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ADAR1 restricts LINE-1 retrotransposition

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ABSTRACT

Adenosine deaminases acting on RNA (ADARs) are involved in RNA editing that converts adenosines to inosines in double-stranded RNAs. ADAR1 was demonstrated to be functional on different viruses exerting either antiviral or proviral effects. Concerning HIV-1, several studies showed that ADAR1 favors viral replication. The aim of this study was to investigate the composition of the ADAR1 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 14 non-ribosomal ADAR1-interacting proteins, most of which are novel. A significant fraction of these proteins were previously demonstrated to be associated to the Long INterspersed Element 1 (LINE1 or L1) ribonucleoparticles and to regulate the life cycle of L1 retrotransposons that continuously re-enter host-genome.

Hence, we investigated the function of ADAR1 in the regulation of L1 activity.

By using different cell-culture based retrotransposition assays in HeLa cells, we demonstrated a novel function of ADAR1 as suppressor of L1 retrotransposition. Apparently, this inhibitory mechanism does not occur through ADAR1 editing activity. Furthermore, we showed that ADAR1 binds the basal L1 RNP complex. Overall, these data support the role of ADAR1 as regulator of L1 life cycle.

INTRODUCTION

Adenosine deaminases that act on RNA (ADARs) catalyze the conversion of adenosine (A) to inosine (I) in double-stranded RNA (dsRNA) substrates (1). Three ADAR enzymes are present in mammals (ADAR1-3). ADAR1 and ADAR2 are expressed in most tissues and appear catalytically active (2), whereas ADAR3 is expressed only in brain and its catalytic activity has not been yet demonstrated (2). Two ADAR1 isoforms are known: the full-length p150 ADAR1 form whose expression is induced by interferon and localizes in both nucleus and cytoplasm, and the short p110 ADAR1 form that is constitutively expressed and found predominantly in the nucleus (3).

ADAR1 p150 and p110 isoforms are both active deaminases, with the catalytic domain at the C-terminal and three double-stranded RNA binding motifs in the central region (dsRBD). One Z-DNA binding motif is present at the N-terminal in p110 ADAR1 and two motifs in p150 ADAR1 (Zβ in p110; Zα and Zβ in p150) (1). Since I is interpreted as guanosine (G) by the cellular machinery, RNA editing within the coding sequence of a mRNA can result in codon changes that may lead to the formation of an altered protein (2,4). Nonetheless, it has been recently demonstrated that most A-to-I RNA editing events occur within non-coding regions, most particularly in Alu elements, affecting different aspects of the RNA metabolism (2,4).

Furthermore, growing evidence supports a role of ADAR1 in the replication process of different viruses (5).

Depending on the type of virus, ADAR1 can either act as antiviral or proviral factor (5). In particular, ADAR1 favors replication of vesicular stomatitis virus (6,7), hepatitis D virus (8), dengue virus (9), human T-cell leukemia virus type 1 and type 2 (10) and many other viruses (5). ADAR1 exerts an antiviral effect on viruses, such as lymphocytic choriomeningitis virus (11), bovine viral diarrhea virus (12) and hepatitis C virus (13). For some viruses, such as Influenza A Virus (9,14,15), there are some discrepancies in the results obtained so far and therefore additional studies are required to completely understand the role of ADAR1 in their life cycle.

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Concerning lentiviruses, most of the published data support the hypothesis that ADAR1 exerts a positive effect on Human Immunodeficiency type 1 Virus or HIV-1 (16–19) and Equine Infectious Anemia Virus replication (20). In particular, over-expressed ADAR1 increases the accumulation of HIV-1 proteins with a mechanism that is editing-independent (17–19). Moreover, virions produced in the presence of over-expressed ADAR1 are released more efficiently and display enhanced infectivity (17). Furthermore, it was clearly demonstrated that ADAR1 binds and edits viral transcripts in different regions, such as the 5′ untranslated region, within the Tat and Rev coding sequences and in the vicinity of Rev responsive element within the Env transcript (16,17). Finally, silencing of ADAR1 expression in Jurkat T cells causes an impairment of HIV-1 replication (19).

Long interspersed elements 1 (LINE1 or L1) are the most abundant autonomous retrotransposon, accounting for ∼17% of human DNA. L1 elements have the potential of shaping the human genome by their own retrotransposition, using a 'copy and paste' process through an RNA intermediate to relocate in genomic DNA, and by mobilization of non-autonomous elements (Alu, SVA and processed pseudogenes) (21). The full-length L1 transcript is ∼6 kb long and contains a 5′ untranslated region (5′UTR), two non-overlapping open reading frames (ORF1 and ORF2) that are essential for L1 mobilization and a 3′ UTR. ORF1p is a 40 kDa RNA binding protein with nucleic acid chaperone activity (22), whereas ORF2p is a 150 kDa protein that contains both endonuclease and reverse transcriptase activities (21,23,24). Recently, a third ORF, ORF0, has been found in the 5′ UTR sequence, expressed from an antisense promoter (25) and with a still unknown function. It seems that ORF0 marginally influences the retrotransposition process (25). The 3′UTR contains a weak polyadenylation signal. The LINE1 RNA ends with a poly(A) tail that is critical for an efficient retrotransposition process (26).

Although most of the L1 copies are functionally inactive due to 5′ truncation, internal deletions and other mutations (27), about 80–100 copies are still retrotransposition competent in the human genome (27,28). L1 requires host factors in order to complete its life cycle (29). At the same time, most of the L1 insertions are detrimental to the host, therefore several mechanisms have evolved to restrict the retrotransposition events (21).

The aim of the work described here was to examine the composition of the ADAR1 ribonucleoprotein (RNP) proteome during HIV-1 expression. To this end, ADAR1-binding proteins were isolated using a dual-tag affinity purification system in HIV-1-expressing cells and identified by mass spectrometry. Fourteen non-ribosomal proteins were identified using this strategy and strikingly we found that a good fraction of them were previously reported as associated with the L1–RNP complexes (30–32). Moreover, some of these proteins were also shown to regulate the L1 life cycle. We therefore sought to investigate whether ADAR1 was able to affect L1 retrotransposition and found that this enzyme may act as restriction factor by limiting L1 mobility. Overall our results underpin the role of ADAR1 in regulating both exogenous viruses and endogenous parasites and pave the way toward a better comprehension of the mechanisms that lead to the control of the retrotransposon activity.

MATERIALS AND METHODS

Cells and transfection

293T cells and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM). The complete medium was supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml of streptomycin and 2 mM L-glutamine. Depending on the assay, cells were transfected by using transit-LT1 (Mirus) or lipofectamine2000 (Invitrogen) reagents according to the protocol provided by the manufacturer.

Plasmids

pADAR1-p150-V5 (expressing the full-length p150 ADAR1 wt enzyme fused with a C-terminal V5/6xHis double tag) and pADAR1-Dcat (expressing an ADAR1 p150 mutant that lacks the deaminase domain and fused with a C-terminal V5/6xHis double tag) and pV5 vector (pcDNA3.1/V5 empty vector) were previously described (18). HIV-1 proviral DNA (pNL4-3) was obtained through the NIH AIDS Reagent Program. Retrotransposition cassettes pJM101/L1.3 (containing an active human L1 [L1.3] equipped with a *mneoI* retrotransposition indicator cassette) and pJM105/L1.3 (identical to pJM101/L1.3 but containing a missense mutation in the RT domain of the *ORF2* gene) and pES2TE1 (similar to pJM101/L1.3, containing a T7 *gene10* epitope tag on the C-terminus of ORF1p and a FLAG-HA tag on the C-terminus of ORF2p) were previously described elsewhere (33–35). Retrotransposition cassettes pYX014 (containing an active human $L1_{RP}$ equipped with a *Fluc* retrotransposition indicator cassette) and pYX015 (containing a missense mutation in the ORF1 sequence) were previously described (36). The pc-L1-FH is an expression construct of $L1_{RP}$ containing an ORF1 fused to a HA-FLAG double tag, as previously described (30). Three anti-ADAR1 unique 29mer shRNA constructs and a scrambled non-effective shRNA cassette (scr shRNA) cloned in retroviral GFP vector (pGFP-V-RS plasmid) pre-designed and tested and the empty vector pGFP-V-RS plasmid were purchased from OriGene. The sequences of the shRNAs directed against ADAR1 are the following:

shRNA5:CCTGTGGAATCCAGTGACATTGTGCC TAC; shRNA6:AAGACAGCAACTCCACATCTGCCT TGGAA; shRNA7:AGACTCCGTACCATGTCCTGTA GTGACAA;

Antibodies

The following antibodies or beads were employed in this study: Anti-V5-tag magnetic beads (MBL), NiNTA beads (Qiagen), anti-V5 (Invitrogen), anti-ADAR1 (Santa Cruz Biotechnology), anti-SFPQ/PSF (abcam), anti-NONO/p54nrb (Bethyl), anti-hnRNP L (abcam), anti-NCL (Santa Cruz Biotechnology), anti-PABP (Santa Cruz Biotechnology), anti-HSP70 (Santa Cruz Biotechnology), anti-GAPDH (Millipore), anti-FLAG M2 (Sigma-Aldrich), anti-T7 epitope tag (Millipore), anti-

phospho-eIF-2α (Ser51) and anti- eIF-2α (Cell Signaling) and control IgG (Santa Cruz Biotechnology).

