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RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer–TRBP complex

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MicroRNAs (miRNAs) mediate translational repression or degradation of their target messenger RNAs by RNA interference (RNAi). The primary transcripts of miRNA genes (pri-miRNAs) are sequentially processed by the nuclear Drosha–DGCR8 complex to approximately 60–70 nucleotide (nt) intermediates (pre-miRNAs) and then by the cytoplasmic Dicer–TRBP complex to approximately 20–22 nt mature miRNAs. Certain pri-miRNAs are subject to RNA editing that converts adenosine to inosine (A→I RNA editing); however, the fate of edited pri-miRNAs is mostly unknown. Here, we provide evidence that RNA editing of pri-miR-151 results in complete blockage of its cleavage by Dicer and accumulation of edited pre-miR-151 RNAs. Our results indicate that A→I conversion at two specific positions of the pre-miRNA foldback structure can affect its interaction with the Dicer–TRBP complex, showing a new regulatory role of A→I RNA editing in miRNA biogenesis.

Keywords: RNA editing; ADAR; microRNA; Dicer; RNA interference
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INTRODUCTION

Numerous microRNAs (miRNAs) have been identified in vertebrates, invertebrates and plants. These small noncoding RNAs regulate diverse processes such as development, apoptosis and cancer through the RNA interference (RNAi) mechanism that mediates translational repression or messenger RNA degradation (Bartel, 2004; He & Hannon, 2004; Du & Zamore, 2005; Hammond, 2006). The primary transcripts of miRNAs (pri-miRNAs) are processed sequentially by two RNase III family members, Drosha and Dicer (Kim, 2005). Nuclear Drosha, together with double-stranded RNA (dsRNA)-binding protein DGCR8, initiates cleavage of pri-miRNAs, releasing approximately 60 nucleotide (nt) intermediates (pre-miRNAs), which are then exported to the cytoplasm by exportin-5 and GTP-bound Ran (RanGTP). Cytoplasmic Dicer, with dsRNA-binding protein TRBP (the human immunodeficiency virus transactivating response-RNA binding protein), cleaves the pre-miRNAs to approximately 22 nt RNA duplexes. The presence of a post-transcriptional mechanism that regulates the Drosha cleavage step (Thomson *et al*, 2006) or the Dicer cleavage step (Obernosterer *et al*, 2006) has been recently proposed; however, the details of such regulatory mechanisms remain to be determined.

In addition, recent studies have shown that certain pri-miRNAs undergo RNA editing that converts adenosine residues in dsRNA to inosine (A→I RNA editing; Luciano *et al*, 2004; Blow *et al*, 2006; Yang *et al*, 2006; Kawahara *et al*, 2007). This A→I RNA editing is catalysed by adenosine deaminases that act on RNA (ADARs; Reenan, 2001; Bass, 2002; Nishikura, 2006). Although the significance of pri-miRNA editing remains largely undetermined, processing of pri- to pre-miRNA is suppressed by editing in at least one case (Yang *et al*, 2006). A→I editing of two specific adenosine residues within the foldback dsRNA structure of pri-miR-142 completely blocks cleavage by the Drosha–DGCR8 complex, leading to a reduction in the expression levels of mature miR-142 RNAs (Yang *et al*, 2006). Here, we report that A→I RNA editing of pri-miR-151 at two specific positions within its foldback dsRNA structure completely inhibits its cleavage by the Dicer–TRBP complex, leading to an accumulation of edited pre-miR-151 intermediate RNA. Our results indicate a new function for A→I RNA editing in the regulation of the miRNA biogenesis pathway.

RESULTS AND DISCUSSION

RNA editing of pri-miR-151 in human and mouse tissues

A recent survey of human pri-miRNA sequences identified six pri-miRNAs that undergo A→I editing in various human tissues (Blow *et al*, 2006). Among those found to undergo A→I RNA editing, miR-151 belongs to a class of miRNA that is derived from genomic repetitive sequences—that is, LINE2 (Smalheiser & Torvik, 2005). Both strands of the pri-miR-151 dsRNA are processed, resulting in the expression of miR-151-5p and miR-151-3p RNAs (Kim *et al*, 2004; Fig 1A).

