SUPPLEMENTARY DATA

Binding of RNA by APOBEC3G Controls Deamination-Independent Restriction of Retroviruses

Kasandra Bélanger[1], Mathieu Savoie[1], Maria Carla Rosales Gerpe[1],
Jean-François Couture[1,2] and Marc-André Langlois[1,3,4*]

[1]Department of Biochemistry, Microbiology and Immunology, [2]Ottawa Institute of Systems Biology,
[3]Emerging Pathogens Research Centre, [4]Department of Pathology and Laboratory Medicine,
University of Ottawa, Ottawa, Ontario, Canada.

SUPPLEMENTARY TABLES

Table S1. Primers and probes

Primer or Probe	Sequence
W94A-Fwd	5'-ACCTGGTACATATCCGCGAGCCCCTGCACAAAG-3'
W94A-Rev	5'-CTTTGTGCAGGGGCTCGCGGATATGTACCAGGT-3'
W127A-Fwd	5'-CGCCTCTACTACTTCGCGGACCCAGATTACCAG-3'
W127A-Rev	5'-CTGGTAATCTGGGTCCGCGAAGTAGTAGAGGCG-3'
HA-NheI-Fwd	5'-GAATTCGCTAGCACCACCATGGGATACCCATACGACGTCCCAGACTACGCTATGAAGCCTCACTTCAGA-3'
HpaI-Rev	5'-ATAAACAAGTTAACAACAACAA-3'
3G-NheI-Fwd	5'-ACGTATCCGCTAGCACCACCATGGGATACCCATACGACGTCCCAGACTACGCTCCAGAGATGAGATTCTT-3'
3G-EcoRI-Rev	5'-TTCTAGAGAATTCTTATCATGCAACAAAGATGGTCAGGGT-3'
EG-NheI-Fwd	5'-ACGTATCCGCTAGCACCACCATGGGATACCCATACGACGTCCCAGACTACGCTGTGAGCAAGGGCGAG-3'
EG-EcoRI-Rev	5'-TGGATCCGAATTCTTATCAGGGCACGGGCAGCTTGC-3'
eGFP-Fwd	5'-GTGAGCAAGGGCGAGGAGCTGTTCA-3'
eGFP-Rev	5'-CTTGTACAGCTCGTCCATGCCGAGA-3'
qeGFP-Fwd	5'-GTGAGCAAGGGCGAGGAGCTGTTCA-3'
qIN-eGFP-Fwd	5'-TGCTGCTGCCCGACAAC-3'
qIN-eGFP-Rev	5'-CGTCCATGCCGAGAGTGAT-3'
qIN-eGFP-**Probe**	5'-FAM-CACCCAGTCCGCC-3'
qAlu-1	5'-TCCCAGCTACTGGGGAGGCTGAGG-3'
qAlu-2	5'-GCCTCCCAAAGTGCTGGGATTACAG-3'
qB1-1	5'-CCAGGACACCAGGGCTACAGAG-3'
qB1-2	5'-CCCGAGTGCTGGGATTAAAG-3'
qHIV-Fwd	5'-CAAGTAGTGTGTGCCCGTCTGT-3'
qHIV-Rev	5'-CGAGTCCTGCGTCGAGAGA-3'
qHIV-**Probe**	5'-FAM-CAGTGGCGCCCGAA-3'
qMoMLV-Fwd	5'-TTGGGAGGGTCTCCTCTGAGT-3'
qMoMLV-Rev	5'-GAGTGGTAACAGTCTGGCCCATA-3'
qMLV-**Probe**	5'-FAM-ATTGACTACCCGTCAGCGG-3'
7SL-Fwd	5'-ATCGGGTGTCCGCACTAAG-3'
7SL-Rev	5'-CACCCCTCCTTAGGCAACCT-3'
Alu-Fwd	5'-TCACGCCTGTAATCCCAGCA-3'
Alu-Rev	5'-GATCTCGGCTCACTGCAAG-3'
hY1-Fwd	5'-CTGGTCCGAAGGTAGTGAG-3'
hY1-Rev	5'-CTAGTCAAGTGCAGTAGTG-3'
hY3-Fwd	5'-GCTGGTCCGAGTGCAGTGGTGTTTAC-3'
hY3-Rev	5'-AGGCTAGTCAAGTGAAGCAGTG-3'
β-actin-Fwd	5'-GCCCCCCTGAACCCCAAGGCCAACCG-3'
β-actin-Rev	5'-GAAGTCCAGGGCGACGTAGCACAG-3'
Vpr-Xho1-Fwd	5'-GAGACCTCGAGATGCCATACAATGAATGGACACT-3'
Vpr-Xho1-Rev	5'-GAGACCTCGAGTCTCCTCTGTCGAGTAACG-3'
Vpr(ΔRNA)-Rev	5'-GAGACCTCGAGCTGTCGAGTAACG -3'

