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## Inhibition of LINE-1 and Alu retrotransposition by exosomes encapsidating APOBEC3G and APOBEC3F

Atanu K. Khatua, Harry E. Taylor, James E. K. Hildreth, and Waldemar Popik\*

Meharry Medical College, Center for AIDS Health Disparities Research, Nashville, TN 37208

### Abstract

Human cytidine deaminases, including APOBEC3G (A3G) and A3F, are part of a cellular defense system against retroviruses and retroelements including non-LTR retrotransposons LINE-1 (L1) and Alu. Expression of cellular A3 proteins is sufficient for inhibition of L1 and Alu retrotransposition, but the effect of A3 proteins transferred in exosomes on retroelement mobilization is unknown. Here, we demonstrate for the first time that exosomes secreted by CD4<sup>+</sup> H9 T cells and mature monocyte-derived dendritic cells encapsidate A3G and A3F and inhibit L1 and Alu retrotransposition. A3G is the major contributor to the inhibitory activity of exosomes, however, the contribution of A3F in H9 exosomes cannot be excluded. Additionally, we show that exosomes encapsidate mRNAs coding for A3 proteins. A3G mRNA, and less so A3F, was enriched in exosomes secreted by H9 cells. Exosomal A3G mRNA was functional *in vitro*. Whether exosomes inhibit retrotransposons *in vivo* requires further investigation.

### Keywords

Exosomes; APOBEC3G; APOBEC3F; LINE-1; Alu; Retrotransposon

### Introduction

Long interspersed elements (L1) are the only active autonomous non-LTR retrotransposons in the human genome. While L1s make up about 17% of the human genome, only 80 to 100 of the over 500,000 copies can retrotranspose (Kazazian, 2004). L1s are about 6 kb long, harbor an internal polymerase II promoter and encode two open reading frames (ORFs): ORF1p coding for a 40 kDa protein with RNA binding and RNA chaperone activity (Holmes, Singer, and Swergold, 1992; Kolosha and Martin, 2003; Martin, Li, and Weisz, 2000), and ORF2p coding for a 150 kDa protein with endonuclease (Feng et al., 1996), reverse transcriptase (Mathias et al., 1991), and C-terminal cysteine-rich domains (Fanning and Singer, 1987). Upon translation, L1 RNA assembles with ORF1p and ORF2p and the formed ribonucleoprotein complex moves to the nucleus, where an endonuclease makes a single-stranded nick. The reverse transcriptase uses the nicked DNA to prime reverse transcription of the L1 RNA, leading ultimately to integration of L1 cDNA (Babushok and Kazazian, 2007). In contrast to L1, Alu elements are non-autonomous and are mobilized by the L1 retrotransposition

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\*Corresponding author. Mailing address: Center for AIDS Health Disparities Research, Meharry Medical College, 1005 D. B. Todd Blvd., Nashville, TN 37208. Phone: 615-327-6734; Fax: 615-963-3146; wpopik@mmc.edu.

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machinery (Dewannieux, Esnault, and Heidmann, 2003; Ostertag and Kazazian, 2001). As insertions of both Alu and L1 elements into the human genome may cause a number of human genetic disorders (Callinan and Batzer, 2006; Wallace, Belancio, and Deininger, 2008), under normal conditions retrotransposons are silenced. Mechanisms protecting cells against retrotransposition include repression of transcription by DNA methylation (Roman-Gomez et al., 2005), premature polyadenylation (Perepelitsa-Belancio and Deininger, 2003), aberrant splicing (Belancio, Hedges, and Deininger, 2006) or RNA interference (Yang and Kazazian, 2006). Another strategy is editing of nucleic acids by the APOBEC3 (A3) family of cellular cytidine deaminases.

The most prominent member of this family, APOBEC3G (A3G), first identified through its ability to inhibit replication of *vif*-deficient HIV-1 (Sheehy et al., 2002), also functions as a post-entry restriction factor against wild-type viruses (Chiu et al., 2005; Khatua et al., 2009; Pion et al., 2006; Vetter and D'Aquila, 2009; Wang et al., 2008a). Although deaminase-independent activities of A3G are postulated to contribute to its antiviral function (Bishop, Holmes, and Malim, 2006; Holmes et al., 2007; Newman et al., 2005), there is strong evidence that HIV-1 restriction requires A3G deaminase activity when the protein is not overexpressed (Browne, Allers, and Landau, 2009; Miyagi et al., 2007; Schumacher et al., 2008). In mammals, A3G also restricts replication of a variety of endogenous retroviruses (Esnault et al., 2005; Esnault et al., 2006; Esnault et al., 2008; Schumacher et al., 2008) and retroelements including Alu (Bogerd et al., 2006b; Chiu et al., 2006; Hulme et al., 2007) and L1 (Bogerd et al., 2006b; Chen et al., 2006; Kinomoto et al., 2007; Muckenfuss et al., 2006; Niewiadomska et al., 2007; Schumann, 2007; Stenglein and Harris, 2006; Tan et al., 2009; Turelli, Vianin, and Trono, 2004). The expansion of a single A3 gene in mice to the seven A3 genes found in primates correlates with the significant enhancement of restriction of endogenous retroelements seen in primates. This supports the suggestion that A3 proteins provide a cellular defense mechanism against endogenous and exogenous invaders (Esnault et al., 2005).

We recently reported that functional A3G associated with exosomes, is secreted by CD4+ H9 T cells (Khatua et al., 2009). This was corroborated by the observation that a fraction of cellular A3G associates with multivesicular bodies (MVB)/late endosomes (Alce and Popik, 2004), organelles that release intraluminal vesicles as exosomes after fusion with the plasma membrane (de Gassart et al., 2004; Stoorvogel et al., 2002; Thery, Zitvogel, and Amigorena, 2002). This observation suggests that A3G and possibly other A3 proteins are transmitted between cells, in exosomes, and contribute to an antiretroviral defense mechanism (Khatua et al., 2009).

