The RNA editing enzyme ADAR2 restricts L1 mobility

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ABSTRACT
Adenosine deaminases acting on RNA (ADARs) are enzymes that convert adenosines to inosines in double-stranded RNAs (RNA editing A-to-I). ADAR1 and ADAR2 were previously reported as HIV-1 proviral factors. The aim of this study was to investigate the composition of the ADAR2 ribonucleoprotein complex during HIV-1 expression. By using a dual-tag affinity purification procedure in cells expressing HIV-1 followed by mass spectrometry analysis, we identified 10 non-ribosomal ADAR2-interacting factors. A significant fraction of these proteins was previously found associated to the Long Interspersed Element 1 (LINE1 or L1) ribonucleoparticles and to regulate the life cycle of L1 retrotransposons. Considering that we previously demonstrated that ADAR1 is an inhibitor of LINE1 retrotransposon activity, we investigated whether also ADAR2 played a similar function. To reach this goal, we performed specific cell culture retrotransposition assays in cells overexpressing or ablated for ADAR2. These experiments unveil a novel function of ADAR2 as suppressor of L1 retrotransposition. Furthermore, we showed that ADAR2 binds the basal L1 RNP complex. Overall, these data support the role of ADAR2 as regulator of L1 life cycle.

INTRODUCTION
Almost half of the human genome is composed of transposable elements (TEs) [1–3]. These are sequences that have been able or are still able to mobilize from one genomic location to another. TEs include retrotransposons, that move (retrotransposition) by a ‘copy and paste’ mechanism through an RNA intermediate [4]. Long interspersed element-1 sequences (LINE-1s or L1s) are the only active autonomous retrotransposon and comprise ~17% of human DNA [4–6].

There are about 500,000 L1 copies in the human genome, but the vast majority of them are retrotransposition defective, due to 5′ truncations, rearrangements, mutations or splicing [5,7]. However, about 80–100 copies preserve the capacity to retrotranspose, thus still shaping the evolution of the human genome [7–9]. Moreover, the L1 machinery also drives in trans the retrotransposition of short interspersed elements (SINEs), SINE-VNTR-Alu (SVA) and processed pseudogenes [10–12].

A full-length human L1 element is about 6 kb long and contains a 5′ untranslated region (UTR) harbouring an RNA polymerase II sense and an antisense promoter, followed by two open reading frames (ORFs), ORF1 and ORF2, and a 3′ UTR that ends in a poly(A) tract [13,14].

ORF1 encodes a ~40-kDa protein (ORF1p) with RNA-binding and nucleic acid chaperone activities [15–18].

ORF2 encodes an ~150-kDa protein (ORF2p) with endonuclease and reverse transcriptase activities [19,20]. L1 retrotransposition starts with the transcription of a full length bicistronic L1 mRNA from the sense strand promoter [21–23] in the nucleus, followed by translocation of the L1 mRNA to the cytoplasm where translation occurs leading to the synthesis of both ORF1p and ORF2p proteins [24,25]. These proteins preferentially bind, with a poorly understood mechanism, their encoding L1 mRNA [cis preference; 26, 37], thus forming the L1 ribonucleoprotein complex (L1 RNP). The L1 RNP complex then moves to the nucleus where a new copy of L1 element is integrated into the host genome by a target primed reverse transcription mechanism (TPRT) [28,29].

Recently, another ORF (ORF0) was identified [30]. ORF0 is primate-specific and it is transcribed from the antisense 5′UTR promoter sequence. There is published evidence showing that ORF0p may enhance L1 retrotransposition [30].

L1 retrotransposons, even though contributed to the evolution of the human genome, are also potent insertion
mutagenic agents that may cause genomic instability, genetic disorders and cancers [31,32]. For this reason, to limit the L1 deleterious effects cells evolved multiple mechanisms of defence ranging from epigenetic regulation to the action of host restriction factors [31,32]. Among these restriction factors, a role for the DNA and RNA deaminases was recently demonstrated [33, 34, and 35].

ADARs are adenosine deaminases acting on RNA, a family of enzymes that upon binding to double-stranded RNA (dsRNA) catalyse the conversion of Adenosines (A) in Inosines (I) (RNA editing A-to-I) [36,37]. Three ADAR enzymes are expressed in mammals, two that are catalytically active (ADAR1 and ADAR2) and one inactive (ADAR3). These enzymes share some common structural features, such as the catalytic domain at the C-terminus and two or three double-stranded RNA binding motifs (dsRBDMs) in the central or N-terminal region [36,37]. Since inosines are recognized as guanosines by most cellular machinery, RNA editing can lead to the formation of an altered protein (recoding) or may affect different aspects of the RNA metabolism [36,38,39]. RNA editing efficiency differs greatly depending on the target transcript and the different tissue. By perturbing ADAR enzymes in human cells, it has been suggested that ADAR2 catalyzes editing of specific adenosines (site-selective editing), whereas ADAR1 mainly mediates editing of several adenosines on dsRNAs (promiscuous editing) [40,41]. The vast majority of the RNA editing A-to-I occurs within repetitive elements, in particular in SINE (Alu in primates) present in tandem and in inverse orientation that gives rise to dsRNA in the non-coding sequence of mRNAs (introns and 3’UTRs).

Of note, ADARs have more functions besides RNA editing [42] thus exeracting a deep impact on the transcriptome. Moreover, it is well characterized that these enzymes affect microRNAs and other non-coding RNAs (ncRNAs) metabolism [36].

