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The dsRBP and inactive editor, ADR-1, utilizes dsRNA binding to regulate A-to-I RNA editing across the *C. elegans* transcriptome

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SUMMARY

Inadequate adenosine-to-inosine editing of noncoding regions occurs in disease, often uncorrelated with ADAR levels, underscoring the need to study deaminase-independent control of editing. *C. elegans* have two ADAR proteins, ADR-2 and the theoretically catalytically inactive ADR-1. Using high-throughput RNA sequencing of wild-type and *adr* mutant worms, we expanded the repertoire of *C. elegans* edited transcripts over 5-fold and confirmed that ADR-2 is the only active deaminase *in vivo*. Despite lacking deaminase function, ADR-1 affects editing of over 60 adenosines within the 3' UTRs of 16 different mRNAs. Furthermore, ADR-1 interacts directly with ADR-2 substrates, even in the absence of ADR-2; and mutations within its dsRNA binding domains abolished both binding and editing regulation. We conclude that ADR-1 acts as a major regulator of editing by binding ADR-2 substrates *in vivo* and raises the possibility that other dsRNA binding proteins, including the inactive human ADARs, regulate RNA editing by deaminase-independent mechanisms.

INTRODUCTION

RNA editing is a posttranscriptional process that introduces changes in RNA sequences and structures (Gott and Emeson, 2000). The most prevalent form of RNA editing in metazoa is

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SUPPLEMENTAL INFORMATION

Supplemental information includes Extended Experimental Procedures, two figures and four tables and can be found with this article online at xxx. RNA-Sequencing data has been deposited in the GEO database (accession #GSE51556).

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the hydrolytic deamination of adenosine (A) to inosine (I) (Nishikura, 2010). Adenosine deaminases that act on RNA (ADARs) bind to double-stranded regions of RNA and catalyze this type of editing (Goodman et al., 2012; Savva et al., 2012). Although RNA editing was initially thought to be restricted to a few select mRNAs in the central nervous system, it is now clear that adenosine deamination is widespread, with current estimates of 400,000–1,000,000 A-to-I edits in the human transcriptome (Ramaswami et al., 2013).

Adenosine and inosine have different base-pairing properties; therefore, editing alters RNA structure. Furthermore, as inosine is recognized as guanosine by cellular machinery, RNA editing can modify splice sites, alter the amino acid encoded by a codon and redirect miRNAs and siRNAs to new targets (Hundley and Bass, 2010; Rosenthal and Seeburg, 2012). As the extent of RNA editing varies during development and between cell types (Wahlstedt et al., 2009), this type of modification dynamically regulates gene expression (Tan et al., 2009).

The molecular diversity generated by ADARs is most pronounced in the brain transcriptome (Blow et al., 2004; Paul and Bass, 1998). Consistent with this, deletion of ADARs in lower organisms, such as *C. elegans* and *Drosophila*, results in behavioral defects (Palladino et al., 2000; Tonkin et al., 2002), indicating that RNA editing is required for proper neuronal function. Furthermore, alterations in editing levels have been observed in a number of neuropathological diseases, including epilepsy, depression, amyotrophic lateral sclerosis, and brain tumors (Farajollahi and Maas, 2010; Tariq and Jantsch, 2012).

In both development and disease, ADAR expression levels do not directly correlate with the extent of editing (Maas et al., 2001; Wahlstedt et al., 2009), implying that other mechanisms exist to regulate ADAR-mediated RNA editing. Both alternative splicing (Lai et al., 1997; Rueter et al., 1999) and post-translational modification (Desterro et al., 2005) of ADARs generate less active variants of ADARs. Likewise, editing can be inhibited by sequestration of ADAR in the nucleolus (Sansam et al., 2003) or enhanced by proteins that promote nuclear localization of ADARs (Marcucci et al., 2011; Ohta et al., 2008). In addition to proteins that directly regulate ADARs, it has recently been demonstrated that both the local RNA structure (Daniel et al., 2012) and RNA binding protein (RBP) landscape of individual transcripts (Tariq et al., 2013) regulate ADAR activity. To date, none of these mechanisms have been linked to reduced RNA editing activity in disease (Orlandi et al., 2012). Furthermore, it is unlikely that regulators of specific transcripts will play a key role in the global hypoediting of transcripts observed in many human cancers and neurological diseases.

To identify mechanisms that could decrease global RNA editing levels, we focused on the role of catalytically inactive ADAR family members. The *C. elegans* genome encodes two proteins with the common ADAR family domain structure (ADR-1 and ADR-2). However, ADR-1 lacks several key amino acids required for deaminase activity. Worms lacking the *adr-2* gene, have no detectable editing of the six known edited endogenous mRNAs (Tonkin et al., 2002), suggesting that ADR-2 is the catalytically active ADAR protein in worms. However, initial studies of worms lacking *adr-1* revealed alterations in the editing efficiency of all six endogenous mRNAs examined (Tonkin et al., 2002). In addition, recent deep sequencing of *C. elegans* small RNAs identified over 30 small RNAs that are edited *in vivo*, and each have altered editing levels in worms lacking *adr-1* (Warf et al., 2012). These prior observations suggest ADR-1 regulates editing. However, it is also possible that background mutations in the strains lacking *adr-1* contribute to alterations in editing or that loss of *adr-1* indirectly affects editing by ADR-2. To directly address these concerns, we developed a quantitative assay to measure *in vivo* editing levels of worms expressing *adr-1* transgenes. About 40% of adenosines within three known edited mRNAs were affected by loss of *adr-1*.