Direct sequencing of reverse transcription–PCR (RT–PCR) products derived from mouse and human pri-miR-151 RNAs identified an extra adenosine residue at the −1 site that underwent A→I RNA editing at relatively low levels (less than 10%; Fig 1A; supplementary Fig 1A online), in addition to the +3 site reported previously for human pri-miR-151 (Blow *et al*, 2006). The editing

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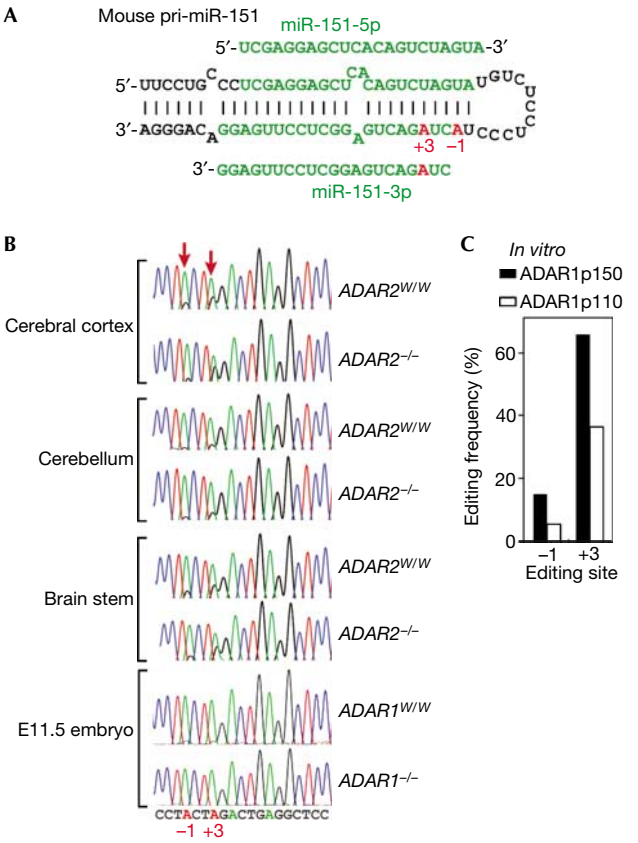


Fig 1 | A→I RNA editing of pri-miR-151 RNA. (**A**) The two editing sites of mouse pri-miR-151 are shown in red. The regions known to be processed into the mature miR-151-5p and miR-151-3p are shown in green. An individual editing site is indicated by a number, with the 5′ end of the mature miR-151-3p sequence counted as +1. (**B**) Editing of pri-miR-151 in mouse brain tissues of wild-type (*ADAR2*$^{W/W}$) and *ADAR2*$^{-/-}$ mice and embryonic day (E) 11.5 wild-type (*ADAR1*$^{W/W}$) and *ADAR1*$^{-/-}$ embryos. DNA sequencing chromatograms of RT–PCR products derived from pri-miR-151 RNAs are shown. An A→I RNA editing site is detected as an A→G change in the complementary DNA sequencing chromatogram. The positions of the two editing sites (−1 and +3) are indicated by red arrows. (**C**) A quantitative summary of the editing efficiency shown by direct sequencing analysis of RT–PCR products corresponding to pri-miR-151 RNAs edited *in vitro* by ADAR1p150 or ADAR1p110. Editing frequency is represented as a percentage estimated from the ratio of 'G' peak over the sum of 'G' and 'A' peaks of the sequencing chromatogram. A→I, RNA editing that converts adenosine to inosine; ADARs, adenosine deaminases that act on RNA; miRNA, microRNA; pri-miRNA, primary transcripts of miRNA genes; RT–PCR, reverse transcription–PCR.

level of the +3 site ranged from high (more than 50%) in human cerebral cortex, amygdala and thymus to low (5–15%) in cerebellum, lung and spleen. Editing of the +3 site was also detected in the fetal tissues that were examined. However, no editing of pri-miR-151 was detected in human HeLa epithelial carcinoma and human embryonic kidney 293 cell lines (supplementary Fig 1B,C online). Editing of mouse pri-miR-151 was restricted to the central nervous systems (Fig 1B), although unedited pri-, pre- and mature miR-151 RNAs were detected in many non-brain tissues, including lung (Fig 2A). Expression levels of enzymatically active *ADAR* gene family members—ADAR1 and ADAR2—among different tissues are similar between humans and rodents (Reenan, 2001; Bass, 2002; Nishikura, 2006); therefore, we have no satisfactory explanation for the absence of pri-miR-151 RNA editing in non-brain tissues of mice.