The biological role of exosomes is not fully understood. Secretion of transcription factors (Ratajczak et al., 2006a; Ratajczak et al., 2006b), morphogenic proteins (Liegeois et al., 2006) as well as cellular mRNAs and miRNAs (Bruno et al., 2009; Deregibus et al., 2007; Valadi et al., 2007) in association with exosomes suggests that these vesicles may epigenetically reprogram the recipient cells and may play a role during embryogenesis. Although retrotransposition occurs sporadically in somatic cells, L1 and Alu transpositions are most active in germ cells and during early embryogenesis (Garcia-Perez et al., 2007; Prak et al., 2003; van den Hurk et al., 2007). We speculate that secretion of exosomes including A3G or other A3 proteins, in combination with functional mRNAs coding for these proteins, provides an additional line of defense against retroelements, especially in recipient cells that do not express or express only suboptimal levels of these proteins.

In the present study, we tested whether exosomes secreted by cells expressing A3G can restrict retrotransposition in recipient cells. We demonstrate that exosomes secreted by cells expressing A3G and A3F potently restrict L1 and Alu retrotransposition. This leads us to speculate that exosomes contribute to anti-retroelement defenses *in vivo*.

## Materials and methods

### Cells

SupT1, H9 and peripheral blood mononuclear cells (PBMCs) were propagated in RPMI supplemented with 10% FCS and gentamicin (50 µg/ml; complete RPMI). HeLa and 293T cells were cultivated in DMEM supplemented with 10% FCS and gentamicin (complete DMEM). PBMCs were obtained by Ficoll-Paque (Amersham) density centrifugation from healthy blood donors (New York Blood Center). Monocyte-derived dendritic cells (M-DC) were obtained from the plastic adherent fraction of PBMC. After 3h adherence to plastic dishes, adherent cells were washed, collected by scraping and plated in T75 flask at  $2 \times 10^6$  cells/ml. The cells were cultivated for 6 days with 5 ng/ml interleukin-4 (IL-4; Humzyme) and 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Humzyme) in complete RPMI. Maturation of M-DC was induced by incubation of the immature M-DC with 10 ng/ml of lipopolysaccharide (LPS; *E.coli* strain O55:B5, Sigma-Aldrich) as described (Wang et al., 2007a). The M-DCs were more than 94% pure by HLA-DR and CD11c staining (FACS analysis).

### Plasmids

Human A3-HA expression vectors were obtained from B. R. Cullen and A3H-V5 from Y. Zheng. L1<sub>RP</sub>-EGFP and EFO5J were obtained from E. T. L. Prak and H. H. Kazazian. Alu neo<sup>Tet</sup> and p220.CMV L1-<sub>RP</sub> was provided by T. Heidmann.

### L1 retrotransposition assay

293T cells were plated one day before transfection in 12-well plates at  $2 \times 10^5$  cells/well in complete DMEM. The cells were transfected using the PolyFect reagent (Qiagen) and 1 µg of the L1 retrotransposition reporter plasmid without or with 0.5 µg of A3-HA or A3H-V5-His (Dang et al., 2008). The total amount of transfected DNA was adjusted to 1.5 µg with control pcDNA3.1 vector. After 3 h, the transfection medium was replaced with complete DMEM for another 3h. Exosomes (20 µg) were added where indicated and incubated with cells for 24 h. The following day, new aliquots of exosomes were added and incubated with cells for another 24 h. Three days post-transfection, the cells were trypsinized and transferred to 60-mm dishes. On day 5–7 post-transfection, the cells were analyzed for GFP expression by fluorescent microscopy and flow cytometry (FACSCalibur, BD). FACS analysis was performed on fixed cells and retrotransposition frequency was determined by gating GFP-positive cells.

### L1-mediated Alu retrotransposition assay

HeLa cells ( $1.1 \times 10^5$  cells/well) were cotransfected using PolyFect (Qiagen) in 12-well plates with 1 µg of neo<sup>Tet</sup>-marked Alu and 0.5 µg pCMV-L1 (p220 L1-<sub>RP</sub>). In control experiments, the cells were additionally cotransfected with A3G-V5 or GFP vectors. Negative controls were mock transfected or transfected with Alu, L1, or Alu and GFP. The transfected cells were incubated with different exosome preparations (20 µg), starting 6 h post-transfection with an additional dose of exosomes added next day. Three days post-transfection, the cells were trypsinized and transferred to 60-mm dishes. Starting at day 7 post-transfection, the cells were selected in the presence of G418 (1 mg/ml; Invitrogen) and the media were changed daily. One week later, the G418-resistant cell foci were fixed with 3% paraformaldehyde, stained with 1% crystal violet, washed, air-dried and counted using Quantity One software (ChemiDoc XRS, Bio-Rad).

### Quantitative real-time RT-PCR analysis

Total RNA was extracted and purified using a total RNA purification kit (Norgen) and contaminating DNA was digested with Turbo DNA-free DNase (Ambion). Purified RNA (200

ng) was reverse transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad) and subjected to real-time PCR using primers specific for human A3 genes (SABiosciences). Amplification of A3 genes was normalized to GAPDH as an endogenous control. Normalized expression of A3C in H9 cells or H9 exosomes was set as 1.0. The experiment was repeated twice with triplicate cDNA samples used for PCR.

### Exosome isolation protocol

Exosome preparation was performed as previously described (Khatua et al., 2009). In short, cells were grown in culture media supplemented with 10% FCS depleted of endogenous exosomes. Culture supernatants were collected every 24 h and were first centrifuged at  $300 \times g$  for 10 min to pellet cells. Cell debris was removed from the resulting supernatant by centrifugation at  $2000 \times g$  for 15 min followed by 30 min centrifugation at  $10,000 \times g$ . The clarified supernatant was filtered through a  $0.45 \mu\text{m}$  filter and ultracentrifuged at  $100,000 \times g$  using a SW32 rotor to pellet exosomes. Exosomes were washed by resuspension in PBS and pelleted for 1 h at  $100,000 \times g$  using a SW41 rotor. After suspension in a small volume (50–100  $\mu\text{l}$ ) of PBS, purified exosomes were aliquoted and stored at  $-80^\circ\text{C}$ . Protein concentration of exosome preparations was determined using Micro BCA protein assay (Thermo Scientific).