Table S2. Analysis of putative deamination target sites in the Env-eGFP fusion protein of MoMLV

	MoMLV Env (N-terminus)	eGFP
Nucleotide range:	1 - 888	889 - 1605
Codons in segment:	296	239
Dinucleotides (5'-**G**G):	72	53
Trinucleotides (5'-**GG**G):	28	12
Tryptophan (*Trp*) codons (T**G**G):	13	1
Trp followed by a G (T**GG**.G):	6	0

The number of 5'-GG and 5'-GGG nucleotide repeats was determined for the coding sequence of the eGFP and the N-terminal Env segments of the Env-eGFP fusion protein as read on the coding strand DNA. The number of TGG (tryptophan) codons, and TGG codons followed immediately by a guanine (TGG.G) were also determined. Deamination of cytidines on the complementary DNA strand to a TGG codon will result in the generation of a T**A**G termination (*stop*) codon. Deamination opposite to the TGG.G sequence will result in a T**A**G, TG**A** or T**AA** termination codon. Letters indicated in **bold** in the table are opposite to deoxycytidines targeted by A3G.

SUPPLEMENTARY FIGURES

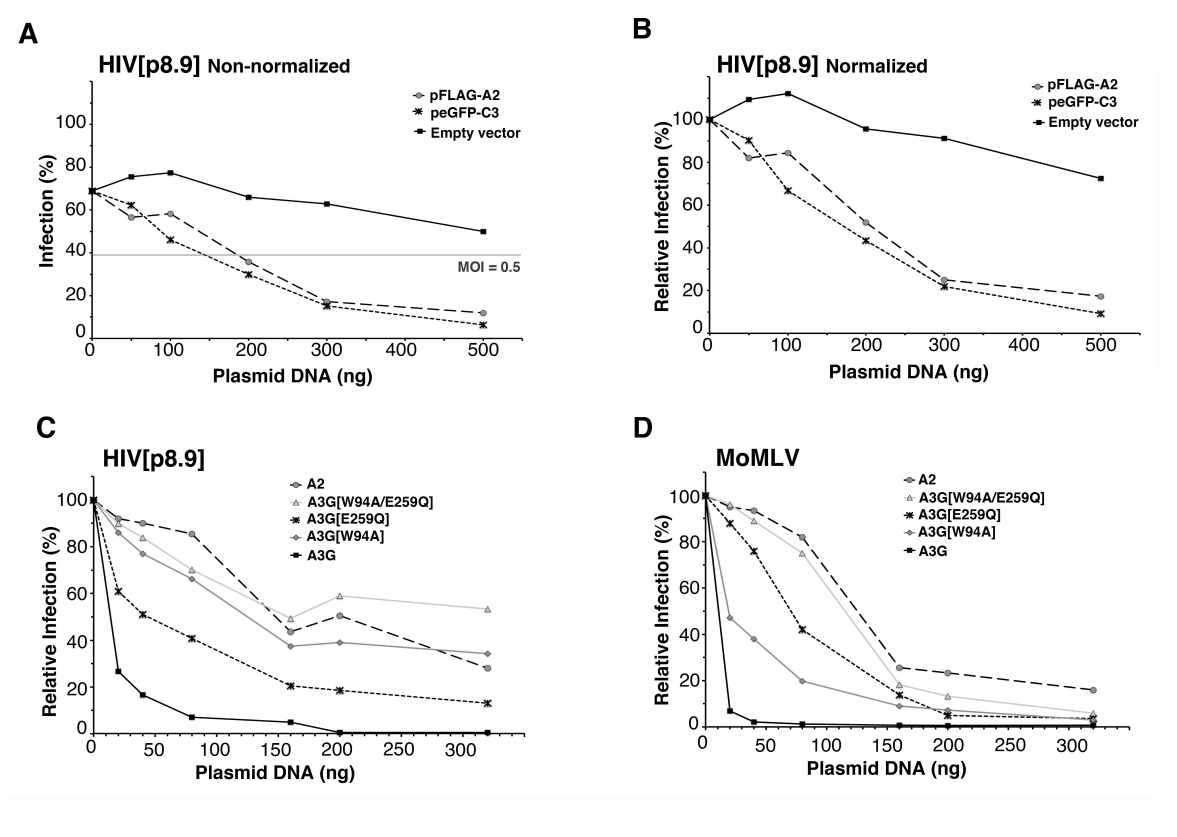

