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Dimerisation of APOBEC1 is dispensable for its RNA editing activity

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ABSTRACT

Among the AID/APOBECs -a family of DNA and RNA deaminases- APOBEC1 physiologically partakes into a complex that edits a CAA codon into UAA Stop codon in the transcript of Apolipoprotein B (ApoB), a protein crucial in the transport of lipids in the blood. Catalytically inactive mutants of APOBEC1 have a dominant negative effect on its activity, as they compete for the targeting to the ApoB mRNA. Here we show that catalytically inactive chimeras of APOBEC1 restricted to different compartments of the cell present different abilities to titrate APOBEC1-mediated RNA editing, and that the ability of APOBEC1 to interact with these mutants is the main determinant for its activity. Our results demonstrate that dimerisation, a feature common to other APOBECs targeting DNA, is not required for APOBEC1 activity on mRNA. Furthermore, APOBEC1-mediated RNA editing is a dynamic process where interplay among the components of the editing complex is regulated through the balance between availability of A1CF, one of APOBEC1 cofactors, and nuclear degradation of APOBEC1.

INTRODUCTION

The AID/APOBECs are a family of DNA and RNA deaminases that target endogenous and exogenous genetic material mostly in the context of the immune processes (1). Their aberrant mutagenic activity has been linked to the onset and progression of cancer (2). While all enzymatically active AID/APOBECs target nucleic acids without site-specificity, Apolipoprotein B Editing Complex, catalytic subunit 1 (APOBEC1) is an mRNA editing enzyme that has been characterised for its ability to target physiologically the cytosine in a CAA codon (Q2180) of the Apolipoprotein B (ApoB) transcript to generate a Stop codon (3–5). APOBEC1-mediated RNA editing leads to the synthesis of a truncated ApoB (ApoB48), whose balance with the long isoform (ApoB100) in liver and small intestine regulates the formation of lipoprotein particles and the transport of cholesterol in the

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bloodstream. In the years, a number of other targets have been identified (6–12), suggesting that APOBEC1-mediated RNA editing could be a widespread phenomenon to increase transcriptome heterogeneity: C>U editing can recode transcripts, create novel miRNA targets or disrupt existing ones. While the full-complexity of physiological APOBEC1-mediated RNA editing is still not clear, it is possible that aberrant RNA editing by APOBEC1 could be linked to the onset of disease (11, 13, 14). Higher-molecular complexes are formed by APOBEC1 in the cell (3, 15, 16), likely due to non specific interactions. Yet, the core editing complex is defined by APOBEC1 partaking into interactions with either APOBEC1 Complementation Factor (A1CF) or RNA-Binding-Motif-protein-47 (RBM47) (17–20), RNA-binding proteins that target APOBEC1 to the specific mRNAs. APOBEC1-mediated RNA editing is a predominantly nuclear process that involves APOBEC1 shuttling from the cytoplasm to the nucleus (21, 22) and acting as a carrier for A1CF (23).

APOBEC1 is able to form homodimers (24), and this characteristic has been hypothesised to be important for its function. Indeed, APOBEC1 deletion mutants lacking the C-terminal domain are unable to edit RNA and they do not self-interact (25). Contradictory results using constructs with partially deleted C-terminal domains to edit RNA *in vitro* indicate that the relation between RNA editing and dimerisation is not clear (26, 27).

It has been shown that, while a catalytically inactive APOBEC1 mutant unable to coordinate Zinc can act as a dominant negative, mutations or deletions in the C-terminal region do not (25). This suggests that interaction through the C-terminal region with at least one other factor, be it the target RNA or another protein, is necessary for APOBEC1 function.

In order to analyse the function of APOBEC1 in live cells, here we exploit two chimeric APOBEC1 constructs where the position of EGFP, at the N- or the C-terminus, forces the localisation of the chimera to the cytoplasm or the nucleus, respectively (28). These chimeric molecules, coherently with the notion that APOBEC1-mediated RNA editing is a nuclear process, display different RNA-editing activities. By testing the interplay between wild-type APOBEC1 and these chimeric mutants in live cells using a FACS-based RNA editing assay (28), we find that APOBEC1 dimerisation is dispensable for its activity on RNA, and that proteasomal degradation likely modulates APOBEC1 activity.