### PolyA<sup>+</sup> mRNA isolation and *in vitro* translation

PolyA<sup>+</sup> mRNA was isolated directly from H9 exosomes using Oligotex direct mRNA protocol according to the manufacturer's specifications (Qiagen). One  $\mu\text{g}$  of polyA<sup>+</sup> mRNA was used for *in vitro* translation using human *in vitro* protein expression kit according to manufacturer's recommendation (Thermo Scientific). A sample without added RNA was used as a negative control. GFP mRNA was used as a positive control. Translation reactions (25  $\mu\text{l}$ ) were performed for 90 min at  $30^\circ\text{C}$  and total reaction volumes were subjected to Western blot analysis.

### Western blot analysis

Proteins were separated on SDS-10% polyacrylamide gels and analyzed as described (Khatua et al., 2009). Antibodies for Western blotting were obtained from the following sources; rabbit anti-Alix from H. Stenmark (Oslo, Norway), rabbit anti-A3G from K. Strebel (NIH), rabbit anti-GFP, monoclonal anti-HA and anti-V5 antibodies from Invitrogen, rabbit anti-Rab7 from Santa Cruz Biotechnology. Rabbit anti-A3F antibodies were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

### Statistical analysis

All experiments were repeated at least three times with similar results. The variations in single experiments were expressed by calculating the standard deviation (SD) from triplicate samples and presented as the mean values  $\pm$  SD.

## Results

### Exosomes secreted by H9 CD4<sup>+</sup> T cells and mature monocyte-derived dendritic cells (M-DCs) inhibit L1 retrotransposition

Previous reports have shown that multiple human A3 proteins inhibit retrotransposition of L1 to different degrees, with A3A, A3B, A3C and A3F being the most effective and A3DE, A3G, and A3H having a smaller or negligible effect on L1 mobilization (Bogerd et al., 2006b; Chen et al., 2006; Kinomoto et al., 2007; Muckenfuss et al., 2006; Niewiadomska et al., 2007; Stenglein and Harris, 2006; Turelli, Vianin, and Trono, 2004). We previously showed that A3G is specifically packaged and secreted by cells in association with exosomes that confer resistance to HIV-1 after internalization by recipient cells (Khatua et al., 2009). To determine

whether exosomes secreted by cells expressing A3G or other A3 proteins show inhibitory activity against retroelements, we analyzed the effect of isolated exosomes on L1 retrotransposition using an established 293T cell culture-based retrotransposition reporter assay (Brouha et al., 2003; Ostertag et al., 2000; Wei et al., 2000).

For this assay, we used a construct in which an active L1<sub>RP</sub> (L1) element (Kimberland et al., 1999; Ostertag et al., 2000) was tagged with a retrotransposition indicator EGFP cassette. The EGFP gene, under control of a CMV promoter, was interrupted by a  $\gamma$ -globin intron and was cloned in a transcriptional orientation opposite to L1. EGFP was expressed only after the full-length L1 was transcribed, the intron was removed by cellular splicing machinery, and the RNA was reverse transcribed and integrated into the host genome. 293T cells were transfected with the L1 reporter construct alone or in combination with plasmids expressing various human A3 proteins (Fig. 1). In addition, cells transfected with L1 were exposed to exosomes secreted by H9 CD4<sup>+</sup> T cells, activated M-DC, and SupT1 CD4<sup>+</sup> T cells, or exosomes isolated from the blood plasma of normal blood donors. 293T cells were also transfected with EF05J, a negative control derived from L1<sub>RP</sub> containing disabling mutations in ORF1 (Farkash et al., 2006).

Retrotransposition events were monitored by the appearance of EGFP-positive cells 5–7 days after transfection (Fig. 1A) and quantitated by flow cytometry (Fig. 1B). Expression of A3 proteins, used as controls, showed an anticipated inhibition of L1 retrotransposition, with cells containing A3A, A3B, and A3F having the most potent inhibitory activity. Retrotransposition events were also inhibited by A3C (about 43%) and by A3G (about 30%). A3H showed no activity against L1 under the conditions of assay. Observed differences in L1 inhibition were most likely A3 dependent since all A3 proteins were expressed in 293T cells at roughly comparable levels (Fig. 1C). Similarly, exosomes isolated from various cellular sources had different anti-L1 activities. While the effect of transient expression of A3G on L1 was only moderate (30% decrease) (Fig. 1B), H9 and M-DC exosomes with A3G inhibited L1 retrotransposition (in contrast to A3G-negative exosomes secreted by SupT1 cells or derived from blood plasma). In particular, exosomes secreted by A3G-positive H9 cells inhibited L1 transposition by about 75%, while exosomes released from mature M-DCs inhibited L1 transposition by about 50%. Together, these results suggest that in addition to A3G, other A3 proteins present in exosomes contribute to L1 inhibition.

Since A3F expressed in 293T cells was more potent in inhibiting L1 than A3G (Fig. 1B), we determined if A3F was detectable in H9-, SupT1-, mature M-DC-, and plasma-derived exosomes, and thus might contribute to the capacity of these exosomes to inhibit L1 retrotransposition. As shown by Western blot analysis (Fig. 2), A3G was not only expressed by H9 T cells and mature M-DC, but also detected in exosomes secreted by these cells. Two forms of A3G were detected in H9 exosomes, while only faster migrating A3G was present in H9 cells (Khatua et al., 2009). In addition to H9 cells and M-DC, A3F was also detectable in both SupT1 cells and exosomes, as well as in blood exosomes derived from one of the two blood donors. However, unlike H9 exosomes that appeared to concentrate A3F about 3-fold in comparison to H9 cells, the expression of A3F in exosomes secreted by M-DC and SupT1 cells was lower than in their respective cell samples. Comparable levels of Rab7 expression in cells and exosomes suggest that observed differences in A3G and A3F were not caused by unequal protein loading on a gel. Different forms of Alix, a typical exosomal marker, were also detected in exosomes and in cells, with slower migrating forms of Alix detected predominantly in exosomes (Khatua et al., 2009). Together, H9 exosomes expressed about 10 times more A3F protein than SupT1 or M-DC exosomes. Thus, the presence of both A3G and A3F in exosomes may translate into the higher potency of H9 exosomes against L1 mobilization.



