Selectively Constrained RNA Editing Regulation Crosstalks with piRNA Biogenesis in Primates

Xin-Zhuang Yang,†,1 Jia-Yu Chen,†,1 Chu-Jun Liu,1 Jiguang Peng,1 Yin Rei Wee,2,3 Xiaorui Han,1 Chenqu Wang,1,4,5 Xiaoming Zhong,† Qing Sunny Shen,1 Hsuan Liu,2,3 Huiqing Cao,1 Xiao-Wei Chen,1,4,5 Bertrand Chin-Ming Tan,‡,2,3 and Chuan-Yun Li*,1

1Beijing Key Laboratory of Cardiometabolic Molecular Medicine, Institute of Molecular Medicine, Peking University, Beijing, China
2Department of Biomedical Sciences and Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan
3Molecular Medicine Research Center, Chang Gung University, Tao-Yuan, Taiwan
4Peking-Tsinghua Center for Life Sciences, Beijing, China
5Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, China

These authors contributed equally to this work.

*Corresponding author: chuanyunli@pku.edu.cn; btan@mail.cgu.edu.tw.

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Abstract

Although millions of RNA editing events have been reported to modify hereditary information across the primate transcriptome, evidence for their functional significance remains largely elusive, particularly for the vast majority of editing sites in noncoding regions. Here, we report a new mechanism for the functionality of RNA editing—a crosstalk with PIWI-interacting RNA (piRNA) biogenesis. Exploiting rhesus macaque as an emerging model organism closely related to human, in combination with extensive genome and transcriptome sequencing in seven tissues of the same animal, we deciphered accurate RNA edithome across both long transcripts and the piRNA species. Superimposing and comparing these two distinct RNA edithome profiles revealed 4,170 editing-bearing piRNA variants, or epiRNAs, that primarily derived from edited long transcripts. These epiRNAs represent distinct entities that evidence an intersection between RNA editing regulations and piRNA biogenesis. Population genetics analyses in a macaque population of 31 independent animals further demonstrated that the epiRNA-associated RNA editing is maintained by purifying selection, lending support to the functional significance of this crosstalk in rhesus macaque. Correspondingly, these findings are consistent in human, supporting the conservation of this mechanism during the primate evolution. Overall, our study reports the earliest lines of evidence for a crosstalk between selectively constrained RNA editing regulation and piRNA biogenesis, and further illustrates that such an interaction may contribute substantially to the diversification of the piRNA repertoire in primates.

Key words: RNA editing, piRNA biogenesis, rhesus macaque, population genetics, whole-genome sequencing, RNA-Seq.

Introduction

RNA editing is a core cotranscriptional process through which nucleotides are modified to generate transcript sequence different from that encoded by the genomic DNA. In the past few years, studies of the RNA editing regulation have been accelerated dramatically by the development of next generation sequencing (NGS) technology, which facilitates genome-wide determination and comparison of DNA and RNA sequences in a precise and cost-effective manner (Li et al. 2009; Ju et al. 2011; Bahn et al. 2012; Peng et al. 2012; Bazak et al. 2014; Chen et al. 2014). Despite the consequent revelation of millions of new RNA editing sites in mammals (largely A-to-I editing), only dozens of editing sites with recoding potential are known to have functional implications (Li et al. 2009). It remains controversial presently as to whether the immensely larger number of editing sites in noncoding regions (>$99.9%) represents functional entity or is merely neutral transcriptional noise (Gommans et al. 2009). To this end, our recent comparative genomics study revealed that these noncoding RNA (ncRNA) editing sites are under evolutionary constraints, lending support to the functional significance of at least a proportion of these sites (Chen et al. 2014). However, the exact biological relevance of these conserved editing events in the noncoding regions remains largely unknown.

Intriguingly, considering the widespread occurrence of RNA editing in repetitive regions (e.g., >95% on primate-specific Alu elements), as well as the testis-biased expression profile of ADAR1 (a member of the adenosine deaminases acting on RNA, or ADAR, family) (Zhang et al. 2013, 2014; Chen et al. 2014), a crosstalk between RNA editing and the germ line-specific, transposons-targeting PIWI-interacting RNA (piRNA) remains a formal but as yet unexplored possibility. piRNAs are a family of small RNA species that was first identified by virtue of association with the PIWI clade of the Argonaute (AGO) proteins (Aravin, Sachidanandam, et al. 2007; Brennecke et al. 2007; Thomson and Lin 2009; Siomi...
et al. 2011; Luteijn and Ketting 2013). Unlike microRNAs (miRNAs) and other endogenous small interfering RNAs (siRNAs), these regulatory RNA molecules exhibit enormous sequence diversity, a predominantly gonadal expression, a strong bias for uridine at position 1 (1U bias), and unique congregation into genomic regions called piRNA clusters (Aravin et al. 2006). Moreover, no particular secondary structures, such as the stem-loop configuration in miRNA precursors, are detected in regions surrounding mature piRNAs (Seto et al. 2007). piRNAs are further distinct from other cellular small RNAs with respect to their biogenesis pathways—the primary piRNAs are first generated by a Dicer-independent processing from long, single-stranded transcripts transcribed from piRNA clusters, and may subsequently be amplified by a secondary, or “ping-pong,” cycle. In the latter mechanism, transcripts complementary to the primary piRNA sequences are cleaved by the Slicer activity of PIWI proteins, producing new secondary piRNAs to the primary piRNA sequences are cleaved by the Slicer cycle. In the latter mechanism, transcripts complementary to the primary piRNAs are cleaved by the Slicer activity of PIWI proteins, producing new secondary piRNAs that have strong bias for adenosine at the tenth nucleotide (10A bias) and further serve as guides for piRNA amplification (Brennecke et al. 2007; Aravin et al. 2008). Functionally, piRNAs and the PIWI proteins form active piRNA-induced silencing complex, a highly conserved mechanism that targets mobile transposable elements in the germ line. This protective function thus provides defense against genome instability and critically underlies gonadal development and organism fertility (Vourekas et al. 2012).

Despite the seemingly straightforward connection between RNA editing and piRNAs, issues such as the restricted expression of these pathways, their distinct association with primate-specific Alu elements, the stringent requirements for high-quality tissue samples across different tissues and individuals, as well as the computational challenges in accurately identifying and verifying these events hamper further understanding of any possible mechanistic interaction between the two regulatory levels in primates.

In this study, we performed this interrogation in rhesus macaque, a close evolutionary relative of human. By combining transcriptome sequencing of multiple tissues from the same animal and its whole-genome sequencing, we deciphered accurate RNA editome across both long transcripts and the piRNA species, and further uncovered editing-bearing piRNA variants (epiRNAs). These epiRNAs are primarily processed from edited long transcripts, representing the regions where the RNA editing regulations potentially intersect piRNA biogenesis and diversify the piRNA repertoire in primates. Our population genetics analyses in human and rhesus macaque populations further showed that these epiRNA-associated RNA editing events are under selective constraints, providing the earliest clues for the functionality of such an editing–piRNA crosstalk in primates.