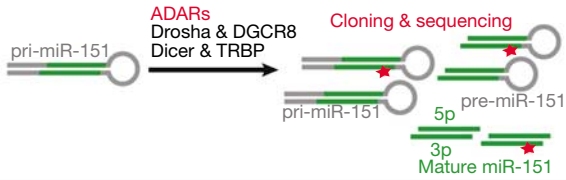
ADAR1 as a responsible enzyme for editing of pri-miR-151

ADAR1$^{-/-}$ mouse embryos die at embryonic day (E) 12.0 owing to widespread apoptosis (Hartner *et al*, 2004; Wang *et al*, 2004), whereas *ADAR2*$^{-/-}$ mice are viable (Higuchi *et al*, 2000). To determine the ADAR responsible for pri-miR-151 RNA editing, we examined the editing frequency in brain tissues of adult *ADAR2*$^{-/-}$ mice and *ADAR1*$^{-/-}$ E11.5 embryos. The levels of pri-miR-151 RNA editing at both −1 and +3 sites in brain tissues of adult *ADAR2*$^{-/-}$ mutant mice were comparable to those of wild-type (*ADAR2*$^{w/w}$) mice. By contrast, no editing was detected in *ADAR1*$^{-/-}$ embryos, whereas low but recognizable levels of pri-miR-151 RNA editing were confirmed in wild-type (*ADAR1*$^{w/w}$) embryos (Fig 1B). Two isoforms of ADAR1—a cytoplasmic 150-kDa protein (ADAR1p150) and a nuclear 110-kDa protein (ADAR1p110)—are synthesized, owing to transcription from different promoters followed by alternative splicing, leading to translation initiation at alternative methionine codons (Patterson & Samuel, 1995). Editing of pri-miR-151 was examined *in vitro* using recombinant ADAR1p150 and ADAR1p110 proteins. The +3 site was edited by both ADAR1p150 and ADAR1p110, but at different levels. In addition, the –1 site was also edited by ADAR1p150 and ADAR1p110 at lower levels compared with the +3 site (Fig 1C). Together, these results indicate that editing of the −1 and +3 sites is probably carried out by ADAR1.

Accumulation of edited pre-miR-151 RNAs in brains

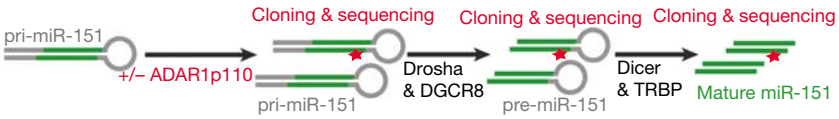
Editing of the −1 and +3 sites replaces an A:U Watson–Crick pair with an I·U wobble pair (Fig 1A). Conversion of an A:U to an I·U pair reduces the overall stability and changes the dsRNA structure, possibly affecting pri- to pre- and/or pre- to mature miR-151 RNA processing pathways. We investigated whether edited pri-miR-151 RNAs would be processed into mature miR-151 RNAs by the strategy designed for the cloning of all unedited and edited pri-, pre- or mature miR-151 molecules (supplementary Fig 2 online). No complementary DNA isolates corresponding to edited mature miR-151-3p RNAs were found among more than 50 clones each examined in human amygdala, mouse cerebral cortex and mouse lung, regardless of the different levels of pri-miR-151 RNA editing detected in these tissues. However, some heterogeneity in the 5′ end of unedited mature miR-151-3p RNAs—that is, 2% extended to the −1 site and 12% to the −2 site in amygdala—was noted (Fig 2A). We then examined cDNA clones corresponding to pre-miR-151 RNAs. Surprisingly, all pre-miR-151 molecules detected in human amygdala and mouse cerebral cortex were completely edited at the +3 site (Fig 2A). A closer examination of the cDNA sequence of the edited pre-miR-151 showed the 3′-end structure expected from cleavage by Drosha, indicating that they are not nonspecific degradation products of edited pri-miR-151 RNAs

A



In vivo		Amygdala (human) (%)	Cerebral cortex (mouse) (%)	Lung (mouse) (%)
	–1 +3			
pri-miR-151	..CUACUAGAC..	59	71	100
	..CUACUIGAC..	38	13	0
	..CUICUAGAC..	0	8	0
	..CUICUIGAC..	3	8	0
pre-miR-151	..CUACUAGAC..	0	0	100
	..CUACUIGAC..	100	94	0
	..CUICUAGAC..	0	0	0
	..CUICUIGAC..	0	6	0
miR-151-3p (5′-end)	CUAGAC..	86	98	96
	ACUAGAC..	2	0	2
	UACUAGAC..	12	2	2

B



In vitro	ADAR1p110	– (%)	+ (%)
	–1 +3		
pri-miR-151	..CUACUAGAC..	100	73
	..CUACUIGAC..	0	23
	..CUICUAGAC..	0	0
	..CUICUIGAC..	0	4
pre-miR-151	..CUACUAGAC..	100	62
	..CUACUIGAC..	0	38
	..CUICUAGAC..	0	0
	..CUICUIGAC..	0	0
miR-151-3p (5′-end)	CUAGAC..	ND	75
	ACUAGAC..	ND	0
	UACUAGAC..	ND	25