ADAR1 plays also a role as a suppressor of the interferon (IFN) signalling and response [43-46]. Mouse models and human cell lines lacking ADAR1 expression showed that its RNA editing activity (in particular of the interferon-inducible p150 isoform) plays a critical role in the innate immune response by avoiding that endogenous 'self' dsRNAs (probably long dsRNA formed by Alu:Alu hybrids) improperly stimulate cytosolic RNA sensing receptors melanoma differentiation-associated gene 5 or MDA5 [47] and activate innate immune proinflammatory responses via the MAVS adaptor [44-46,48]. Specifically, RNA editing A-to-I suppresses filament formation of MDA5 on dsRNAs [48]. MDA5 is one sensor of virus presence and is activated by RNA, recognizing the internal duplex structure [47].

The main function of ADAR2 is the editing of the coding sequence of transcripts found mostly in the brain, in particular the recoding of the Q/R site in the glutamate receptor Gria2 transcript [49]. Impairment of this editing leads to early-onset epilepsy and eventual death in mouse models [50]. Adar2 knockout mice die early after birth due to seizures caused by Gria2 underediting [51]. This phenotype can be rescued by knocking-in a G residue in the genomic DNA at the edited Q/R codon of Gria2, mimicking the constitutive recoding at this site [51].

As widely reported, RNA editing is deregulated in a variety of human diseases, such as cancer, neurodegenerative disease, interferonopathies and viral infection [37,39,52]. There is growing evidence showing that ADARs, in particular ADAR1, affect the life cycle of viruses exerting either an antiviral or a pro-viral activity depending on the type of virus and host [53,54]. Very recently, we demonstrated that ADAR1 plays a role also in controlling the activity of endogenous parasites, such as L1 and Alu retrotransposons [33,34, and, data not shown]. Herein, we provide evidence that also ADAR2 is a LINE-1 restriction factor.

**RESULTS**

**Nano-LC MS analysis of ADAR2-binding proteins**

Based on previous studies showing a role of ADAR2 as HIV-1 proviral factor [55], we searched for ADAR2 interacting factors that may affect this novel function by means of a dual-tag affinity purification procedure. To this end, we generated an ADAR2-expressing vector (pADAR2-V5) to express a V5 tagged protein that we used as a bait for the affinity purification. The expression and the efficient catalytic activity of the ADAR2-V5 was tested in 293 T cells overexpressing an artificial RNA editing substrate (data not shown). 293 T cells were co-transfected with HIV-1 proviral DNA (pNL4-3), together with pADAR2-V5 or pV5 empty vector (as a control).

Total cell extract was prepared from the transfected cells and subjected to dual-tag affinity procedure. Ruby-stained electrophoretic gels of the immunocomplex recovered showed relatively few bands in pV5 empty vector lane compared with those in pADAR2-V5 lane (Fig. 1); then, proteins were in-gel digested and analysed by mass spectrometry (nano-LC-MALDI-TOF/TOF). Using this approach, 10 putative non-ribosomal ADAR2-associated factors were identified (Table 1). Interestingly, NONO, PSF, DHX15 and NCL, all nuclear/nucleolar proteins, were previously found as ADAR1-associated factors by using the same experimental approach [33]. Of note, half of the identified factors, specifically ADAR1, TOP1, NCL, hnRNP C1/C2 and PSF were previously reported as associated factors to the L1 RNP complex or to its components (ORF1p, ORF2p and L1 RNA) [33,56,57], whereas ZNF317 is a member of the large family of Krüppel-associated box (KRAB) zinc finger proteins, well-known transcription factors that repress the expression of endogenous retroelements [58,59]. These results point-out a possible functional correlation between ADAR2 and LINE-1 elements, further strengthened by the previously published data showing that ADAR1, the other active deaminase in mammals, is an inhibitor of L1 retrotransposition in vitro.

Co-immunoprecipitation (co-ip) experiments were performed in order to validate the mass spectrometry (MS) results. Thus, 293 T cells were co-transfected as described above and total cell extract prepared and subjected to co-ip experiments using anti-V5 tag magnetic beads followed by western blot (WB) analysis using specific antibodies. As shown in Fig. 2A, the putative endogenous interacting factors analysed (five out of ten identified) were confirmed to co-
immunoprecipitate with ADAR2. Notably, their association with ADAR2 was found to be mostly RNA dependent because disappeared or dropped dramatically after RNase treatment (Fig. 2A). The same co-ip experiments were performed without co-transfection of the HIV-1 proviral DNA (pNL4-3) (Fig. 2B), providing overlapping results and thus suggesting that the interaction of ADAR2 with these endogenous protein factors does not require active HIV-1 replication.

**ADAR2 restricts L1-retrotransposition**

The dual-tag affinity procedure/MS analysis and the validation by co-ip experiments uncovered novel ADAR2 interactors and prompted us to explore the role of ADAR2 in L1 life cycle.

To reach this goal, we employed well-characterized L1 retrotransposition cell culture assays [60].

In particular, we used the pYX014 plasmid (Fig. 3A) that contains a full-length L1 element harbouring the firefly luciferase gene (Fluc) inserted in the 3’UTR of L1 in the opposite direction of the retrotransposon coding sequence, in which the Fluc coding sequence is disrupted by an intron in the sense orientation.