### H9 cells secrete exosomes enriched in A3G and A3F mRNAs

In addition to hundreds of proteins, exosomes encapsidate numerous cellular mRNAs that can be delivered and translated into corresponding proteins and in turn profoundly affect gene expression in target cells (Deregibus et al., 2007; Ratajczak et al., 2006a; Skog et al., 2008; Valadi et al., 2007). To investigate the possibility that H9 exosomes incorporate mRNAs coding for A3 proteins, total RNA was isolated from H9 and SupT1 cells and exosomes and analyzed for the presence of A3 specific mRNAs using real-time RT-PCR. Expression of A3 mRNAs was normalized against GAPDH mRNA. Since the expression of A3C mRNA was the highest among tested A3 mRNAs in H9 cells and exosomes, it was set as 1 (100%) and the levels of other A3 mRNAs were expressed relative to the level of A3C mRNA. H9 cells expressed A3G and A3F mRNAs at a level of about 48% and 27%, respectively, of A3C mRNA (Fig. 3A). In contrast, SupT1 cells expressed significantly lower levels of A3 mRNAs with the highest levels of A3C, A3DE and A3F mRNAs ranging between 7 and 10% of H9 A3C mRNA level. In SupT1 exosomes, A3 mRNAs were almost undetectable with the exception of A3F mRNA (Fig. 3B). The very low levels of A3 mRNAs in SupT1 exosomes is in keeping with the low expression of these proteins in these cells. H9 exosomes, on the other hand, were enriched in A3G and A3F mRNAs in addition to A3C mRNA, with A3F mRNA expressed at 38% and A3G expressed at 97% of A3C mRNA level in H9 exosomes. To investigate whether exosomal A3G mRNA is functional, we performed an *in vitro* translation assay using polyA<sup>+</sup> mRNA isolated directly from H9 exosomes (Fig. 3C). Using rabbit anti-A3G antibodies, we detected a protein product around 43 kDa that corresponds to A3G. Low intensity bands at around 37 kDa and stronger bands around 20 kDa were detected in all *in vitro* reactions even in the absence of added mRNA, suggesting that they represent non-specific proteins. In conclusion, H9 exosomes encapsidate several A3 mRNAs and some of them can be translated *in vitro* into proteins. In contrast, exosomes secreted by SupT1 cells were generally depleted of A3 mRNAs.

### A3G is a major determinant of restriction of Alu retrotransposition by exosomes

Recent studies have shown that A3G strongly restricts Alu retrotransposition, possibly by sequestering Alu RNAs in cytoplasmic high molecular mass (HMM) ribonucleoprotein complexes thus preventing access of Alu RNAs to the nuclear L1 machinery (Bogerd et al., 2006b; Chiu et al., 2006; Gallois-Montbrun et al., 2008; Hulme et al., 2007). To examine the ability of exosomes to inhibit retrotransposition of Alu in human cells, we performed an L1-dependent Alu retrotransposition assay using the *neo*<sup>Tet</sup> marked Alu reporter construct as described (Dewannieux, Esnault, and Heidmann, 2003). HeLa cells were cotransfected with an Alu reporter and L1 expression vector p220.CMV-L1 (Esnault et al., 2006) and treated for 48 h with exosomes of different cell origins. The appearance of neomycin-resistant colonies provided a direct measure of successful Alu retrotransposition. The colonies were stained with crystal violet and counted two weeks later. To ensure measurement of L1-dependent Alu transposition, we included negative controls by transfecting HeLa cells with Alu and a GFP expression vector or transfecting the cells only with either the *neo*<sup>Tet</sup> marked Alu reporter or with L1 vector. No retrotransposition events were detected in negative controls (Fig. 4).

Consistent with previous observations (Bogerd et al., 2006b; Chiu et al., 2006; Hulme et al., 2007), expression of A3G from transfected vector inhibited over 98% of Alu retrotransposition events. H9 exosomes were almost equally potent and inhibited on average 92–96% of Alu retrotranspositions. To investigate the contribution of A3G to the observed Alu inhibition by H9 exosomes, we first tried down-regulating the expression of A3G in H9 cells by using short interfering RNA. However this approach did not substantially reduce A3G protein expression in either H9 cells or in exosomes (Khatua et al., 2009), and thus did not appreciably reduce the inhibitory effect previously seen on Alu transposition (not shown).

To overcome this problem, we transfected A3G-negative SupT1 cells with GFP-A3G or GFP expression vectors and collected exosomes to be used in retrotransposition assays. Exosomes secreted by SupT1 cells transfected with GFP-A3G (SupT1/A3G exo) inhibited Alu retrotransposition by 68%, while exosomes derived from GFP-transfected cells (not shown) or parental A3G-negative SupT1 cells inhibit Alu retrotransposition only marginally (Fig. 4). Similarly, exosomes isolated from peripheral blood plasma samples of 2 different healthy donors showed no inhibitory effect on Alu retrotransposition. In contrast, exosomes secreted by mature M-DC that expressed A3G (Fig. 1C) inhibited Alu transposition by 55%. Since exosomes isolated from blood plasma (Fig. 1C) and secreted by SupT1 (Fig. 2) did not express detectable levels of A3G, these results suggest that the inhibitory effect of exosomes against Alu retrotransposition are affected, at least in part, by the presence of encapsidated A3G.