Figure S1. Determination of plasmid controls and titration of the effect of A3G variants on the infectivity of HIV[p8.9] and MoMLV. (A) Percentage of 293T target cells infected with HIV[p8.9] virus produced with increasing amounts of each control plasmid; Empty vector is pcDNA 3.1; peGFP-C3 expresses eGFP; pFLAG-A2 expresses human APOBEC2. The horizontal line intersects the curves at an MOI of 0.5. (B) Relative percentages of infected target cells normalized to HIV[p8.9] virus alone produced without control plasmids. (C and D) Relative infection of 293T target cells with HIV[p8.9] (C), or MoMLV (D) produced in the presence of increasing amounts of APOBEC expression plasmids. Normalizations were performed to infection values for virus alone.

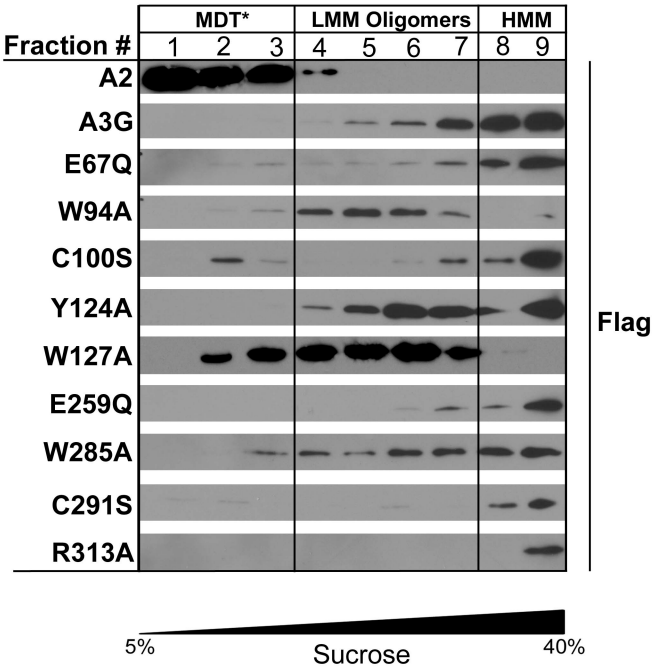

Figure S2. Effect of various amino acid substitutions on A3G HMM complex assembly. Lysates of transfected 293T cells were resolved by velocity sedimentation in non-denaturing 5% to 40% sucrose gradients and analyzed by Western blot using an anti-FLAG antibody. *MDT: RNA-independent monomers, dimers, tetramers.