Thus, Fluc activity can be measured only after one round of retrotransposition and serves as read-out of L1 retrotransposition efficiency. A Renilla Luciferase (Rluc) expression cassette cloned in the backbone of the pYX014 plasmid was used to normalize for transfection efficiency (Fig. 3A).

HeLa cells were co-transfected in triplicate with pYX014 plasmid together with pV5 or different amounts of pADAR2-V5, and four days after transfection, cells were lysed for luminescence analysis and L1 activity measured as the Fluc/Rluc ratio.

As shown in Fig. 3, overexpression of ADAR2 (Fig. 3C) caused a reduction of retrotransposition efficiency compared to the control (cells transfected with the pV5 empty vector) (Fig. 3B).

This result points to a role of ADAR2 as inhibitor of L1 retrotransposition.

To further confirm this hypothesis, we tested the effect of depleting endogenous ADAR2 expression on L1 retrotransposition activity. To this aim, we employed the CRISPR/Cas9 gene-editing technology and generated single 293 T clones partially knocked-out for ADAR2 expression, with the intracellular level of the enzyme reduced by 70 to 80% compared to the parental 293 T cell line (KO1 and KO2 clones, Fig. 4A). Moreover, we derived a control wild-type clone (CTR), expressing ADAR2 at the same level as in parental 293 T cell line (Fig. 4A).

Next, ADAR2 KO clones and the control clone were transfected in triplicate with the pYX104 plasmid. Four days post-transfection, cells were lysed for luminescence analysis and L1 activity measured. As shown in Fig. 4B, ablation of ADAR2 expression, even if partial, causes an increase in retrotransposition efficacy.

To confirm this result, we carried out another retrotransposition assay by using the 99-PUR-RPS-pBlaster1 plasmid, which contains a blastidin S deaminase gene reporter cassette which activates the blastidin-resistant gene after retrotransposition (Fig. 4C). Again, ADAR2 KO clones and controls were transiently transfected with 99-PUR-RPS-pBlaster1 and 96 h post-transfection cells were selected with blastidin. After 15 days of selection cells were stained and

![Figure 1. Isolation of the ADAR2 native complex by dual-tag affinity purification. Total cell extract was prepared from 293 T cells transfected with either pADAR2-V5 or pV5 empty vector and the proviral NL4-3 genome and then subjected to two steps of immunoprecipitation (IP), first with the NINTA Magnetic Beads and then the eluted His-tagged native protein complex was subjected to a second step of IP using the anti-V5-tag magnetic beads. The resulting magnetic beads were resuspended in SDS loading buffer and the protein separated by SDS-PAGE, visualized by Sypro–Ruby staining and subjected to nano-LC-MALDI-TOF/TOF analysis. ADAR2-V5 and all the 10 non-ribosomal proteins identified as putative ADAR2-interacting factors are indicated.](image-url)
counted. The resulting number of drug-resistant colonies provides a readout of retrotransposition activity. In all experiments, a co-transfection with plasmid pEGFP was carried out in parallel as control for transfection efficiency. As shown in Fig. 4C and Fig. 4D, the number of blastidicin-resistant colonies increased in ADAR2 KO clones compared to the control. ADAR2 KO clones and the control were also transfected with the pCDNA6 plasmid containing a constitutively expressed blastidicin-resistance gene. As shown in Fig. 4D, the blastidicin-resistant colonies are similar in all the transfected clones.

Overall, these results strongly support a role of ADAR2 as an inhibitor of L1 retrotransposition.

**The ADAR2-catalytic domain is not required for L1 suppression**

Since ADAR2 is an active deaminase, it is conceivable that its RNA editing activity might be necessary to suppress L1 activity. To investigate this possibility, we employed a catalytically inert ADAR2 mutant [ADAR2 E/A, 61]. ADAR2 E/A was generated by a single-point mutation in the catalytic domain changing a glutamate residue (E396) into an alanine.

HeLa cells were co-transfected in triplicate with pYX014 plasmid together with either pADAR2-V5 or pADAR2 E/A-V5 or pV5 together with the pES2TE1 plasmid, was subjected to immunoprecipitation (IP) with an anti-V5 antibody. The pES2TE1 plasmid contains the full-length L1.3 retrotransposon sequence that allows expression of a T7-tagged ORF1p protein [62]. A fraction of the resulting immunocomplex was analysed by WB using specific antibodies showing that both the ADAR2-V5 and ADAR2 E/A-V5 proteins co-immunoprecipitated with the ORF1p-T7 protein (Fig. 6A, Fig. S1A). The interaction between ADAR2-V5 and ORF1p-T7 occurs in an RNA-dependent manner since it was

**ADAR2 associates with L1 RNP complex**

To elucidate the mechanism responsible for L1 inhibition, we tested whether ADAR2 associates with the basal L1 RNP complex.

