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POINT OF VIEW

Restricting retrotransposons: ADAR1 is another guardian of the human genome

Elisa Orecchini, Loredana Frassinelli, and Alessandro Michienzi

Department of Biomedicine and Prevention, University of Rome 'Tor Vergata', Rome, Italy

ABSTRACT

ADAR1 is an enzyme that belongs to the Adenosine Deaminases Acting on RNA (ADARs) family. These enzymes deaminate adenosines to inosines (RNA editing A-to-I) within double-stranded RNA regions in transcripts. Since inosines are recognized as guanosines by the cellular machinery, RNA editing mediated by ADARs can either lead to the formation of an altered protein (recoding) or affect different aspects of RNA metabolism.

Recently, a proteomic analysis led to the identification of novel ADAR1-associated factors and found that a good fraction of them is shared with the Long Interspersed Element 1 (LINE-1 or L1) ribonucleoparticles (RNPs). This evidence suggested a possible role of ADAR1 in regulating the L1 life cycle. By taking advantage of the use of cell culture retrotransposition assays, a novel function of this deaminase as an inhibitor of L1 retrotransposition was demonstrated. These results pave the way toward a better comprehension of the mechanisms of restriction of retrotransposons.

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Introduction

Retrotransposons, are the predominant class of Transposable Elements (TEs) in most mammalian genomes and can be subdivided into those sequences that contain the long-terminal repeats (LTR) and those that do not (non-LTR).[1-2] The Long INterspersed Element-1 (LINE-1s or L1s) is an autonomous non-LTR retrotransposon that continues to generate both intra-and inter-individual genetic variations in the human population.[1]

A typical retrotransposition-competent human LINE-1 element is ~6 kb in length and contains a 5′ untranslated region (UTR), open reading frames (ORFs) and a 3′ UTR.[3-4]

The promoter region of these elements is within the 5′ UTR, driving the transcription of a bicistronic L1 RNA, which contains 2 non-overlapping ORFs (ORF1 and ORF2).[5]

ORF1p is a ~40 kDa polypeptide with nucleic acid chaperone activity,[6-7] and ORF2p is a ~150 kDa protein with endonuclease and reverse transcriptase functions.[8-10] Upon translation the ORF1p and ORF2p proteins bind their own L1 RNA (*cis*-preference) forming a ribonucleoprotein (RNP) complex in the cytoplasm.[11-12] Upon translocation of the RNP to the nucleus, it may generate a new chromosomal insertion via a target primed reverse transcription mechanism (TPRT).[13] L1 can also function *in trans* to mobilize the non-autonomous Short Interspersed Elements (SINEs).

Even though more than 500 000 copies of L1 exist in the human genome, most L1s are inactive due to point mutations, rearrangements, or truncations, with only 80–100 elements potentially active in any individual.[14-15]

The uncontrolled retrotransposition of LINE-1 can be deleterious for the host genome thereby the cell has evolved several mechanisms of defense against these endogenous parasites. The repression of retrotransposons occurs both at the transcriptional and post-transcriptional levels.[16-17]

The major control occurs by limiting the expression of L1 through histone modification and DNA methylation.[18-25]

Furthermore, several cellular trans-acting restriction factors regulate the L1 life cycle with different mechanism.[16-17] The list of these factors is growing fast and interestingly many of these proteins are involved in nucleic acid metabolism, and some are induced by type I interferons.

During evolution, the cell has developed different mechanisms of defense to protect against the danger of endogenous and exogenous parasites, thus it is not surprising that many of the known anti-retrotransposon restriction factors are also anti-retroviral. Of note, different enzymes belonging to the family of the APOBEC3 cytidine deaminase were reported to restrict LINE-1 retrotransposition. In particular, APOBEC3A can inhibit retrotransposition through the deamination of the single-stranded DNAs that are exposed transiently during the LINE-1 TPRT process,[26] whereas APOBEC3B, APOBEC3C, and APOBEC3DE seem to inhibit retrotransposition by diverse deamination-independent mechanisms,[27-29] thus showing the complexity of the restriction processes. Moreover, we recently provided evidence showing that another deaminase, ADAR1, is a suppressor of LINE-1 retrotransposition.[30]