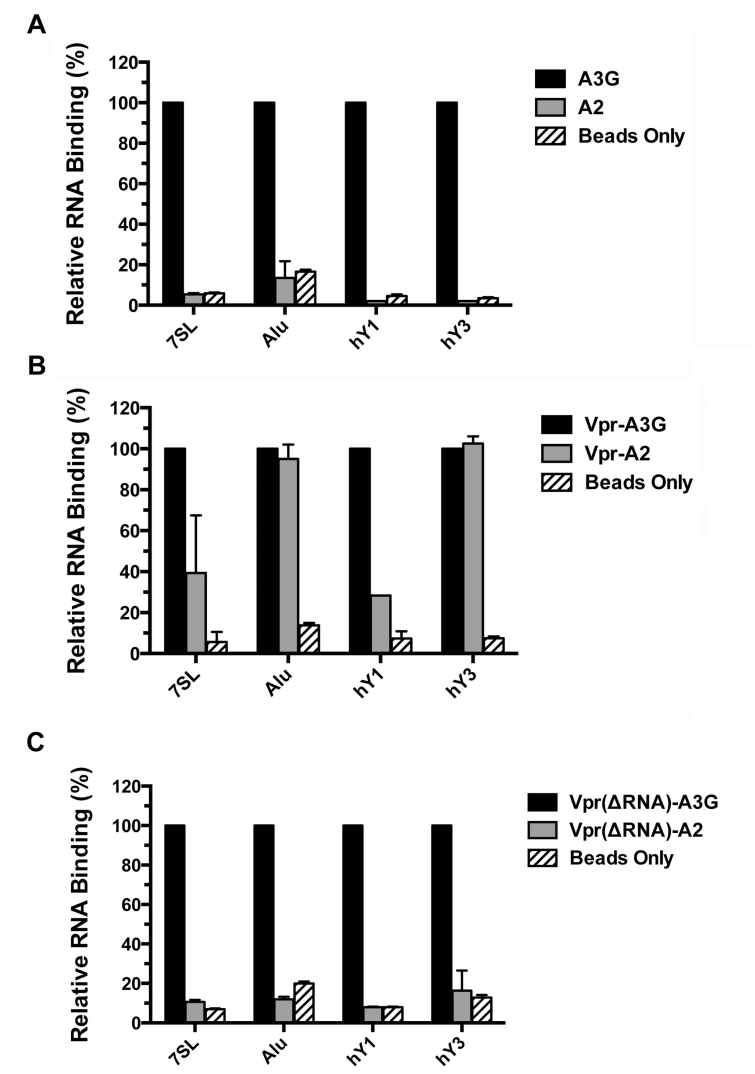

Figure S3. Non-specific binding of RNA to agarose beads. Binding of 7SL, *Alu*, hY1 and hY3 RNAs to: (A) A3G, A2 and beads only; (B) Vpr-A3G, Vpr-A2 and beads only, and (C) Vpr(ΔRNA)-A3G, Vpr(ΔRNA)-A2 and beads only. Relative binding to either A3G, Vpr-A3G or Vpr(ΔRNA)-A3G is depicted. Results represent the mean ± SD of triplicate values from three independent transfection experiments.