**Results**

**Accurate and Quantitative Catalogs of RNA Editome and piRNAome in Primates**

Considering the widespread occurrence of RNA editing in repetitive regions and the testis-enriched expression profile of ADAR1 (Chen et al. 2014), we speculated a link of RNA editing to the germ cell-specific piRNA regulation. To consider this possibility, we first profiled an accurate and more comprehensive RNA editome in rhesus macaque, by refining our previously reported RNA editing calling pipeline (Chen et al. 2014) and applying it on the seven-tissue (prefrontal cortex, cerebellum, heart, kidney, lung, muscle, and testis), poly(A)-positive RNA-Seq data of a rhesus macaque animal (100MGP-001) and its whole-genome resequencing data (tables 1 and 2, fig. 1, and see Materials and Methods). In total, 274,054 candidate editing sites were identified by this transcriptome-wide approach (http://www.rhesusbase.org/download/RNAedit/rna_edit_info.xlsx, last accessed September 12, 2015). Seventy-three of the 78 randomly selected candidate sites (93.6%) were experimentally verified by polymerase chain reaction (PCR) amplification and Sanger sequencing of both DNA and the corresponding cDNA (supplementary fig. S1, Supplementary Material online). The high validation rate suggested that most of the sites identified by the refined identification pipeline are verifiable (supplementary fig. S1, Supplementary Material online). In addition, multiple features of these candidate sites further supported that they represent bona fide RNA editing events mediated by ADARs (Ramaswami et al. 2012; Chen et al. 2014): 1) Predominant representation of the A-to-G conversion (98.2%, or 269,087 editing sites) (fig. 2A), 2) prevalent association with the Alu repeat elements (270,985 of 274,054, or 98.9%) (http://www.rhesusbase.org/download/RNAedit/rna_edit_info.xlsx, last accessed September 12, 2015), 3) a conserved local sequence context (fig. 2B), and 4) quantitative correspondence of the tissue-biased profile of the RNA editome to the expression of ADARs (fig. 2C, and see Materials and Methods) (Li and Church 2013; Chen et al. 2014).

We further set out to identify and characterize the piRNA repertoire in rhesus macaque, by performing high-quality small RNA deep sequencing on the corresponding tissues of the same animal (MGP-001) (fig. 1 and table 1). After excluding small RNAs mapped to the annotated ncRNAs in rhesus macaque (see Materials and Methods), a class of small RNAs with length ranging from 24 to 32 bp was observed specifically in testis (fig. 2D and E), represented by 58,571,712 reads (or 24,121,526 unique tags; see Materials and Methods). These small RNAs verified known features of piRNAs in mammals, including testis-exclusive tissue distribution (fig. 2D and E), 5′ uridine bias for the nucleotide composition (fig. 2F), the signature of the ping-pong biogenesis mechanism (fig. 2G) (Aravin, Sachidanandam, et al. 2007; Brennecke et al. 2007; Yan, Hu, et al. 2011), an overrepresentation in intergenic regions (fig. 2H) (Vourekas et al. 2012), as well as the clustered distribution of the small RNAs with identical transcriptional orientation as the long transcripts across the region (fig. 2I and supplementary table S1, Supplementary Material online) (Girard et al. 2006).

To facilitate cross-species comparative analyses, we also performed small RNA-Seq for the corresponding seven tissues from human. piRNAs and piRNA clusters with similar features were identified accordingly (table 1 and supplementary table S1 and fig. S2, Supplementary Material online).
Together, the informative editome and piRNA profiles established across multiple tissues from the same animal, as well as the corresponding data in human, constitute the basis for systematic investigation of the relationship between the two layers of RNA regulation in the context of primate evolution.

**Table 1.** Statistics of the RNA-Seq Data Used in This Study.

| Sample          | Total Reads (M) | Length | Q20 (%) | Mapped (uniquely mapped [%]) | Reference            |
|-----------------|-----------------|--------|---------|------------------------------|----------------------|
| **Small RNA-Seq** |                 |        |         |                              |                      |
| 100MGP-001 Testis | 94.0            | 49 bp  | 100     | 85 (69)                      | This study           |
| 100MGP-001 Prefrontal cortex | 58.2 | 49 bp  | 100 | 83 (17)                     | This study           |
| 100MGP-001 Cerebellum | 62.2 | 49 bp  | 100 | 92 (16)                      | This study           |
| 100MGP-001 Heart | 61.1            | 49 bp  | 100     | 81 (11)                      | This study           |
| 100MGP-001 Kidney | 62.6            | 49 bp  | 100     | 91 (19)                      | This study           |
| 100MGP-001 Lung | 57.8            | 49 bp  | 100     | 83 (18)                      | This study           |
| 100MGP-001 Muscle | 81.8            | 49 bp  | 100     | 85 (18)                      | This study           |
| 100MGP-002 Testis | 96.2            | 49 bp  | 100     | 82 (80)                      | This study           |
| 100MGP-003 Testis | 86.9            | 49 bp  | 100     | 80 (70)                      | This study           |
| 100MGP-004 Testis | 86.3            | 49 bp  | 100     | 85 (58)                      | This study           |
| Human (A) Testis | 94.8            | 49 bp  | 100     | 92 (54)                      | This study           |
| Human (A) Prefrontal cortex | 62.6 | 49 bp  | 100 | 93 (22)                     | This study           |
| Human (A) Cerebellum | 57.0 | 49 bp  | 100 | 93 (23)                     | This study           |
| Human (A) Heart | 70.1            | 49 bp  | 100     | 91 (23)                      | This study           |
| Human (A) Kidney | 71.2            | 49 bp  | 100     | 92 (25)                      | This study           |
| Human (A) Lung | 74.6            | 49 bp  | 100     | 94 (15)                      | This study           |
| Human (A) Muscle | 80.0            | 49 bp  | 100     | 93 (17)                      | This study           |
| Human (B) Testis | 74.3            | 52 bp$^a$ | 100  | 83 (71)                      | This study           |
| 1411H | 68.7            | 51 bp  | 98      | 88 (24)                      | This study           |
| **Poly(A)-positive RNA-Seq** |                 |        |         |                              |                      |
| 100MGP-001 Prefrontal cortex | 142.1   | 90 bp × 2 | 97 | 86 (90)                      | Chen et al. (2014)  |
| 100MGP-001 Cerebellum | 129.0   | 90 bp × 2 | 96 | 87 (93)                      | Chen et al. (2014)  |
| 100MGP-001 Heart | 123.7   | 90 bp × 2 | 97 | 79 (80)                      | Chen et al. (2014)  |
| 100MGP-001 Kidney | 95.7    | 90 bp × 2 | 97 | 84 (86)                      | Chen et al. (2014)  |
| 100MGP-001 Lung | 113.6   | 90 bp × 2 | 97 | 89 (96)                      | Chen et al. (2014)  |
| 100MGP-001 Muscle | 120.0   | 90 bp × 2 | 97 | 82 (91)                      | Chen et al. (2014)  |
| 100MGP-001 Testis | 100.7   | 90 bp × 2 | 97 | 87 (93)                      | Chen et al. (2014)  |
| 100MGP-002 Testis | 128.4   | 100 bp × 2 | 98 | 88 (85)                      | This study           |
| 100MGP-003 Testis | 109.6   | 100 bp × 2 | 98 | 87 (83)                      | This study           |
| 100MGP-004 Testis | 104.6   | 100 bp × 2 | 100 | 84 (80)                      | This study           |
| 1411H siADAR$^b$ | 61.9    | 51 bp  | 99      | 75 (80)                      | This study           |
| 1411H Mock$^c$ | 54.7    | 51 bp  | 99      | 80 (83)                      | This study           |