ADAR1 is a member of the Adenosine Deaminases Acting on RNA (ADARs) family and catalyzes the conversion of adenosine to inosine (A-to-I) within double-stranded RNAs.[31-32] There are 2 different ADAR1 isoforms generated by the use of alternative promoters and alternative splicing.[33-35] The short form of ADAR1 (p110) is constitutively

CONTACT Alessandro Michienzi alessandro.michienzi@uniroma2.it Biomedicine and Prevention, University of Rome "Tor Vergata", via Montpellier 1, Rome 00133, Italy.

expressed and localizes mainly to the nucleus, whereas the long form of ADAR1 (p150) is interferon-inducible and shuttles between the nucleus and cytoplasm.[31] ADAR1 can edit both cellular and viral RNAs and since inosines are recognized as guanosines by the cellular machinery, RNA editing mediated by ADARs can lead to the formation of an altered protein if editing occurs within the coding sequence of mRNAs.[31,36] However, recently it has been clearly demonstrated that most of the RNA editing occurs within non-coding regions, most particularly Alu elements, affecting different aspects of the RNA metabolism, such as RNA stability, translation, splicing, and interaction with specific protein factors.[36] Moreover, RNA editing is deregulated in a variety of human diseases.[32,37-41] Therefore, ADAR1 has a deep impact on gene expression regulation. Moreover, ADAR1 plays a role as suppressor of interferon (IFN) signaling.[42-43] Recently, Mannion and colleagues proposed that ADAR1 editing may mark endogenous dsRNAs as "self" to distinguish them from exogenous, mostly viral, "non self" dsRNAs thus avoiding the induction of an aberrant type I IFN response.[44] This hypothesis was further confirmed by others[45-47]

ADAR1 is an inhibitor of LINE-1 retrotransposition

Our study started with the affinity purification of the ADAR1 RNP complexes followed by mass spectrometry analysis of 293T cells expressing HIV-1 with the initial goal of identifying the ADAR1-interacting factors that could contribute to the proviral activity of the deaminase previously reported.[30,48-50] This analysis led to the identification of 14 non-ribosomal ADAR1-associated factors, among which a good fraction, such as PABPC1, hnRNP L, HSPA1A, nucleolin and TOP1 were previously reported as L1 RNP-associated factors.[51-53] Moreover, 3 of these proteins (nucleolin, hnRNP L and PABPC1) were shown to affect L1 retrotransposition.[51,54-55] This result prompted us to test whether ADAR1 too is involved in the L1 life cycle. First, we confirmed by co-immunoprecipitation experiments the interaction between ADAR1 and the L1 RNP-associated proteins identified by mass spectrometry. Moreover, we found that these interactions occur also in the absence of HIV-1 expression, thus suggesting that we identified general interactors of the deaminase, most of which are novel. Furthermore, we tested whether ADAR1 is a regulator of L1 retrotransposition by taking advantage of the use of different and widely used cell culture retrotransposition assays.[56-58]

By using these assays in HeLa cells silenced for ADAR1 expression, we observed an increase of LINE-1 retrotransposition. We further extended our analysis and confirmed that overexpression of ADAR1 decreases L1 retrotransposition.[30] Overall, these results suggest a novel function for ADAR1 as a general repressor of retrotransposition.

Possible mechanism for ADAR1 anti-retrotransposon activity

What could be the mechanism that drives the inhibition of L1 retrotransposition mediated by ADAR1?

a) RNA editing model

It was previously suggested that L1 RNAs harbor some double-stranded (ds) RNA-binding elements for Microprocessor,[59] thus the most logical answer to this question is that ADAR1 upon binding to L1 dsRNA, catalyzes the conversion of adenosines to inosines, thus potentially either altering the folding of some dsRNA elements or mutating sequences that are critical for retrotransposition activity (Fig. 1A).