Furthermore, using a combination of high-throughput RNA sequencing of transgenic worms and probabilistic modeling we were able to identify 48 novel edited transcripts and demonstrate that loss of *adr-1* affects editing of at least half of these newly identified ADAR targets. Using an RNA immunoprecipitation (RIP) assay, we demonstrate that ADR-1 directly binds to known editing targets *in vivo*, that disrupting this binding alters editing of the mRNAs, and that ADR-1 and ADR-2 co-occupy transcripts *in vivo*. In summary, we demonstrate that catalytically inactive ADR-1 acts as a global regulator of editing by binding to target mRNAs and modulating the accessibility of ADR-2 for target adenosines.

RESULTS

ADR-1 significantly alters RNA editing of multiple *C. elegans* mRNAs

To determine the ability of ADR-1 to directly regulate RNA editing *in vivo*, we established a quantitative assay to measure changes in editing in worms lacking *adr-1* and then tested if these changes were rescued by an ADR-1 transgene. First, we examined editing levels at 50 individual adenosines within three known edited mRNAs: *C35E7.6*, *lam-2* and *pop-1*. These three mRNAs were chosen based on their diverse cellular functions and length of the double-stranded 3' UTR, which range from 517 to 1423 nucleotides (nts). RNA was isolated from three independent biological replicates of wild-type and *adr-1(-)* adult worms. After reverse transcription, PCR amplification and Sanger sequencing, editing efficiency was quantitatively measured using the Bio-Edit program. Technical replicates of the editing assay suggest that editing at each site can be determined with <1% error (Figure S1A), which is consistent with published data on the accuracy of measuring editing efficiency by Sanger sequencing (Eggington et al., 2011). Of the 50 edited adenosines, we observed statistically significant differences in editing levels between wild-type and *adr-1(-)* worms at 22 individual sites (Figure 1A). The bulk of the statistically significant sites (91%) had decreased editing, ranging from 3–35%, in the absence of *adr-1*.

To demonstrate that these sites are directly regulated by ADR-1, a 3X FLAG tagged genomic version of *adr-1* was re-introduced to *adr-1(-)* worms by microinjection. Importantly, this transgenic worm rescues a known *adr-1* dependent effect on neuronal protein expression (Hundley et al., 2008), indicating that the transgene expresses functional ADR-1 protein (Figure S1B). As the transgenic worms express FLAG-ADR-1 from an extrachromosomal array that is transmitted to progeny at a high frequency, but not 100%, a neuronal GFP marker was co-injected and flow cytometry was used to purify worms containing the ADR-1 transgene. In addition, to reduce effects of developmental timing on editing efficiency all worms were also sorted by size to obtain young adults. The quantitative editing assay showed that FLAG-ADR-1 significantly restored editing to 15 of the 22 editing sites altered in *adr-1(-)* worms (Figure 1B). It is important to note, that editing changes in the FLAG-ADR-1 worms are not a general phenomenon, as editing sites that are not affected by loss of *adr-1* are not altered by the transgene (Figure S1C). The 15 ADR-1 regulated sites include both adenosines that have increased and decreased editing in the absence of *adr-1*. Together, these data indicate that ADR-1 alters editing of multiple transcripts, but the effects vary depending upon the individual adenosines examined.

ADR-1 binds directly to ADR-2 target mRNAs *in vivo*

As the effects of *adr-1* on editing are site specific, we hypothesized that ADR-1 is capable of regulating editing by utilizing two dsRNA binding domains (dsRBDs) to bind to potential editing substrates and alter accessibility of ADR-2 to particular nucleotides. To determine if ADR-1 could bind ADR-2 editing targets *in vivo*, we developed an RNA-immunoprecipitation (RIP) assay for ADR-1. As a previously generated polyclonal antibody to ADR-1 was incapable of immunoprecipitating ADR-1 efficiently, the 3x FLAG-tagged

ADR-1 transgenic worm was utilized. To measure specific binding of ADR-1 to target mRNAs *in vivo*, we compared immunoprecipitates (IPs) from FLAG-ADR-1 and *adr-1(-)* worms that were subjected to UV irradiation (Fig 2A). The IP samples were treated with Proteinase K to degrade FLAG-ADR-1 and release ADR-1 associated RNAs into the supernatant. RNA was extracted from the supernatant, reverse transcribed and quantified using real-time PCR (qRT-PCR). Primers that amplify the three mRNAs tested in Figure 1, produced 3–15 fold more cDNA in the FLAG-ADR-1 IPs compared to *adr-1(-)* IPs (Fig 2B). In contrast, an mRNA that lacks dsRNA, *gpd-3*, is not enriched, indicating that, *in vivo*, ADR-1 specifically binds to these double-stranded ADR-2 target mRNAs.

As these three mRNAs have both adenosines that are inhibited and enhanced by ADR-1, these data support the hypothesis that ADR-1 modulates editing via a direct interaction with dsRNA. However, in order to regulate editing, ADR-1 needs to bind to the dsRNA before it is edited. To test this possibility, we performed the RIP assay in cells expressing FLAG-ADR-1, but lacking *adr-2* and RNA editing. FLAG-ADR-1 was expressed and immunoprecipitated to a similar level in the presence and absence of *adr-2* (Fig 2C). Compared to the *adr-1(-)* worms, all three ADAR target mRNAs were enriched to a similar extent in the FLAG-ADR-1 IPs in the presence and absence of *adr-2* (Fig 2D), indicating that binding of ADR-1 to known edited mRNAs is independent of ADR-2. Furthermore, as these mRNAs have no detectable editing in *adr-2(-)* worms, we conclude that ADR-1 binds unedited mRNAs in the cell.