MATERIAL AND METHODS

Plasmids

The GFP-APOBEC1 and FLAG-APOBEC1 expression plasmids were described in Conticello et al (29). APOBEC1-GFP, APOBEC1-GFP(Y66H), GFP(Y66H)-APOBEC1, pEGFP mCherry-ApoB-GFP and the A1CF expression plasmids are described in Severi and Conticello (28). The FLAG-tagged A1CF expression plasmid was generated by inserting a FLAG expressing adaptor (CTAGACCATGGACTACAAGGACGATGATGACAAGCTTAAG and TCGACTTAAGCTTGTCATCATCGTCCTTGTAGTCCATGGT) 5’ to the A1CF coding sequence (NheI/SalI). The untagged rat APOBEC1 construct was generated by excising the coding sequence from the plasmid described in Severi and Conticello (28) (NheI/BglII) and inserting it into a pEGFP-C3 backbone in which the EGFP had been removed (NheI/BamHI). The expression constructs for the

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catalytic inactive APOBEC1 (E63A) were generated by PCR amplification of the coding sequence in the plasmid described in Severi and Conticello (28) (aaagctagcatgagtTCCGAGACAGGCCCTGTA and aaatgtacaagatcTCATTTCAACCCTGTGGC) and cloned into either pEGFP-C3, pEGFP-N1 or an pEGFP-C3 without GFP backbone as described in Severi and Conticello (28).

Cell cultures, transfections, and flow cytometry

HEK293T cells were maintained in DMEM (Euroclone) supplemented with 10% FBS (Carlo Erba), 2mM L-Glutamine (Carlo Erba) and 1mM penicillin/streptomycin (Carlo Erba) at 37°C in 5% CO2. Transient transfections were carried out using either Lipofectamine LTX (Invitrogen - catalog #15338100) according to the manufacturer's instructions or PolyEthylenimine (PEI, Polyscience - catalog #23966) following Durocher et al (30) using a DNA:PEI ratio of 1:3. The amount of plasmid constructs used in the titration experiments was determined based on the best separation between the titration induced by the empty plasmid or by the E63A mutant (Supplementary Figure 1).
FACS analysis was performed on an Accuri C6 Cytometer (BD) after 30-48 hours from transfection.

Immunofluorescence and microscopy analysis

Transfected cells were plated 24 hours after transfection on coverslips pretreated with poly-D-Lysine hydroxide (Sigma) and fixed at 48 hours. Following fixation with ice cold methanol, cells were stained using either rabbit anti-A1CF (1:400, Sigma - catalog # HPA037779) and anti-rabbit IgG conjugated with Alexa Fluor 647 (1:800, Life technologies - catalog # A21246), or goat anti-APOBEC1 (1:400, Santa Cruz - catalog # 11739) and donkey anti goat IgG H&L conjugated with Alexa Fluor 647 (1:800, Abcam - catalog # 150131). Nuclei were stained with DAPI.
Cells were visualised on an Axio Observed Z1 (Zeiss) inverted microscope. Localisation analysis was performed as described in McCloy et al. (31). Using ImageJ (v1.50i, NIH), for each cell the outline was drawn for the cytoplasmic and nuclear regions. For each region, as well as for 5 adjacent background readings, area and integrated intensity were measured, and the corrected total cellular fluorescence [CTCF; integrated density – (area of selected region × mean fluorescence of background readings)] was calculated. The percentage of nuclear localisation for each cells was then calculated as the percentage of nuclear CTCF over total CTCF (cytoplasmic + nuclear).

Protein preparation and western blotting

To monitor association by coimmunoprecipitation, extracts were prepared by lysing cells in buffer (50mM Tris-HCl [pH7.4], 150 mM NaCl, 5% Glycerol, 1mM EDTA, 0.2% sodium deoxycolate, 1% Triton-100) containing complete protease inhibitor cocktail (Roche - catalog # 11873580001) as well as 1mM PMSF (Sigma - catalog # 93482). Immunoprecipitations were performed by incubating cleared lysates at 4ºC for 4 hours using 15 µl of FLAG M2 Affinity Gel (Sigma-Aldrich - catalog #F2426) with gentle mixing. The affinity gel was then washed according to manufacturer's instructions. In order to minimise release of proteins bound to the gel aspecifically, elution of immunoprecipitated proteins was obtained using FLAG peptide (Sigma - catalog # F4799) for 2 hours at 4°C according to manufacturer's instructions.

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In order to obtain nuclear and cytoplasmic fractions, cells were incubated at 4ºC for 30 minutes in hypotonic buffer A (10 mM HEPES [pH 7.6], KCl 15mM, 2mM MgCl2, 0.1mM EDTA) supplemented with protease inhibitors. After a further 5 minutes incubation on ice in presence of 1% NP40, samples centrifuged for 15' at 1000g at 4°C. The supernatant was then cleared by centrifugation and represented the cytoplasmic fraction. After two washes with Buffer A, 1% NP40 and protease inhibitors, the pellet was dissolved in Buffer B (100mM Tris HCl pH 7.4, 100mM NaCl 2mM vanadate, 2 mM EDTA, 10% Glycerol, 1% Triton X-100, 0.1% SDS) for 30 minutes on ice with mixing. The nuclei were then subjected to 8 strokes in Dounce homogenizers and cleared by centrifugation (20000g for 10 minutes at 4°C).
Protein samples were then denatured in sample buffer (0.4ml 2-Mercaptoethanol, 0.8 gr SDS, 2ml Tris HCl 1M [pH 6.8], 4ml Glycerol, 8 mg Bromophenol Blue) at 95ºC for 10 minutes.
Formaldehyde crosslinking has been performed as described in Klockenbusch et al. (32). We performed crosslinking either before or after cell lysis, with overlapping results.
After SDS-PAGE, proteins were visualised by western blots using either goat anti-APOBEC1 antibody (Santa Cruz - catalog # sc-11739), rabbit anti-A1CF (Sigma - catalog # HPA037779), mouse anti-HSP90 (F-8) (Santa Cruz - catalog # sc-13119), goat anti- GAPDH V-18 (Santa Cruz - catalog # sc-20357), or rabbit anti-Lamin A (H102) (Santa Cruz - catalog # sc-20680). The primary antibodies were associated with relevant HRP-conjugated antibodies: anti-goat IgG (Santa Cruz - catalog # sc-2768), anti-rabbit IgG (Cell Signalling - catalog # 7074S), anti-Mouse IgG (Cell Signalling - catalog # 7076).