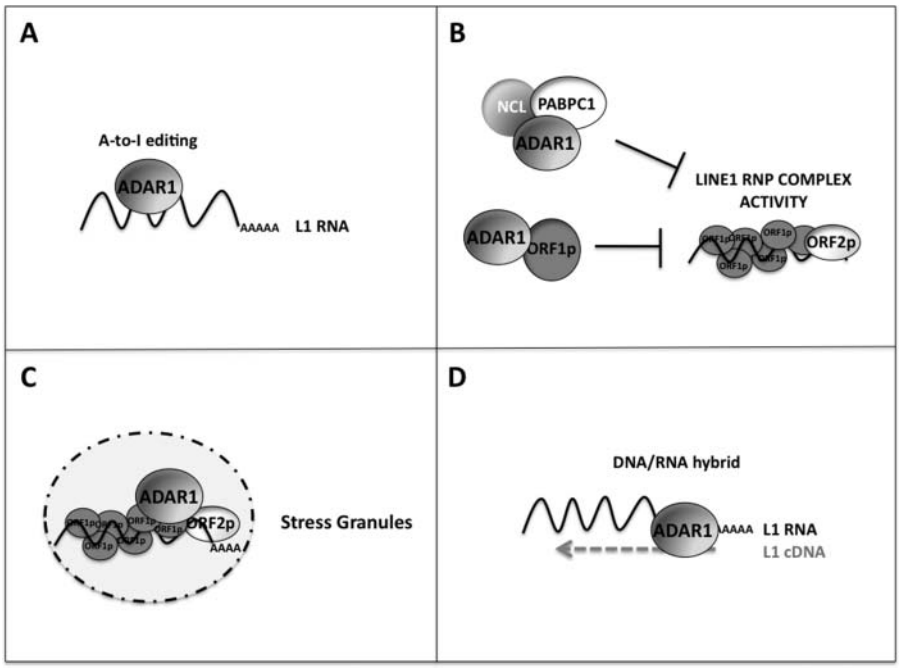

Figure 1. Possible models for how ADAR1 inhibits LINE-1 retrotransposition. (A) L1 RNAs are edited by ADAR1 causing nucleotide changes in the ORF sequence or altering the dsRNA elements harbored within L1 RNAs. (B) ADAR1 by binding the basal L1 RNP complex and/or its associated proteins impairs its functionality. (C) ADAR1 sequesters L1 RNP complexes in the stress granules. (D) ADAR1 binds L1 DNA/RNA hybrids during the RT step of the L1 life cycle and either impairs the formation of these hybrids or edits the DNA.

We tested this hypothesis by using a deletion mutant of ADAR1 that lacks the deaminase domain, and showed that this protein represses L1 retrotransposition to a similar extent as the wild-type protein.[30] Moreover, we did not find A-to-I editing events in the ectopically expressed L1 RNAs isolated from cells over-expressing ADAR1 (almost half of the full-length 6 kb analyzed).[30] All together, these results suggest that ADAR1 inhibits retrotransposition by a mechanism that is RNA-editing independent, even though a more detailed analysis is required to fully address this issue. In fact, we cannot exclude that editing is indeed occurring at a very low frequency below the threshold of detection of our assay (3–4%), although not sufficiently to affect the retrotransposition process. Moreover, we cannot rule out that editing is occurring in the L1 RNA regions not sequenced.

To further complicate this issue, recently, by using inosine-specific cyanoethylation combined with Sanger sequencing (ICE method) and deep sequencing (ICE-seq), several A-to-I editing events were identified in the human brain transcriptome, and some of them lie in the 3′UTRs of transcripts (i.e. TAF1, ACBD7, GPLD1, TAF1, NBPF11 mRNAs) containing dsRNA structures formed by LINE sequences (partial, not full length) repeated in tandem and in inverse orientation.[60] Therefore, LINE-1 sequences embedded in other transcripts and forming dsRNA structures can be targets of ADAR enzymes.

b) Restriction of L1-RNP complex

In our study, we also reported that ADAR1 binds to the basal L1 RNP complex, in particular ORF1p and the L1 RNA.[30] The correct assembly of the L1 RNP complex is pivotal for retrotransposon activity, thus it is plausible that ADAR1 binding to the L1 RNP complex might directly or indirectly interfere with its activity resulting in the inhibition of retrotransposition (Fig. 1B). We first assayed whether ADAR1 binding to the L1 RNP complex might affect the intracellular accumulation of L1 RNA and ORF1p components, as shown for other L1 restriction factors.[53,61-64]