## Discussion

Retrotransposons constitute about 45% of the human genome. To protect genome stability, eukaryotic cells have developed several strategies to restrict proliferation of transposable elements. One of the strategies adopted by cells is silencing the mobilization of retroelements by cytidine deaminases belonging to the family of A3 proteins. Human A3 proteins show some preferences with regard to the inhibition of non-LTR transposons, L1 or Alu. For instance, A3A and A3B inhibit both L1 and Alu retrotransposition, A3C and A3F mostly inhibit L1 (Bogerd et al., 2006b; Chen et al., 2006; Muckenfuss et al., 2006; Stenglein and Harris, 2006), while A3G preferentially inhibits Alu mobilization, with a marginal effect on L1 (Bogerd et al., 2006b; Chiu et al., 2006; Hulme et al., 2007). However, a stronger A3G mediated inhibitory activity on L1 retrotransposition has also been reported (Kinomoto et al., 2007; Niewiadomska et al., 2007). Recently, an inhibitory activity against Alu retrotransposition has been attributed to A3H and A3DE (Tan et al., 2009) as well as A3F (Gallois-Montbrun et al., 2008). Since cells may express suboptimal levels or different forms of A3 proteins, it is likely that the supply of specific A3 proteins in exosomes may influence the extent and specificity of anti-retroelement activity in the recipient cell. Indeed, we have shown that A3G transmitted in exosomes and functionally expressed in HIV-1 susceptible cells strongly inhibits replication of HIV-1 in exosome-receiving cells (Khatua et al., 2009).

Exosomes are small membrane vesicles released by various types of cells, and carry molecular imprints of cells from which they were secreted. Exosomes originate from intraluminal vesicles of multivesicular bodies (MVBs) that form during the inward budding of limiting membranes of endosomes. Fusion of MVBs with the plasma membrane releases exosomes into intercellular space where they are believed to play a role in intercellular communication by transferring biologically active molecules to recipient cells. Detection of mRNA and microRNA in exosomes has led to the proposition that exosomes are also involved in exchange of genetic information between cells (Valadi et al., 2007).

In this study, we tested the hypothesis that exosomes secreted by cells expressing A3G and A3F restrict mobilization of L1 and Alu retroelements in target cells. Using an *in vitro* assay to quantify the frequency of L1 and Alu retrotransposition, we have confirmed that transiently overexpressed A3 proteins inhibit these non-LTR retroelements to different extents, with A3A, A3B and A3F restricting L1, and A3G strongly inhibiting Alu retrotransposition. Somewhat surprisingly, H9 exosomes that were originally found to encapsidate A3G and inhibited HIV-1 replication (Khatua et al., 2009) had a strong inhibitory activity against L1. Since A3G only weakly reduced L1 activity in our assays, this suggests that H9 exosomes encapsidate other A3 protein(s) that are able to inhibit L1. Indeed, we have identified A3F in H9 exosomes suggesting that this cytidine deaminase, alone or in combination with A3G or other restriction factors, is responsible for the observed inhibitory effect on L1 retrotransposition (Muckenfuss et al., 2006). Therefore, the moderate activity of exosomes secreted by mature M-DC may be

explained by the lower levels of A3F detected in these exosomes. However, it is possible that other A3 proteins enhance the activity of H9 exosomes against L1 retrotransposition. One such candidate could be A3C which modestly inhibits L1 (Bogerd et al., 2006b; Kinomoto et al., 2007; Muckenfuss et al., 2006). The possibility that exosomal mRNAs are translated in recipient cells after exosome internalization has been well documented (Deregibus et al., 2007; Ratajczak et al., 2006a; Skog et al., 2008; Valadi et al., 2007). We have found that H9 exosomes encapsidate mRNAs coding for A3C, A3F and A3G. Further analysis showed that A3G mRNA isolated from exosomes is functional and supports protein synthesis in an *in vitro* translation system. Based on these observations, we speculate that transfer of functional A3 proteins and corresponding mRNAs modulate or enable cells to resist invading or endogenous retroelements.

Alu retrotransposition was profoundly inhibited by H9 exosomes and moderately inhibited by M-DC exosomes. Since H9 exosomes encapsidated not only A3G but also A3F, we investigated whether A3G in exosomes inhibits Alu mobilization. A short interfering RNA (siRNA) approach to downregulate A3G in H9 cells was unsuccessful. However, introduction of A3G gene into A3G-negative CD4<sup>+</sup> SupT1 cells that expressed low levels of A3F showed that exosomes secreted by the cells reduced Alu retrotransposition by 68%. This result confirms that exosomal A3G inhibits Alu but does not rule out the possibility that additional factors (e.g. A3F), likely in combination with A3G, limit Alu activity. Blood-derived microvesicles were negative for A3G and one preparation (donor 2) contained small amounts of A3F. However, all tested blood-derived microvesicles (3 different donors) did not show any effect on L1 or Alu retrotransposition.

The mechanism of exosome entry and delivery of its cargo into the cell cytoplasm without degradation in the lysosomal compartment is unknown. However, the observation that some cellular receptors (Mack et al., 2000), transcription factors (Ratajczak et al., 2006a) or mRNAs (Deregibus et al., 2007; Ratajczak et al., 2006a; Valadi et al., 2007) transferred by microvesicles or exosomes remain functional in recipient cells, supports the notion that molecules delivered by exosomes escape degradation.