C

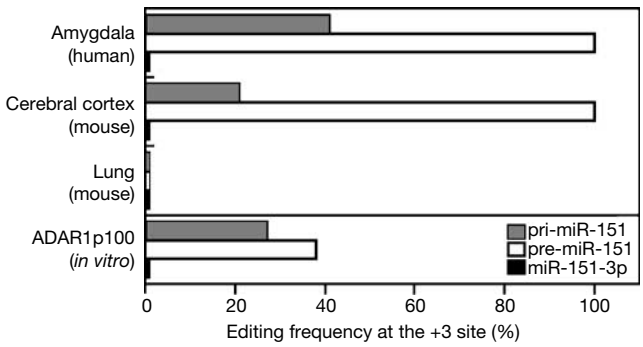


Fig 2 | Characterization of pri-, pre- and mature miR-151 RNAs edited and processed by Drosha and Dicer. (**A**) Editing and processing of endogenous miR-151 RNAs were monitored by cloning and sequencing complementary DNA isolates corresponding to pri-, pre- and mature miR-151 RNAs. (**B**) Analysis of RNA molecules derived from pri-miR-151 RNAs that were edited *in vitro* by ADAR1p110 and then processed by Drosha–DGCR8 and/or Dicer–TRBP complexes. The recombinant ADAR1p110 protein or the Drosha–DGCR8 complex was removed before the next reaction by proteinase K digestion and subsequent phenol extraction. (**A**,**B**) Editing frequency was estimated as the percentage ratio of the cDNA clones containing the A→G change over the total number of cDNA clones examined. The inosine residue introduced by A→I editing at the +3 site is indicated by a red star. (**C**) Summary of *in vivo* and *in vitro* editing and/or processing products of pri-miR-151. ADARs, adenosine deaminases that act on RNA; miRNA, microRNA; pre-miRNA, precursor miRNA; pri-miRNA, primary transcripts of miRNA genes; TRBP, the human immunodeficiency virus transactivating response-RNA binding protein.

(data not shown). By contrast, all pre-miR-151 cDNA isolates derived from mouse lung contained the unedited pre-miR-151 sequence, as expected by the fact that no editing of pri-miR-151 occurred in this tissue (Fig 2A). The results indicate that editing of pri-miR-151 at the −1 and/or +3 site is unlikely to affect the Drosha cleavage step. Furthermore, processing of pre- to mature miR-151 RNAs might be blocked by editing, resulting in the accumulation of edited pre-miR-151 RNAs possibly owing to inhibition of their nuclear export or cleavage by Dicer in the cytoplasm.

Inhibition of Dicer cleavage by editing of miR-151 RNAs

Next, we investigated the effect of editing on miR-151 processing pathways *in vitro*. Individual pri-, pre- and mature miR-151 RNAs that were edited and/or processed by the Drosha–DGCR8 and/or Dicer–TRBP complexes were examined by sequencing of cDNA isolates (Fig 2B). First we examined more than 50 cDNA isolates derived from pri-miR-151 RNAs that were edited *in vitro* by ADAR1p110. We then analysed cDNA clones derived from pre- and mature miR-151 RNAs that were further processed *in vitro* with the purified Drosha–DGCR8 and/or Dicer–TRBP complexes.

Sequencing analysis showed that pri-miR-151 RNA edited only at the +3 site, and at both the −1 and +3 sites represented 23% and 4%, respectively, of the total cDNA isolates examined (Fig 2B). Similarly, analysis of cDNA isolates showed that 38% of pre-miR-151 RNAs were edited at the +3 site, whereas the remaining precursor molecules remained unedited (Fig 2B). The ratio of edited pri- to pre-miRNA molecules detected (0.7) indicates that editing of the +3 site has no inhibitory, if not enhancing, effect on cleavage of pri- to pre-miR-151 by the Drosha–DGCR8 complex (Fig 2C). Although a small fraction (4%) of pri-miR-151 molecules edited at both the −1 and +3 sites was detected, the corresponding pre-miR-151 RNAs edited at both sites were not found among the pre-miR-151 cDNA isolates examined. However, it is unlikely that editing of this minor site has suppressive effects on the Drosha–DGCR8 action because pre-miR-151 RNA edited *in vivo* at both sites was detected in mouse cerebral cortex (Fig 2A). Interestingly, 25% of miR-151-3p RNAs contained two additional nucleotides (AU) extended to the −2 position, as noted for 5′-end analysis of endogenous, mature miR-151-3p RNAs (Fig 2A), indicating ambiguity for *in vivo* and *in vitro* selection of the Dicer cleavage site. However, no cDNA isolates corresponding to mature miR-151-3p molecules edited at the +3 site were detected *in vitro* (Fig 2B,C). This excludes the possibility that endogenous, edited, mature miR-151 RNAs might be generated but rapidly degraded *in vivo*.