L1 retrotransposition assay

For the Fluc assay, HeLa cells were seeded in 24-well plates and grow up to 60% confluence and then transfected in triplicate with 250 ng of the different anti-ADAR1 shRNA plasmids (shRNA5-7) or the relative controls (empty vector and scr shRNA). Forty-eight hours later cells were further transfected with the pYX014 plasmid. Twenty-four hours post-transfection puromycin (2.5 μg/ml) was added to culture medium. Four days after pYX014 transfection, cells were lysed for luminescence analysis using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instruction. L1 activity was measured as the Fluc/Rluc ratio as previously reported (36). To confirm that the Fluc signals result from retrotransposition, we used the pYX015 cassette (that carries a missense mutation that dramatically impairs retrotransposition) in place of the pYX014 following the same experimental procedure.

For the G418-resistance-based retrotransposition assay, HeLa cells were seeded into 24-well plates and the day after transfected in triplicate with 250 ng of the different anti-ADAR1 shRNA plasmids or the relative controls (empty vector and scr shRNA). Twenty-four hours post-transfection, puromycin (2.5 μg/ml) was added in complete medium. Twenty-four hours later, cells were trypsinized, resuspended in DMEM medium and seeded in 12-well plates. The day after, cells were further transfected with 500 ng of pJM101/L1.3 cassette and 72 h later cells were trypsinized, resuspended in DMEM medium and seeded in T25 flasks. One day later, transfected cells were supplemented with DMEM with G418 (800 μg/ml). After 10–12 days of continued G418 selection, the remaining cells were fixed with 0.4% paraformaldehyde, stained with 0.1% crystal violet solution and the foci number counted. Within each experiment results were normalized for transfection efficiency. In particular, HeLa cells were first transfected with the shRNA plasmids as described above and after 48 h were further transfected with pJM101/L1.3 and pDsRed-Express plasmid (Clontech) followed by FACS analysis after 72 h.

In each retrotransposition experiment, some wells of transfected HeLa cells were lysed and used for Western blot (WB) analysis to monitor the down-regulation of ADAR1 throughout the experiment. Furthermore, to confirm the specificity of the results obtained, the pJM105/L1.3 cassette (identical to pJM101/L1.3 but containing a point mutation that dramatically impairs retrotransposition) or pcDNA3.1 plasmid were transfected in place of the pJM101/L1.3 following the same experimental procedure.

For experiments addressing the effect of ADAR1-V5 over-expression on L1 retrotransposition, HeLa cells were transiently co-transfected with 250 ng of pYX014 cassette and different amounts of pADAR1-p150-V5 (ranging from 1:1 to 25:1 ratio between the two plasmids) or pV5 empty vector. Twenty-four hours post-transfection, puromycin (2.5 μg/ml) was added and four days after pYX014 transfection, cells were lysed for luminescence analysis using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instruction. L1 activity was measured as the Fluc/Rluc ratio as previously reported (36).

Dual tag affinity purification

293T cells were plated in 100 mm dishes. The following day cells were co-transfected with 8 μg of HIV-1 proviral DNA (pNL4-3) together with 12 μg of either pADAR1-p150-V5 or pV5 vector.

Transfected 293T cells were lysed in NP40 buffer (0.5% NP40, 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 50 mM NaH_2PO_4, pH 8, cocktails of protease and phosphates inhibitors from Roche and Sigma-Aldrich, respectively) supplemented with 10 mM imidazole for 30 min on ice. A total of 30 mg of cell 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 with 20 mM imidazole) and elution of His-tagged native protein complex from the beads (using NP40 buffer with 250 mM imidazole). The eluted protein complex was then subjected to a second step of immunoprecipitation (IP) using 200 μl of the anti-V5-tag MBL for 2 h at 4°C followed by several washes with NP40 buffer. The resulting bead-associated complexes were resuspended in SDS loading buffer and stored at –80°C.

RESULTS

Nano-LC MS analysis of ADAR1-binding proteins

In order to characterize ADAR1-binding partners in HIV-1 expressing cells, we employed a dual tag affinity purification procedure that allows identification of target-associated native protein complexes with low background. To this aim, we co-transfected 293T cells with HIV-1 proviral DNA (pNL4-3), together with either pADAR1-p150-V5 or pV5 empty vector. Forty-eight hours post-transfection, total cell extract was prepared and 30 mg subjected to dual-tag affinity procedure.

Sypro Ruby-stained electrophoretic gels of the immunocomplex recovered showed relatively few bands in pV5 empty vector lane compared with those in pADAR1-p150-V5 (Figure 1); therefore, proteins were in-gel digested and analyzed by mass spectrometry (nano-LC MS) as described in the Supplementary Materials and Methods.

Table 1 lists 22 proteins identified as putative ADAR1-binding factors. Fourteen putative ADAR1-binding proteins are either components of cytoskeleton (TUBB4B and TUBB) or heat-shock proteins (HSPA1L, HSPA1A/1B and HSPA8) or DNA/RNA binding proteins (TOP1, ADAR2, PSF, DHX15, NCL, PABPC1, NONO and hnRNP L) most of which are involved in different aspects of RNA metabolism or belong to the Stress Granules (SG) complex (G3BP2, PABPC1). Seven ribosomal proteins were also identified along with URB1, i.e. the URB1 ribosome biogenesis 1 homolog of *S. cerevisiae* involved in 60S ribosomal subunit biogenesis (37), which are likely contaminants of the affinity purification procedure and therefore were excluded from further analysis (38,39). As expected, some of the proteins indentified, such as ADAR2 and NONO, were previously reported to interact with ADAR1 (40–42),