Another unanswered question is how A3 proteins are packaged into exosomes. One possibility is that cytoplasmic molecules may be engulfed during the inward budding of limiting membranes of MVBs. However, this non-specific mechanism does not explain specific enrichment of some molecules (e.g. A3F or A3G mRNA) in exosomes. The ability of A3 proteins to interact with certain mRNAs and small noncoding RNAs (Chiu et al., 2006; Gallois-Montbrun et al., 2008; Khan et al., 2007; Kozak et al., 2006; Wang et al., 2007b; Wang et al., 2008b) suggests that A3 proteins may localize into exosomes indirectly by interaction with cellular RNAs. Although packaging of A3G into HIV-1 particles is mediated by HIV-1 Gag and cellular RNAs (Alce and Popik, 2004; Cen et al., 2004; Douaisi et al., 2004; Luo et al., 2004; Navarro et al., 2005; Schafer, Bogerd, and Cullen, 2004; Svarovskaia et al., 2004; Zennou et al., 2004), packaging into exosomes obviously does not require HIV-1 Gag. Our findings that exosomes encapsidate large quantities of 7SL RNA (not shown) together with previous observations that A3G interacts with 7SL RNA (Bach et al., 2008; Wang et al., 2007b; Wang et al., 2008c), suggests that 7SL RNA may mediate A3G encapsidation into exosomes in a Gag-independent manner. Since A3G is able to interact with diverse retrotransposon Gag proteins (Bogerd et al., 2006a; Dutko et al., 2005), we cannot dismiss the possibility that A3G interacts with endogenous retroviral Gag and cellular RNA (including 7SL RNA) complexes to gain access to exosomes. The mechanism by which other A3 proteins gain access into exosomes is presently unknown.