Finally, the inhibitory effect of editing at the −1 and +3 sites on Dicer cleavage was tested *in vitro*, using a set of synthetic pre-miR-151 RNAs: one unedited, and the other with A→I substitutions at the −1 and/or +3 site. Precursor RNAs were labelled with ^{32}P either at the 5′ end (supplementary Fig 3 online) or at the +2 site (Fig 3A). The Dicer–TRBP complex cleaved the unedited pre-miR-151 to generate mature miR-151-5p (5′-end-labelled, data not shown) and miR-151-3p RNAs (Fig 3B). By contrast, the A→I substitution at the −1 or +3 site substantially inhibited cleavage of the edited pre-miR-151 by the Dicer–TRBP complex. Editing at both −1 and +3 sites essentially resulted in no processing of mature miR-151 molecules (Fig 3B). Binding of Dicer–TRBP to pre-miR-151 seemed to be unaffected by editing, as seen from a set of representative electrophoresis mobility shift assay (EMSA) gels (Fig 3C). The nearly identical K_d values (approximately 1.5 nM) for binding to unedited and edited (−1 and/or +3 sites) pre-miR-151 RNAs were estimated from analysis of several EMSA gels. These *in vitro* and *in vivo* analyses together showed that Dicer cleavage is the step inhibited by editing of pri-miR-151 at the major +3 and minor −1 sites.

Editing of pre-miR-151

The complete (100%) editing frequency of pre-miR-151 RNAs *in vivo* in human amygdala and mouse cerebral cortex indicates that A→I editing of the +3 site might occur not only on pri-miR-151 but also on pre-miR-151. To test this possibility, further *in vitro* experiments were carried out. *In vitro* prepared pri-miR-151 was first cleaved to pre-miR-151 by the Drosha–DGCR8 complex, followed by editing of the processed products by ADAR1p150 or ADAR1p110. Finally, unedited and edited pre-miR-151 RNA molecules were analysed by cloning and sequencing of cDNA isolates (Fig 4). The results indicated that editing of pre-miR-151 did indeed occur at high levels. For example, 91% of pre-miR-151 RNAs treated with ADAR1p150 were edited at the +3 site, leaving only 9% of the precursor molecules unedited. Editing frequency of pre-miR-151 RNAs at the +3 site by ADAR1p110 was also very high (86%), especially in comparison with that of pri-miR-151 editing by this shorter isoform of ADAR1 (20–30%; Figs 1C,2B). The results indicate that pre-miR-151 could certainly be edited by the nuclear ADAR1p110 and also by the cytoplasmic ADAR1p150.

In summary, we have shown that pri- and pre-miR-151 RNAs are edited at two specific positions, −1 minor and +3 major sites, by ADAR1. Our *in vitro* and *in vivo* studies clearly indicate that A→I editing of pri-miR-151 inhibits cleavage of pre- to mature miR-151 RNAs by the Dicer–TRBP complex, although it has no effect on the processing of pri- to pre-miR-151 by the Drosha–DGCR8 complex. Consequently, no expression of edited mature miR-151 RNAs was detected. Furthermore, analyses of *in vitro* edited/processed or endogenous pre-miR-151 RNAs indicate that editing at the +3 site might occur even after processing of pri- to pre-miR-151 RNAs, possibly by interferon-inducible ADAR1p150 in the cytoplasm (Fig 5).

Editing of pri- or pre-miRNA could have a regulatory role in adjusting mature miRNA expression levels by suppressing their processing, as has been shown for editing of pri-miR-142 and the consequent inhibition of Drosha cleavage (Yang *et al*, 2006), and reported in this study for editing of pri-miR-151 and inhibition of Dicer cleavage. Alternatively, A→I editing might alter the 'effective' miRNA strand (Bartel, 2004; He & Hannon, 2004; Du & Zamore, 2005). Finally, editing of certain pri-miRNAs might result in the expression of edited mature miRNAs and silencing of a set of genes different from those targeted by the unedited miRNAs, as reported for editing of miR-376 cluster RNAs (Kawahara *et al*, 2007). Editing of these miR-376 RNAs occurs within the 'seed sequence'—that is, the 5′ half (+2 to +8) of the miRNA sequence (Kawahara *et al*, 2007) that is important for pairing with the target mRNA (Bartel, 2004; He & Hannon, 2004; Du & Zamore, 2005). Future studies are likely to show other cases of A→I RNA editing that alter the expression and functions of miRNA.