Total cell extract prepared from 293 T cells previously transiently co-transfected with either pADAR2-V5 or pADAR2 E/A-V5 or pV5 together with the pES2TE1 plasmid, was subjected to immunoprecipitation (IP) with an anti-V5 antibody. The pES2TE1 plasmid contains the full-length L1.3 retrotransposon sequence that allows expression of a T7-tagged ORF1p protein [62]. A fraction of the resulting immunocomplex was analysed by WB using specific antibodies showing that both the ADAR2-V5 and ADAR2 E/A-V5 proteins co-immunoprecipitated with the ORF1p-T7 protein (Fig. 6A, Fig. S1A). The interaction between ADAR2-V5 and ORF1p-T7 occurs in an RNA-dependent manner since it was
Figure 3. Overexpression of ADAR2 inhibits L1 retrotransposition. A) Schematic representation of the pYX014 cassette and the rational of L1 retrotransposition assay, as previously described [60]. The Fluc indicator cassette is cloned into the L1 3’ UTR in antisense orientation relative to L1 transcription. This cassette has its own promoter (P2) and the Fluc coding sequence is interrupted by a gamma globin intron. An Rluc cassette, containing its own promoter (P3), is incorporated into the backbone of the plasmid and allows measurement of the transfection efficiency. The Fluc gene can be expressed only when the L1 transcript (L1 pre-mRNA) is spliced (L1 mRNA), reverse transcribed and inserted into genomic DNA. B) HeLa cells were co-transfected in triplicate with two different amounts of the vector pADAR2-V5 or the control empty vector pV5, together with the retrotransposition cassette pYX014. After three days of puromycin selection the total cell extract was prepared and the level of both Firefly luciferase and Renilla luciferase were measured by a luminometer. The ratio Fluc/Rluc was used to measure the retrotransposition efficiency. The different samples are indicated in the X-axis. The data are calculated as the means ± SD from three independent experiments and normalized to the control pV5 that is set at 100%. P-values were calculated by two-tailed t test and they are indicated above each histogram (**P < 0.01). C) 48 h post-transfection the expression of ADAR2-V5 was analysed by WB, using the specific antibody anti-V5. The antibody anti-Actin was used to normalize the expression of ADAR2-V5.

abolished by RNase treatment of the cell extract prior to immunoprecipitation (Fig. 6A).

From the remaining immunocomplex, total RNA was isolated and subjected to conventional RT-PCR to assay for the presence of the ectopically expressed L1 RNA. As shown in Fig. 6B and S1B, ADAR2-V5 and ADAR2 E/A-V5 immunoprecipitated with the L1 RNA but not the actin mRNA, used as negative control.

Overall, ADAR2 associates with components of the basal L1 RNP suggesting that through this interaction it might interfere with retrotransposon activity.

To get insight into the mechanism that may cause L1 restriction, we tested whether the interaction between ADAR2 and L1 RNA may affect accumulation of the retrotransposon transcripts. To this aim, we transiently transfected ADAR1 KO and control clones with pES2TE1 retrotransposition cassette. Four days post-transfection, total RNA was isolated from the transfected cells and subjected to an RT-qPCR analysis to measure the level of the ectopically expressed L1 transcripts by using specific primers. As shown in Fig. S1C, partial ablation of ADAR2 expression does not affect significantly the accumulation of L1 RNA.

To further confirm the association between ADAR2 and the basal L1 RNP, subcellular localization of ADAR2 and ORF1p in HeLa cells expressing ADAR2-V5 and ORF1p-T7 was analysed by using confocal laser scanning microscopy (FV1000, Olympus). As previously reported ORF1p accumulates in cytoplasmic foci such as stress granules in most cells [62,63], whereas a small percentage of cells show also a nucleolar localization [64, Fig. S2A]. ADAR2 is a nuclear/nucleolar protein [65, Fig. S2B]. Notably, as shown in Fig. 7 and Movie S1 (https://figshare.com/s/ae5f8012b2131598ad7) in HeLa cells co-expressing ORF1p-T7 and ADAR2-V5, the
ADAR2 binds but does not edits L1 RNA

Next, we tested whether ADAR2 binding to L1 transcripts causes deamination of specific adenosines. To this aim, the L1 RNAs ectopically expressed in cells overexpressing ADAR2 were sequenced to identify any putative A-to-I editing events. In particular, 293 T cells were co-transfected with pADAR2-V5 and pES2TE1 plasmid and taking advantage of specific Tag sequences (T7 and FLAG-HA) cloned downstream the ORF1 and ORF2 sequence respectively, fragments of the exogenous L1 RNA were RT-PCR amplified and sequenced by Sanger method. This partial analysis (50% of the 6kb sequence assayed) failed to detect any A-to-I editing events in the L1 RNA sequence. Thus, we cannot rule out that some RNA editing events occurred elsewhere in the retrotransposon transcripts.

DISCUSSION

It was previously reported the roles of ADAR2 as HIV-1 proviral factor, stimulating some steps of viral replication with mechanisms that are both RNA editing-dependent and -independent [55]. To unveil and characterize the ADAR2-associated factors that may contribute to its proviral function, we employed a dual-tag affinity procedure followed by MS analysis in HIV-1 positive cells. This approach led to the identification of 10 non-ribosomal interacting factors, most of which are novel. Four of these factors, NONO, PSF, DHX15 and NCL, were previously found also in the ADAR1 native complex isolated by using the same experimental approach [33]. The association with the nuclear DNA Topoisomerase 1 (TOP1), suggests the localization of ADAR2 in close proximity of chromatin, thus further
strengthening the hypothesis of co-transcriptional RNA editing activity of ADARs [66].