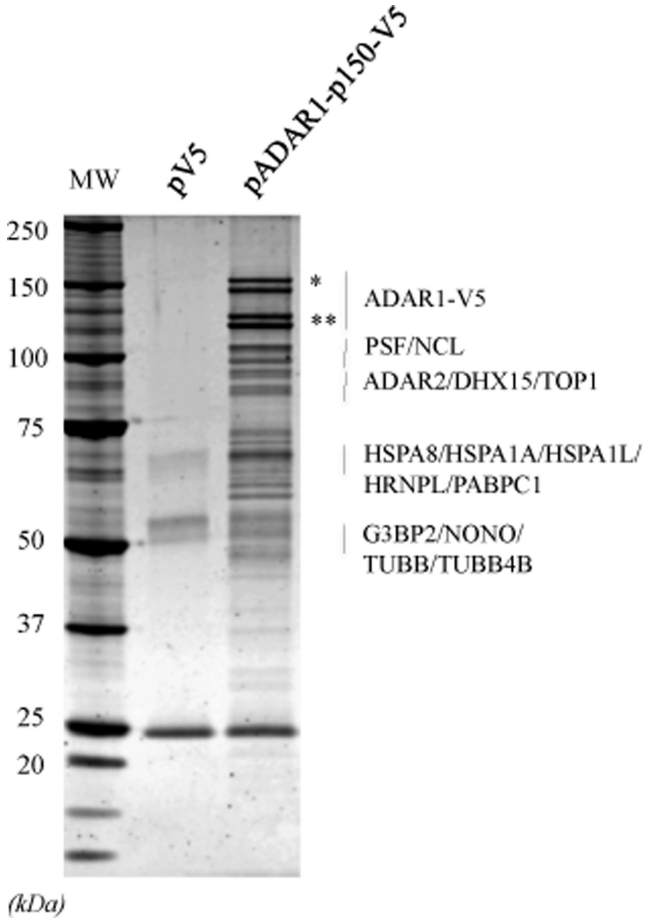

Figure 1. Results of the dual tag affinity purification procedure. Whole cell lysates from 293T cells transfected with either pADAR1-p150-V5 or pV5 empty vector and the proviral NL4-3 genome were subjected to immunoprecipitation (IP) with the NiNTA Magnetic Beads followed by several washes and elution of His-tagged native protein complex from the beads. The eluted protein complex was then subjected to a second step of IP using the anti-V5-tag magnetic beads followed by several washes with NP40 buffer. The resulting beads were resuspended in SDS loading buffer and the proteins were separated by SDS-PAGE, visualized by Sypro–Ruby staining and subjected to LC-MS/MS. The single asterisk indicates the p150 isoform of ADAR1-V5, the double asterisk corresponds to the ADAR1-V5 protein of about 110 kDa produced by translation machinery from a second methionine at position 296, as previously reported (69). Both isoforms migrate as doublets of bands possibly due to post-translation modification as originally observed (18). All the 14 non-ribosomal proteins identified as putative ADAR1-interacting factors are indicated.

whereas the other binding proteins identified are novel interactors.

Validation of the mass spectrometry results by co-immunoprecipitation assay

Strikingly, TOP1, PABPC1, NCL, hnRNP L and HSPA1A proteins were previously reported as associated with the L1 ORF1 and/or ORF2 proteins and their ribonucleoproteins (RNPs) (30–32). Moreover, NCL, hnRNP L, PABPC1 were also demonstrated to play a role in the regulation of the L1 activity (43,44). These results suggest a functional link between ADAR1 and the L1 retrotransposon that we further investigated. First of all, to confirm the interaction of ADAR1 with the identified L1 RNP-associated proteins and overall to validate the mass spectrometry results, these selected proteins and other two putative ADAR1-interactors that are well-known RNA- and DNA-binding proteins (PSF and NONO) were analyzed for their capacity to specifically bind to ADAR1-V5. To this aim, we co-transfected 293T cells as described above and total cell extract prepared and subjected to co-immunoprecipitation experiments (Co-IP) using anti-V5 tag magnetic beads followed by WB analysis.

As shown in Figure 2, the putative interactors were confirmed to Co-IP with ADAR1-V5 and interestingly their association with ADAR1 was found to be mostly RNA-dependent and disappeared or reduced dramatically after RNase treatment (Figure 2, left panel). The only exceptions are NONO and HSPA1A that interacted with ADAR1 in an RNA-independent manner (Figure 2, left panel). Complete degradation of total RNA from cell lysate after RNase-treatment was confirmed (Figure 2, right panel). Notably, all these associations occur also in the absence of HIV-1 expression since they can be recapitulated in total cell extracts prepared from 293T cells transfected only with pADAR1-p150-V5 vector (Supplementary Figure S1). Unfortunately, due to the low immunoblotting and immunoprecipitation efficiency of various TOP1 antibodies employed in this study, we could not test the interaction between ADAR1-V5 and TOP1.

Next, we assayed the ability of the ADAR1-binding factors to associate with endogenous ADAR1. To this aim, we performed Co-IP experiments using total cell extract of 293T cells and specific antibodies against the putative ADAR1-interactors (NCL, hnRNP L, HSPA1A, PABPC1, PSF and NONO) followed by WB analysis with anti-ADAR1 antibody. As shown in Figure 3, the selected proteins associate with the endogenous ADAR1.

Overall, the Co-IP data strongly support MS sequencing results and validate the use of tagged-ADAR1 construct for discovering cellular proteins that potentially associate with ADAR1. Furthermore, we identified novel ADAR1-interacting factors, a good fraction of which is shared with the L1 RNP complexes.

ADAR1 regulates retrotransposition in a cell culture assay

Next, we sought to investigate whether ADAR1 is involved in the regulation of L1 life cycle. We used previously established cell culture L1 retrotransposition assays (34–36) to determine the effect of down-regulation of endogenous ADAR1 expression on the activity of engineered L1 retrotransposons. First, to knockdown ADAR1 expression, we transiently transfected HeLa cells with the three different short hairpin (shRNA) plasmids and 72 h post-transfection total cell extract was prepared and analyzed by WB using specific antibodies (Figure 4A). HeLa cells transfected with anti-ADAR1 shRNA plasmids showed a reduction of ADAR1 protein level of at least 60–70% compared to the level in cells transfected with suitable controls (a plasmid expressing the scramble shRNA or the empty vector) (Figure 4A). A GFP expression cassette cloned in the backbone of the shRNA plasmids was used to normalize transfection efficiency.

Table 1. ADAR1-RNPs proteins in HIV-1 expressing cells identified by nano-LC MS/MS. ProteinPilot Unused ProtScore (Conf) >1.30 (95.0%). All the 14 non-ribosomal proteins indentified as putative ADAR1-interacting factors are separated from the ribosomal proteins and URB1 protein considered as contaminants of the affinity purification procedure

Accession number[a]	Gene name[a]	Protein Name
P55265	**ADAR1 (DSRAD)**	**Double-stranded RNA-specific adenosine deaminase**
P23246	PSF (SFPQ)	Splicing factor, proline-and glutamine-rich
P78563	ADAR2 (ADARB1)	Double-stranded RNA-specific editase 1
O43143	DHX15	Pre-mRNA splicing factor ATP-dependent RNA helicase DHX15
P19338	NCL	Nucleolin
P11142	HSPA8	Heat shock cognate 71 kDa protein
P11387	TOP1	DNA topoisomerase I
P11940	PABPC1	Polyadenylate-binding protein 1
Q15233	NONO (p54 nrb)	Non-POU domain containing octamer-binding protein
P08107	HSPA1A/1B	Heat shock 70 kDa protein 1A/1B
P14866	HNRNPL	Heterogeneous nuclear ribonucleoprotein L
P34931	HSPA1L	Heat shock 70 kDa protein 1-like
P07437	TUBB	Tubulin beta chain
Q9UN86	G3BP2	Ras-GTPase-activating protein-binding protein 2
P68371	TUBB4B	Tubulin beta-4B chain
O60287	URB1	Nucleolar pre-ribosomal-associated protein 1
P05388	RPLP0	60S acidic ribosomal protein P0
P62906	RPL10A	60S ribosomal protein L10a
P61313	RPL15	60S ribosomal protein L15
P50914	RPL14	60S ribosomal protein L14
P18124	RPL7	60S ribosomal protein L7
P62241	RPS8	40S ribosomal protein S8
P26373	RPL13	60S ribosomal protein L13

[a]According to the UniProtKB/Swiss–Prot entry.