RESULTS

Differential inhibition of APOBEC1 activity by various catalytically inactive constructs

As titration of APOBEC1 activity using various mutants has been shown in vitro (25), we first aimed to port it in live cells using a fluorescence-based assay previously described (28). In short, HEK293T cells are transiently transfected with APOBEC1, its A1CF cofactor, and an mCherry-ApoB-EGFP construct bearing the editing target for APOBEC1 in frame with the chimeric coding sequence. In presence of APOBEC1-mediated RNA editing, a CAA codon in the ApoB segment is edited to a Stop codon (UAA), thus leading to loss of EGFP fluorescence that can be visualised by FACS analysis.
In order to set-up the titration using different amounts of plasmidic constructs, we first assessed the efficiency of the assay in presence of increasing amounts of competitor plasmid (Supplementary Figure 1). Indeed, the activity of APOBEC1-mediated RNA editing decreased with the amount of transfected DNA, and the dominant negative effect of a catalytically inactive APOBEC1 (E63A) was appraisable only at lower DNA concentrations. This is probably due both to the excessive amount of DNA hindering the efficiency of the transfection and to transcriptional competition among the various constructs. In order to avoid such effects we therefore decided to use varying competitor/APOBEC1 ratios and fixed amounts of total plasmidic DNA (4 µg, Figure 1, Supplementary Figure 2). Titration of APOBEC1 activity using the catalytically inactive APOBEC1 (E63A) resulted in a decrease of its activity, an observation analogous to what described in Oka et. al (25). Indeed, inactive APOBEC1-EGFP chimera (E63A-GFP) induced an even more pronounced titration of APOBEC1 activity. This

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can be reasoned with E63A-GFP localisation, as RNA editing takes place in the nucleus, and a nuclear mutant is probably more available to outcompete wild-type APOBEC1 in the editosome. On the other hand, inactive EGFP-APOBEC1 chimera (GFP-E63A) resulted completely unable to titrate APOBEC1 activity. This was completely unexpected: (a) APOBEC1 dimerises, therefore dimerisation of APOBEC1 with the cytoplasmic chimera should interfere with its nuclear import; (b) the interaction of APOBEC1 with A1CF begins in the cytoplasm, from where they shuttle together into the nucleus (23), thus GFP-E63A should induce cytoplasmic retention of A1CF. One possibility is that the GFP-A1 construct had lost its ability to interact with the other components of the editosome.

All APOBEC1 constructs interact with A1CF

We thus assessed whether the observed differences could be due to loss of interaction with A1CF, one of APOBEC1 cofactors responsible for its targeting to the ApoB transcript. We first used a FLAG-tagged A1CF to immunoprecipitate the various APOBEC1 constructs from total lysates obtained from transiently cotransfected HEK293T cells (Figure 2A). In these experiments, while immunoprecipitation products were present for both APOBEC1 and the chimeras, the APOBEC1-GFP construct, the nuclear one capable to titrate APOBEC1 activity, seemed to show the weaker interaction (especially if compared to that of a cleaved product which is enriched in the same IP lane).
In parallel, since A1CF shuttles into the nucleus in presence of APOBEC1 (23), we analysed the cellular localisation of A1CF in presence of the various APOBEC1 constructs in transiently cotransfected cells (Figure 2B, 2C). As expected, A1CF accumulates in the cytoplasm of HEK293T cells, and it becomes more nuclear when the cells are cotransfected with APOBEC1. In a similar way, also A1-GFP increases the percentage of nuclear A1CF, albeit less than wild-type APOBEC1, in line with the weaker interaction observed in the coimmunoprecipitation experiments. In the case of GFP-A1 the accumulation of A1CF in the cytoplasm is even stronger, as expected given the cytoplamic retention of GFP-A1. Thus, the interaction of GFP-A1 with A1CF cannot explain its inability to titrate APOBEC1 activity.