$^a$Median length of reads by Ion Torrent sequencing.

$^b$1411H cells transfected with ADAR1 siRNAs.

$^c$1411H cells transfected with siRNA negative control.

epiRNAs: A New Class of piRNAs Processed from Edited Long Transcripts

To assess whether RNA editing is associated with piRNAs, we first examined their positional overlap by comparing the genomic locations of both the poly(A)-positive RNA-associated editing sites and the uniquely mapped piRNAs, which presumably represent the loci of piRNA origin. Interestingly, 7,758 A-to-G mRNA editing sites were found to reside in the origin loci of piRNAs, of which 6,357 sites (81.9%) are transcribed in the same strand as piRNAs. Among these sites, small RNA-Seq reads further corroborated A-to-G variation on piRNAs at 1,243 positions, with a total of 3,038 piRNA reads (or 2,150 piRNA tags) harboring these variations (supplementary table S2, Supplementary Material online). Across these piRNA sequences, overrepresentation of the A-to-G variation was evident specifically at the mRNA-edited positions, but not the nonedited positions, suggesting that these variations were derived from A-to-G editing rather than technical errors (fig. 3A).

Although a total of 3,038 uniquely mapped epiRNAs were identified by this initial approach, the size of epiRNA repertoire was likely to be underestimated due to bias related to reads mapping. For instance, considering the pervasive clustering editing (multiple sites in the vicinity), identification of
As a proof of concept, we performed independent experimental verification of the epirNAs by amplifying and sequencing randomly selected epirNAs and their corresponding gDNA and cDNA regions in macaque samples. For example, editing position on chr12:70000511 (rheMac2) was verified to be homozygous A allele in the gDNA sample, whereas in the corresponding cDNA sample it was heterozygous with a G allele at 21.1% frequency. This was in close agreement with the genome sequencing and poly(A)-positive RNA-Seq data (fig. 4A). Small RNAs were also amplified and cloned, and further subjected to sequencing according to a small RNA-specific verification approach (see Materials and Methods). Our data subsequently supported the existence of both epirNAs and the corresponding wild-type piRNA (fig. 4A). We also confirmed the existence of another epirNA spanning clustered editing sites (fig. 4B).

As mature piRNAs are structurally unsuitable for ADARs binding, editing detected on these epirNAs is most likely transmitted from the precursor transcripts that are targeted by ADARs prior to processing. Several lines of evidence further supported this notion: 1) The possibility of observing the edited allele “G” on piRNAs is largely accounted for (67.8%) by using features of the piRNA abundance and the editing levels estimated by short piRNA reads (fig. 3B and see Materials and Methods); 2) the abundance of epirNAs were in accordance with the expression levels of the corresponding long transcripts (fig. 3C), and the editing levels estimated by short piRNA reads were closely commensurate with those of the corresponding long transcripts, as estimated by the poly(A)-positive RNA-Seq reads (fig. 3B and see Materials and Methods); and 3) for long transcripts with clustered editing sites (multiple editing within a 32-bp window), similar combinatorial distributions of editing were detected on the corresponding piRNA reads, an observation that also implies an editing-elicited diversification of piRNA sequences (supplementary table S3, Supplementary Material online).

Table 2. Statistics of the Whole-Genome Sequencing Data Used in This Study.

| Sample                  | Total Reads (M) | Length | Total Bases | Q30 (%) | Mapped Reads (%) |
|-------------------------|-----------------|--------|-------------|---------|------------------|
| 100MGP-001 Prefrontal cortex | 2,718.4 | 90 bp x 2 | 244.7G | 90 | 94 (82) |
| 100MGP-002 Blood         | 905.5 | 151 bp x 2 | 135.8G | 90 | 91 (81) |
| 100MGP-003 Blood         | 786.3 | 151 bp x 2 | 117.9G | 89 | 91 (81) |
| 100MGP-004 Blood         | 1,542.3 | 151 bp x 2 | 230.5G | 90 | 91 (81) |
| 100MGP-005 Blood         | 1,643.5 | 151 bp x 2 | 246.5G | 89 | 91 (81) |
| 100MGP-006 Blood         | 1,450.3 | 151 bp x 2 | 215.7G | 92 | 91 (82) |
| 100MGP-007 Blood         | 1,704.2 | 151 bp x 2 | 255.4G | 89 | 91 (81) |
| 100MGP-008 Blood         | 1,303.5 | 151 bp x 2 | 194.2G | 90 | 91 (81) |
| 100MGP-009 Blood         | 711.8  | 151 bp x 2 | 106.8G | 90 | 91 (81) |
| 100MGP-010 Blood         | 627.6  | 151 bp x 2 | 94.1G  | 90 | 92 (82) |
| 100MGP-011 Blood         | 695.5  | 151 bp x 2 | 104.3G | 90 | 91 (81) |
| 100MGP-012 Blood         | 614.5  | 151 bp x 2 | 92.2G  | 89 | 91 (82) |
| 100MGP-013 Blood         | 736.2  | 151 bp x 2 | 110.4G | 90 | 91 (81) |
| 100MGP-014 Blood         | 704.4  | 151 bp x 2 | 105.7G | 89 | 91 (81) |
| 100MGP-015 Blood         | 679.2  | 151 bp x 2 | 101.9G | 90 | 91 (81) |
| 100MGP-016 Blood         | 697.1  | 151 bp x 2 | 104.6G | 90 | 91 (81) |
| 100MGP-017 Blood         | 706.3  | 151 bp x 2 | 105.9G | 89 | 91 (81) |
| 100MGP-018 Blood         | 826.2  | 151 bp x 2 | 123.9G | 90 | 91 (81) |
| 100MGP-019 Blood         | 892.8  | 151 bp x 2 | 133.9G | 90 | 90 (81) |
| 100MGP-020 Blood         | 728.3  | 151 bp x 2 | 109.2G | 89 | 90 (81) |
| 100MGP-021 Blood         | 855.2  | 151 bp x 2 | 128.0G | 90 | 91 (82) |
| 100MGP-022 Blood         | 676.5  | 151 bp x 2 | 101.5G | 89 | 91 (81) |
| 100MGP-023 Blood         | 706.0  | 151 bp x 2 | 105.9G | 90 | 90 (80) |
| 100MGP-024 Blood         | 750.9  | 151 bp x 2 | 112.6G | 90 | 91 (82) |
The above findings are also correspondingly consistent in human (supplementary fig. S3 and table S2, Supplementary Material online). Taken together, our studies for the first time verified experimentally the existence of epiRNAs in primates, and further showed that they may represent piRNAs generated from previously edited long transcripts. These findings, although not unexpected given the pervasive distribution of A-to-I RNA editing on long RNA transcripts, are actually nontrivial due to multiple technical challenges intrinsic to this type of analyses in primates (see Discussion). Considering the relatively small number of identified epiRNAs, it is then essential to discriminate next whether these epiRNAs represent infrequently degradation fragments of the edited long transcripts, or a functional crosstalk between RNA editing and piRNA biogenesis during the primate evolution.