Strikingly, 5 out of the 10 identified factors are also associated with the L1 RNP components thus implying that ADAR2 might be involved, as ADAR1, in regulating the L1 retrotransposition. To elucidate the role of ADAR2 in L1 activity, we employed well-characterized cell culture retrotransposition assays showing that the overexpression of ADAR2 causes a decrease in L1 retrotransposition efficiency, whereas partial depletion of the endogenous enzyme causes, as expected, a corresponding increase of L1 activity. These results underpin the role of ADAR2 as an L1 restriction factor. To test whether the catalytic activity of ADAR2 is required to suppress retrotransposition, we employed a catalytic inactive ADAR2 mutant (ADAR2 E/A) and found that its overexpression affects the L1 activity even though probably to a lesser extent compared to the ADAR2 wt. Thus, the ADAR2 RNA editing activity appears to play a minor role in the impairment of L1 mobilization or not to be required at all.

To get insight into the mechanism through which ADAR2 inhibits L1 activity, we investigated whether this enzyme associates with L1 RNP complex or its components, in particular ORF1p and L1 RNA. To reach this goal, we performed immunoprecipitation experiments using an antibody specific for the V5 tag in cell overexpressing the tagged ADAR2-V5 or ADAR2 E/A-V5 proteins and an L1 retrotransposon encoding an ORF1p fused to a T7 tag.

Figure 5. The catalytic domain of ADAR2 is not required for suppression of LINE-1 mobilization A) HeLa cells were co-transfected in triplicate with either the vector pADAR2-V5 or pADAR2 E/A-V5 or pADAR1-V5 or the control empty vector pV5, together with the retrotransposition cassette pYX014. After three days with puromycin selection the total cell extract was prepared and the level of both Firefly luciferase and Renilla luciferase were measured by a luminometer. The ratio Fluc/RLuc was used as readout of the retrotransposition efficiency (Y-axis). The different samples are indicated in the X-axis. The data are calculated as the means ± SD from eight independent experiments and normalized to the control pV5 that is set at 100%. P-values were calculated by two-tailed t-test and they are indicated above each histogram (*P < 0.05, **P < 0.01, ***P < 0.001). B) 48 h post-transfection the expression of ADAR2-V5, ADAR2 E/A-V5 and ADAR1-V5 was analysed by WB, using a specific anti-V5 antibody. Beta-Actin expression was used as loading control.

Figure 6. ADAR2 binds the basal L1 RNP complex. A) Lysates of 293 T cells transfected with either pADAR2-V5 or pV5 empty vector together with pES2TE1 cassette were subjected to IP with anti-V5-tag magnetic beads (IP) followed by WB analysis with anti-V5 and anti-T7 tag antibodies. WB analysis of 10 µg of cell lysate inputs (input) is shown. Total cell lysates were mock-treated or RNase (A+/V1)-treated prior to IP B) Total RNA isolated from a fraction of the immunocomplexes (IP) obtained in A) and total RNA isolated from the co-transfected 293 T cells with pADAR2-V5 or pV5 plasmids together with pES2TE1 cassette (input) were subjected to RT-PCR analysis using specific primers to amplify fragments of the ectopically expressed L1 RNA and actin mRNA.
The resulting immunocomplex was then analysed by WB and RNA immunoprecipitation assay, showing that ADAR2-V5 and ADAR2 E/A-V5 co-immunoprecipitate with both ORF1p-T7 protein and the L1 RNA. Indeed, immunofluorescence (IF) experiments confirmed the association between ADAR2 and L1 RNP components.

In particular, IF experiments showed that in cells where a fraction of ORF1p-T7 localizes in the nucleoli a colocalization with the deaminase in this subcellular compartment can be clearly observed.

As to the mechanisms used by ADAR2 to restrict L1 activity, it could be envisioned that ADAR2 binding to the L1 RNA causes deamination of specific adenosines, which in turn impairs the retrotransposition process. The ORFs or the regulatory sequence of the retrotransposon are the most plausible targets of RNA editing. To test this hypothesis, we sequenced the ectopically expressed L1 RNA in cells overexpressing ADAR2 (more than 50% of the 6 kb full-length sequence analysed) and failed to detect any A-to-I changes.

To explain the lack of evidence of any A-to-I editing events in L1 transcripts, one possibility is that a fraction of L1 RNA is excessively edited (hyper-edited) by ADAR2, and this in turn causes RNA degradation, as shown for other RNA targets. This is the less probable hypothesis, though, as ADAR2 generally catalyses A-to-I editing of specific adenosines, whereas ADAR1 is the enzyme more prone to the promiscuous editing [40]. In addition, it cannot be excluded that the RNA editing events lye in the rest of the L1 RNA sequence not yet analysed. In any event, the most plausible explanation is that L1 RNA is a target for binding but not for the editing of ADAR2 or that RNA editing occurs at very low frequency, below the threshold of detection of the DNA Sanger sequencing analysis and it has a poor impact on L1 activity. This hypothesis is in agreement with the results showing the ADAR2 E/A inhibits the L1 retrotransposition and thus that the catalytic domain exerts a limited or no role in this inhibitory mechanism. Finally, we also analysed whether the interaction of ADAR2 with the L1 RNAs may have an impact on their accumulation. Specifically, we showed that the partial ablation of ADAR2 expression does not cause a significant alteration in the intracellular accumulation of the L1 transcripts.

Overall, the results we obtained suggest that ADAR2 through the binding of the L1 RNP complex might interfere with its retrotransposition activity without affecting neither the accumulation of the retrotransposon transcripts nor by deaminating specific adenosines.