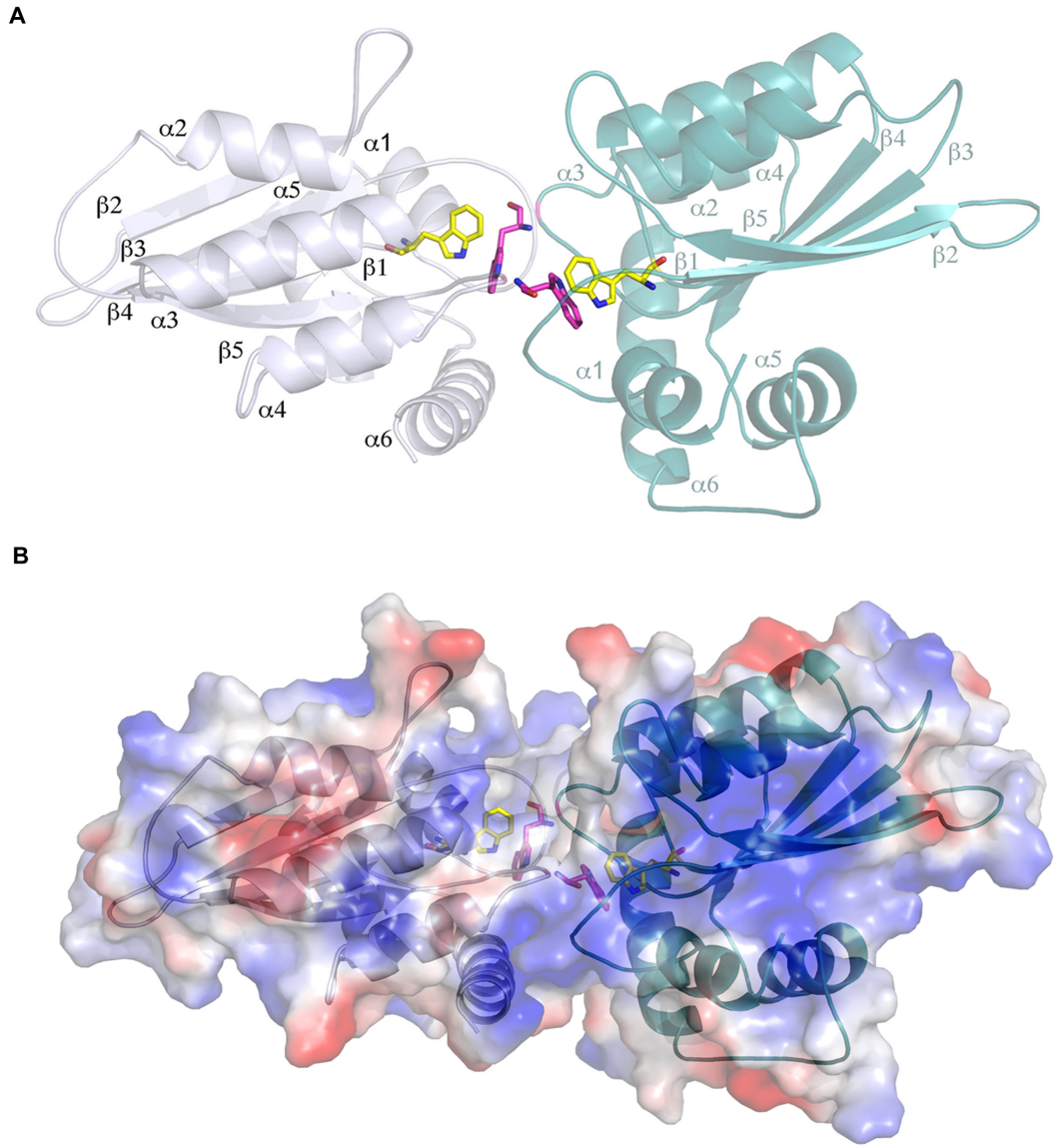

Figure S4. Homology modeling of the A3G head-to-head N-terminal domain (NTD) dimer. (A) Secondary structure of the NTD A3G dimer in which each protomer is colored either in green (right) or grey (left). For clarity, only W94 (yellow) and W127 (pink) residues were rendered in stick. (B) Electrostatic potential of the NTD dimer showing the positively (blue) and negatively (red) charged regions. The homology model was generated using the EasyPred3d using the coordinates of APOBEC2 as template (1). The figures were generated using PyMOL (http://pymol.sourceforge.net/).