APOBEC1-GFP does not dimerise

Considering the correlation between APOBEC1 editing ability and its dimerisation, even though its meaning is controversial (25–27), we wondered whether interaction of the APOBEC1 chimera with APOBEC1 could explain the results of the titration experiments. We tested the ability of APOBEC1 to form heterodimers with the chimeras in transiently transfected HEK293T cells: coimmunoprecipitation of either wild-type APOBEC1, GFP-A1, or A1-GFP using FLAG-tagged APOBEC1 reveals that the only construct with a very limited ability to form heterodimers is A1-GFP (Figure 3A).
We next confirmed the inability of A1-GFP to dimerise by formaldehyde cross-linking in transiently transfected HEK293T cells. After treatment with formaldehyde, cells were lysed and western blotting was performed to visualise APOBEC1 (Figure 3B). A lower band for the wild-type APOBEC1 at the expected molecular weight for monomeric APOBEC1 (27kDa) was associated to a stronger one, presumably the dimeric form (expected molecular weight 54kDa, but crosslinks alter the apparent molecular weight). Other weaker bands were present as well (overexposed image in Supplementary

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Figure 3A), possibly representing other aggregation forms. An analogous pattern was apparent for the GFP-A1 chimera (56kDa for the monomer; 112kDa for the dimeric form), suggesting that both APOBEC1 and the GFP-A1 chimera are able to dimerise. On the other hand only one band corresponding to the monomer was present for the A1-GFP chimera. Even though a much weaker band of high molecular weight was present (overexposed image in Supplementary Figure 3A), this results corroborate the inability of the A1-GFP chimera to dimerise. Coexpression of APOBEC1 together with the chimeras leads to the formation of bands likely representing heterodimers (83kDa , Figure3B). Yet, the ratio between the homodimeric APOBEC1 band and the heterodimer one suggests that the interaction between APOBEC1 and A1-GFP is much weaker than the one between APOBEC1 and GFP-A1. Coexpression of both chimeras together result in a pattern identical to that of GFP-A1 alone.

Crosslinking experiments after nuclear/cytoplasmic fractionation (Figure 3C; Supplementary Figure 3B) reveal that the dimerisation bands of GFP-A1 disappear completely in the nuclear fraction, thus leaving only the monomeric form in the nucleus. While this differential localisation of the GFP-A1 dimeric form could be due to its size being large enough to prevent its nuclear import (albeit a decreased nuclear localisation of the APOBEC1 dimer might be present as well; Supplementary Figure 3B), this reinforces the notion that dimerisation of APOBEC1 is not needed for its activity. On the other hand, treatment with RNAse does not affect dimerisation (Figure 3D). In all samples where the chimeras were expressed a very high molecular weight band was present as well: considering its absence in the sample where APOBEC1 alone is expressed, this band might be due to the presence of EGFP-induced aggregation in the cytoplasm.

While we anticipated that crosslinking experiments performed in presence of A1CF could reveal the composition of the minimal editing complex, all our attempts to visualise crosslinked bands including monomeric or dimeric APOBEC1 together with A1CF have failed, as the only pattern visible for APOBEC1 or its chimeras was identical to the one shown in Fig 3C, and A1CF appeared as a single band of the molecular weight expected for a monomer (data not shown). This could be either an artefact of the overexpression, or it might be an indication of labile interaction.

Dimerisation of APOBEC1 is dispensable for RNA editing and A1 degradation limits RNA editing

Considering that the A1-GFP was the only construct that, while maintaining its ability to trigger RNA editing, had a different behaviour with regards to interacting partners -in particular its inability to dimerise- we wondered how its activity on the RNA editing target would change in presence of the inactive APOBEC1 constructs. As expected, titration of its activity in transiently transfected HEK293T cells with the E63A-GFP chimera is substantially overlapping with that obtained titrating APOBEC1 with its inactive E63A mutant (Figure 4A). Similarly, the GFP-E63A mutant is unable to outcompete the activity of A1-GFP. What is drastically different is the RNA editing activity in presence of the E63A chimera, where an increase was apparent. On one hand, the lack of competition by A1 on the dimerisation-unable A1-GFP proves that dimerisation of A1 is not necessary for its RNA editing. On

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the other one, the activity observed, even higher than that observed in presence of the negative control plasmid, suggests that some other factor affects the activity of APOBEC1 in the nucleus. Since the expression levels of A1-GFP in our experiments were always lower than those of the other constructs, we thought that nuclear A1-GFP could be unstable and the presence of APOBEC1, regardless of its ability to deaminate, could improve A1-GFP availability for editing. Indeed, treatment with the protease inhibitor MG132 of transiently transfected cells induced an increase of A1-GFP (Figure 4B), suggesting that A1-GFP is primarily degraded through the proteasome. Cotransfection of constant amounts of A1-GFP with increasing ones of wild-type APOBEC1 resulted in higher expression levels of A1-GFP (Figure 4C). Conversely, no increase in APOBEC1 levels were perceptible when cotransfected with increasing amounts of A1-GFP, suggesting that this might be the reason for the increase in RNA editing observed when A1-GFP was coupled with catalytically inactive APOBEC1.