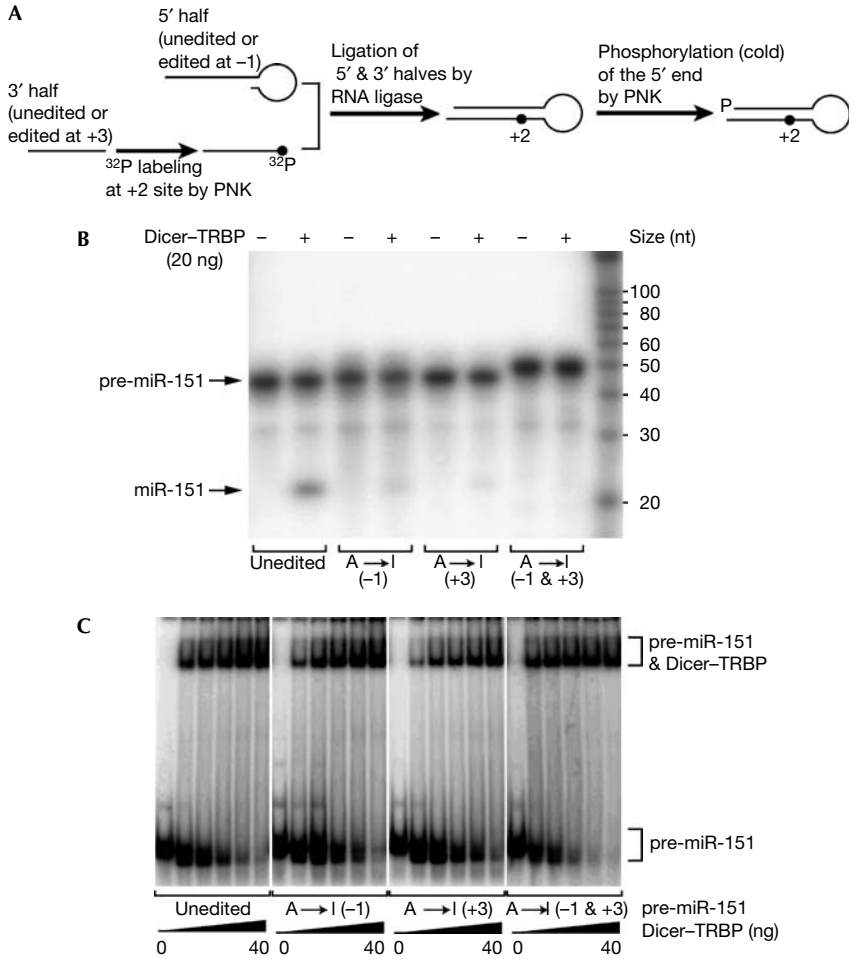
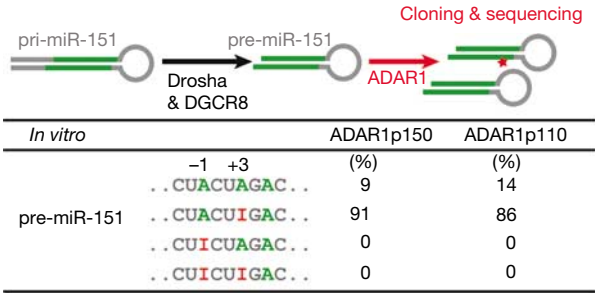


Fig 3 | *In vitro* processing of pre-miR-151 RNAs by Dicer–TRBP. (**A**) Preparation of pre-miR-151 RNAs labelled with ^{32}P specifically at the +2 site is shown. (**B**) *In vitro* Dicer–TRBP cleavage reaction of pre-miR-151. The sizes of the four different pre-miR-151 RNAs prepared seem to be different from each other and shorter than the expected size of 56 nucleotides (nt), perhaps owing to retention of some secondary structure even during denaturing gel electrophoresis (also see Supplementary Fig 3 online). (**C**) Binding of Dicer–TRBP to pre-miR-151. Varying amounts of purified Dicer–TRBP complexes (0, 5, 10, 20, 30 and 40 ng) were examined by electrophoresis mobility shift assay using a native 5% polyacrylamide gel. A→I, RNA editing that converts adenosine to inosine; miRNA, microRNA; PNK, T4 polynucleotide kinase; pre-miRNA, precursor miRNA; TRBP, the human immunodeficiency virus transactivating response-RNA binding protein.