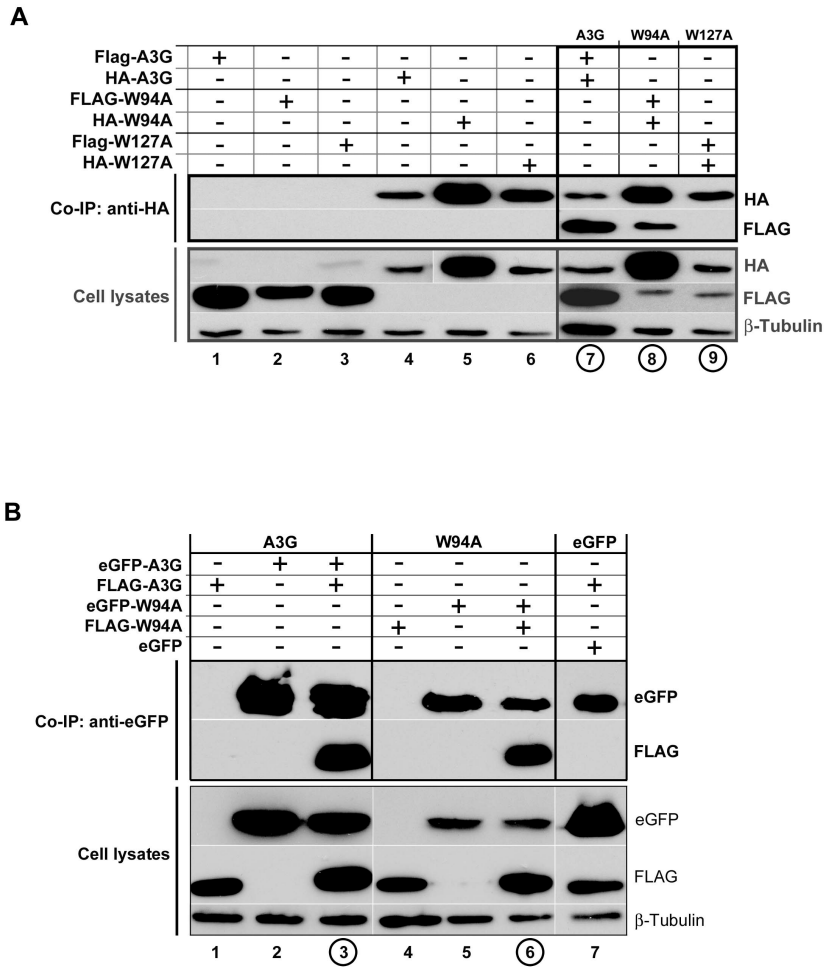

Figure S5. Self-association analysis of W94A and W127A. (A) 293T cells were transfected (lanes 1 to 6) or co-transfected (lanes 7 to 9) with FLAG- or HA-tagged APOBEC3 proteins. Pull-downs were carried out with anti-HA magnetic beads. A3G variants were detected with either anti-FLAG or anti-HA antibodies. (B) 293T cells were co-transfected with FLAG- or eGFP-tagged APOBEC3 proteins. Pull-downs were carried out with anti-eGFP magnetic beads. Detection of the proteins was done by Western blot analysis using either anti-FLAG or anti-eGFP antibodies. No unspecific binding to the magnetic beads (lanes 1 and 4) or to the eGFP reporter protein (lane 7) was detected. Circles indicate lanes depicting the result of the self-association.

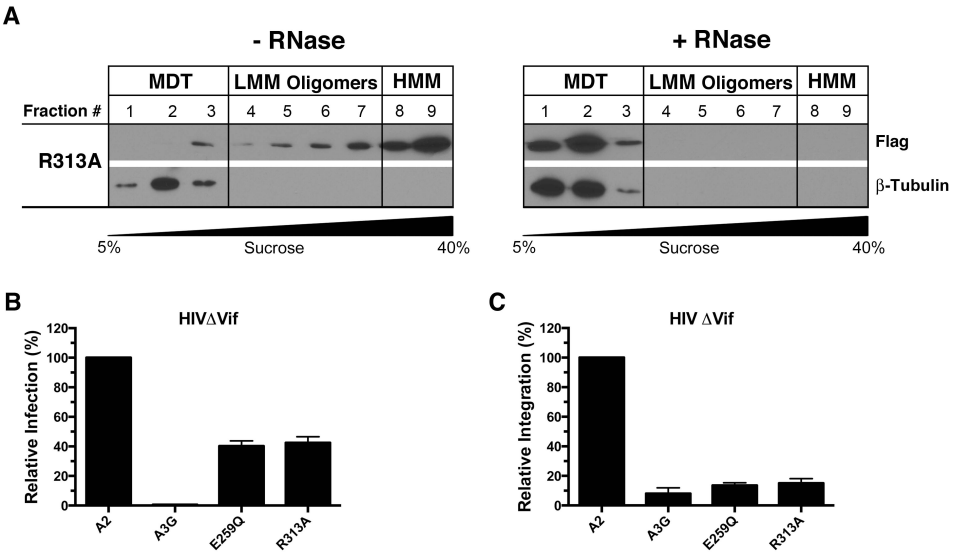

Figure S6. Assessment of the properties of the C-terminal domain (CTD) DNA-binding mutant R313A. (A) Velocity sedimentation analysis showing that R313A associates into RNA-dependent HMM complexes. (B) Restriction of HIVΔVif infection. (C) Inhibition of HIVΔVif integration.

SUPPLEMENTARY MATERIALS AND METHODS

Antibodies and cells

Human embryonic kidney epithelium (293T) and mouse embryonic fibroblast (NIH 3T3) cells were cultured in HyClone DMEM/High Glucose medium (Thermo Fischer Scientific) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100µg/ml streptomycin (Multicell). The following antibodies were used for this study: HRP-conjugated anti-FLAG (A8592, Sigma), anti-p24 (ab9069, Abcam), anti-eGFP (no.632381, Clontech), HRP-conjugated anti-β-tubulin (ab21058, Abcam), and anti-HA (H3663, Sigma).