ADR-1 alters RNA editing via binding to dsRNA *in vivo*

Our results indicate that ADR-1 binds to mRNAs that are targets for editing by ADR-2 *in vivo*. To determine if this binding is required for the ability of ADR-1 to alter editing *in vivo*, we created mutations in the dsRBDs of ADR-1 and examined the effects on endogenous RNA editing. A patch of lysine (K) residues, referred to as the KKxxK motif (x=any amino acid), is required for dsRNA binding proteins to bind dsRNA (Ramos et al., 2000; Ryter and Schultz, 1998). Mutation of the lysines to glutamate (E) and alanine (A) disrupts binding of human ADARs to dsRNA (Valente and Nishikura, 2007). To disrupt ADR-1 dsRNA binding, the KKxxK motif was mutated to EAxxA within both dsRBDs (referred to as the ds1+2 mutant) (Fig 3A). Similar to the aforementioned wild-type ADR-1, the ds1+2 mutant was 3XFLAG tagged and reintroduced in the *adr-1(-)* background. The FLAG-ADR-1 ds1+2 mutant protein is expressed in the transgenic worms to about the same level as transgenic wild-type FLAG-ADR-1 (Fig 3B). To test whether these mutations disrupt ADR-1 binding to dsRNA, the RIP assay was performed with the ds1+2 mutant. In contrast to wild-type ADR-1, the ds1+2 mutant IPs were not enriched for the ADR-2 editing targets (Fig 3C). Thus, the ds1+2 mutant has defects in mRNA binding *in vivo*.

To determine if ADR-1 binding to target mRNAs influences editing efficiency, we compared *in vivo* editing levels of the FLAG-ADR-1 worms to the FLAG-ADR-1 ds1+2 mutant at the 15 sites that were identified as significantly regulated by ADR-1 (Figure 1B). As ADR-1 primarily promotes editing within these target mRNAs, most of the sites exhibit decreased editing in the absence of *adr-1*, with the exception of nt 631 of *lam-2*, which has increased editing in *adr-1(-)* worms (Figure 1A). The ADR-1 ds1+2 mutant failed to significantly restore editing to 11 of these 15 sites, including nt 631 of *lam-2* (Fig 3D). Thus, ADR-1 binding to target mRNAs is required both for its ability to promote and inhibit editing of known edited mRNAs *in vivo*.

Binding of dsRNA by ADR-1 regulates editing across the transcriptome

Our data indicates that ADR-1 binding to target mRNAs alters editing of specific adenosines *in vivo*. To understand the impact of ADR-1 across the transcriptome, we conducted strand-

specific RNA-sequencing (RNA-Seq) of RNA from wild-type (N2), *adr-1(-)*, *adr-2(-)*, *FLAG-ADR-1* and *FLAG-ADR-1 ds1+2 mutant* adult worms and compared the nucleotide changes amongst the strains and the published *C. elegans* genomic sequence (WS220,ce10) (Fig 4A). To distinguish true RNA editing events from single nucleotide polymorphisms (SNPs), we removed annotated SNPs using Illumina's iGenomes collection. Unannotated single-nucleotide variants (SNVs) were further addressed by performing RNA-Seq on RNA from *adr-1(-);adr-2(-)* worms and identifying all SNVs between the *adr-1(-);adr-2(-)* RNA (which lacks all A-to-I editing) and the *C. elegans* genome. These 118,651 SNVs were subtracted from all other RNA-seq datasets. A Bayesian "inverse probability model" was then adapted (Li et al., 2008) to identify high-confidence A-to-I editing sites from the RNA-seq data, where a confidence value based on the number of reads is associated with each predicted site. Empirically, we found that a confidence threshold of 0.995 produced the largest number of predicted sites in all strains: 59 sites in N2, 141 sites in *adr-1(-)*, 71 sites in *FLAG-ADR-1*, 102 sites in *FLAG-ADR-1 ds1+2 mutant*, while identifying the lowest number of edits in the *adr-2(-)* strain (6 sites) that we presumed represented false positives (Table S1).

Of the 270 unique high confidence editing sites that were identified, but not present in *adr-2(-)* worms (Table S1), 250 sites are novel editing events that occur within 48 different transcripts; the remaining 20 high confidence sites were located within the previously identified ADAR targets *C35E7.6*, *lam-2* and *rncs-1* (Morse et al., 2002; Morse and Bass, 1999). The majority (71%) of these candidate-editing events occur within non-coding regions of the genome (Fig 4B). Strikingly, the vast majority of editing events occurred in 3' UTRs, consistent with the hypothesis that A-to-I editing controls gene expression by altering regulatory motifs in these regions. Interestingly, regions of the genome that encode for transposons were the second most highly identified (18%) category of editing events. In addition, we did identify 11 potential editing sites in coding regions of 8 different mRNAs. As editing events in the coding region of *C. elegans* mRNAs had not previously been identified, this suggests that similar to mammalian and *Drosophila* ADARs, *C. elegans* ADARs may also perform site selective editing *in vivo*.