DISCUSSION

Dissection of the mode of action of the AID/APOBECs has always been a central research theme, as their ability to target DNA and RNA can have profound repercussions on the stability of both genetic and transcribed information in the cell.

It is important to discriminate two steps in the mechanism of action of the AID/APOBECs: the targeting of the AID/APOBEC to the nucleic acid in a specific cellular compartment, and its ability to act on the target itself.

With regards to activity on DNA, the quaternary structure of the AID/APOBECs has been directly associated to both of these steps. Aspecific association with RNA induces aggregation of the AID/APOBECs in High Molecular Mass complexes that hinder their targeting, and RNAse treatment, which releases them into Low Molecular Mass complexes, is required in order to obtain active purified proteins (not needed for APOBEC1) (16, 33–42). On the other hand mutants lacking the ability to oligomerize are not packaged in the viruses and cannot act on the lentiviral genome (43–49). In the case of APOBEC1, its ability to oligomerise is important for its packaging into HIV virions (50).

With regards to AID/APOBEC binding to the target DNA and its enzymatic activity, a correlation between dimer formation and their association with the DNA substrate has been observed through several approaches, sometimes with conflicting interpretations (51–62). Structural analysis suggests that dimerisation in individual APOBEC proteins might be different, either an intrinsic property of the AID/APOBECs (57, 63–65) or mediated by nucleic acids (66–69). Yet monomeric APOBECs can target ssDNA and are active in cells and *in vitro* (53, 55, 65, 70–73).

While most studies address the importance of dimer formation towards viral or mobile element restriction, for which binding to the RNA is relevant for the viral packaging, only little is known about targeting to specific RNA transcripts. In the years, it has been hypothesised that APOBEC1 acts as a dimer within the editing complex (26), yet the use of APOBEC1 mutants lacking the ability to dimerise led to contradictory results (25, 27). In this context our results indicate that APOBEC1 ability to dimerise is dispensable for its RNA editing activity.

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The differential titration of RNA editing using the various constructs indicates that several factors are involved in the regulation of APOBEC1 activity. We observe a weaker interaction of A1-GFP with A1CF, with regards both to APOBEC1 and GFP-A1, and to the truncated form of A1-GFP, which appears enriched after coimmunoprecipitation (Figure 2A). Similarly, the A1-GFP constructs induces only a moderate shift in A1CF localisation compared to APOBEC1. This suggests that even a fleeting interaction with A1CF is sufficient to allow significant editing of the transcript by APOBEC1. As it has been observed that a weaker interaction between APOBEC1 and A1CF can modulate hyperediting of the ApoB transcript (74), it will be interesting to assess in a cellular model less reliant on overexpression whether APOBEC1 dimerisation affects hyperediting as well. More important, it remains to be assessed, through identification of loss of dimerisation mutants, whether there is an association between APOBEC1 dimerisation and its ability to interact with A1CF, or the weaker interaction is an independent effect of the chimeric APOBEC1.

Also cellular localisation is important, as the only constructs that interfere with each other are those able to reach the nucleus. Our experimental conditions do not let us discriminate whether there is any link between dimerisation of APOBEC1 and its cellular localisation (the GFP-A1 dimeric chimera could be too large for nuclear import). On the other hand, titrations using APOBEC1 and the nuclear non-dimerising A1-GFP chimera have opposing effects when one or the other is used as a catalytically inactive mutant (Figure 4A). This is not related to their localisation as both constructs have a significant nuclear fraction. Based on the increased levels of A1-GFP either after treatment with the MG-132 proteasomal inhibitor or through coexpression of APOBEC1 (Figure 4B and C), the more straightforward interpretation for such opposite effect is the existence of a correlation between dimerisation of APOBEC1 and its degradation. Similarly to another family member, AID, which is predominantly degraded in the nucleus through the proteasome (75), and whose monomeric and dimeric forms interact with different molecules (76), it could be possible that APOBEC1 dimers are preferentially degraded through their interaction with a factor, be it nucleic acids or proteins, that triggers their degradation. Alternatively the dimer could induce changes in its interactors: e.g. it is known that A1CF is phosphorylated in the nuclear fraction bound to the RNA editing complex (19).

Similar to the other AID/APOBECs, APOBEC1 is also able to act on DNA (33, 77–81), and its mutagenic effect has been linked to cancer (77, 82). It will be interesting to understand whether dimerisation of APOBEC1, which we demonstrate to be dispensable for its activity on RNA, could be relevant with regards to its targeting to DNA as it might represent a layer of protection against its mutagenic activity.