Existence of epiRNAs Signifies a Regulatory Crosstalk between RNA Editing and piRNA Biogenesis

As these epiRNAs are mainly transmitted from the precursor transcripts that are targeted by ADARs prior to processing, they may actually be derived from degradation fragments of the edited long transcripts. To rule out the possibility, we further characterized them in comparison with small RNA-Seq reads detected in other somatic tissues with no definite ncRNA annotations, which potentially are degradation products. These small RNA-Seq reads did not exhibit 5′ uridine bias but showed a strong correlation in tissue expression profile with the corresponding parental transcripts (fig. 5A and supplementary fig. S4, Supplementary Material online, and see Materials and Methods). Conversely, the epiRNAs had strong sequence preference of uridine on the 5′ end (78.0%) and a largely testis-specific presence, irrespective of the tissue expression profiles of the corresponding long transcripts (fig. 5A and B). These observations thereby confirmed that epiRNAs are generated specifically rather than through nonselective degradation.

Upon establishing the piRNA nature of these epiRNAs, we then set out to investigate whether such a crosstalk between RNA editing and piRNA biogenesis in primates could have functional implications at a global level. To this end, we performed whole-genome sequencing, mRNA-Seq and small RNA-Seq for three other macaque testis samples (table 1...
and supplementary tables S1 and S2 and figs. S2 and S3, Supplementary Material online), and subsequently compared the piRNAomes and RNA editomes among the four macaque animals. Of note, for the epRNA-associated RNA editing sites across different tissues from the same animal, RNA editing levels, as well as the expression levels of ADARs, were estimated on the basis of the poly(A)-positive RNA-Seq data, and the relative levels are shown in colors in relation to the color scale (right). Editing sites were further categorized into ADAR1-associated (green) or ADAR2-associated (purple), according to the tissue distributions of editing levels (see Materials and Methods). PFC, prefrontal cortex. The histograms show the length distribution of reads or tags in different macaque tissue types, before (D) or after (E) the exclusion of annotated ncRNA sequences. PFC, prefrontal cortex. (F) Nucleotide distribution (%) of the first 10 nt at the 5'-end of the candidate piRNAs is plotted for small RNAs in different macaque tissue types. A, U, C, and G are shown in blue, red, green, and black, respectively. (G) For each head-to-head overlapping piRNA pair, the length of sequence complementarity (Offset) was calculated. The distributions of the “Offset” values in all seven tissues are shown. (H) Pie chart showing the genomic distribution of piRNAs in different sequence regions. (I) The proportion of piRNAs exhibiting the same strand orientation as the corresponding piRNA cluster, based on the 100MGP-001 data set.

Fig. 2. Accurate catalogs of RNA-editing sites and piRNAome profile in rhesus macaque. (A) Relative representation of RNA editing types in the macaque transcriptome. (B) The enriched (upper) and depleted (lower) nucleotide sequences flanking the focal editing sites are shown by TwoSample Logo, with the level of preference or depletion presented in height proportional to the scale. (C) Hierarchical clustering of RNA editing levels of ADARs-associated editing sites across different tissues from the same animal. RNA editing levels, as well as the expression levels of ADARs, were estimated on the basis of the poly(A)-positive RNA-Seq data, and the relative levels are shown in colors in relation to the color scale (right). Editing sites were further categorized into ADAR1-associated (green) or ADAR2-associated (purple), according to the tissue distributions of editing levels (see Materials and Methods). PFC, prefrontal cortex. The histograms show the length distribution of reads or tags in different macaque tissue types, before (D) or after (E) the exclusion of annotated ncRNA sequences. PFC, prefrontal cortex. (F) Nucleotide distribution (%) of the first 10 nt at the 5'-end of the candidate piRNAs is plotted for small RNAs in different macaque tissue types. A, U, C, and G are shown in blue, red, green, and black, respectively. (G) For each head-to-head overlapping piRNA pair, the length of sequence complementarity (Offset) was calculated. The distributions of the “Offset” values in all seven tissues are shown. (H) Pie chart showing the genomic distribution of piRNAs in different sequence regions. (I) The proportion of piRNAs exhibiting the same strand orientation as the corresponding piRNA cluster, based on the 100MGP-001 data set.

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Given the small representation of epiRNAs in the total population (<0.1%), the proportionally increased abundance of the piRNA pools may not be contributed mainly by the epiRNAs themselves. Interestingly however, the epiRNAs exhibited a larger margin of variation in piRNA tag types in parallel with increased ADAR1 expression (13.4-fold vs. 4.5-fold). In addition, a larger proportion of the clusters with epiRNAs exhibited abundance closely commensurate with ADAR1 expression than that of the total piRNA clusters (98% vs. 87%, fig. 5C). Even for a class of “editing-absent” piRNAs, which have positional overlap with the editing sites on the long transcripts but lack detectable RNA editing on piRNA due to limited sensitivity of the current sequencing depth (fig. 3B and supplementary fig. S7, Supplementary Material online), the majority also showed high correlation between the piRNA expression and the RNA editing activity (95% vs. 87%, fig. 5C). These correlations provide the initial clues that RNA editing may be a causal mechanism for regulating and diversifying the piRNA repertoire in rhesus macaque (see Discussion).