Although ADARs target dsRNA of any sequence, the extent of editing at a particular site depends on the neighboring nucleotides (Wahlstedt and Ohman, 2011). Using the Two Sample Logo software (Vacic et al., 2006), the 270 candidate editing sites had an over-representation of A both immediately 5' and 3' to the edited adenosine, whereas both G and C are under-represented at the positions 5' to the edited adenosine and C is under-represented 3' to the edited adenosine (Fig 4C). Both *in vitro* biochemical studies and transcriptome-wide RNA-Seq data indicates that human ADARs have a similar 5' preference. However human ADARs tend to favor a G at the 3' position to the edited adenosine (Lehmann and Bass, 2000; Riedmann et al., 2008). It is important to note, that because of overlapping specificities of mammalian ADARs, human transcriptome-wide datasets apply to editing by both human ADAR1 and ADAR2. However, as *C. elegans* ADR-2 is responsible for deamination of all of the RNA-Seq sites, our data provides the first *in vivo* nucleotide preferences of a single ADAR acting primarily at noncoding regions.

To validate the potential editing sites, Sanger sequencing editing assays were performed for 9 novel edited transcripts (Figure S2A). Importantly, 50 of the 53 predicted sites were verified by Sanger sequencing, suggesting the false discovery rate of the pipeline is approximately 5.7%. In addition to the 50 editing sites identified from the RNA-Seq analysis, Sanger sequencing of these 9 novel transcripts revealed 179 additional editing sites (Table S2), indicating that our probabilistic model is capable of identifying highly edited transcripts.

To determine if ADR-1 affected editing in the transcriptome, the editing efficiency of the 270 high confidence editing sites was quantified using a novel Bayesian model. To ensure accurate quantification, we processed all the RNA-Seq reads through the bioinformatics pipeline described above (Fig 4A), with one exception: read filter 5d was relaxed from requiring an edit site to be 25 nt from each end down to a less-stringent 5 nt and required a minimum of 5 reads for a site in a given strain. With these criteria, we were able to quantify editing of over 100 sites for each of the four strains, with any two strains having an overlap of between 72–105 editing sites (Figure S2B–E). Pairwise comparison of the editing sites identified from the four RNA-Seq data sets indicated that editing efficiency is most consistent between the wild type and FLAG-ADR-1 strains (Fig 4D, Figure S2F–H). This is consistent with the Sanger sequencing data of known editing sites and provides further evidence that the FLAG-ADR-1 transgene is capable of restoring editing to the *adr-1(-)* strain at most sites. As over-two thirds of the wild-type and FLAG-ADR-1 sites fell within one standard deviation (12%) of the regression line on the scatter plot, we used this threshold to categorize our newly identified sites into ADR-1 and non-ADR-1 regulated (Table S3). As multiple RNA-Seq studies have shown that determination of editing levels increases with read coverage (Bahn et al., 2012; Lee et al., 2013), it is important to note that similar results (>80% overlap) were obtained when we utilized read density to estimate the error of editing at each site (Table S3), suggesting that the editing percent thresholds for ADR-1 regulated and non-regulated sites are accurate. Comparison of editing levels at the 81 sites common between wild-type and *adr-1(-)* RNA-Seq datasets revealed that over half (56%) of the edited adenosines have altered editing levels in the absence of *adr-1* (Table S3). Interestingly, 44 of these 45 sites are located within the 3' UTRs of 13 novel edited transcripts that we identified. This data is consistent with our quantitative Sanger sequencing analysis of the known ADAR targets 3' UTRs (Fig 1A). In addition, at 38 of these ADR-1 regulated sites we were able to quantify editing levels for both the FLAG ADR-1 and FLAG-ADR-1 ds1+2 RNA-Seq datasets. Editing levels at 13 sites located within the 3' UTRs of 8 newly identified ADAR target mRNAs were dependent upon dsRNA binding by ADR-1 (Fig 4E). Together these transcriptome-wide studies indicate that ADR-1 regulates editing of specific adenosines within the 3' UTRs of the majority of *C. elegans* edited mRNAs and dsRNA binding is required for this function.

ADR-1 and ADR-2 co-occupy transcripts *in vivo*

At present it is unclear how ADR-1 binding to mRNAs affects editing by ADR-2. It is possible that ADR-1 and ADR-2 heterodimerize in the cell to edit certain transcripts, whereas others are edited by ADR-2 alone. Alternatively, it is possible that ADR-1 and ADR-2 interact on the same transcripts, but regulate editing in an adenosine-specific manner. To gain insight into these possibilities, we examined the wild-type and FLAG-ADR-1 RNA-Seq datasets to determine whether editing at ADR-1 regulated adenosines occurred on the same reads as edited adenosines that are not affected by loss of *adr-1*. For most of the novel transcripts edited in the 3' UTR (9/12), editing was observed at both adenosines affected by *adr-1* and non-regulated sites, within the same 75 nt read (Table S3).

To provide further evidence that ADR-1 and ADR-2 associate on common targets *in vivo*, we immunoprecipitated FLAG-ADR-1 and tested for the presence of ADR-2 with an ADR-2 specific antibody (Fig 4F). ADR-2 was present in IPs from FLAG-ADR-1 worms, but not FLAG-ADR-1 ds1+2 mutant or *adr-1(-)* worms (Fig 4G). Consistent with an RNA-dependent interaction of ADR-1 and ADR-2, IPs of wild-type ADR-1 treated with RNase also resulted in reduced ADR-2 co-immunoprecipitation (Figure S2J,K). Together, these data suggest that ADR-1 and ADR-2 interact on transcripts *in vivo*, but are not likely to heterodimerize independent of target mRNAs.