Finally, considering the recent interest in artificially inducing DNA and RNA editing by deaminases fused to nucleic acid targeting molecules (e.g. 83, 84), a better understanding of the physiology of APOBEC1-mediated RNA editing could prove useful for biotechnological applications. In these respects, differences in APOBEC1 function mediated by its dimerisation might be taken into account in designing APOBEC1 fusion molecules for DNA or RNA targeting applications.

DATA AVAILABILITY

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Plasmids are available through the Authors or through Addgene (https://www.addgene.org): pEGFP ratAPOBEC1-EGFP, #112857; pEGFP ratA1, #112858; pEGFP mCherry-apoB-EGFP, #112859; PEGFP human A1CF, #112860.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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TABLE AND FIGURES LEGENDS

Figure 1. Dominant negative effects of catalytically inactive mutants on the RNA editing activity of APOBEC1. The plot represents APOBEC1-dependent RNA editing in HEK293T cells transiently cotransfected with plasmids encoding for the mCherry-ApoB-EGFP reporter (1 μg) and A1CF (1 μg), together with 2 μg of a plasmid mix expressing increasing ratios of catalytically inactive APOBEC1 mutants (E63A, GFP-E63A, E63A-GFP, or a control plasmid) and wild-type APOBEC1. APOBEC1-dependent RNA editing of the reporter (percentage of gated cells) has been normalised to the activity of APOBEC1 in absence of competitor DNA. The error bars represent the standard deviation from three experiments.

Figure 2. Immunoprecipitation and localisation of A1CF suggest its interaction with all APOBEC1 chimeras. (A) Immunoprecipitation of FLAG-tagged A1CF brings down APOBEC1 constructs. Lysates from HEK293T cells that had been cotransfected with FLAG-tagged A1CF together with either APOBEC1 or the GFP-tagged APOBEC1 chimeras (A1, GFP-A1, A1-GFP) were subjected to immunoprecipitation with anti-FLAG resin. Following SDS/PAGE, blots were probed with anti-APOBEC1 or anti-FLAG antibodies. Aliquots (7%) of the total-cell extracts were probed with anti-APOBEC1, anti-FLAG, or anti-GAPDH antibodies to control for expression level. The lower band (~28KDa) in the A1-GFP lane as well as the band beneath the main GFP-A1 band represent products

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from proteolytic cleavage of the N-terminal region of the GFP epitope. The western blots are representative of 5 independent experiments. (B) Localisation of A1CF is APOBEC1 dependent. Representative images of HEK293T cells transfected with A1CF alone or in combination with APOBEC1 constructs (A1, GFP-A1, A1-GFP). An empty plasmid was used as a negative control. The localisation of the various proteins was assessed by GFP fluorescence for the GFP-tagged APOBEC1 chimeras, and by anti-APOBEC1 and anti-A1CF antibodies for APOBEC1 and A1CF, respectively. Due to the antibodies used, A1CF and APOBEC1 expression was visualised separately (anti-A1 in the red square). (C) The plot depicts the percentage of nuclear localisation of either A1CF or the APOBEC1 constructs in HEK293T cells transfected with A1CF alone or in combination with APOBEC1 constructs (A1, GFP-A1, A1-GFP). Thirty cells per samples were analysed, the median is indicated in the boxplot.

Figure 3. The APOBEC1-GFP chimera displays reduced dimerisation. (A) Immunoprecipitation of FLAG-tagged APOBEC1 shows a decreased dimerisation of the APOBEC1-GFP chimera. Lysates from HEK293T cells that had been cotransfected with FLAG-tagged APOBEC1 together with either APOBEC1 or the GFP-tagged APOBEC1 chimeras (A1, GFP-A1, A1-GFP) were subjected to immunoprecipitation with anti-FLAG resin. Following SDS/PAGE, western blots were probed with anti-APOBEC1 antibodies. Aliquots (7%) of the total-cell extracts were probed with anti-APOBEC1 or anti-HSP90 antibodies to control for expression level. The Flag-A1 control on the left originates from a different experiment. (B) Crosslinking of the APOBEC1 constructs in whole cell lysates, (C) in cytoplasmic/nuclear fractions, or (D) after treatment with RNAseA. HEK293T cells were transiently cotransfected as indicated and were treated with 2% formaldehyde (10’ at room temperature). The crosslinking was blocked with 1.25M glycin and cells were then lysed. Following SDS/PAGE, western blots were probed with anti-APOBEC1 antibodies. All western blots are representative of at least three independent experiments.