There are similarities and few differences in the modalities used by ADAR1 and ADAR2 to restrict L1 elements. In particular, both deaminases suppress L1 retrotransposition mostly by an RNA-editing independent mechanism, interact and co-localize with ORF1p protein and do not affect L1 RNA accumulation, but ADAR1 probably exerts this function within stress granules (unpublished data), while ADAR2 in the nucleolus. Interestingly, ADAR2 is highly expressed in the brain where L1 retrotransposition is active even in mature neurons, where it is probably involved in the neural plasticity [67]. It could be speculated that ADAR2 in neurons may contribute by limiting L1 activity thus avoiding the detrimental effects of retrotransposition. Of note, very
recently bi-allelic variants in ADAR2 (most of them in the deaminase domain or in close proximity) were found in individuals with microcephaly, intellectual disability, and epilepsy. Some of these variants have reduced editing activity in the Gria2 Q/R-site tested by in vitro assay; thus, the reduced editing at this site might be responsible at least in part for the phenotypes observed in patients, such as the epilepsy [68,69]. It cannot be excluded that mutations in ADAR2 affect other enzyme functions. Thus, it would be worth to test whether in these patients the control of L1 mobilization mediated by ADAR2 might be hindered thus contributing to the onset of the disease. In fact, dysregulation of retrotransposon activity has been detected in neurological disorders [70].

Overall, our results further extend the knowledge on the functional role of ADAR2 in human cells and underline the contribution of this enzyme along with the other host deaminases as a line of defence against endogenous parasites [35].

**MATERIALS AND METHODS**

**Cells and transfection**

Human Embryonic Kidney (HEK) 293 T cells and HeLa cells were grown at 37°C in a humidified incubator and in an atmosphere of 5% CO₂, in Dulbecco’s Eagle’s Medium supplemented with 10% foetal calf serum, 50 U/ml penicillin, 50 mg/ml of streptomycin and 2 mM L-glutamine. Depending on the assay, cells were transfected by using either trans-it-LT1 (Mirus) or lipofectamine 3000 (Invitrogen) reagents according to the protocol provided by the manufacturer.

**Plasmids**

pADAR1-V5 (expressing the full-length p150 ADAR1 wt enzyme fused with a C-terminal V5/6xHis double tag) and pV5 vector (pcDNA3.1/V5 empty vector) were previously described [pADAR1-p150-V5; 71].

To generate the pADAR2-V5 expression vector, a fragment containing the human ADAR2 cDNA was amplified by PCR using as substrate the pGFP-ADAR2 wt [65] and the following pair of primers: ADAR 2 forward 5’-GGGGGGTACCCACATGATTAGAGAAGTGAAG-3’ (Kpn I site is underlined) and ADAR2 reverse 5’-AAAAAAGCCGGCGGCGGCGTGATGAGAAGTGAAG-3’ (Not I site is underlined). The PCR fragment was subcloned into the KpnI and NotI sites of the pcDNA3.1 V5/His and the nucleotide sequence determined by Sanger sequencing. To generate the pADAR2 E/A-V5 the same subcloning procedure described above was followed using the same pair of primers and as PCR substrate the pGFP-ADAR2 E/A [61]. This plasmid encodes for the fusion protein GFP-ADAR2 E/A, containing a single amino acid change in ADAR2 catalytic domain (Glut396 to Ala396), that makes the enzyme catalytically inactive.

pYX014 is a retrotransposition cassette containing an active human LINE-1 (L1RP), and a Fluc retrotransposition indicator gene as previously described [60].

L1 reporter construct 99-PUR-RPS-pBlaster1 contains an active L1 element (L1RP) and a blasticidin S deaminase gene reporter cassette [63].

pcDNA6 myc/his B (Thermofisher). pES2TE1 is a vector containing an active human L1 element expressing ORF1p protein fused to the T7 gene 10 epitope tag at its C-terminus and the ORF2p fused to its C-terminus to a double FLAG-HA tag [as described in 62].

HIV-1 proviral DNA (pNL4-3) was obtained through the NIH AIDS Reagent Program.

The eSpCas9-2A- GFP (PX458) (purchased from GenScript) encodes for the Cas9 enzyme from S. pyogenes with 2A-EGFP tag, and an sgRNA directed against a sequence of exon 4 of ADAR2 gene. In particular, we employed three PX458 plasmids each one encoding a different sgRNA: ADAR2 1: 5’-GCTGAGGCTGTCTCAGCCT-3’ ADAR2 2: 5’-CACGCTCGAGAAATGC-3’ ADAR2 3: 5’-GGTAAGTTGGTACCTG-3’

**Antibodies**

The following antibodies were employed in this study: anti-ADAR2 (Sigma), anti-ACTIN (Promega), anti-V5 (Invitrogen), anti-SFPQ/PSF (Abcam), anti-ADAR1 (Santa Cruz Biotechnology), anti-NCL (Santa Cruz Biotechnology), Anti-P54/NONO (Bethyl), anti-hnRNP-C1/C2 (Santa Cruz Biotechnology), anti-GAPDH (Invitrogen), anti-T7 epitope tag (Millipore), Anti-V5-tag magnetic beads (MBL), NiNTA beads (Qiagen).

**Generation of 293 T cells partially knocked-out for ADAR2 expression**

We transiently transfected 293 T cells with plasmid eSp-Cas9-2A-GFP (PX458, GenScript) expressing the SpCAS9-GFP and the anti-ADAR2 guide RNAs (gRNAs) specific for exon 4. Seventy-two hours post-transfection, single GFP⁺ cells were isolated by flow cytometry and plated in a 96 well plate. Single and expanded ADAR2 KO clones were screened for reduction of ADAR2 expression by WB and disruption of the genomic locus that was confirmed by DNA Sanger sequencing. Single clones expressing wild-type level of ADAR2 protein as in the parental 293 T cells were identified in parallel and used as controls.