DISCUSSION

In this study, we have demonstrated that *C. elegans* ADR-1 utilizes its dsRNA binding function to regulate A-to-I editing levels *in vivo*. Using a high-throughput RNA sequencing approach coupled to probabilistic modeling, we were able to expand the number of known ADAR target mRNAs five-fold, as well as provide the first transcriptome-wide evidence that ADR-1 is a catalytically inactive member of the ADAR family. Furthermore, using both our extensive Sanger sequencing analysis of ADAR targets and quantification of transcriptome-wide RNA-Seq data, we demonstrate that ADR-1 regulates editing efficiency of specific adenosines within most ADAR target 3' UTRs.

We propose that ADR-1 regulates editing by binding to target mRNAs and altering accessibility of ADR-2 for specific adenosines. Multiple recent studies support the idea that the RNA binding protein (RBP) landscape of ADAR target mRNAs affects editing levels (Bhogal et al., 2011; Garncarz et al., 2013; Tariq et al., 2013). However, in most of these studies, RNA binding by the regulators was not shown to be required for A-to-I regulatory activity and these regulators were all single-stranded RBPs that altered editing of specific coding editing events. In contrast, we demonstrate that ADR-1 binds to several target mRNAs via its dsRBDs, and that this binding is required for regulation of editing. This dsRNA binding activity would allow ADR-1 to interact with nearly all the same targets as ADR-2, thus allowing it to serve a more global role in regulating editing within long double-stranded regions. As dsRBDs are the second most abundant RNA recognition motif (Stefl et al., 2010), it is unlikely that this regulatory role is limited to *C. elegans* ADR-1. Consistent with this, 20% of our newly discovered edited transcripts overlap with recently identified targets of another dsRNA binding protein (dsRBP), *C. elegans* Staufen (LeGendre et al., 2013) (Table S1).

Our Sanger sequencing and transcriptome-wide analyses suggest that the regulatory role of ADR-1 is specific to certain adenosines (Fig 1A, Table S3). Although dsRBPs are generally presumed to lack sequence specificity (Tian et al., 2004), recent structural data suggests ADARs recognize specific nucleotides within dsRNA (Stefl et al., 2010). Our RIP assay indicates that ADR-1 binds to *lam-2* and *pop-1* mRNAs to a similar extent in the presence and absence of *adr-2* (Fig 2D). Thus, at least for certain edited mRNAs, ADR-1 does not compete with ADR-2 for binding sites *in vivo*. Consistent with this, the majority of the ADR-1 regulated sites identified in both the RNA-Seq datasets and Sanger analysis have enhanced editing in the presence of *adr-1* (Fig 1A, Table S3), suggesting that ADR-1 functions primarily to promote ADR-2 editing, not compete with ADR-2 for target adenosines. As editing is not required for ADR-1 to bind these mRNAs, we postulate that, *in vivo*, ADR-1 first binds to target mRNAs and then either alters binding of ADR-2 to specific regions and/or regulates the catalytic activity of ADR-2 (See Graphical abstract). Interestingly, it was recently demonstrated that human ADAR1 binding to mRNAs creates binding sites for another RBP, HuR, which results in increased RNA stability of HuR-ADAR1 bound transcripts (Wang et al., 2013). Similar to human ADAR1-HuR, we detected an *in vivo* interaction between wild-type ADR-2 and ADR-1, but not the ADR-1 ds1+2 mutant, which is consistent with ADR-1 and ADR-2 interacting on target mRNA. Interestingly, it has previously been suggested that human ADAR homodimerization on dsRNA is required for efficient editing *in vitro* (Jaikaran et al., 2002). Although our evidence indicates that ADR-1 utilizes dsRNA binding to regulate editing, it is possible that this regulatory function is due to effects of ADR-1 on expression of other RBPs, which in turn alter ADR-2 accessibility to target mRNAs. Future work aimed at both identifying ADR-1 and ADR-2 binding sites on mRNAs *in vivo* and determining the impact of ADR-1 on ADR-2 editing activity *in vitro* will be needed to determine if there is a correlation between binding site specificity and regulation of specific sites. In summary, our results

indicate that ADR-1 utilizes dsRNA binding to regulate A-to-I editing across the *C. elegans* transcriptome. These studies not only suggest a potential biological function for the catalytically inactive ADARs present in humans, but also unveil a potential mechanism for other dsRBPs to regulate RNA editing levels.

EXPERIMENTAL PROCEDURES

Maintenance of worm strains and Transgenics

Worm strains were maintained by growth on NGM plates seeded with *Escherichia coli* OP50. A detailed description of the transgenic strains is given in the Extended Experimental Procedures.

RNA Isolation and Editing Assays

Total RNA was isolated using Trizol (Invitrogen). RNA was further treated with Turbo DNase (Ambion) and then isolated using the RNA Easy Extraction kit (Qiagen). Editing assays were performed using Thermoscript (Invitrogen) for reverse transcription and PFX Platinum DNA Polymerase (Invitrogen) for PCR amplification with gene-specific primers (Table S4). PCR products were gel purified and subjected to Sanger sequencing. For all editing assays, negative controls were conducted without Thermoscript RT to ensure that all DNA subjected to sequencing resulted from cDNA amplification.