We tested this hypothesis by measuring the levels of both ORF1p (by Western Blot analysis) and L1 RNA (by RT-qPCR) ectopically expressed from a retrotransposition cassette in HeLa cells silenced for ADAR1 expression, but we did not find any significant alteration in their levels compared with the controls.[30] This result indicates that in our experimental setting the ADAR1 inhibition of L1 retrotransposition is not caused by a decreased stability/accumulation of the L1 RNP components. Nevertheless, silencing of ADAR1 expression in HeLa cells causes an increase of the endogenous L1 transcripts (measured by RT-qPCR, Orecchini et al. unpublished). We don't know the reason for this discrepancy and additional experiments are required to explain it.

Furthermore, as mentioned above, we have shown the interaction between ADAR1 and some L1 RNP-associated proteins.[30] In particular, PABPC1 and nucleolin proteins were previously reported to exert a positive effect on L1 retrotransposition.[54-55] In fact, PABPC1 is critical for L1 RNP formation, and alteration of its intracellular level affects retrotransposition and subcellular localization of ORF1p.[54] Moreover, nucleolin likely acts as an IRES trans-acting factor to stimulate ORF2 translation of murine L1 RNA.[55]

Therefore, ADAR1 by interacting with these proteins and other L1 RNP-associated factors may affect their stimulatory activity thus impairing L1 retrotransposition (Fig. 1B).

Finally, we have preliminary results showing that ADAR1 may also impair Alu retrotransposition (Orecchini et al., unpublished). In particular, in HeLa cells silenced for ADAR1 expression and co-transfected with an Alu retrotransposition cassette (pAlu-Neo^{tet})[65] together with an ORF2p expression vector (pORF2NoNeo), an increase in Alu retrotransposition was observed. This result suggests that ADAR1 regulates different classes of retrotransposons. Since in this assay the Alu retrotransposition can be achieved only through the overexpression of the LINE-1 ORF2p protein, it would be of great interest to test whether both the inhibition of L1 and Alu retrotransposition mediated by ADAR1 occur through the disruption of ORF2p activity or by reducing its level as previously shown for SAMHD1.[66]

c) Stress Granules as a site for L1 RNP sequestration by ADAR1

It has been previously reported that the L1 RNA, ORF1p and ORF2p proteins accumulate in stress granules (SGs) and in the nucleoli of a small percentage of cells.[67-69] SGs are assemblies of untranslating messenger ribonucleoproteins (mRNPs) that form from mRNAs stalled at translation initiation, and their formation modulates the stress response, viral infection, and signaling pathways.[70]

Of note, ADAR1 p110 isoform is almost exclusively a nuclear/nucleolar protein, while ADAR1 p150 is a shuttling protein and accumulates in the cytoplasm and under particular stress localizes in stress granules (SGs).[71] The Zα domain of ADAR1 p150 is required for such specific subcellular localization.[71] Moreover, we identified stress granule-associated proteins G3BP2 and PABPC1 as novel interactors of ADAR1.[30]

Therefore, it is conceivable that SGs and the nucleoli are the subcellular compartments where the interaction between ADAR1 and the L1 RNPs may take place. To address this issue, we performed immunofluorescence experiments in 293T cells transfected with a retrotransposition cassette containing the full length L1 sequence with the ORF1p fused to a T7 tag (pES2TE1; 69). We have preliminary results showing that the endogenous ADAR1 co-localizes with ORF1p in cytoplasmic granules; this localization is even more evident when cells are treated with an inducer of SGs, such as sodium arsenite (Orecchini et al. unpublished).