In vitro		ADAR1p150 (%)	ADAR1p110 (%)
	−1 +3		
	..CUACUAGAC..	9	14
pre-miR-151	..CUACUIGAC..	91	86
	..CUICUAGAC..	0	0
	..CUICUIGAC..	0	0

Fig 4 | *In vitro* editing of pre-miR-151 RNAs. *In vitro* editing of pre-miR-151 by ADAR1p150 and ADAR1p110 was monitored by cloning and sequencing of complementary DNA isolates. ADARs, adenosine deaminases that act on RNA; pre-miRNA, precursor miRNA; pri-miRNA, primary transcripts of miRNA genes.

METHODS

Determination of pri-miR-151 RNA editing sites. First-strand cDNA was synthesized using 1 μg of total RNA and miR-151-specific RT primers: HpriRV (5′-AATTCAGTGCCTGGG TGACTCT-3′) for human and MpriRV (5′-TGTTCCAATGG TGAAGTCCAAC-3′) for mouse. The resultant cDNA was then amplified by PCR using PCR primers: HpriFW (5′-TCACAGC TGACTAGCCTTCACC-3′) and HpriRV for human, and MpriFW (5′-TCTCTTGGGTTAGGCATGCTC-3′) and MpriRV for mouse. RT–PCR products were sequenced directly or subcloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) followed by sequencing more than 50 cDNA isolates.

Characterization of pre- and mature miR-151 RNAs. Small RNA (less than 200 nt) was extracted from 50 μg of total RNA using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). Preparation of the cDNA library enriched in small RNA has been

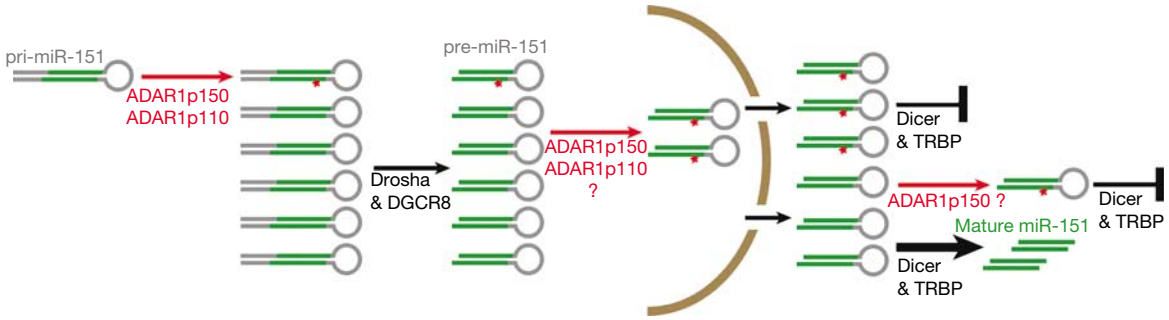


Fig 5 | Effects of pri-miR-151 RNA editing on its processing and expression. The schematic presentation highlights the inhibitory effects of editing on the Dicer–TRBP cleavage step. No detection of unedited pre-miR-151 RNAs in human amygdala and mouse cerebral cortex suggests that *in vivo* cleavage of unedited pre-miR-151 by Dicer–TRBP is highly efficient. Although *in vivo* editing of pre-miR-151 in the nucleus and/or cytoplasm remains to be shown, it might be more efficient than editing of pri-miR-151. ADARs, adenosine deaminases that act on RNA; miRNA, microRNA; pre-miRNA, precursor miRNA; pri-miRNA, primary transcripts of miRNA genes; TRBP, the human immunodeficiency virus transactivating response-RNA binding protein.

described previously (Fu *et al*, 2005; Supplementary Fig 2 online). Briefly, 1.5 μg of small RNA was polyadenylated using the Poly(A) Tailing Kit (Ambion). A 5′ adaptor (5′-CGACUGGAGCACGAG GACACUGACAUGGACUGAAGGAGUAGAAA-3′) synthesized at Dharmacon (Lafayette, CO, USA) was ligated to poly(A)-tailed RNA using T4 RNA ligase, followed by RT using an RT primer: 5′-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30 (A, G or C) (A, G, C or T)-3′. The resultant cDNA was then amplified by PCR. PCR primers for miR-151-3p were as follows: matFW (5′-CTGACATG GACTGAAGGA-3′) and HmatRV (5′-TTTCCTCAAGGAGCTTC A-3′) for human, and matFW and MmatRV (5′-TTTCCTCA AGGAGCCTCA-3′) for mouse. PCR primers for pre-miR-151 were HpreFW1 (5′-CAGTCTAGTATGTCTCATCCCC-3′) and preRV (5′-ATTCTAGAGGCCGAGGCGGCCGACATGT-3′) for human, and MpreFW1 (5′-CAGTCTAGTATGTCTCCTCCC-3′) and preRV1 for mouse. Although these primer sets can amplify PCR products derived from both pri- and pre-miR-151 RNAs, the length of PCR products is different between pri- and pre-miR-151. To be certain, pre-miR-151 was amplified using another set of PCR primers: preFW2 (5′-GGACTGAAGGAGTAGAAATCGA-3′) and HmatRV = HpreRV2 for human, and preFW2 and MmatRV = MpreRV2 for mouse. preFW2 primer contains the first four nucleotides (TCGA) of pre-miR-151 and was confirmed to amplify PCR products from pre-miR-151, but not from pri-miR151. RT–PCR products were subcloned and more than 50 cDNA isolates were sequenced.