The biological significance of exosomes bearing encapsidated A3 proteins and respective mRNAs is unknown. One possibility is that encapsidation of A3 in exosomes may serve to



dispose of excess cytidine deaminases that might otherwise lead to genomic instability (Anant and Davidson, 2003; Pham, Bransteitter, and Goodman, 2005). However, we speculate that exosomes with encapsidated A3 proteins may serve to fine tune the response against transiently expressed retroelements in germ cells or during the early stages of human embryogenesis (Garcia-Perez et al., 2007; Kazazian, 2004; Sawyer, Emerman, and Malik, 2004). In addition, exosomes with encapsidated A3 proteins may control retrotransposition which may occasionally occur in somatic cells (Kubo et al., 2006; Schulz, 2006).

In conclusion, the findings reported here show for the first time that exosomes packaging A3G and A3F potently restrict mobilization of L1 and Alu in recipient cells *in vitro*. Whether exosomes contribute to restriction of mobilization of retroelements *in vivo* will require further investigation.

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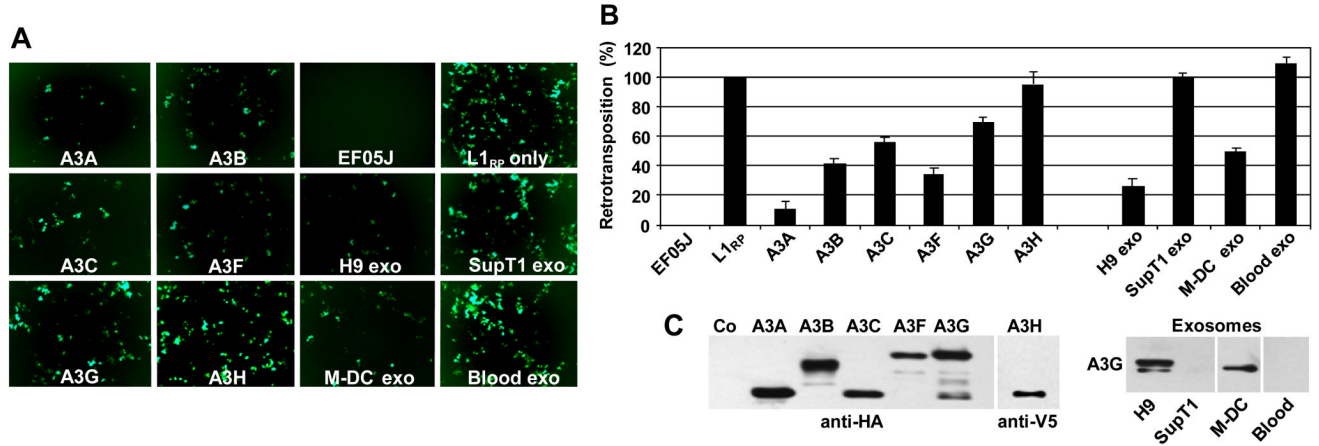
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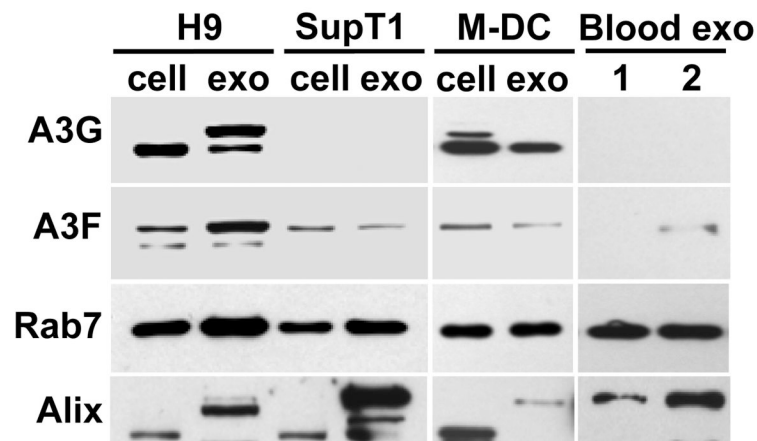
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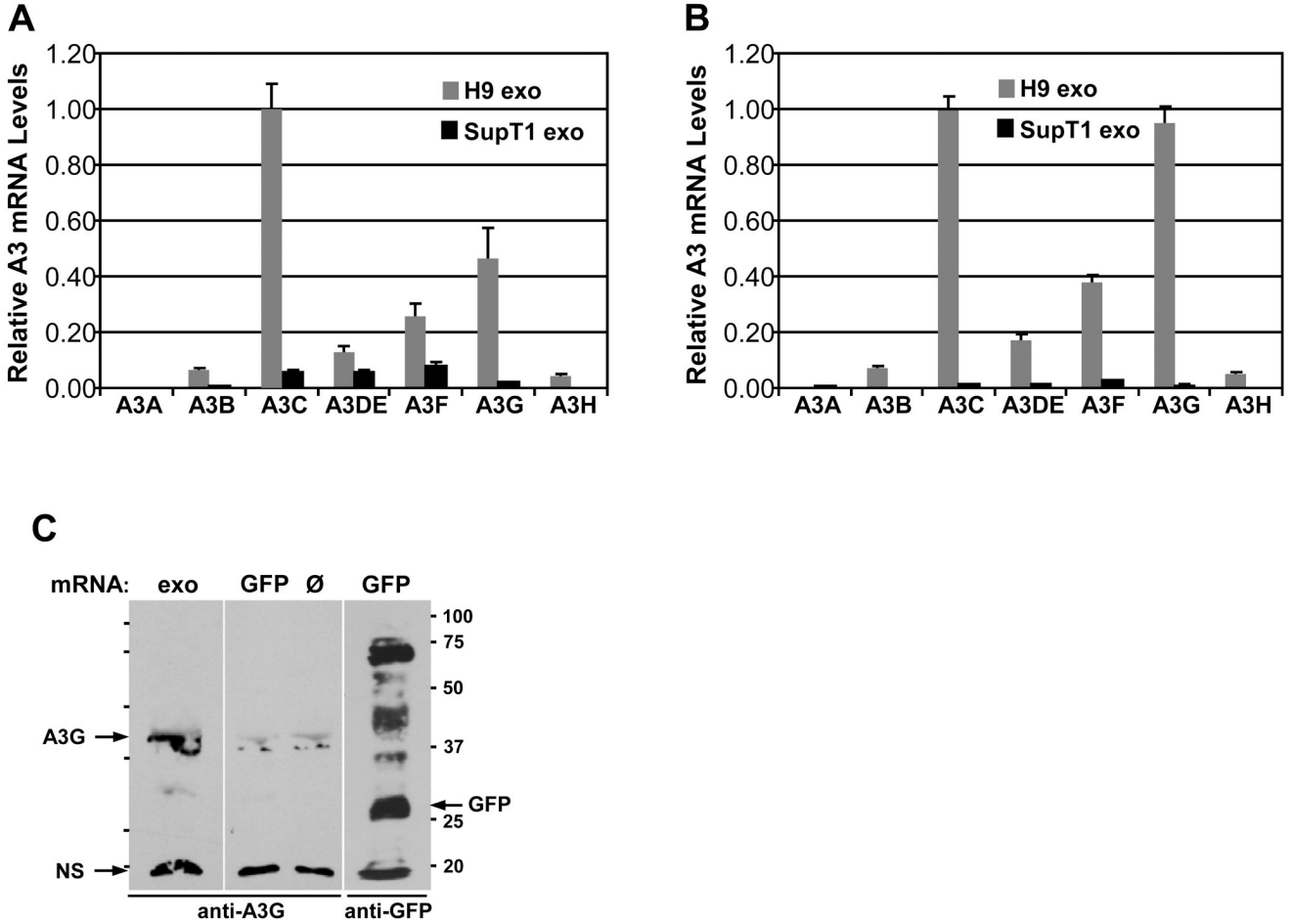
**Fig. 1. Effect of A3 proteins and exosomes on L1 retrotransposition**