Strand-specific RNA sequencing

Strand specific mRNA sequencing libraries were prepared as described previously (Parkhomchuk et al., 2009). Libraries were normalized to 2nM and sequenced for SE76 cycles on either HiSeq2000 (*adr-1(-);adr-2(-)*) or Illumina GAII (all other strains).

Bioinformatics Pipeline

To achieve accurate identification of editing sites, we combined filters from existing pipelines (Chen, 2013; Lee et al., 2013; Levanon et al., 2004; Ramaswami et al., 2012) in a strand-specific manner. Accurate quantification was performed by extending the existing Bayesian method for genomic variant calling used in the 1000 Genomes project (Li et al., 2008) with a custom-designed prior on the editing % (Figure S2I). In addition to leveraging established considerations with regards to read sequencing and alignment errors (Kleinman and Majewski, 2012; Lin et al., 2012; Pickrell et al., 2012) our approach benefits greatly from using the *adr-1(-);adr-2(-)* strain as a powerful filter for unannotated variants. Detailed steps of the pipeline and Bayesian method for variant calling are described in the Extended Experimental Procedures.

RNA Immunoprecipitation (RIP) Assay

After washing with IP Buffer (50mM HEPES, pH 7.4; 70mM K-Acetate, 5mM Mg-Acetate, .05% NP-40 and 10% glycerol), worms were subjected to 3J/cm² of UV radiation using the Spectrolinker (Spectronics Corp.) and stored at -80°C. To obtain cell lysates, frozen worms were ground with a mortar and pestle on dry ice. After thawing, the lysate was centrifuged and protein concentration was measured with Bradford reagent (Sigma). Five micrograms of extract was added to anti-Flag magnetic beads (Sigma) that were washed with wash buffer (WB: 0.5M NaCl, 160mM Tris-HCl pH 7.5). After incubation for 1 hour at 4°C, the beads were washed with ice-cold WB, resuspended in low salt WB (0.11M NaCl), 1µl RNasin (Promega) and 0.5µl of 20mg/ml proteinase K (Sigma) and incubated at 42°C for 15 minutes to degrade protein and release bound RNA. Protein samples were subjected to SDS-PAGE and western blotting with a FLAG antibody (Sigma). RNA samples were

isolated as described above. Following DNase treatment, qRT-PCR for known editing targets was performed as previously described (Hundley et al., 2008).

Flow Cytometry

Flow cytometry was conducted at the IUB Flow Cytometry Facility by a dedicated technician using the COPAS Select (Union Biometrica) large particle sorter. Parameters were adjusted to select adult worms and expressing GFP for transgenic lines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

Identification of >400 novel A-to-I editing sites, primarily within noncoding regions.

ADR-1 regulates editing of specific adenosines within 3' UTRs of diverse transcripts.

ADR-1 regulates RNA editing by directly binding to ADR-2 target mRNAs.

ADR-1 and ADR-2 do not form heterodimers, but co-occupy transcripts *in vivo*.

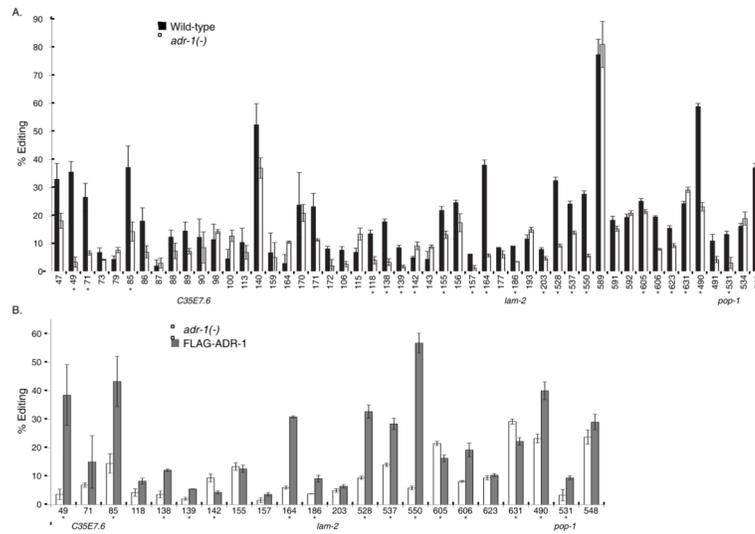


Figure 1. ADR-1 alters editing at specific adenosines in multiple mRNAs
 (A and B) Editing levels at individual nucleotides within the 3' UTRs were measured for 3 biological replicates. Error bars represent standard error of the mean (SEM). Significant changes ($p < 0.05$) in editing levels between (A) wild-type and *adr-1(-)* or (B) *adr-1(-)* and FLAG-ADR-1 are marked with an asterisk.