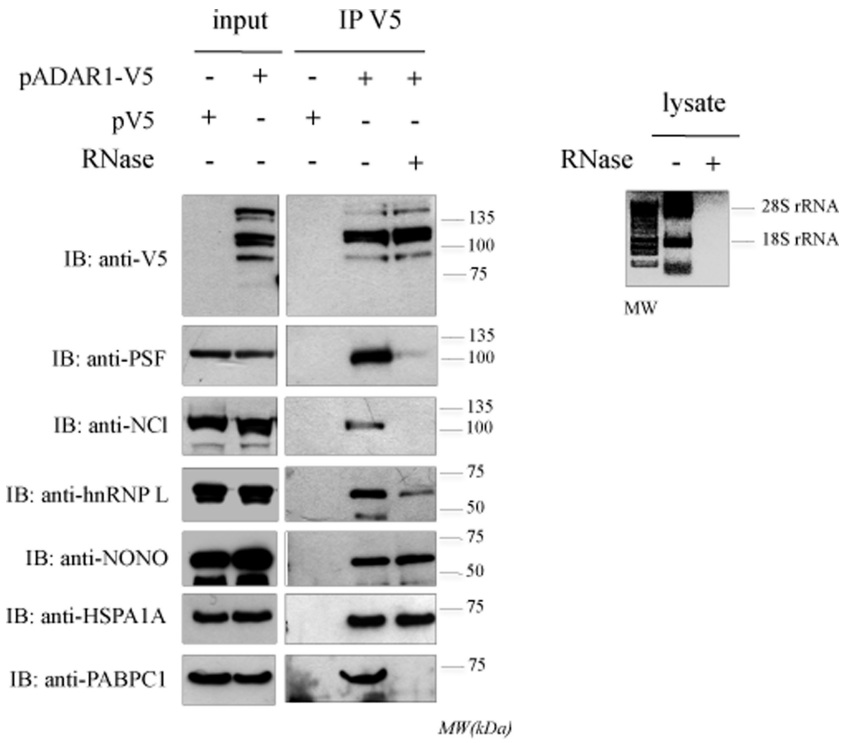

Figure 2. Validation of the results of the nano-LC MS/MS data by co-immunoprecipitation (Co-IP) analysis. Lysates of 293T cells co-transfected with pADAR1-p150-V5 or pV5 empty vector and the proviral NL4-3 genome were subjected to IP with anti-V5-tag magnetic beads (IP V5) followed by Western blot (WB) analysis with anti-V5, anti-NONO, anti-PSF, anti-hnRNP L and anti-NCL, anti-HSPA1A and anti-PABPC1 antibodies (left panel). Total cell lysates were mock-treated or RNase (A+V1)-treated prior to IP. WB analysis of 20 μg of cell lysate inputs (input) is shown. Complete RNA digestion after RNase-treatment of the cell extract (lysate) prior IP was confirmed by loading a fraction of the treated and untreated cell extract onto 1% agarose/formaldehyde gel followed by electrophoresis and ethidium bromide staining (right panel). The 28S and 18S rRNA are indicated.

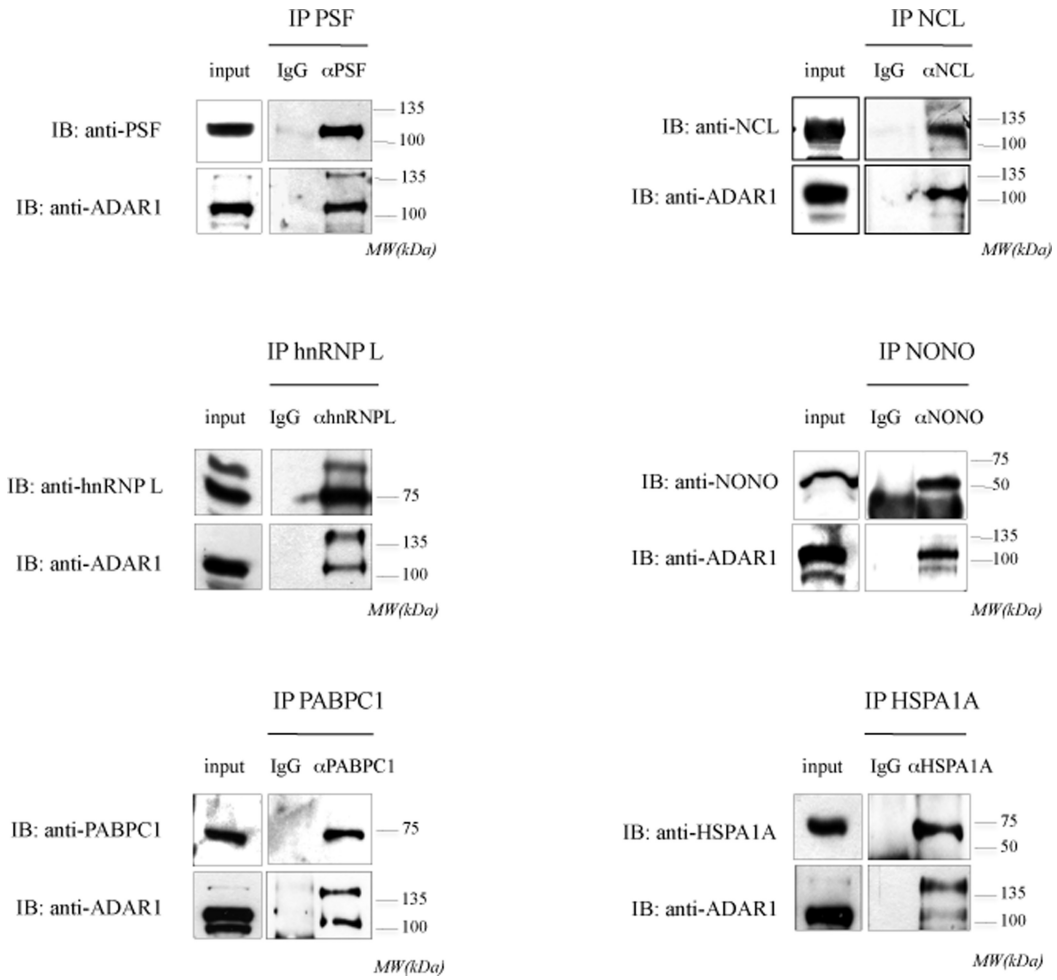

Figure 3. Validation of the results of the nano-LC MS/MS data by reciprocal IP. Lysate of 293T cells was subjected to IP using either IgGs or anti-NONO or anti-PSF or anti- hnRNP L or anti-NCL or anti-HSPA1A or anti-PABPC1 antibodies followed by WB analysis using specific antibodies. WB analysis of 20 μg of cell lysate inputs (input) is shown.

To test the effect of silencing of ADAR1 on L1 retrotransposition, we employed a dual-luciferase retrotransposition assay using the pYX014 plasmid (36). This plasmid is a retrotransposition cassette containing an engineered L1 element harboring the Firefly luciferase (*Fluc*) gene as retrotransposition indicator (36, Figure 4B). The *Fluc* reporter gene was cloned in the antisense orientation (relative to L1) in the 3′-untranslated region (3′-UTR) of L1 sequence and its coding sequence disrupted by an intron in the sense orientation. Therefore, Fluc expression occurs in the transfected cells only after one round of retrotransposition, in particular when the L1 is transcribed, the intron is removed by splicing and the reverse transcription and the integration is followed by transcription of the intact *Fluc* gene. The resulting Firefly luciferase measurement serves as read-out of L1 retrotransposition efficiency. A Renilla Luciferase expression cassette cloned in the backbone of the pYX014 plasmid was used to normalize transfection efficiency (36, Figure 4B). We transfected HeLa cells in triplicate with anti-ADAR1 shRNA plasmids, and 48 h later we further transfected them with the pYX014 plasmid. Four days after pYX014 transfection, we lysed cells for luminescence analysis and L1 activity measured as the Fluc/Rluc ratio. As shown in Figure 4C knockdown of ADAR1 expression in HeLa cells increased L1 activity by 2- to 3-fold when compared to controls (scramble shRNA and empty vector). To confirm that Fluc signal was originated from L1 retrotransposition, we performed experiments using the pYX015 vector (36), instead of the pYX014, carrying a missense mutation in the ORF1 coding sequence that dramatically impairs L1 retrotransposition. Using this vector in ADAR1-silenced HeLa cells, following the same experimental procedure described above, we measured only background levels of Fluc (data not shown). Therefore, these results suggest that ADAR1 is a regulator of L1 retrotransposition activity.