Considering the difficulty in performing ADAR1 knockdown assay in primate models with piRNA regulation (see Discussion), we then focused our analysis on the public small RNA-Seq data from adar mutant C. elegans to further corroborate the causal link between RNA editing and piRNA biogenesis (Warf et al. 2012). Similar with the original report, we noted that the total piRNA reads were decreased by approximately 20% and approximately 40% in adar-1(–/–) and adar-1(–/–);adar-2(–/–) strains as compared with the wild type (supplementary fig. S8A, Supplementary Material online). For majority of piRNA loci (68.7%), the expression levels decreased in adar mutants compared with wild-type strains (supplementary fig. S8B and C, Supplementary Material online). In addition, we identified seven differentially expressed epiRNAs, all of which were downregulated in adar mutant worms (supplementary table S5, Supplementary Material online). These findings thus hinted at a direct crosstalk between ADARs and piRNA biogenesis in worms, as well as its conservation between evolutionarily divergent species.

(epiRNA-Associated RNA Editing Events Are under Selective Constraints in Primates)

Given the restricted expression of these regulatory pathways, as well as their distinct association with primate-specific Alu elements, direct elucidation of the functional implications of the link between RNA editing and piRNA biogenesis remains technically challenging. Alternatively, a population genetics approach, that is, characterizing polymorphisms in the epiRNA-associated genomic regions and comparing the patterns to that of the nearby regions as negative control, could potentially provide evolutionary clues to the functional significance of this crosstalk. Our previous study has proposed that for functional editing sites, the regions nearby the focal editing sites should be under selective constraints, owing to the requisite formation of local secondary structures for ADARs recognition and the evolutionary necessity to

(Zhang et al. 2013, 2014), by the strong quantitative correspondence between RNA editing level and ADAR1 expression level across individuals (supplementary fig. S5, Supplementary Material online), as well as by the ADAR1 knockdown assay in a testis-origin cell line (supplementary fig. S6 and table S4, Supplementary Material online). In fact, the type of piRNA tags in animal with the highest ADAR1 expression was 4.5-fold higher than that with the lowest ADAR1 expression (at comparable sequencing depths) (fig. 5C). In line with this finding, for 87% of these piRNA clusters, the relative expression levels were correlated with ADAR1 expression (Spearman Correlation Coefficient > 0.5).
maintain such a structure for a functional editing event (Chen et al. 2014). Based on this notion, we then performed population genetics analyses to assess whether selective constraints are applied to these epiRNA-associated RNA editing events in the populations of rhesus macaque and human.

We first profiled a set of polymorphism sites in rhesus macaque populations, on the basis of whole-genome sequencing in 24 independent macaque animals from different subpopulations (see Materials and Methods). Totally, 23.7 billion paired-end reads were generated with high quality, of which 19.2 billion reads (81.2%) were uniquely mapped to the macaque genome, yielding high sequencing coverage (ranging from 26- to 70-folds) (table 2 and supplementary table S6, Supplementary Material online). Utilizing these deep

**Fig. 4.** Experimental verification of epiRNAs. (A, B) For two selected epiRNA candidates, the deep sequencing raw data as well as the Sanger sequencing results corresponding to the genome, mRNA and small RNA in the macaque animal (100MGP-001) testis sample are shown. The detected editing sites are highlighted by black box (in deep sequencing) or red arrows (in Sanger sequencing).
sequencing data (a total sequencing output of 3,382 Gb), as well as seven public data sets for macaque genomes (Fang et al. 2011; Yan, Zhang, et al. 2011; Gokcumen et al. 2013), we profiled 54,079,575 single nucleotide polymorphic sites across the macaque genome (see Materials and Methods). To examine the functional significance of the epiRNA-associated RNA editing regulation, we then compared the distributions of macaque polymorphic sites in epiRNA regions adjacent to the focal editing sites with those in more distal regions (see Materials and Methods). As the adjacent and distal regions are both located within the same Alu element, they should have the same rate of mutation accumulation at a neutral expectation. However, our analysis evidenced substantially lower single nucleotide polymorphism (SNP) density and nucleotide diversity for regions adjoining the editing foci, thus demonstrating the presence of purifying selection on these genomic regions (supplementary fig. S9, Supplementary Material online).

However, considering the established role of piRNAs in repressing transposable elements in primates, these sequence features might be attributed to a positional effect that is potentially contributed by the underlying selectively constrained piRNAs (supplementary fig. S10, Supplementary Material online). To rule out this possibility, a subset of RNA editing sites with the 5’ and 3’ 4-bp nearby regions distributed in the central region of epiRNAs were selected for further analyses \( (n = 325) \) (see Materials and Methods). The population genetics parameters of these RNA editing sites fell between those of the synonymous and nonsynonymous sites, thus indicating a moderate level of purifying selection on these sites (fig. 6A and B). Furthermore, in terms of the frequency spectra of derived allele, we discovered an excess of low-frequency mutations for editing-proximal regions in

Fig. 5. Interaction of RNA editing and piRNA biogenesis. (A) The plot shows the percentages of epiRNAs and mRNA degradation fragments with the nucleotide uridine at the 5’-end of the respective sequences. (B) Heatmaps showing the relative expression levels across seven different macaque tissues for long transcripts (left) and small RNAs (right) corresponding to the epiRNA-associated regions, with reference to the color scale on top. (C) The differences in ADAR expression levels and normalized piRNA tag types across four different animals are shown in linear graphs on the left. The heatmap on the right depicts the relative expression levels of piRNAs in each piRNA cluster of the four animals, organized and scaled in rows. These piRNA clusters were categorized into three groups: epiRNAs-expressing clusters (yellow), "editing-absent" epiRNA clusters (overlapping with editing sites on the long transcripts but lack detectable epiRNAs; orange), and canonical piRNA clusters (red), as indicated by the color bar on top.
comparison with the distal piRNA regions as a control (fig. 6C).

Notably, compared with canonical piRNAs (i.e., editing is absent on both piRNA and the associated mRNA), epiRNAs seemed to be under stronger selective constraints in rhesus macaque, probably due to the existence of these functional editing sites (fig. 6D, Wilcoxon one-tailed test, \( P = 0.02 \)). Considering that epiRNAs with higher expression levels seemed to be under stronger selective constraints (supplementary fig. S11A, Supplementary Material online), we further investigated whether their abundance might contribute to the different strengths detected for the selection signal. However, we did not observe significant difference in the expression levels between epiRNAs and the canonical piRNAs (Wilcoxon one-tailed test, \( P = 0.806 \), supplementary fig. S11B, Supplementary Material online). More importantly, even the lowly expressed epiRNAs were under yet stronger selective constraints than the canonical piRNAs (supplementary fig. S11A, Supplementary Material online). These findings thus strengthen the notion that existence of these functional editing events, rather than the expression levels, contributes to the detection of stronger selective constraints on epiRNAs versus canonical piRNAs.