**L1 retrotransposition assay**

We performed the L1 retrotransposition assay following the previously published protocol [33]. Briefly, HeLa cells were seeded in a 24-well plate and grown up to ~ 60% confluence and then co-transfected in triplicate with either pV5 empty vector or pADAR2-V5 or pADAR2 E/A-V5 or pADAR1-V5, together with pYX014. 24 h post-transfection puromycin (2.5 μg/ml; Sigma-Aldrich) was added in complete media. Three days later, cells were lysed for luminescence analysis
using the Dual-Luciferase Reporter Assay System (Promega)

following the manufacturer’s instructions. LINE-1 activity was

measured as the Fluc/Rluc ratio [33,60].

For the Fluc retrotransposition assay in partially depleted

ADAR2 cells, ADAR2 KO (KO1 and KO2) and control clones

(CTR) were seeded in a 24-well plate at a density of 9 × 10^4
cells/well and grow up to ~80% confluence. The day after
clones were transfected in triplicate with the pYX014 plasmid.
After 24 h puromycin (1.5 μg/ml; Sigma-Aldrich) was added in
complete media and three days later cells were lysed for

luminescence analysis using the Dual-Luciferase Reporter
Assay System (Promega) following the manufacturer's
instructions.

For the blasticidin resistance-based retrotransposition

assay, control and ADAR2 KO clones (KO1 and KO2) were
seeded in 12-well plates and the day after transfected in
triplicate with the 99-PUR-RPS-pBlaster1 plasmid.

Seventy-two hours post-transfection were detached and
~200,000 cells were resuspended in complete DMEM
medium and seeded in T25 flasks.

The day after, cells were supplemented with blasticidin
(7 μg/ml) (Santa Cruz Biotechnology). After 15 days of selection,
resistant cells were fixed with methanol 100%, stained with
0.1% crystal violet solution and counted. To take into account
any toxic effect of the ADAR2 KO clones and control
clone, these cells were seeded into 24-well plates and the day
after transfected in triplicate with the pcDNA6 myc/his B plasmid
encoding a constitutively expressed blasticidin-resistance
gene. Twenty-four hours post-transfection cells were detached and ~1000 cells were seeded in T25 flasks.

One day later, cells were supplemented with blasticidin
(7 μg/ml). After 8–10 days of selection, cells were fixed with
methanol 100%, stained with 0.1% crystal violet solution and the
foci number counted. The colony numbers obtained with
pcDNA6 transfection were used to normalize those with the
99-PUR-RPS-pBlaster1 construct to calculate the retrotrans-
position efficiency.

In each experiment, one well was transfected with pGFP
plasmid for monitoring the transfection efficiency.

**Dual-Tag Affinity Purification (DTAP)**

293 T cells were plated in 100 mm dishes. 24 h after they
were transfected with 8 μg of HIV-1 proviral DNA (pNL4-3)
together with 12 μg of either pADAR2-V5 or pV5 empty
vector. Transfected cells were lysed in NP40 buffer (0.5%
NP40, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20, 50 mM NaH2PO4, pH 8) supplemented with
10 mM imidazole, protease and phosphatase inhibitor cocktail
(Roche and Sigma-Aldrich, respectively) for 30 minutes
on ice. Thirty milligrams of cells extracts were incubated
with 120 μl of the NiNTA Magnetic Beads (Qiagen) for 3 h
at 4°C followed by several washes (using NP40 buffer sup-
plemented with 20 mM imidazole). The His-tagged native
complex was then eluted with Elution Buffer (0.5% NP40,
50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20,
50 mM NaH2PO4, 250 mM imidazole, pH 8) and subjected to
a second step of immunoprecipitation (IP) using 200 μl of
the anti-V5-tag magnetic beads (MBL) for 2 h at 4°C
followed by several washes with NP40 buffer. The resulting
beads were resuspended in SDS loading buffer and stored at
~80°C. The pADAR2-V5 or pV5 eluted complex proteins
were separated on 4–12% gradient gel (Invitrogen), stained
with Sypro Ruby staining (BioRad Laboratories) and visualized
using a Typhoon 9200 Laser scanner (GE Healthcare).
Every lane was cut in ten sections and then digested to isolate
proteins, that were then analysed by MS using MALDI TOF/
TOF analyser (AB Scie, Foster City, CA) following the same
experimental procedure previously described [33].

**Protein and RNA immunoprecipitation (IP)**

293 T cells transfected with 5–12 μg of pADAR2-V5 or pV5
empty vector with or without 5 μg of HIV-1 proviral DNA
(pNL4-3) were lysed in NP40 buffer for 30’ on ice. Two
milligrams of cell extracts were pre-cleared on IgG/dynabeads
protein G (Invitrogen) and incubated for 1 h at 4°C with
rotation and then incubated for 3 h or overnight at 4°C with
either anti-V5 antibody (Invitrogen) or anti-V5-tag magnetic
beads (MBL) or control IgG followed by incubation with
dynabeads protein G (Invitrogen) for 1–3 h at 4°C with
rotation. After 3–4 washes with NP40 buffer, the resulting
beads and 10 μg of the total cell extract (input) were resolved
by SDS-PAGE and transferred onto nitrocellulose membrane
and then analysed by immunoblotting using specific antibodies.