APOBEC expression vectors

FLAG-tagged and eGFP-tagged A3G and A2 expression plasmids were constructed previously (54). pFLAG-W94A, pFLAG-W94A/E259Q, pFLAG-W127A, pFLAG-W127A/E259Q and pFLAG-W94A/W127A were generated from the corresponding pFLAG-A3G plasmids by PCR-based site-directed mutagenesis. The sequences for all primers and probes used in this study can be found in Table S1. HA-APOBEC expression vectors were made by amplifying the corresponding APOBEC coding sequence with a forward primer containing the HA sequence. The HA-NheI-FWD and HpaI-REV primers were used to amplify the coding sequence. The PCR product was then digested and inserted into the NheI and HpaI cloning sites of peGFP-C3 (Clontech). pTrc99a-APOBEC expression vectors were generated by amplifying the corresponding APOBEC coding sequences from pFLAG-APOBEC plasmids with a forward and a reverse primer containing an XhoI and PstI restriction site respectively. The PCR product was then digested and inserted into the pTrc99a vector. pTrc99a-W94A and pTrc99a-W127A were generated by site-directed mutagenesis from the corresponding pTrc99a-A3G plasmid.

For Vpr-APOBEC fusion constructs, the sequence coding for amino acids 14 to 88 of Vpr was amplified by PCR from the HIV-1 pNL4-3 plasmid using forward and reverse primers containing Xho1 restriction sites (Table S1). The PCR product was then digested and inserted in-frame into the Xho1 site of pFLAG-A3G located between the FLAG epitope tag and the APOBEC coding sequence. Fusion proteins with the Vpr(ΔRNA) polypeptide (Vpr_{14-86}) were generated in a similar way.

Fluorescence microscopy

293T cells were seeded in 35mm glass bottom dishes (MatTech) and transfected with plasmids expressing eGFP-A3G or the various eGFP-A3G mutants. The cells were washed 24h post transfection and media was replaced with DMEM without phenol red. Images of live cells were captured as Z-stacks using a Zeiss Axio Observer.Z1 inverted fluorescent microscope and a Plan-Apochromat 63x/1.4 oil immersion objective. Acquired images were deconvolved to achieve the best image quality using the constrained Iterative Maximum Likelihood Algorithm. (http://www.zeiss.ca/). Acquisition and deconvolution were performed using the AxioVision software (Zeiss).

Rif^R bacterial mutator assay

Rifampicin resistance has been used extensively to measure the catalytic activity of A3G proteins (2,3). To obtain rifampicin resistant (Rif^R) mutants, the *E. coli* uracil excision-defective strain BW310 was transformed with pTrc99a or an APOBEC-expressing pTrc99a derivative and plated overnight on ampicillin containing plates. Single transformants were picked and used to inoculate 6 independent liquid media cultures supplemented with 1mM IPTG and 100µg/ml ampicillin. These were then grown to saturation in a 37^{o}C shaker. To obtain Rif^R mutants, 300µl of the resultant culture was spread onto low salt LB media containing 100µg/ml rifampicin and grown at 37^{o}C. 100µl of a 10^{-6} dilution was spread onto plates containing 100µg/ml ampicillin to assay for the number of viable cells. Rifampicin and ampicillin resistant colonies were counted 24 hours later and mutation frequencies were calculated accordingly.

Western blotting

Cells were harvested 48h after transfection, washed twice with cold PBS and lysed on ice for 30 minutes with NP40 lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% NP40, 0.1% Na-deoxycholate) supplemented with cOmplete, EDTA-free, protease inhibitor cocktail (Roche). Lysates were resuspended in Laemmli loading buffer, boiled and then cleared by centrifugation at 17,000 x g for 5min. Finally the samples were resolved on 10% SDS-PAGE gels, transferred to PVDF membranes and processed for immunoblot analysis.

Virus encapsidation assays

Viruses were produced as described earlier. Viral supernatants were cleared from cell debris, filtered and then layered over a 20% sucrose cushion and concentrated by ultra-centrifugation at 100,000 x *g* for 1h in a Beckman TLA 100.3 rotor. Virus pellets were dissolved in RIPA buffer (150mM NaCl, 1% NP-40, 0.2% sodium dodecyl sulfate (SDS), 1mM EDTA, 0.5% Na-Deoxycholate, 50mM Tris-HCL pH 8.0) supplemented with cOmplete, EDTA-free, protease inhibitor cocktail (Roche) and incubated on ice for 10min. Viral lysates were then processed for immunoblot analysis as described above.