To further validate these results, we employed a different and widely used pJM101/L1.3 retrotransposition cassette (33,34). This cassette is similar to pYX014 described above (Figure 4B), but the retrotransposition indicator *Fluc* gene is substituted by the neomycin phosphotransferase gene (*mneoI*). We measured retrotransposition efficiency by counting the numbers of G418-resistant cells as a result of new L1 insertions as previously described (33,34). In par-

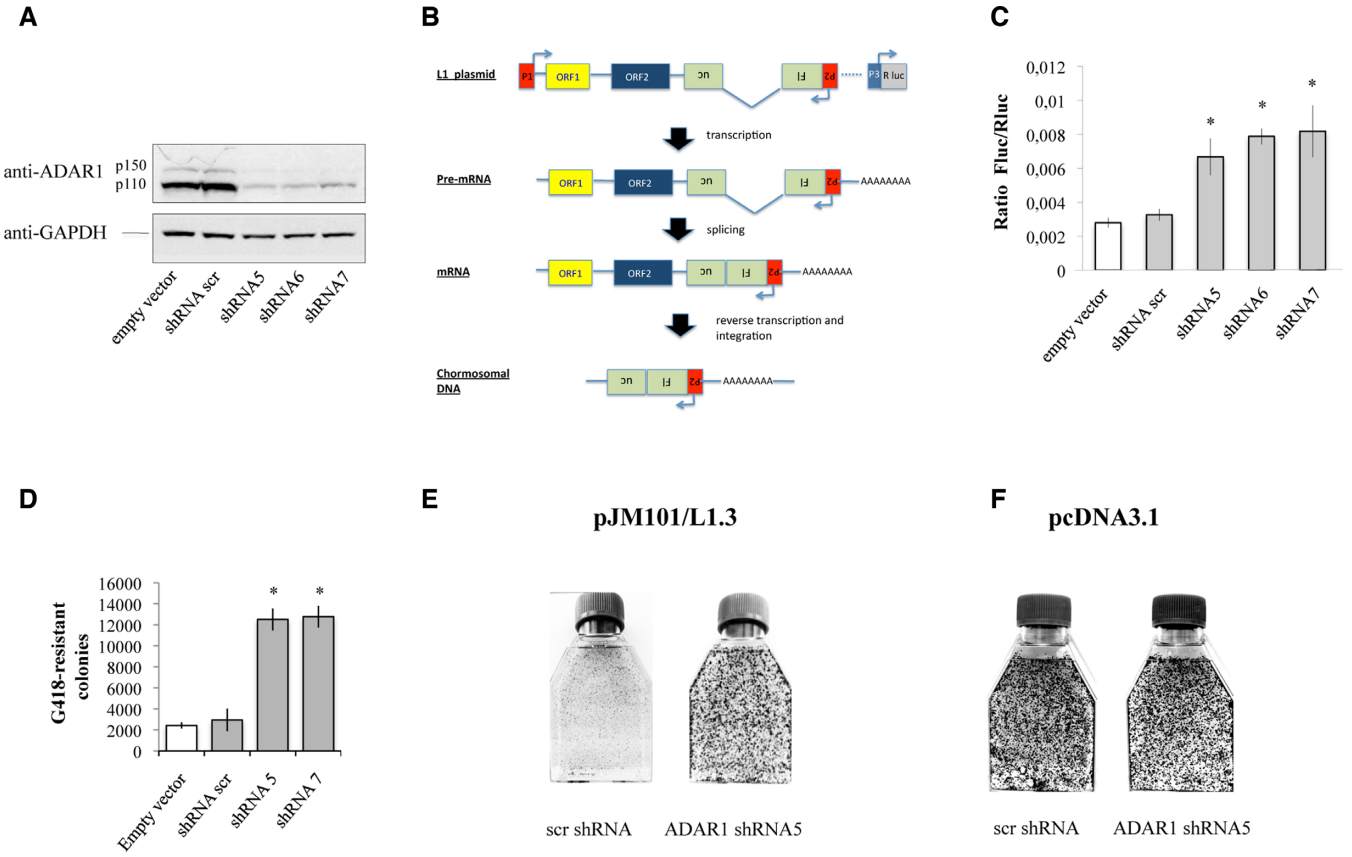

Figure 4. ADAR1 regulates L1 retrotransposition. (**A**) HeLa cells were transiently transfected with specific anti-ADAR1 shRNA plasmids or controls (empty vector or scramble shRNA plasmid). WB analysis using anti-ADAR1 and anti-GAPDH antibodies of total cell extract prepared from the transfected cells confirms the specific knock-down of ADAR1 protein 72 h after transfection. (**B**) Schematic representation of the pYX014 cassette and the rational of L1 retrotransposition assay, as previously described (36). 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 by expressed only when the L1 transcript (L1 pre-mRNA) is spliced (L1 mRNA), reverse transcribed and inserted into genomic DNA. (**C**) Representative Fluc retrotransposition assay results: HeLa cells previously transfected with anti-ADAR1 shRNA plasmids or control plasmids (empty vector or scramble shRNA plasmid) were then transfected with the luciferase assay vector pYX014. Four days after pYX014 transfection and puromycin selection, cells were lysed for luminescence analysis. The X-axis indicates the construct name. The Y-axis indicates the L1 activity measured as the ratio of the Fluc/Rluc values as previously reported (36). Data are reported as the mean ± SD from three independent experiments. Asterisks indicate statistically significant differences from the scr shRNA sample (*P*-values were calculated by two-tailed t-test and are indicated above each histogram, $*P < 0.05$). (**D**) Representative G418 retrotransposition assay results: HeLa cells previously transfected with anti-ADAR1 shRNA plasmids or control plasmids (empty vector or scramble shRNA plasmid) were further transfected with the pJM101/L1.3 cassette. Seventy-two hours later, cells were grown in media supplemented with G418 and after ∼10 days of G418 selection, the remaining cells were fixed and then stained with crystal violet to facilitate the visualization and allow the counting of the colonies formed for individual retrotransposition events. The X-axis in the graph indicates the constructs name. The Y-axis indicates the number of G418-resistant foci per cell culture dish. Data are reported as the mean ± SD from three technical replicates of a single representative experiment. Asterisks indicate statistically significant differences from the scr shRNA sample (*P*-values were calculated by two-tailed t-test and are indicated above each histogram, $*P < 0.05$). The experiment was conducted five times (biological replicates) with similar results. (**E**) Representative T25 flasks with crystal violet-stained G418-resitant HeLa colonies of the experiments described in (D) are shown. (**F**) Representative T25 flasks with crystal violet-stained G418-resitant HeLa colonies of experiments performed as described in (D) by substituting the pJM101/L1.3 cassette with the pcDNA3.1 plasmid are shown.

ticular, using an experimental protocol similar to that described above we transfected HeLa cells in triplicate first with the anti-ADAR1 shRNA plasmids or control plasmids (empty vector and scramble shRNA vector), and 72 h post-transfection, we further transfected them with the pJM101/L1.3 cassette. Forty eight hours later, cells were grown in media supplemented with G418 and after ∼10 days of G418 selection, the remaining cells were fixed and then stained with crystal violet to facilitate the visualization and allow the counting of the colonies formed for individual retrotransposition events. As shown in Figure 4D, E and Supplementary Figure S2, knockdown of ADAR1 expression in HeLa cells increased L1 retrotransposition efficiency by at least 6-fold when compared to the controls (scramble shRNA and empty vector) in five independent transfection experiments. In order to confirm that the results obtained are due to the reduction of ADAR1 expression acting specifically on L1 retrotransposition, and not to either a general or toxic effect of ADAR1 knockdown, we carried out the same experiments described above using the pcDNA3.1 vector instead of the pJM101/L1.3 cassette. The pcDNA3.1 is a plasmid that confers to the transfected

cells resistance to G418 without the need of retrotransposition. The G418-resistant colonies were counted in the HeLa cells silenced or not for ADAR1 expression after ∼10 days of G418 selection and their numbers were equivalent (Figure 4F and data not shown). Moreover, we performed also experiments using the pJM105/L1.3 cassette that is identical to pJM101/L1.3 but contains a missense mutation in the RT domain of the ORF2 gene that dramatically reduces L1 retrotransposition efficiency. Using the pJM105/L1.3 cassette in ADAR1 silenced HeLa cells we selected no or just few G418-resistant colonies (data not shown). Overall, these results demonstrate that the knockdown of the endogenous ADAR1 expression in HeLa cells causes an increase in L1 retrotransposition efficiency thus unveiling a novel role for ADAR1 as regulator of L1 retrotransposition.

The retrotransposition assays employed in this study are based on the removal of the gamma globin intron for the expression of the reporter gene (*Fluc* or *mneoI*)) allowing the measurement of the retrotransposition efficiency (Figure 4B). Since native L1 lacks introns, this raises the possibility that alteration of endogenous ADAR1 protein level by using specific shRNAs may not affect directly the L1 life cycle but merely alters the efficiency in the removal of the artificial intron contained within the cassettes. To exclude this possibility, we employed a previously described strategy based on RT-qPCR experiments (Supplementary Figure S3, 44) to measure the splicing efficiency of the globin intron harbored within the pJM101/L1.3 cassette in HeLa cells previously knocked-down for ADAR1 expression. As shown in Supplementary Figure S3, silencing of ADAR1 expression does not affect significantly the splicing efficiency when compared to specific controls (HeLa cells transfected with scr shRNA and empty vector), thus indicating that this mechanism cannot account for the increased L1 retrotransposition activity observed (Figure 4C and D) and confirming a specific role of ADAR1 in the L1 life cycle.