Moreover, to assay the physical association between
ADAR2 and the L1 RNP components, such as ORF1p and
L1 RNA, 293 T cells were transiently co-transfected with 5 μg
of either pADAR2-V5 or pADAR2 E/A-V5 or pV5 vector

with 5 μg of the pES2TE1 retrotransposon cassette.
72 h post-transfection the cell lysates were prepared by using
the NP40 buffer supplemented with 80 U of RNase inhibitors
(NEB) and the immunoprecipitation with an anti-V5 anti-
body was performed as described above. A fraction of the
immunocomplex was analysed by WB analysis using specific antibodies.

From the remaining immunocomplex total RNA was iso-
lated and analysed by RT-PCR to amplify fragments of the
exogenous LINE-1 RNA and actin mRNA. To isolate RNA,
beads were incubated with NP40 buffer supplemented with
5 mM EDTA, 0.5% SDS and 50 μg proteinase K (Promega) for
20 minutes at 37°C, followed by phenol-chloroform extraction
method, and ethanol precipitation. After DNase treatment
(NEB) each RNA sample was subjected to RT-PCR experi-
ments using random hexamers (Invitrogen) and M-MLV
reverse transcriptase (Invitrogen) according to manufacturer’s
instructions. The resulting cDNA was amplified by PCR using
these two pairs of primers: LINE-1 forward: 5′-CACCCGGATATTCCTACAATAGG-3′, LINE-1 reverse: 5′-
GCACCTTCCAGGTCAAGGA-3′, Actin forward: 5′-
CCACACCTTTCTAATAGGC-3′, Actin reverse: 5′-
CGTCATACTCCTGCTTGCT-3′.

**RNase treatment**

When indicated, before the co-IP, RNase V1 (0.002 U/μl, Life
Technologies) was added to the cell extract and after an
incubation at RT for 15 minutes, we added the RNase A (0.1 μg/μl, Sigma-Aldrich), with a further incubation at 37°C for 15 minutes. The complete digestion of the RNA by RNase treatment was confirmed by loading a fraction of the treated and un-treated lysates on 1% agarose gel, followed by electrophoresis and ethidium bromide staining.

**Immunofluorescence and confocal microscopy**

HeLa cells were plated on a pre-sterilized coverslip in a 6-well plate at $60 \times 10^3$ cells per well with complete media and transfected the next day with pADAR2-V5 or pADAR2 E/A-V5 or pV5 and/or pES2TE1 plasmids. Two days after transfection, cells were rinsed with 1X phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT), followed by 3 washes with PBS 1X. Next the cells were permeabilized with 0.1% Triton X-100 in PBS 1X for 5 minutes at RT, and then incubated with a bovine serum albumin in PBS 1X containing (BSA) 5% solution for 30 minutes. For dual immunofluorescence staining, the cells were incubated with anti-V5 antibody (Invitrogen) and anti-T7 epitope tag (Millipore) for 90 minutes at 37°C. After 3 further washes with PBS 1X, the cells were stained with the secondary Alexa Fluor 488-labelled anti-rabbit antibody (Invitrogen) and the secondary Alexa Fluor 555-labelled anti-mouse antibody (Invitrogen) at room temperature for 60 minutes. After 3 washes with PBS 1X, the coverslip was mounted on a glass slide using one drop of dapi-mountant (Thermo Fisher Scientific). Samples were analysed under a confocal laser-scanning microscope (FV1000, Olympus) after excitation at 405, 488, and 543 nm wavelengths for blue, green, and red channel acquisition, respectively. Volume reconstruction (3D rendering) and iso-surface for blue and red channels of xyz confocal image was performed using Imaris software (Bitplane).

**Real-time RT-qPCR**

Total RNA was prepared from the transfected cells using TRI reagent (Zymo Research) according to manufacturer’s instruction followed by DNase treatment (DNase I, NEB). cDNA was synthesized with M-MLV RT (Invitrogen) following the manufacturer instructions and 25 ng was used as template in 20 μl qPCR reactions performed with Luna Universal qPCR Master mix (NEB). All the reactions were performed with biological triplicates using StepOnePlus instrument (Applied Biosystem). Reactions were incubated at 95°C for 1 min, then for 40 cycles at two-step cycling (95°C for 15", 60°C for 30") and melting curves were used to confirm the specificity of each amplification product. Negative controls (-RT) were included for each experiment and showed an insignificant background.

**Analysis of accumulation of L1 RNA**

ADAR2 KO and control clones were seeded into 24-well plates and the day after transfected in triplicate with 500 ng of pES2TE1 cassette. Four days post-transfection, total RNA was extracted and analysed by RT-qPCR using primers designed to detect the L1 RNA generated from the pES2TE1 cassette. In particular, these primers pair the ORF2 gene and the downstream FLAG-HA tag sequence in the pES2TE1 cassette:

Flag-HA rev: 5’-GAGCTTTGTATCGTGTCCTTTGAG-3’;
ORF2 for: 5’-CACCGCATATTCTCACTCATAG-3’.

The following primers were used to detect the actin mRNA:

ACT for: 5’-GACACTTCCAGCCTTCC- 3’;
ACT rev: 5’-TGTCACGTCACACITCAGTI- 3’.

Data were analysed with the $2^{-\Delta\Delta C_T}$ method. Three biological replicates were performed.

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No potential conflict of interest was reported by the author(s).

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