RNA-binding analysis by quantitative real-time PCR (qPCR)

Lysates of 293T cells transfected with FLAG-APOBEC expression vectors were incubated for 3h at 4ºC with anti-FLAG conjugated agarose beads pre-treated with salmon sperm DNA (500μg/ml) to reduce non-specific binding of cellular nucleic acids. After a gentle 3h agitation, the beads were washed four times with 50mM Tris-HCl (pH 8.0), 150mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and then washed a final time with the same buffer containing 50mM NaCl. Bound complexes were then eluted from the beads with 100μl of 0.1M triethylamine, pH 11.8 to which 3ul of 1M MES buffer, pH 3.0 was added to neutralize the samples prior to RNA isolation.

Methods for RNA amplification and quantification were performed as described in a previous report (4). Briefly, RNA was purified from normalized quantities of FLAG-tagged APOBEC immunoprecipitates using Trizol (Sigma). After DNase treatment of the samples, RNA was reverse transcribed using random hexamers and the ImProm-II™reverse transcriptase according to the manufacturer's recommendations (Promega). The resulting cDNA was used for qPCR using GoTaq Green Master Mix (Promega) and specific primers for 7SL, *Alu*, hY1, hY3, β-actin as previously described (4). Plasmid DNA was added as an internal standard in each reaction for quantitation purposes (4). All primers used can be found in Table S1. The reactions were performed under the following conditions: 50ºC for 2min and 95ºC for 10min, followed by 40 cycles of 95ºC for 15s and 60ºC for 1min on an AB ViiA7 System. RQ was computed using A3G as the comparative reference.

Protein self-association analysis

Pull-downs for eGFP and HA epitope tags were performed according to the manufacturer's specifications detailed in the uMACS™ Epitope Tag Protein Isolation kits (Miltenyi Biotec).

Hypermutation analysis

Mutation analysis was performed by a commonly used approach (5,6). Target cells from the infection assays were harvested after 24h and the coding sequence of the internal eGFP reporter gene for each virus was amplified by PCR, cloned and sequenced. Viral supernatants were treated with the endonuclease *DpnI* prior to infection to avoid plasmid contamination of the target cells. DNA was extracted using the Wizard genomic DNA purification kit (Promega). A 717bp sequence of the eGFP reporter gene was amplified using the high fidelity polymerase Prime Star (Takara Bio Inc.) with the following primers: eGFP-FWD, eGFP-REV. Purified PCR products were then cloned using the TOPO-Blunt cloning kit (Invitrogen) and sequenced using M13 reverse primers. Sequencing was performed at the genotyping platform of McGill University and Génome Québec Innovation Centre. Primers for the amplification were designed to target regions with no or few A3G deamination consensus sites to avoid an amplification bias of unmutated sequences due to poor primer annealing.

SUPPLEMENTARY REFERENCES

1. Prochnow, C., Bransteitter, R., Klein, M.G., Goodman, M.F. and Chen, X.S. (2007) The APOBEC-2 crystal structure and functional implications for the deaminase AID. *Nature*, **445**, 447-451.

2. Chen, K.M., Harjes, E., Gross, P.J., Fahmy, A., Lu, Y., Shindo, K., Harris, R.S. and Matsuo, H. (2008) Structure of the DNA deaminase domain of the HIV-1 restriction factor APOBEC3G. *Nature*, **452**, 116-119.

3. Newman, E.N., Holmes, R.K., Craig, H.M., Klein, K.C., Lingappa, J.R., Malim, M.H. and Sheehy, A.M. (2005) Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. *Current biology : CB*, **15**, 166-170.

4. Bach, D., Peddi, S., Mangeat, B., Lakkaraju, A., Strub, K. and Trono, D. (2008) Characterization of APOBEC3G binding to 7SL RNA. *Retrovirology*, **5**, 54.

5. Dapp, M.J., Holtz, C.M. and Mansky, L.M. (2012) Concomitant lethal mutagenesis of human immunodeficiency virus type 1. *Journal of molecular biology*, **419**, 158-170.

6. Langlois, M.A., Beale, R.C., Conticello, S.G. and Neuberger, M.S. (2005) Mutational comparison of the single-domained APOBEC3C and double-domained APOBEC3F/G anti-retroviral cytidine deaminases provides insight into their DNA target site specificities. *Nucleic acids research*, **33**, 1913-1923.