To further confirm the suppression of LINE-1 retrotransposition by ADAR1, it was evaluated whether overexpression of this enzyme causes an impairment of L1 retrotransposition.

Unfortunately, the ectopic expression of ADAR1 may lead to erroneous result interpretation.

In fact, it has been previously demonstrated that coexpression of ADAR1 increases any exogenous plasmid-based gene expression independently of the type of the promoter or reporter present in the plasmid (45). This effect is caused by the reduction of the PKR (protein kinase regulated by RNA) activation and phoshorylation of the PKR-substrate eIF-2α mediated by the ectopic ADAR1 expression. This, in turn, leads to an increase of the exogenous gene expression at the protein level up to 20- to 50-fold (45).

In the attempt of overcoming this problem, we assayed the amount of the pADAR1-p150-V5 plasmid that transfected in HeLa cells is sufficient to show an effect on L1 retrotransposition efficiency without affecting the eIF-2α phosphorylation at Ser51 (normalized quantification of the level of phospho-eIF-2α relative to the level of total eIF-2α).

To this aim, we transiently transfected HeLa cells with a constant amount of the pYX014 retrotransposition cassette and a decreasing amount of pADAR1-p150-V5 plasmid ranging from a 1:1 to 25:1 ratio. Four days after transfection, we lysed transfected cells for both luminescence analysis to measure L1 activity as the Fluc/Rluc ratio and for WB analysis. As shown in Figure 5A, overexpression of ADAR1-V5 (bottom panel) at all the amounts of pADAR1-p150-V5 plasmid transfected caused a reduction of L1 retrotransposition efficiency compared to control (cells transfected with pV5 empty vector) (Figure 5A, top panel).

We repeated the transfection experiment described above using a 5:1 ratio of the two plasmids and by luminescence analysis confirmed the inhibition of L1 retrotrasposition mediated by the ADAR1 over-expression and by WB analysis we demonstrated that no alteration of the level of phosphorylation of eIF-2α protein occurred compared to its level in control cells (cells transfected with pV5, Figure 5B middle panel).

Therefore over-expression of ADAR1, as expected, causes inhibition of L1 retrotransposition.

ADAR1 deaminase domain is not required to restrict LINE-1 retrotransposition

We examined whether the RNA editing activity of ADAR1 was required for suppression of retrotransposition. To this aim, we employed the pADAR1-Dcat plasmid that allows the expression of an ADAR1 mutant deleted in the catalytic deaminase function (Figure 5B, bottom panel). As shown in Figure 5B (top panel), when HeLa cells were transfected with pADAR1-p150-V5 or pADAR1-Dcat plasmids together with the pYX014 cassette (1:5 ratio to avoid effects on eIF-2α phoshorylation level, Figure 5B middle panel) we observed a reduction of L1 retrotransposition efficiency compared to the control (cells transfected with pV5 plasmid). Only a slight difference, not statistically significant, in reducing L1 retrotransposition was measured between cells expressing ADAR1-V5 and ADAR1-Dcat mutant (Figure 5B, top panel) suggesting that the deaminase domain of ADAR1 is either dispensable or plays a minor role in inhibiting retrotransposition.

ADAR1 binds the L1 RNP complex

To get further insight into the mechanism used by ADAR1 to inhibit L1 retrotransposition, we investigated whether ADAR1 associates with the basal L1 RNP complex, as previously demonstrated for its putative binding-factors identified in this study, and hence possibly interferes with its activity.

To this aim, we immunoprecipitated with anti-V5 antibody total cell lysate prepared from 293T cells previously co-transfected with pADAR1-V5 or pV5 together with the pES2TE1 plasmid. The pES2TE1 is an expression vector of L1.3 retrotransposon containing an ORF1 fused to a T7-Tag at the C terminus (35). We analyzed half of the resulting immunocomplex by WB with antibodies specific for the V5 and T7 tags showing that ADAR1-V5 does co-immunoprecipitate the ORF1 protein (Figure 6A), even though this association seems to be weak. From the remaining immunocomplex, we isolated total RNA that was subjected to RT-PCR analysis with specific primers for the

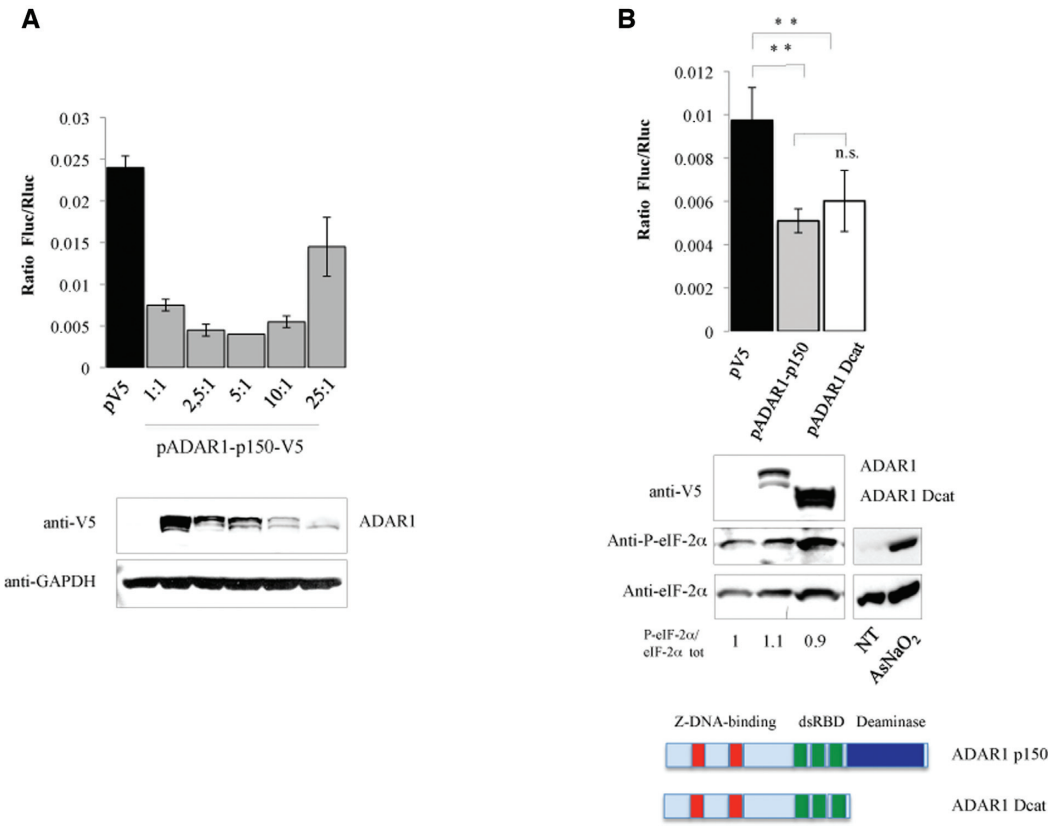

Figure 5. Inhibition of LINE-1 retrotransposition by ADAR1 over-expression. (**A**) Representative Fluc retrotransposition assay results: Hela cells were co-transfected with a constant amount of the pYX014 retrotransposition cassette and a decreasing amount of pADAR1-p150-V5 plasmid ranging from a 1:1 to 25:1 ratio. Four days post-transfection, cells were lysed for both luminescence analysis to measure L1 activity (top panel) and for WB analysis (bottom panel) with anti-V5 and anti-GAPDH antibodies. In the top panel, the X-axis indicates the construct name and the plasmids ratio. The Y-axis indicates the L1 activity measured as the ratio of the Fluc/Rluc values as previously reported (36). Data are reported as the mean ± SD from three independent experiments. (**B**) HeLa cells were co-transfected with the pYX014 retrotransposition cassette together with a 5:1 ratio of either pADAR1-p150-V5 plasmid or pADAR1-Dcat plasmid expressing a mutant of ADAR1 lacking the deaminase domain (bottom panel) or pV5 empty vector. Four days post-transfection, cells were lysed for both luminescence analysis to measure L1 activity (top panel) and for WB analysis (middle panel) with anti-V5, anti- eIF-2α and anti- P- eIF-2α antibodies. As positive control, HeLa cells were treated with 1 μM sodium arsenite for 48 h previously reported to induce eIF-2α phoshorylation (middle panel). The ratio P- eIF-2α /total eIF-2α is indicated. In the top panel, the X-axis indicates the construct name. The Y-axis indicates the L1 activity measured as the ratio of the Fluc/Rluc values as previously reported (36). Data are reported as the mean ± SD from four independent experiments. Asterisks indicate statistically significant differences from the pV5 sample (*P*-values were calculated by two-tailed t-test and are indicated above each histogram, $^{**}P < 0.005$).

amplification of fragments of the ectopically expressed L1 RNA and endogenous actin mRNA, used as negative control. As shown in Figure 6B, ADAR1-V5 Co-IP the L1 RNA but not the actin mRNA. This result suggests that ADAR1 may affect L1 retrotransposition by binding the basal L1 RNP complex. Additional experiments showed that ADAR1-V5 Co-IP also the endogenous L1 transcripts (data not shown).