293T cells were transfected with a pL1<sub>RP</sub> tagged with an EGFP retrotransposition indicator cassette and HA- or V5-tagged A3 expression plasmids. Cells transfected with pL1<sub>RP</sub>-EGFP only were exposed to exosomes (20 µg/ml) secreted by H9, SupT1, M-DC, or isolated from peripheral blood plasma (Blood exo). (A) After 5 days, cells were observed by fluorescence microscopy and (B) trypsinized, fixed with 3% paraformaldehyde and subjected to flow cytometry analysis. The retrotransposition level in cells transfected with pL1<sub>RP</sub>-EGFP (in the absence of A3 proteins or exosome treatment) was set as 100%. EF05J = negative control with inactive pL1<sub>RP</sub>-EGFP. Error bars, mean values ± SDs of triplicate samples. Similar results were obtained in 4 independent experiments. (C) Expression of A3G proteins in transfected cells (left panel) or in exosomes (right panel) was analyzed by SDS-PAGE followed by immunoblotting with anti-HA or anti-V5. A3G in exosomes was detected with anti-A3G antibodies.



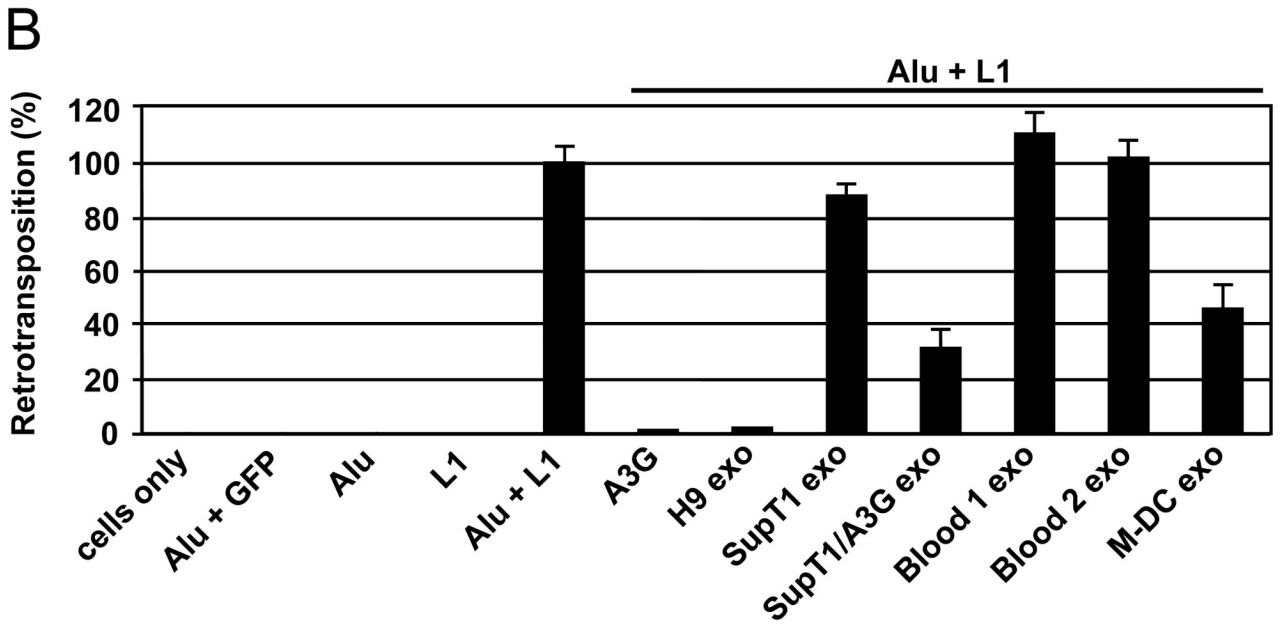
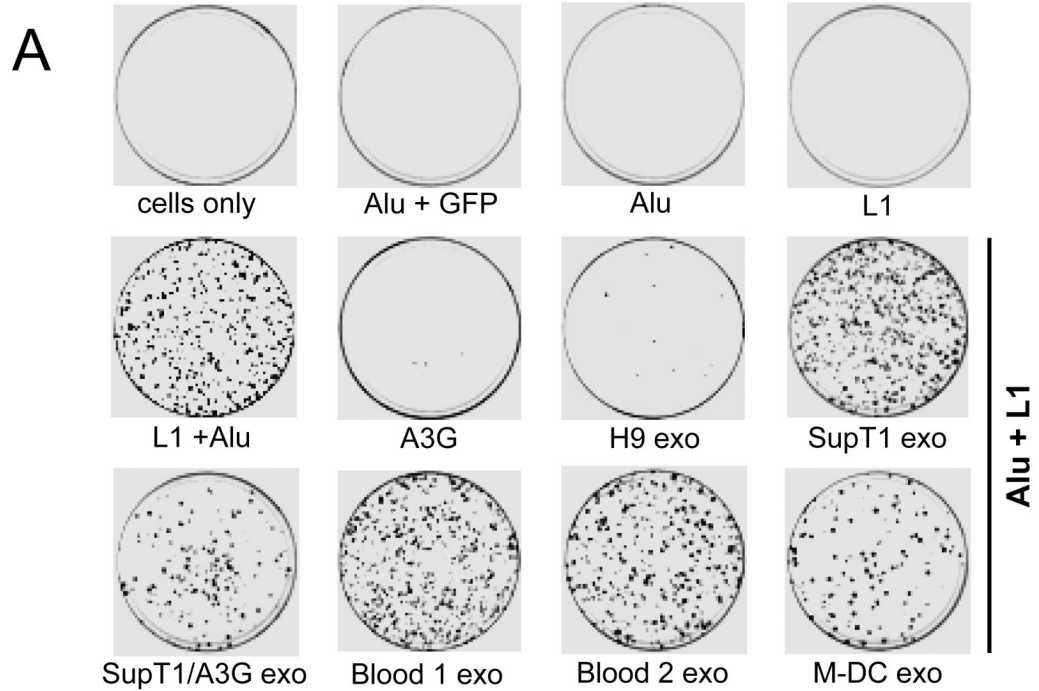
**Fig. 2. Expression of A3G and A3F in exosomes**

Exosomes were purified from cell culture supernatants or from blood plasma of 2 different healthy blood donors. Cell and exosome lysates (20 and 10  $\mu$ g/lane, respectively) were separated by SDS-PAGE and analyzed by Western blotting for the presence of A3G, A3F, and exosome markers Rab7 and Alix. In addition to the 70–75 kDa form of Alix detected predominantly in cells, higher molecular forms of Alix (95–105 kDa) were detected, particularly in exosomes. A slower migrating form of A3G was specifically detected in H9 exosomes. Note that A3F was detected in exosomes purified from blood plasma of donor 2 but not donor 1.



**Fig. 3. Expression of A3 mRNAs in CD4<sup>+</sup> T cells and exosomes**

Total RNA was extracted from H9 and SupT1 cells and from exosomes secreted by these cells and subjected to real-time RT-PCR. Levels of A3 mRNAs in cells (A) and in exosomes (B) were normalized against GAPDH mRNA levels and were expressed relative to A3C mRNA levels that were set as 1.0 in both H9 cells and H9 exosomes. Error bars, mean values  $\pm$  SD of triplicate samples. (C) A3G mRNA present in H9 exosomes is functional. PolyA<sup>+</sup> mRNA (1  $\mu$ g) isolated from H9 exosomes (exo) was subjected to an *in vitro* translation using a human *in vitro* protein expression assay. Samples without RNA ( $\emptyset$ , negative control) or containing GFP mRNA (positive control) were included. Translation reaction products were separated by SDS-PAGE and analyzed by Western blotting for the presence of A3G and GFP. Molecular weight markers (in kDa) are shown. NS, non-specific protein.



**Fig. 4. Effect of exosomes on L1-mediated Alu retrotransposition**

HeLa cells were cotransfected with an Alu construct tagged with the neo<sup>Tet</sup> retrotransposition indicator cassette and an L1 construct (Alu + L1), with or without an A3G expression vector. Cells transfected with Alu + L1 were exposed with different exosomes (20 µg/ml). Three days post-transfection, cells were subjected to selection in G418 for 14 days and resistant colonies were fixed, stained with crystal violet (A) and counted (B). As negative controls, cells were mock transfected (cells only) or transfected with L1 alone, Alu neo<sup>Tet</sup> alone (Alu) or together with irrelevant GFP gene (Alu + GFP). Retrotransposition efficiency was expressed relative to the positive control (Alu + L1), which was set as 100%. Values represent the means ± SD of triplicate samples. Similar results were obtained in 4 independent experiments.