Taken together, these population genetics attributes should lend support to the functionality of these epiRNA-associated RNA editing events in rhesus macaque. Moreover, given that these regions are rarely linked to other gene regulatory processes such as splicing or gene expression, a piRNA-associated regulation represents a plausible scenario for these RNA editing events. Importantly, all of these findings are correspondingly consistent in human, further supporting the conservation of this mechanism during the primate evolution (supplementary fig. S12, Supplementary Material online). Our results thus provide the earliest evolutionary clues to the functionality of the crosstalk between RNA editing regulation and piRNA biogenesis in primates.

Discussion

An Intersection between RNA Editing Regulation and piRNA Biogenesis in Primates

Although a substantial number of editing sites have been identified across primate transcriptome, evidence for functional significance of this process is largely lacking, particularly for the overwhelmingly large group of editing sites in noncoding regions (> 99.9% of total). To this end, a growing body of evidence has linked RNA editing to the small ncRNA species of miRNAs, alterations of which are known to have developmental and pathological implications (Pfeffer et al. 2005; Singh 2012; Shoshan et al. 2015). Interestingly, RNA editing regulation shares multiple spatial features with another noncoding component of the transcriptome, the piRNA-based regulation—both processes target the Alu elements, and the main enzymes for both regulatory pathways show some degree of testis-biased expression (fig. 2C and supplementary fig. S13, Supplementary Material online). Considering these shared spatial features, a link between these two regulatory mechanisms might conceivably exist. Further considering the pervasive distribution of A-to-I RNA editing in the primate genome, it is rational to speculate the existence of edited piRNAs that are derived from Alu sequences, identification of which could further substantiate the crosstalk between the two regulatory levels.

However, it is technically challenging to directly test the hypothesis, in part due to the restricted expression of these pathways, the intensive association of these regulations with primate-specific Alu elements, the stringent requirements for high-quality tissue samples across different tissues and individuals, as well as the computational challenges in accurately identifying and verifying these regulations. Exploiting the emerging primate model of rhesus macaque, our present study presents to our knowledge the first documentation of "epiRNAs" in primates, a new class of piRNA that serves as evidence for the RNA editing–piRNA interplay.

Consistently with the reports that piRNA-like small RNAs exist in other nongermline tissues (Yan, Hu, et al. 2011), we also detected a class of small RNAs with the length of 24–32 bp in the macaque somatic tissues. However, these small
RNAs only represented a minor proportion of all small RNA reads (fig. 2D), a profile that is also consistent with the previous characterization of piRNA-like small RNAs in somatic tissue (Yan, Hu, et al. 2011). Moreover, considering that these piRNA-like small RNAs do not verify the known features of canonical piRNAs in our study (fig. 2D), and that PIWI proteins were hardly expressed in these somatic tissues (supplementary fig. S13, Supplementary Material online), we focused our studies on the link of RNA editing to the germ cell piRNA regulation. Nevertheless, our present data do not fully exclude the possibility that RNA editing regulation may also crosstalk with these somatic piRNA-like small RNAs.

RNA Editing Regulation May Diversify the piRNA Repertoire in Primates

The observed correlations between the piRNAome and RNA editing in rhesus macaque, together with the additional evidence in C. elegans ADAR-KO model (supplementary fig. S5, Supplementary Material online), provide initial clues that RNA editing regulations may crosstalk with piRNA biogenesis in multiple species. However, unequivocal demonstration of this causal relationship in primates remains challenging, mainly owing to the testis-selective nature of these regulations. Due to the restricted expression of piRNA-based pathways and limited availability of corresponding cell models, further clarification of the functional implications of these epiRNAs in primates may rely on tools such as CRISPR/Cas9-based transgenic monkeys (Niu et al. 2014), which now represent effective means of genetic functional assays.

We noted that the number of piRNA tag types in animal with the highest ADAR1 expression was 4.5-fold higher than that with the lowest ADAR1 expression (fig. 5C). However, based on the small number of epiRNAs detected in this study (<0.1% of the total pool), it seems plausible that these editing events may not directly contribute to the diversity and increased abundance of piRNA repertoire in correlation with ADAR1 expression (fig. 5C). There are two possible explanations to these seemingly contradictory observations. First, although 4,170 epiRNAs have been defined in this study, this number may still represent an insufficient account of the cellular epiRNA repertoire owing to the following reasons: 1) For the presumably “editing-absent” piRNA clusters that share positional overlap with RNA editing sites in the long transcripts but do not express epiRNA variants, a fraction may actually represent bona fide epiRNAs that were undetected due to the limited sensitivity provided by the current sequencing depth (fig. 3B and supplementary fig. S7, Supplementary Material online); 2) this underestimation could also be contributed by the ambiguity in small RNA sequencing reads alignment—many small RNA reads corresponding to putative epiRNAs were mapped to multiple positions across transcriptome and thus excluded initially; and 3) as calling mRNA editing events in primates is as yet a refined approach (Bazak et al. 2014), the complexity and contribution of epiRNAome may be far more extensive than expected from the current analysis. Second, the piRNA clusters that give rise to epiRNAs may represent the initial sites of priming reaction for a genome-wide regulation on piRNA biogenesis in primates. It is thus possible that these piRNAs triggered by the RNA editing regulation, including the editing-associated epiRNAs, may further activate canonical piRNA loci across the genome through the “ping-pong” biogenesis mechanism. In this regard, experimental characterization of the true genomic region distribution of piRNA targets is currently an unresolved challenge, mainly due to the difficulty in obtaining high-quality PIWI ChIP-seq data sets (Marinov et al. 2015). More efforts are thus needed to elucidate the regulatory landscape of these epiRNA. Taken together, our results are in line with the notion that piRNA-associated editing sites, which are possibly underestimated in the present study, likely contribute to the biogenesis and diversification of the total piRNA pool.

Link of RNA Editing to the Purifying Selection Signals Detected on epiRNA Regions

Our population genetics analyses showed that the epiRNA-associated RNA editing regulation is under selective constraints in primates (fig. 6 and supplementary fig. S9, Supplementary Material online), strengthening the evolutionary significance, and thus functionality, of these ADAR1-directed regulations across the primate transcriptome. Several observations further support that RNA editing is directly associated with these selectively constrained regulatory events. First, the population genetics analysis pinpointed the sequences immediately nearby the focal editing sites as regions with substantially decreased SNP density and nucleotide diversity—a sequence context that implies functional selection for the targeting by the catalytic domain, rather than the RNA binding domain, of the adenosine deaminase. Although it remains likely that these signals of purifying selection may actually correspond to the necessity in maintaining a stable ADAR1 binding to the substrates, we favor a simpler explanation that this selectively constrained regulation is manifested in an editing catalysis-associated functionality. Second, given that these regions with purifying selection signals are rarely linked to other functional regulations, such as splicing or gene expression regulations (Chen et al. 2014), occurrence of RNA editing may thus directly exerts functional impact on these piRNAs.