L1 RNA is not edited by ADAR1

Finally, we investigated whether ADAR1 binding to L1 RNA resulted in editing of retrotransposon sequences. To this aim, total RNA isolated from 293T cells co-transfected with pADAR1-V5 or pV5 together with the pcL1-FH retrotransposition cassette was subjected to reverse transcription and polymerase chain reaction (PCR) using specific primers for the amplification of different fragments of the ectopically expressed L1 RNA followed by DNA Sanger sequencing. Inosine is interpreted as guanosine during reverse transcription, therefore the A-to-I changes in the RNA appear as A-to-G changes in the corresponding PCR products. By using this experimental approach we did not identify any editing event within the L1 sequence analyzed (more than 2.5 kb) (data not shown). This result is in agreement with the experiments described above (Figure 5B), carried out by using a plasmid expressing an ADAR1 mutant lacking the catalytic domain (ADAR1-Dcat) and showing the negligible role of this domain in ADAR1 inhibition of L1 retrotransposition.

This result supports a model in which ADAR1 affects L1 retrotransposition mainly by a mechanism that is editing-independent.

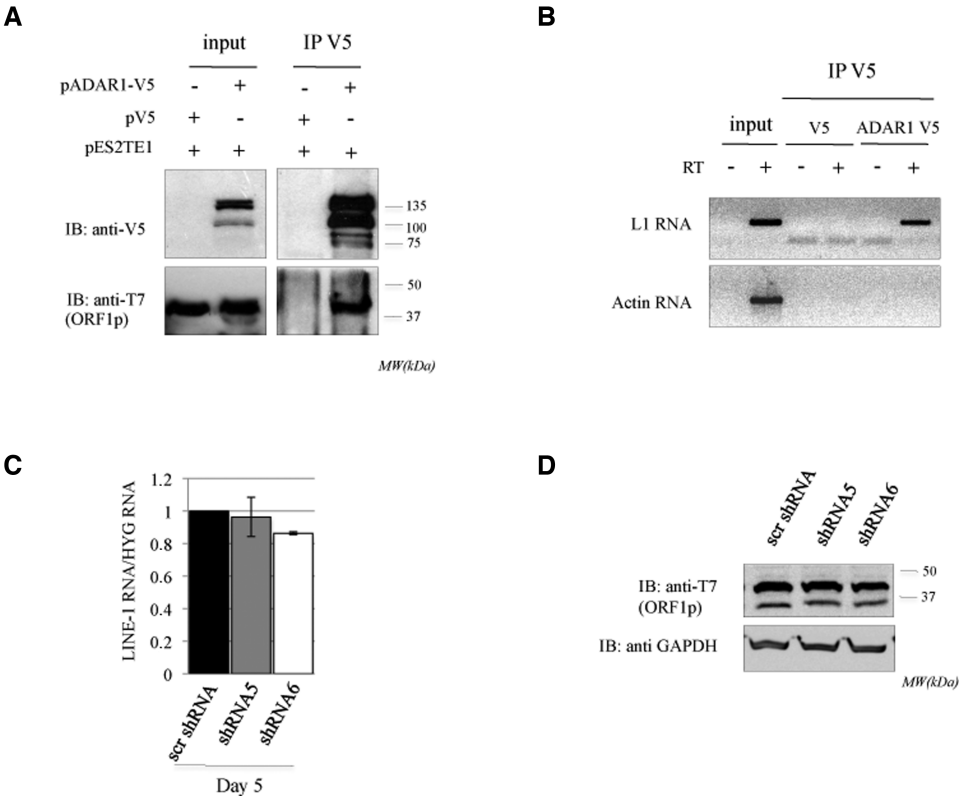

Figure 6. ADAR1 binds the basal L1 RNP complex without affecting the accumulation of its components. (**A**) Lysates of 293T cells transfected with either pV5-ADAR1-p150 or pV5 empty vector together with pES2TE1 cassette were subjected to IP with anti-V5-tag magnetic beads (IP V5) followed by WB analysis with anti-V5 and anti-T7 tag antibodies. WB analysis of 7 μg of cell lysate inputs (input) is shown. (**B**) Total RNA isolated from a fraction of the immunocomplexes (IP V5) obtained in (A) and total RNA isolated from the co-transfected 293T cells with pV5-ADAR1-p150 plasmid 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. (**C**) Results of the RT-qPCR experiments: HeLa cells previously transfected with anti-ADAR1 shRNAs or scr shRNA plasmids were further transfected with the pES2TE1 cassette. Five days post-transfection the ectopically expressed L1 RNA level was determined. The X-axis indicates the construct name. The Y-axis indicates the relative level of L1 RNA generated from the pES2TE1 cassette and normalized for the hygromycin mRNA level generated from the same cassette. The ratio L1 RNA/HYG mRNA of the scr shRNA sample is assigned as 1. Data are reported as the mean ± SD from three independent experiments. (**D**) ORF1p protein expression: total cell extract prepared from the HeLa cells transfected as described in (C) were analyzed by WB using the anti-T7 and the anti-GAPDH antibodies. One WB analysis representative of three independent experiments is shown.

ADAR1 does not affect the accumulation of full length L1 RNA and ORF1p

Since ADAR1 binds the basal L1 RNP, we next asked whether this interaction alters the activity of the complex by affecting the accumulation of both the L1 RNA and the translated ORF1p. To reach this goal, we transiently transfected HeLa cells previously knocked-down for ADAR1 expression with the pES2TE1 retrotransposition cassette followed by hygromicyn selection. Five days post-transfection, total RNA and total cell extract were prepared from the transfected cells. Total RNA was subjected to an RT-qPCR analysis to measure the level of L1 RNA by using specific primers to amplify a fragment that span the ORF2/Flag-HA sequence. These primers were designed to amplify exclusively the exogenous L1 RNA derived from the pES2TE1 cassette. Primers specific for the hygromycin phosphotransferase gene (HYG) present on the pES2TE1 plasmid were used to amplify a fragment of the corresponding mRNA that was used for normalization.

As shown in Figure 6C silencing of ADAR1 expression in HeLa cells does not affect significantly the L1 RNA accumulation. Moreover, the WB analysis of the total cell lysates using specific antibodies anti-T7 tag and anti-GAPDH, showed no significant alteration of the ORF1p protein in cells silenced for ADAR1 expression compared to control cells. Overall these results show that the knock-down of ADAR1 expression in HeLa cells does not affect the accumulation of the L1 RNA or ORF1p protein.

It is reasonable to envision that, even though ADAR1 binds the L1 RNP complex, suppression of the LINE-1 activity occurs by a mechanism that does not cause an alteration in the level of the L1 RNP components but impairs some functions of the complex critical for the retrotransposition.

DISCUSSION

Herein we presented the first comprehensive study aimed at identifying candidate ADAR1-interacting factors in HIV-1 expressing cells using a dual-tag affinity approach followed by mass spectrometry analysis. Using this approach, we identified 22 proteins. Excluding 7 ribosomal proteins and the nucleolar pre-ribosomal-associated protein 1 (URB1)

that are possibly contaminants of the affinity purification procedure, the other 14 candidates include three heat shock proteins, two components of the cytoskeleton and a large number of proteins with a reported DNA and/or RNA binding capacity. Some of these proteins are well known to interact with HIV-1 components and/or affect viral replication thus suggesting that through their association with ADAR1 may contribute to or modulate its proviral activity.