Figure 4. Dimerisation and degradation of APOBEC1 modulate RNA editing. (A) Effects of catalytically inactive mutants on the RNA editing activity of APOBEC1-GFP. The plot represents APOBEC1-dependent RNA editing in HEK293T cells transiently cotransfected with plasmids encoding for the mCherry-ApoB-EGFP reporter (1 µg) and A1CF (1 µg), together with 2 µg of a plasmid mix expressing increasing ratios of catalytically inactive APOBEC1 constructs (E63A, GFP-E63A, E63A-GFP) and the APOBEC1-GFP chimera. APOBEC1-dependent RNA editing of the reporter (percentage of gated cells) has been normalised to the activity of APOBEC1-GFP in absence of competitor DNA. The dotted lines report the RNA editing activity shown in Figure 1. The error bars represent the standard deviation from three experiments. The experiments shown in Figure 1 and Figure 4 were performed concurrently. (B) Representative western blots showing the expression levels various the APOBEC1 constructs after treatment with proteasome inhibitor MG132. (C) Representative western blots showing the expression levels of fixed amounts (0.5 µg) of either APOBEC1-GFP or APOBEC1 in presence of increasing amounts of the other one as indicated. HEK293T cells were transiently cotransfected with the indicated plasmids and lysed after 36 hours. All western blots are representative of at least three independent experiments.