It has been well established that piRNAs are not conserved across different species, possibly a response to the quick turnover rates of their lineage-specific targets (Lu and Clark 2010). The findings that RNA editing may crosstalk with piRNA biogenesis to diversify the piRNA repertoire, as well as the fact that the crosstalk is maintained by purifying selection in the populations of human or rhesus macaque, infer that these editing–piRNAs interplay may have significant functions in controlling lineage-specific transposable elements.

Redirection of miRNA-Like piRNA Targeting by RNA Editing

Recent studies have revealed that piRNAs may inhibit mRNA expression through a similar mechanism as siRNA-mediated silencing, and that their 5′ regions are important for target
within the first 10 bp of the piRNA 5′-end. Moreover, 89.6% of these epiRNAs constitute a significant proportion of the expression of the corresponding piRNAs loci (>5%, supplementary fig. S14, Supplementary Material online). This finding is then in line with the scenario that these piRNA-associated RNA editing events may also redirect piRNA targeting to extended Alu subfamilies through sequence pairing (Aravin, Hannon, et al. 2007; Kelleher and Barbash 2013) (supplementary fig. S14, Supplementary Material online), which is analogous to the effects of mutations on miRNA seed regions (Blow et al. 2006).

Materials and Methods

Ethics Statement

Rhesus macaque tissue samples were provided by the internationally accredited (Association for Assessment and Accreditation of Laboratory Animal Care, AAALAC) animal facility at the Institute of Molecular Medicine in Peking University. All animal studies were approved by the Institutional Animal Care and Use Committee of Peking University. Commercial human RNA samples were obtained from Clontech Laboratories, Inc. and Ambion, Inc.

Sample Preparation and Data Analysis for Whole-Genome Sequencing, RNA-Seq, and Small RNA-Seq

Strand-specific, poly(A)-positive RNA-Seq libraries were prepared from seven rhesus macaque tissues (prefrontal cortex, cerebellum, heart, kidney, lung, muscle, and testis) derived from a 5-year-old animal (100MGP-001) as previously reported (Xie et al. 2012; Chen et al. 2014), and testis tissues from three other animals (100MGP-002–100MGP-024) (supplementary table S6, Supplementary Material online), for which sample preparation was done as previously reported (Chen et al. 2014). NGS was performed on Illumina HiSeq sequencing systems, with 90/100/151-bp paired-end reads mode, or 49-bp single-end reads mode (tables 1 and 2).

Whole-genome sequencing data in this study were combined with the published genome resequencing data from the same macaque sample (Chen et al. 2014). The genome sequencing and poly(A)-positive RNA-Seq data were then aligned to the rhesus macaque genome (rheMac2) by BWA (v0.5.9-r16), using parameters as outlined in our previous study (Chen et al. 2014). For small RNA sequencing data, reads were collapsed into one tag if they were totally identical. Small RNA tags were then aligned to the genome of human (hg19) or rhesus macaque (rheMac2) using Bowtie (v0.12.8) with one mismatch allowed, according to a pipeline described previously (Yan, Hu, et al. 2011). Deep sequencing data in this study are available at NCBI Gene Expression Omnibus and SRA under accession numbers GSE34426, GSE42857, SRP039366, and SRP049394.

Identification and Characterization of RNA Editing Sites

To identify mRNA-associated editing sites, several improvements were incorporated into our previous pipeline (Chen et al. 2014): 1) On the basis of the recent report on variant calling (Ramaswami et al. 2012), instead of using Samtools pipelines, variants on RNA molecules were directly identified by piling up poly(A)-positive RNA-Seq reads and distinguishing discrepancies between RNA sequences and the corresponding DNA sequences. The sensitivity in variant identification was thus dramatically increased. 2) To deal with potential false positives stemming from sequencing errors or mismapping, only RNA variants that satisfy specified criteria, including the minimal number of supporting reads for variant allele (2/3 for Alu/non-Alu editing sites), minimal allele frequency (0.02/0.10 for Alu/non-Alu editing sites), and required base quality (Phred score ≥ 25), were taken into consideration. 3) Candidate editing sites in non-Alu regions were further excluded if they were located in simple repeats or within a 4-bp intronic regions adjacent to the splicing junctions (Ramaswami et al. 2012). To test whether these editing sites represent bona fide editing sites, 78 candidate sites in testis with editing levels ≥ 10%, which is the approximate detection limit of Sanger sequencing, were randomly selected. PCR amplification and Sanger sequencing of both DNA and cDNA samples from testis tissues were then performed (supplementary fig. S1, Supplementary Material online).

Editing levels were estimated only for sites covered by ≥ 10 sequencing reads. The normalized RNA editing levels across seven tissues of the same macaque animals were clustered by heatmap package in R (v3.0.1); and ADARs-associated sites as defined by linear regression model in our previous study (Chen et al. 2014) were used in the hierarchical clustering analyses. Overall, 32.5% and 15.3% of the A-to-G editing sites showed significantly positive correlation with ADAR2 and ADAR1 expression, respectively, in terms of tissue distribution of levels (fig. 2C).

Mapping and Definition of piRNA and piRNA Clusters

We first removed reads derived from annotated ncRNAs by filtering mappable small RNA reads against the ncRNA databases of human or rhesus macaque. Briefly, human miRNA annotated in miRBase (v20) and other ncRNAs (ribosomal RNA, transfer RNA, small nucleolar RNA, small nuclear RNA, small conditional RNA, and misc-RNA) annotated in UCSC and Ensembl were downloaded and integrated into a comprehensive data set for annotated ncRNAs in human.
Subsequently, human–macaque pairwise alignment was performed to infer a list of ncRNAs in rhesus macaque, from which alignments of matched macaque sequences having >70% and <130% coverage of the query sequence were retained (Yan, Hu, et al. 2011). A small RNA-Seq read was excluded if any of its mapped locations overlaps with the regions encoding annotated ncRNAs. The remaining 24–32 nt reads were then defined as candidate piRNAs.

We next defined the piRNA clusters in the 100MGP-001 testis. Using a 3-kb-long window with 0.5-kb sliding steps, regions that encompass at least 300 uniquely mapped reads in the same orientation, with at least 60% of the reads also containing 5′ uridine sequence, were identified. Positive sliding windows were merged into a larger piRNA cluster if they located within 1 kb of each other (Yan, Hu, et al. 2011). Similar approach was used to pinpoint the piRNA clusters in other samples, with the cutoff for uniquely mapped reads being optimized according to the total sequencing depth of the respective samples.