These proteins include Nucleolin that interacts with p55 Gag and it has been recently demonstrated to affect viral transcription (46,47). Moreover, Hsp70 proteins mediate Tat activation and viral transcription, and are released in HIV-1 virions (48–50). Both PSF and NONO bind the HIV-1 RNA instability element (INS), but only PSF causes a down-regulation of the Rev-dependent *gag* and *env* transcripts (51).

Finally, the Ras GTPase-activating protein-binding protein 2 (G3BP2) is one of the main components of the cytoplasmic SGs (52,53) and G3BP proteins play a role in the replication process of different viruses (54). Interestingly, it has been recently reported that the HIV-1 Gag protein inhibits SG assembly (55).

Strikingly, 5 out of the 14 identified ADAR1-interacting factors, such as TOP1, PABPC1, NCL, hnRNP L and HSPA1A, were previously reported as associated with the L1 ORF1p and/or ORF2p proteins and their ribonucleoproteins (30–32,43).

This unexpected result suggested a possible involvement of ADAR1 in L1 life cycle and this hypothesis was further investigated.

First, we assayed the interaction between ADAR1 and these L1 RNP-associated proteins by Co-IP experiments. Actually, we also tested additional proteins (NONO and PSF) to validate the overall results of mass spectrometry analysis. In particular, the tagged ADAR1-V5 was able to Co-IP the selected endogenous ADAR1-interactors (hnRNP L, NONO, PSF, PABPC1, HSPA1A and NCL) and by treatment with an RNase mix it was determined that most of these associations are RNA-dependent. The only exceptions are the interactions between ADAR1 and the NONO and HSPA1A proteins.

Moreover, the interactions we validated are maintained also in the absence of HIV-1 expression, thus suggesting that ADAR1 interacts with the L1 RNP-associated proteins independently from viral replication. However, it is reasonable to expect that some other interactors distinct from those validated by Co-IP associate with ADAR1 only during viral replication. Finally, by using specific antibodies raised against PABPC1, HSPA1A, hnRNP L, NONO, PSF and NCL the endogenous ADAR1 was Co-IP.

Of note, ADAR2 and NONO were previously described to interact with ADAR1 (40–42), thus corroborating the results of our experimental approach. Nevertheless, other previously described ADAR1-binding factors were not identified in our study, such as PKR (6), PACT (19), NF90 proteins (56), HuR (ELAVL1, 57), Tudor (58) and Dicer (59) and others. The only plausible explanation for this discrepancy is the different experimental settings used in these studies. In fact, prior to this study most of the ADAR1-binding factors were identified by single IP experiments, instead of two sequential IP experiments employed here, or by the use of differently tagged-ADAR1 proteins/antibodies, different cell lines and only another study employed the mass spectrometry analysis (60).

It is likely that our approach allowed the identification of the most stable associations in 293T cells, as indicated by the limited numbers of putative ADAR1-binding factors identified. In support of this hypothesis, in our validation Co-IP experiments performed with lysate prepared from 293T cells expressing the ADAR1-V5 protein, we were able to Co-IP, using the anti-V5 antibody, the endogenous PKR protein (not found in the mass spectrometry analysis), also confirming that this interaction is strictly RNA-independent (data not shown).

The interaction of ADAR1 with proteins shared with the L1 RNP complexes suggests that this enzyme might play a regulative role in L1 life cycle. This hypothesis is in agreement with recent published results showing that cellular factors that restrict L1 retrotransposition are also involved in the regulation of exogenous viruses (TREX1, APOBEC3 proteins, SAMHD1, MOV10, RNase L and ZAP) (32,61–63), thus indicating a sort of selection during the evolution of some host proteins to defend cells from endogenous and exogenous parasites. To investigate whether ADAR1 affects L1 activity, we employed two distinct and well-established retrotransposition assay systems (33,34,36). By using these assays, we demonstrated that silencing of ADAR1 expression in HeLa cells causes an increase in L1 retrotransposition. Conversely, ADAR1 over-expression inhibits L1 retrotransposition.

An ADAR1 mutant lacking the deaminase domain (ADAR1-Dcat) still acts as an L1 inhibitor at similar extent compared to the wt protein, suggesting that RNA editing plays a marginal role in this novel ADAR1 function.

This result is in agreement with the analysis of L1 RNA sequence carried out by RT-PCR followed by DNA Sanger sequencing that failed to detect any A-to-I change in the retrotransposon sequence. The RT-PCR analysis was performed using primer pairs designed to amplify specific fragments of the ectopically expressed L1 RNA, with a significant fraction of full length RNA competent for retrotransposition thus avoiding any contamination with the mixed population of mostly truncated endogenous L1 RNAs.

Unfortunately, there are only few unique sequences in the full-length ectopically expressed L1 RNA, thus this analysis was limited to almost half of the retrotransposon sequence. For this reason, we can't exclude that A-to-I editing might occur in the L1 regions we could not sequence. Moreover, it should be taken into account the possibility that a small fraction of L1 RNA can be edited by ADAR1 or that editing occurs at very low frequency below the threshold of detection by using an RT-PCR approach. In any event, it seems that if editing occurs within L1 RNA sequence this modification might affect only marginally the retrotransposition efficiency.

The use of a deep sequencing approach to conduct an unbiased genome-wide screening of A-to-I editing sites in the transcriptome of human adult brain, revealed the presence of editing sites within the LINE sequences (64). They are generally located in truncated LINE-1 elements that reside in 3′ UTR of transcripts in tandem repeats allowing the for-

mation of extensive dsRNA structures that are good target for ADAR1 binding and editing.

Hence, we can't exclude that retrotransposition of endogenous intragenic and full-length LINE-1 elements might be restricted through RNA editing of their sequence.

In addition, we demonstrated that ADAR1 binds the basal L1 RNP complex, in particular the L1 RNA (both endogenous and ectopically expressed) and the ORF1p protein. The interaction between ADAR1 and ORF1p protein seems to be weak, requiring prolonged exposure of the WB filters to detect it, indicating that probably this enzyme interacts more stably with the LINE-1 RNA. In agreement with this result, Bahn *et al*. (65) recently reported the first global study of ADAR1–RNA interaction in human cells using CLIP-seq and demonstrated an interaction between endogenous ADAR1 and L1 RNA that was further confirmed by RNA immunoprecipitation (RIP)-PCR experiments (65).

Notably, both L1 RNA and ORF1p protein were previously reported to localize in SGs (35,66). ADAR1 was shown to localize within the same granules in HeLa cells following several type of stress (58). Moreover, it was shown that the Zα-DNA-binding domain of the p150 isoform is required for this specific localization (67). Thus, it is conceivable that ADAR1 p150 may interact with the L1 RNP complex within SG. To further strength this hypothesis, in this study we report the interaction between ADAR1 and two main components of the SG, G3BP2 and PABPC1 (53). Interestingly, it has been recently reported that SAMHD1 inhibits L1 retrotransposition by promoting SG formation and sequestration of the retrotransposon complex in these granules, thus interfering with the nuclear import of LINE-1 RNA and its subsequent reverse transcription and integration into genomic DNA (68).

Further experiments are required to investigate whether ADAR1 and other retrotransposon restriction factors may inhibit L1 activity using the same mechanism.

To investigate how ADAR1 inhibits LINE-1 activity, we analyzed whether the interaction of ADAR1 with the L1 RNP complex may cause a reduction in the accumulation of the L1 RNA and ORF1p protein. This analysis showed that the knockdown of ADAR1 expression in HeLa cells does not cause a significant alteration in the accumulation of the L1 RNP components.

Hence, the more plausible explanation for the inhibitory effect of ADAR1 on L1 retrotransposition is that the binding of the enzyme to the basal L1 RNP complex might interfere with its activity without affecting the accumulation of its components.

Further experiments are required to determine this inhibitory activity of ADAR1 thus paving the way toward a better comprehension of the cell defense against exogenous and endogenous parasites.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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