***In vitro* RNA editing assay for pri-miR-151.** The plasmids pHpri151 and pMpri151 used for *in vitro* transcription contained 341- and 261-bp genomic DNA fragments of human and mouse pri-miR-151, respectively. The *in vitro* editing reaction mixture, containing 5 fmol of pri-miR-151 RNA and 50 ng of ADAR1p150 or ADAR1p110 protein, was incubated at 30 °C for 60 min, as described previously (Yang *et al*, 2006).

Analysis of *in vitro* edited and processed pri-, pre- and mature miR-151 RNAs. The *in vitro* pri-miRNA processing assay has been described previously (Yang *et al*, 2006). The *in vitro* editing reaction mixture containing recombinant ADAR1 protein was treated with proteinase K and then subjected to phenol extraction to prevent the carryover of protein to the next *in vitro* processing reaction. The *in vitro* edited pri-miR-151 RNA was subjected to the Drosha–DGCR reaction at 37 °C for 60 min followed by proteinase K digestion and subsequent phenol extraction, in some experiments, and then the Dicer–TRBP reaction at 37 °C for 90 min. The reaction products were examined by cloning and sequencing more than 50 cDNA isolates.

Preparation of ^{32}P-labelled pre-miR-151 RNAs. The two 5′ halves (unedited 5′ half, 5′-UCGAGGAGCUCACAGUCUAGUAU GUCUCCUCCCUAC-3′, and 5′ half edited at the −1 site, 5′-UCGAGGAGCUCACAGUCUAGUAUGUCUCCUCCCUIC-3′) and the two 3′ halves (unedited 3′ half, 5′-UAGACUGAGGCU CCUUGAGG-3′, and 3′ half edited at the +3 site, 5′-UIGAC UGAGGCUCCUUGAGG-3′) of mouse pre-miR-151 RNAs were synthesized at Dharmacon (Lafayette, CO, USA). 5′ ends of two 3′ halves of pre-miR-151 were phosphorylated by T4 polynucleotide kinase (PNK) in the presence of 50 μCi [γ-^{32}P]ATP. 5′-^{32}P-labelled 3′ half molecules were then ligated with 5′ half molecules by T4 RNA ligase, resulting in four different pre-miR-151 RNAs (unedited and A→I substitution at the −1 and/or +3 site). These pre-miR-151 RNAs, labelled with ^{32}P specifically at the +2 position, were gel purified and then phosphorylated at the 5′ end by PNK in the presence of 1 mM ATP (cold).

***In vitro* cleavage of synthetic pre-miR-151 by the Dicer–TRBP complex.** Approximately 100 fmol of ^{32}P-labelled pre-miR-151 was subjected to the *in vitro* processing reaction using 20 ng of the Dicer–TRBP complex at 37 °C for 90 min. The reaction products were fractionated on 15% (w/v) polyacrylamide and 8 M urea gel.

RNA binding assays. EMSA was carried out by incubating 50 fmol of ^{32}P-labelled pre-miR-151 RNA with the Dicer–TRBP complex (up to 40 ng) in a 20-μl reaction mixture containing 10 mM Tris–HCl (pH 8.0), 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol and 10% glycerol at 25 °C for 20 min as described previously (Yang *et al*, 2005). The fraction of pre-miR-151 bound to Dicer–TRBP was determined by dividing the radioactivity measured in the pre-miR-151:Dicer–TRBP complex band by the radioactivity in the free and complex bands. K_d is defined as the protein concentration required for 50% binding (Yang *et al*, 2005). The concentration of the Dicer–TRBP complex was estimated by assuming that the complex is a dimer consisting of one molecule each of Dicer (200 kDa) and TRBP (40 kDa).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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COMPETING INTEREST STATEMENT

The authors declare no competing financial interests.

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