. *RNA* **7**, 846–858 (2001).
- Rieder, L. E. & Reenan, R. A. The intricate relationship between RNA structure, editing, and splicing. *Semin. Cell. Dev. Biol.* **23**, 281–288 (2012).
- Stern, M., Kreber, R. & Ganetzky, B. Dosage effects of a *Drosophila* sodium channel gene on behavior and axonal excitability. *Genetics* **124**, 133–143 (1990).
- Larschan, E. *et al.* X chromosome dosage compensation via enhanced transcriptional elongation in *Drosophila*. *Nature* **471**, 115–118 (2011).
- Toor, N., Keating, K. S., Taylor, S. D. & Pyle, A. M. Crystal structure of a self-spliced group II intron. *Science* **320**, 77–82 (2008).
- Harris-Kerr, C. L., Zhang, M. & Peebles, C. L. The phylogenetically predicted base-pairing interaction between alpha and alpha' is required for group II splicing in vitro. *Proc. Natl Acad. Sci. USA* **90**, 10658–10662 (1993).
- Garber, G., Smith, L. A., Reenan, R. A. & Rogina, B. Effect of sodium channel abundance on *Drosophila* development, reproductive capacity and aging. *Fly* **6**, 57–67 (2012).
- Rodriguez, J., Menet, J. S. & Rosbash, M. Nascent-seq indicates widespread cotranscriptional RNA editing in *Drosophila*. *Mol. Cell.* **47**, 27–37 (2012).
- McManus, C. J. & Graveley, B. R. RNA structure and the mechanisms of alternative splicing. *Curr. Opin. Genet. Dev.* **21**, 373–379 (2011).
- Zhang, J., Kuo, C. C. & Chen, L. GC content around splice sites affects splicing through pre-mRNA secondary structures. *BMC Genomics* **12**, 90 (2011).
- de la Mata, M. *et al.* A slow RNA polymerase II affects alternative splicing in vivo. *Mol. Cell.* **12**, 525–532 (2003).
- Brink, M. F., Verbeet, M. P. & de Boer, H. A. Formation of the central pseudoknot in 16S rRNA is essential for initiation of translation. *EMBO J.* **12**, 3987–3996 (1993).
- Blackburn, E. H. & Collins, K. Telomerase: an RNP enzyme synthesizes DNA. *Cold Spring Harb. Perspect. Biol.* **3**, 1–9 (2011).
- Brierley, L., Pennell, S. & Gilbert, R. J. Viral RNA pseudoknots: versatile motifs in gene expression and replication. *Nat. Rev. Microbiol.* **5**, 598–610 (2007).
- Ohman, M., Kallman, A. M. & Bass, B. L. In vitro analysis of the binding of ADAR2 to the pre-mRNA encoding the GluR-B R/G site. *RNA* **6**, 687–697 (2000).
- Michel, F., Umesono, K. & Ozeki, H. Comparative and functional anatomy of group II catalytic introns—a review. *Gene* **82**, 5–30 (1989).
- Cisse, I. I., Kim, H. & Ha, T. A rule of seven in Watson-Crick base-pairing of mismatched sequences. *Nat. Struct. Mol. Biol.* **19**, 623–627 (2012).
- Chen, A. A. & Garcia, A. E. Mechanism of enhanced mechanical stability of a minimal RNA kissing complex elucidated by nonequilibrium molecular dynamics simulations. *Proc. Natl Acad. Sci. USA* **109**, E1530–E1539 (2012).
- Wulff, B. E., Sakurai, M. & Nishikura, K. Elucidating the inosinome: global approaches to adenosine-to-inosine RNA editing. *Nat. Rev. Genet.* **12**, 81–85 (2011).
- Li, M. *et al.* Widespread RNA and DNA sequence differences in the human transcriptome. *Science* **333**, 53–58 (2011).
- Bass, B. *et al.* The difficult calls in RNA editing. *Nat. Biotechnol.* **30**, 1207–1209 (2012).
- Shah, S. P. *et al.* Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution. *Nature* **461**, 809–813 (2009).
- Lyddon, R., Dwork, A. J., Keddache, M., Siever, L. J. & Dracheva, S. Serotonin 2c receptor RNA editing in major depression and suicide. *World J. Biol. Psychiatr.* **1–12** (2012).
- Hideyama, T. *et al.* Profound downregulation of the RNA editing enzyme ADAR2 in ALS spinal motor neurons. *Neurobiol. Dis.* **45**, 1121–1128 (2012).
- Renton, A. E. *et al.* A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron* **72**, 257–268 (2011).

58. Baloh, R. H. How do the RNA-binding proteins TDP-43 and FUS relate to amyotrophic lateral sclerosis and frontotemporal degeneration, and to each other? *Curr. Opin. Neurol.* **25**, 701–707 (2012).
59. Burnett, J. C., Zaia, J. A. & Rossi, J. J. Creating genetic resistance to HIV. *Curr. Opin. Immunol.* **24**, 625–632 (2012).
60. Rieu, I. & Powers, S. J. Real-time quantitative RT-PCR: design, calculations, and statistics. *Plant Cell* **21**, 1031–1033 (2009).

Acknowledgements

We thank Dr. Y. Savva and Dr. J. Jepson for the *dADAR^{hyp}* and *dADAR^{5G1}* *Drosophila* lines and for manuscript input, M.A. Reyna for assistance with figures, R. Whitaker for qRT-PCR guidance, K. Sierzputowska for Fig. 3e and Dr. E. Larschan for manuscript input. Dr. B. Ganetzky supplied the *para^{rk5}*;*para dup/Ci* stock. This material is based upon work supported by a National Science Foundation Graduate Research Fellowship under grant number (DGE 0228243).

Author contributions

L.E.R. and R.A.R. conceived and designed experiments and L.E.R. performed all experiments. R.A.R. designed, constructed and performed transgenesis of HR constructs. C.J.S. assisted in HR construct transgenesis and mapping. B.H. cloned and sequenced species introns.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>

How to cite this article: Rieder, L. E. *et al.* Tertiary structural elements determine the extent and specificity of messenger RNA editing. *Nat. Commun.* **4**:2232 doi: 10.1038/ncomms3232 (2013).