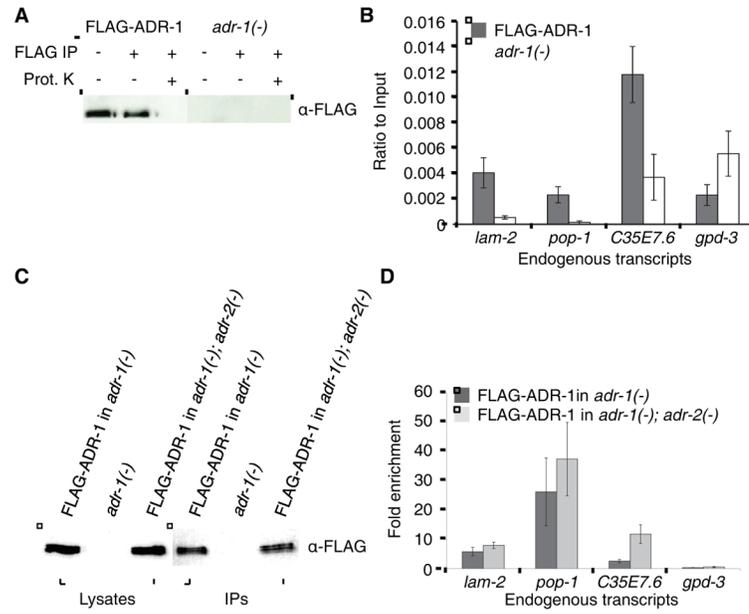


Figure 2. ADR-1 binds ADR-2 substrates *in vivo*

(A) Lysates from the indicated worm lines were subjected to FLAG IP and treatment with Proteinase K (Prot. K). A portion of the untreated lysate (IP–, Prot. K–), IP (IP+, Prot. K–) and beads after Prot. K treatment (IP+, Prot. K–) were subjected to immunoblotting for the FLAG epitope.

(B) cDNA levels for the indicated endogenous mRNAs were measured using qRT-PCR. Values from the IP samples of FLAG-ADR-1 in *adr-1(-)* and the negative control *adr-1(-)* were divided by their respective input levels. Error bars represent SEM for three biological replicates.

(C) Lysates from the indicated worm lines were subjected to immunoprecipitation with magnetic FLAG resin. A portion of the input lysate and IPs were subjected to immunoblotting for the FLAG epitope.

(D) cDNA levels for the indicated endogenous mRNAs were measured using qRT-PCR. Ratios of the cDNAs present in the IP samples of the indicated strains were divided by their respective input levels and normalized to the negative control *adr-1(-)* to give a fold enrichment. Error bars represent SEM for three biological replicates.

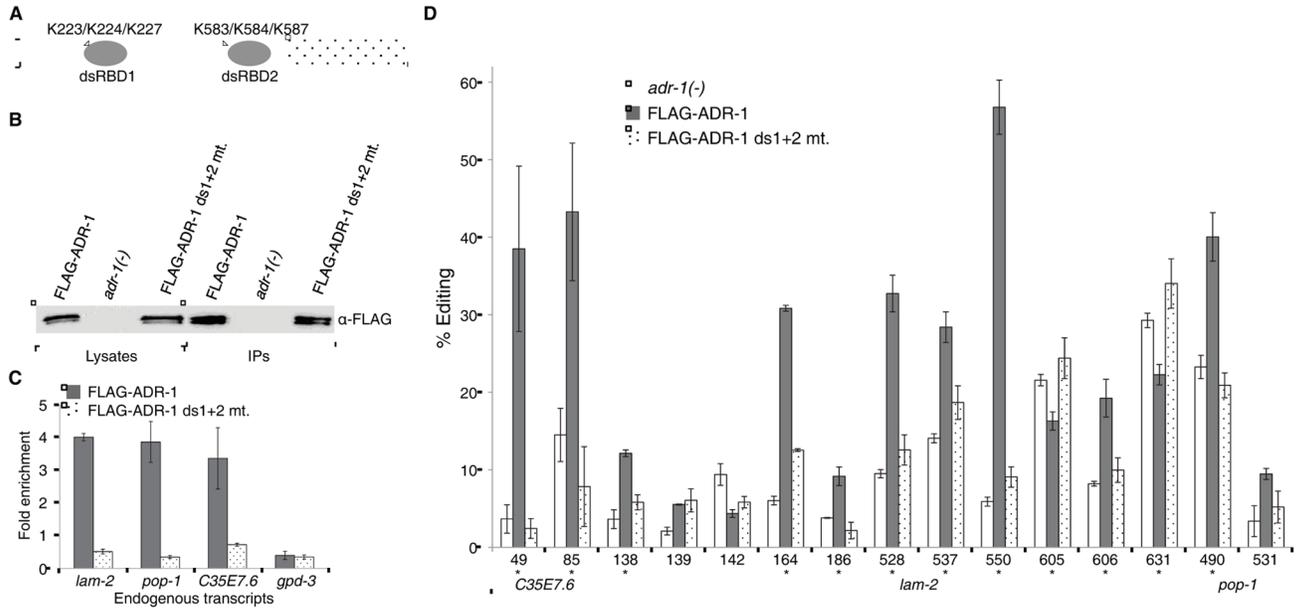


Figure 3. Mutation of the KKxxK Motif within the dsRBDs of ADR-1 abolishes dsRNA binding and editing regulation

(A) Schematic of ADR-1 protein with dsRBDs (grey ovals) and deaminase domain (patterned rectangle). Lysine (K) residues mutated are indicated above each dsRBD. (B) FLAG Immunoblotting of lysates and IPs of the indicated strains. (C) Ratio of the cDNA present in the IP samples divided by the input cDNA levels for the indicated strains were divided by the IP:input ratio of the *adr-1(-)* worms. Error bars represent SEM for three biological replicates. (D) Calculated percent editing in the indicated strains for the endogenous mRNAs of *C35E7.6*, *lam-2* and *pop-1*. Error bars represent SEM of 3 biological replicates. Significant changes ($p < 0.05$) in editing levels between FLAG-ADR-1 and FLAG-ADR-1 ds1+2 mutant are marked with an asterisk.