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Figure 1

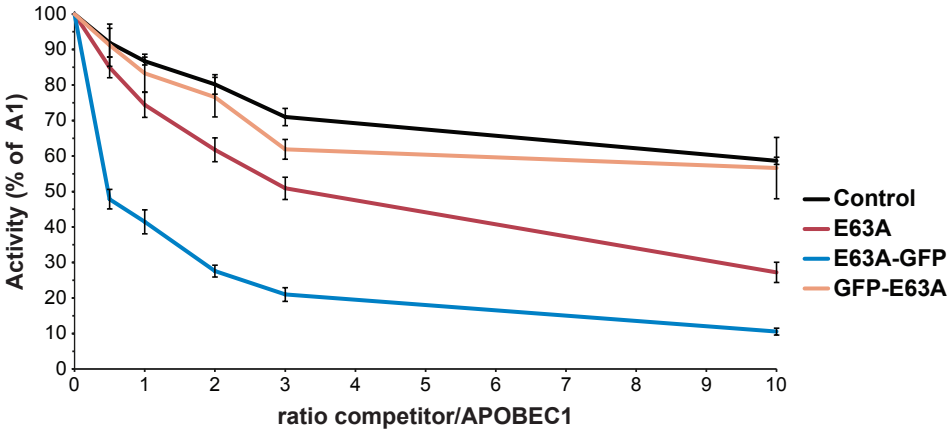

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Figure 2

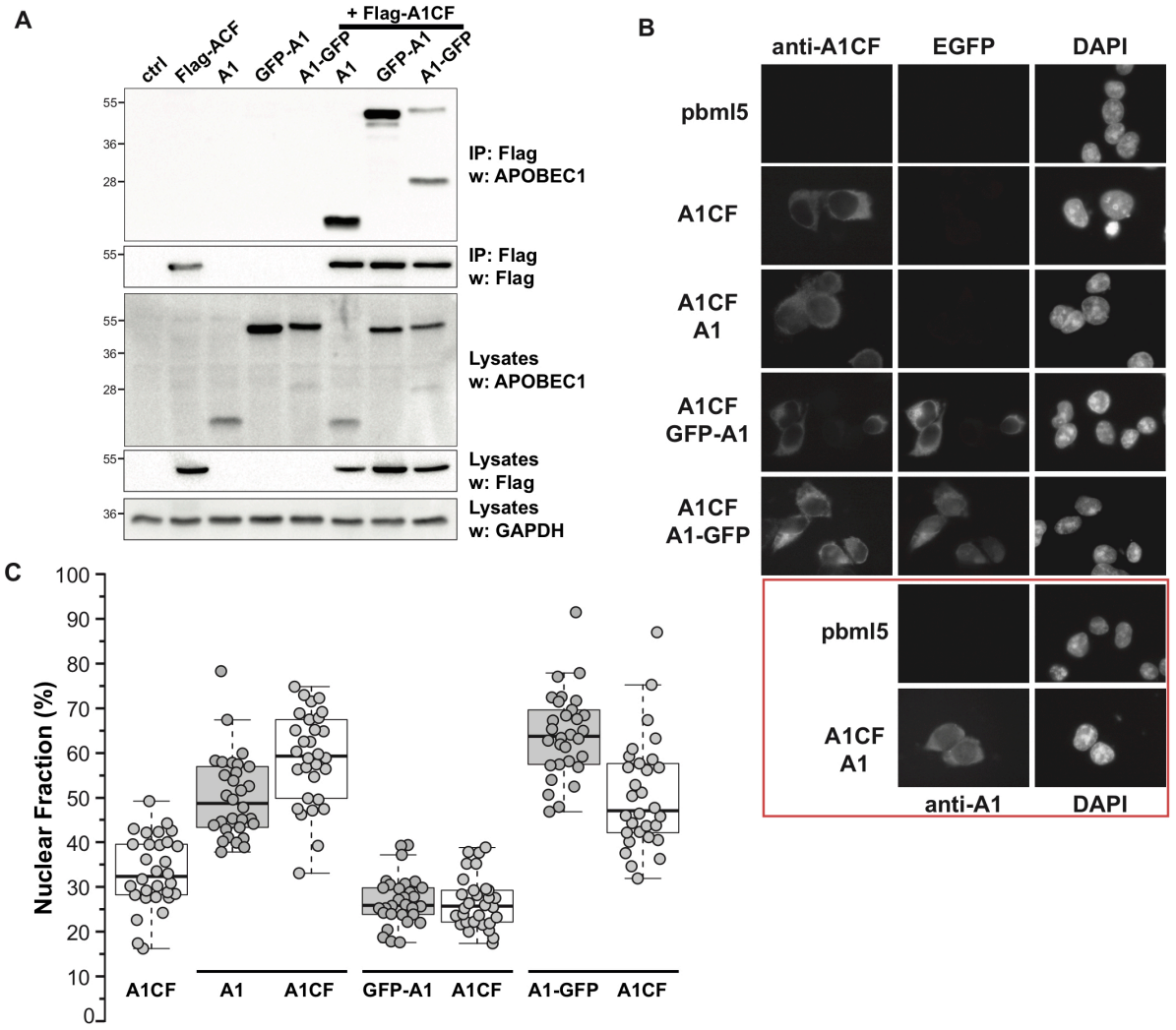

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Figure 3

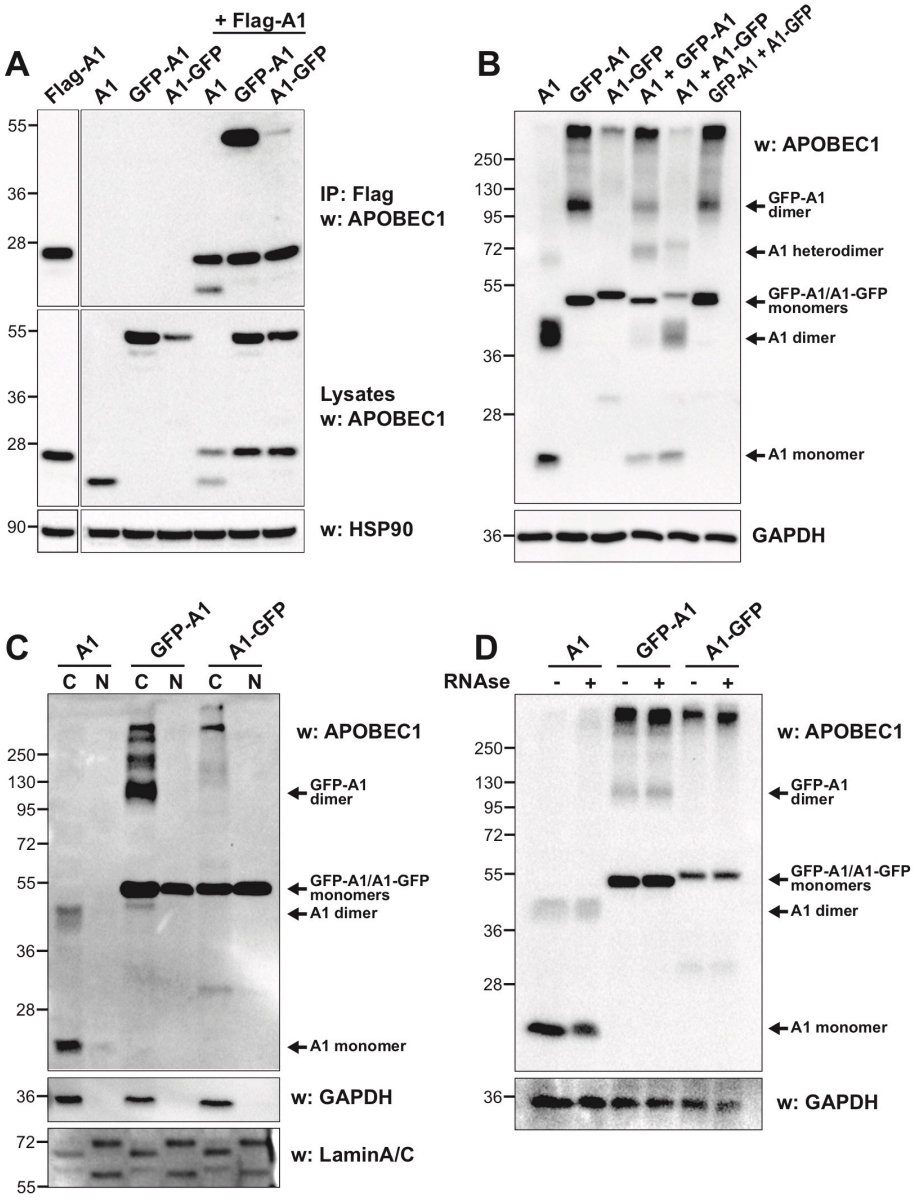

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Figure 4