Interestingly, other L1 RNP-associated factors localize in SGs, such as MOV10, ZAP, PABPC1 and APOBEC3 proteins.[53,61,62,67,72] Notably, Hu and collaborators proposed a novel mechanism whereby SAMHD1 enhances assembly of cytoplasmic stress granules that then sequester L1 RNPs and prevent their retrotransposition.[73]

Therefore, we cannot exclude that ADAR1, in particular the p150 isoform, may suppress L1 retrotransposition through a similar mechanism (Fig. 1C). In any event, co-localization of ADAR1 p150 with the ORF1p protein suggests that this isoform may be critical for L1 restriction.

d) DNA/RNA hybrids

Finally, it has been recently demonstrated that ADARs can deaminate 2′-deoxyadenosines in the DNA strands of DNA/

RNA hybrids *in vitro*, thus expanding the possible biologic functions of ADARs.[74] The RNA-DNA hybrid is an essential intermediate of reverse transcription during the retrotransposition process of LINE-1, thus it can be envisioned that ADAR1, by simply binding such RNA-DNA hybrids and/or by mutating the L1 DNA sequence may affect retrotransposition (Fig. 1D). Based on the results described above using the mutant of ADAR1 lacking the deaminase domain we suppose that the first hypothesis is more plausible.

Aicardi-Goutières syndrome

Aicardi-Goutières Syndrome (AGS) is an inflammatory encephalopathy that exhibits a neurologic dysfunction characterized by increased production of type I interferon (IFN).[75]

AGS can be caused by mutations in any of 7 genes (TREX1, RNaseH2A, RNaseH2B, RNaseH2C, SAMHD1, ADAR1 and IFIH1) that carry out diverse functions of intracellular nucleic acid metabolism and sensing. A deficiency of these proteins is thought to result in the accumulation of self-derived nucleic acid species that are recognized as danger signals by sensors of the innate immune system, triggering the pathogenic type 1 interferon (IFN) response.[75]

The source of endogenous nucleic acids that are hypothesized to induce such a response remains uncertain, but may relate to retroelements.

Indeed, the products of 6 out the 7 AGS-related genes can be placed in a common pathway of metabolism of retroelements. In TREX1-deficient cells, type I IFN activation has been attributed to an increased amount of reverse transcribed DNA derived from endogenous retroelements.[76] Furthermore, it has been recently shown that TREX1 inhibits L1 retrotransposition by depleting ORF1p protein and AGS-related TREX1 mutants are deficient in this activity.[64] Moreover, SAMHD1 has been demonstrated to be a potent inhibitor of L1 activity and importantly it was found that AGS-related SAMHD1 mutants are defective in L1 retrotransposition inhibition.[66] It has been suggested that the RNaseH2 may be involved in the suppression of endogenous retroelements,[77] and we recently demonstrated that ADAR1 is an inhibitor of LINE-1 retrotransposition.[30] In addition, it has been reported that L1 activity is a potential inducer of interferon expression and autoimmune disorders,[77-79] thus providing a link between the dysregulation of L1 retrotransposition and the pathogenesis of AGS.

Therefore, we cannot exclude that in AGS patients containing mutations in ADAR1 (AGS6; 80), an increased level of L1 transcripts could trigger an aberrant IFN activation. However, Mannion and collaborators[44] analyzed repetitive element transcript levels by RNA-Seq in Adar1$^{-/-}$ embryos and no substantial differences were found compared with the control besides an increased expression of individual members of ERV and IAP families in the mutant mice. This result is in disagreement with our results showing an increase of L1 transcripts in HeLa cells silenced for ADAR1 expression, but it may suggest a general role of this enzyme in the metabolism of different retroelements. Therefore, it would be of paramount importance to assay the transcript level of all the different classes of retroelements (with particular attention to the HERV) in AGS6 patients to determine whether their expression is altered in that specific context.

Concluding remarks

Recent studies have provided a deeper knowledge of the different mechanisms causing the restriction of L1 retrotransposition. We found that ADAR1 is among the protein factors that inhibit retrotransposition, probably at the post-transcriptional level. Future investigations are required to shed light on the mechanism through which ADAR1 inhibits retrotransposition and to elucidate whether this enzyme is active against the mobilization of other retroelements.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ORCID

Elisa Orecchini http://orcid.org/0000-0002-0654-4696
Loredana Frassinelli http://orcid.org/0000-0001-6138-3963
Alessandro Michienzi http://orcid.org/0000-0002-9133-8482

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