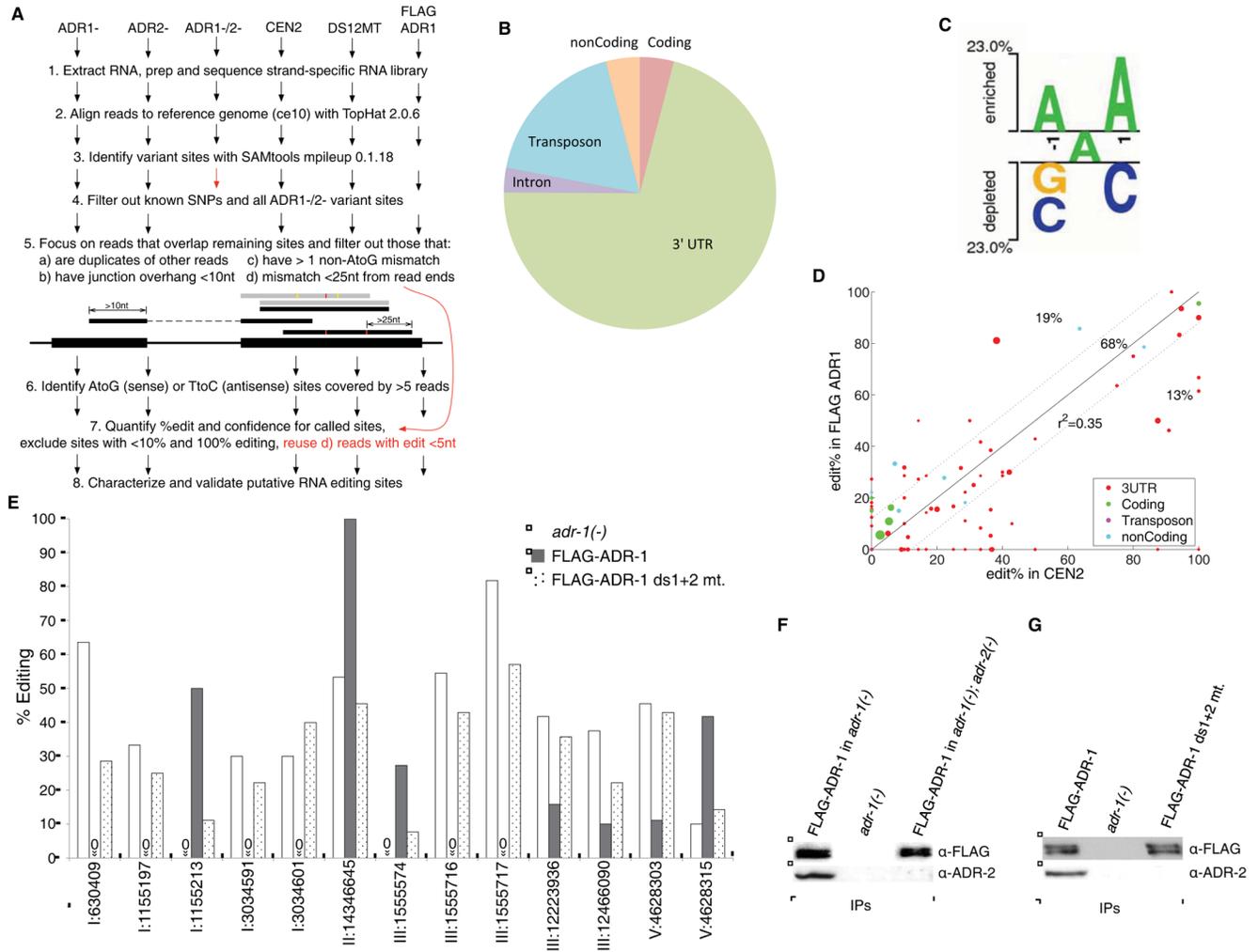


Figure 4. Impact of dsRNA binding by ADR-1 on the editing transcriptome

(A) Bioinformatics strategy depicting the major steps for processing RNA-seq data into A-to-I sites for each strain.

(B) Distribution of identified RNA editing sites within annotated transcriptome regions.

(C) Nucleotide preferences for the 270 candidate editing sites were calculated compared to a randomized control. Enriched and depleted nucleotides are shown above and below the axis, respectively. The level of conservation is represented by letter height. Logos were generated using a t-test with $P < .005$ and no Bonferroni correction.

(D) Scatter plots of percent editing of quantified sites that overlap in the wildtype (CEN2) and FLAG-ADR-1 datasets. The r^2 fit to the $y=x$ line (black diagonal). The margin (dotted line) between no-change and differentially-edited sites equals 12 units of change in the edit % (one standard deviation).

(E) Editing levels for 13 sites from the RNA-seq data where editing levels between *adr-1(-)* and FLAG-ADR-1 and between FLAG-ADR-1 and FLAG-ADR-1 ds1+2 mutant were greater than 12% (Table S3). Adenosines that had no observed editing are marked with a zero above the x-axis.

(F and G) Immunoblotting analysis of FLAG IPs from the indicated strains. IPs were performed as previously stated except worms were not subjected to UV-crosslinking and only light salt washes were employed.