We then investigated whether these candidate piRNAs recapitulate the known features of piRNAs, such as 5′ uridine bias, complementary piRNAs sequences as a result of the “ping-pong” biogenesis mechanism, as well as distinctive genomic distributions. Briefly, the nucleotide compositions of the first 10 bp on 5′-end of these candidate piRNAs were determined to assess the extent of 5′ uridine bias (fig. 2F). To assess the contribution of “ping-pong” mechanism to the generation of these candidate piRNAs, we first selected candidate piRNAs with both 5′ uridine (feature of primary piRNA) and adenosine at the tenth position (feature of secondary piRNA). We then used piRNAs with 5′ uridine (presumably the primary piRNAs) as the reference to identify from the remaining candidate piRNAs any complementary piRNAs (putative secondary piRNAs). We then calculated the lengths of the complementary regions and compared the length distribution in different samples, using an approach as previously proposed (Yan, Hu, et al. 2011) (fig. 2G). These candidate piRNAs were then assigned to different types of genomic regions on the basis of RhesusBase (V2) annotations (Zhang et al. 2014). For piRNAs at positions with multiple definitions, they were given a single assignment according to the order of repeat, exon, intron, UTR, and intergenic regions (fig. 2H). For candidate piRNAs located in clusters, the proportions of piRNAs showing the same strand orientation as the corresponding piRNA cluster were also calculated (fig. 2I).

**Cell Culture and RNA-Seq Studies of 1411H Cell Line**

To experimentally examine the role of ADAR1 on RNA editing sites in testis, as well as the functional implications of piRNAs in cell lines, RNA-Seq studies were performed on the testis-origin 1411H cell line (Public Health England, 06011805). 1411H cells were cultured in complete growth medium DMEM (Hyclone, UT), supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37 °C and 5% CO₂. Proliferative cultures were transfected with synthetic single-strand small RNA oligos (GenePharma, Shanghai, China): 1) siRNA negative control (50 nM) and 2) mixture of three ADAR1 siRNAs (50 nM; si-seq1, si-seq2, si-seq3) (supplementary table S4 and fig. S6, Supplementary Material online). These siRNAs were designed for ADAR1 specifically, and their sequences contain multiple mismatches relative to the ADAR2 sequence (supplementary fig. S6D, Supplementary Material online). The silencing efficiency was further assessed by quantitative PCR (qPCR) and Western blotting (supplementary fig. S6A and B, Supplementary Material online). Briefly, total RNA samples for qRT-PCR experiment were prepared using Trizol (Invitrogen), and reverse transcribed into cDNA using
SuperScript™ II Reverse Transcriptase (Invitrogen). qPCR was performed on AB Step One Plus (Applied Biosystems) with ADAR1 primers (ATCACGGGGCTGTAGAATATG and AAACCTCTCGCCATTTGATGAC). Total protein samples were also extracted using Cell Lysis Buffer (CST) containing PMSF (sigma) and 1 x protease inhibitors (Roche) for Western blotting with antibodies for ADAR1 and GAPDH purchased from Santa Cruz Biotechnology (sc-73408) and EASYBIO (BE0023), respectively. Upon RNA extraction, poly(A)-positive RNA-Seq libraries and small RNA-Seq libraries were also prepared and sequenced on a Hiseq 2500 sequencing system with 51 single-end reads mode.

The orthologous editing sites in human were identified on the basis of pairwise genome alignment between human and rhesus macaque (Karolchik et al. 2014), and sites with the edited form encoded in the human reference genome were excluded. The editing levels were determined only for sites covered by ≥ 10 sequence reads in both the control and the experimental groups. The expression levels of genes were estimated in the form of RPKM, as shown in our previous study (Xie et al. 2012).

Experimental Verification of epiRNAs

Total RNAs were isolated from frozen testis tissues using Trizol reagent (Invitrogen, CA). Genomic DNA was also obtained from the same tissue samples. Editing sites on the mRNA transcripts were validated by means of PCR amplification and Sanger sequencing of both the DNA and corresponding RNA. For epiRNA verification, 1 μg of total RNA was first polyadenylated by poly(A) polymerase (NEB, MA, UK) in a 20-μl reaction mixture at 37 °C for 1 h, and subsequently isolated by phenol–chloroform extraction and ethanol precipitation. Then, 0.5 μg 3′ RACE adapter GCGAGCA CAGATTAATACGACTCACTATAGGT12VN and 200 U SuperScript™ II Reverse Transcriptase (Invitrogen) were used to reversely transcribe the poly(A) tail-added small RNA molecules (Shi and Chiang 2005). Using the resultant cDNAs as template, specific epiRNA sequences were amplified by EasyTaq DNA polymerase (TransGen), with piRNA-specific 5′ primers (TGCAATGCACGGCATGATCTC in fig. 4A or TCCCGAGCAGCTAGGACT in fig. 4B) and the 3′ RACE adapter outer primer (GCCGAGCACAGATAATACGACT). PCR products were then cloned in T1-vector for Sanger sequencing.

Population Genetics Analysis

Whole-genome sequencing data of seven macaque animals (Fang et al. 2011; Yan, Zhang, et al. 2011; Gokcumen et al. 2013) downloaded from SRA (SRA023856, SRA037810, and ERP002376), together with the whole-genome sequencing data of 24 independent macaque animals generated in this study, were mapped to the macaque genome (rheMac2) by BWA (0.7.10-r789). Macaque polymorphism sites in this population of 31 animals were then profiled by the standard GATK (V3.2-2) pipelines with Unified Genotyper. To examine whether piRNAs and epiRNAs are under purifying selection, we first retrieved sequences of equal lengths that are upstream and downstream to the corresponding piRNAs/epiRNAs, and used them as the references. Both piRNA/epiRNA and the control adjacent regions were then individually divided into 15 subregions for the calculation and comparison of the SNP densities and nucleotide diversity, with any missing alleles substituted with the reference alleles. For the estimation of the frequency spectra of a given derived allele, as defined by the EPO pipeline (Paten et al. 2008), 1,000 times of bootstrap were performed to estimate the confidence intervals of the proportions of polymorphism sites. Similar population genetics analyses were performed to analyze evolutionary significance of the editing sites on epiRNAs, in terms of the SNP densities, nucleotide diversity, and the frequency spectra of derived allele for the focal regions (8-nt regions surrounding the editing sites) versus the reference regions (8-nt sequences upstream and downstream of the focal regions) (fig. 6). To investigate the functionality of epiRNAs in human, we also profiled human polymorphism data of 67 individuals with high sequencing coverage from the 1000 Genomes project (Genomes Project Consortium et al. 2012; Chen et al. 2015), and performed similar population genetics analyses (supplementary fig. S12, Supplementary Material online).

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