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Movements of HIV-1 Genomic RNA-APOBEC3F Complexes and PKR Reveal Cytoplasmic and Nuclear PKR Defenses and HIV-1 Evasion Strategies

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Abstract

APOBEC3 cytidine deaminases and viral genomic RNA (gRNA) occur in virions, polysomes, and cytoplasmic granules, but have not been tracked together. Moreover, gRNA traffic is important, but the factors that move it into granules are unknown. Using *in situ* hybridization of transfected cells and protein synthesis inhibitors that drive mRNAs between locales, we observed APOBEC3F cotrafficking with gRNA without altering its movements. Whereas cells with little cytoplasmic gRNA were translationally active and accumulated Gag, suprathreshold amounts induced autophosphorylation of the cytoplasmic double-stranded RNA (dsRNA)-dependent protein kinase (PKR), causing eIF2 α phosphorylation, protein synthesis suppression, and gRNA sequestration in stress granules. Additionally, we confirmed recent evidence that PKR is activated by chromosome-associated cellular dsRNAs after nuclear membranes disperse in prophase. By arresting cells in G2, HIV-1 blocks this mechanism for PKR activation and eIF2 α phosphorylation. However, cytopathic membrane damage in CD4- and coreceptor-positive cultures infected with laboratory-adapted fusogenic HIV-1_{LAI} eventually enabled PKR entry and activation in interphase nuclei. These results reveal multiple stages in the PKR-HIV-1 battleground that culminate in cell death. We discuss evidence suggesting that HIV-1s evolve *in vivo* to prevent or delay PKR activation by all these mechanisms.

Keywords

HIV-1; PKR; APOBEC3F; stress granules; P-bodies; nuclear pores; mitosis

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1. Introduction

The anti-HIV-1 cytidine deaminases APOBEC3F (A3F) and APOBEC3G (A3G), which invade assembling virions and lethally hypermutate reverse transcripts, occur in the cytoplasm associated with large ribonucleoprotein(s) (RNPs) as well as in a low M_r form (Alce and Popik, 2004; Cen et al., 2004; Chable-Bessia et al., 2009; Chiu et al., 2006; Gallois-Montbrun et al., 2008; Izumi et al., 2013; Khan et al., 2007; Kozak et al., 2006; Marin et al., 2008; Nathans et al., 2009; Phalora et al., 2012; Wichroski et al., 2006). Immunocytochemical analyses indicate that A3F and A3G partially colocalize with mRNAs in P-bodies (PBs) and stress granules (SGs) (Gallois-Montbrun et al., 2008; Kozak et al., 2006; Marin et al., 2008; Phalora et al., 2012; Wichroski et al., 2006). Although PBs and SGs share many components and interact closely, they also have distinct features (Kedersha et al., 2005). PBs occur in normal proliferating cells and are involved in mRNA turnover. In contrast, SGs efficiently sequester Cap-dependent mRNAs released from polysomes in stressed cells that become dormant and proapoptotic. Analyses of tandem affinity purified A3F and A3G complexes by mass spectrometry identified many mRNA associated proteins including ribosomal proteins and Staufen1 that could be released by RNase A (Chiu et al., 2006; Gallois-Montbrun et al., 2008; Kozak et al., 2006). Using these methods, we reported that the high M_r RNPs associated with A3G and A3F are predominantly polysomes, and that polysomal APOBEC3s are bound to mRNAs (Kozak et al., 2006; Marin et al., 2008). A3G cosediments in sucrose gradients with polysomes but not with single ribosomes that lack mRNA. Protein synthesis inhibitors that release mRNA from polysomes cause A3G release, whereas cycloheximide (CHX), which slows translational elongation and drives mRNA from storage granules into polysomes, recruits A3G back into polysomes (Kozak et al., 2006). Messenger RNAs including unspliced HIV-1 genomic RNA (gRNA) and cognate APOBEC3 mRNAs coimmunoprecipitate with A3G and A3F from cell extracts (Gallois-Montbrun et al., 2008; Gallois-Montbrun et al., 2007; Kozak et al., 2006). Protein synthesis inhibitors including arsenite (Ars) that drive mRNAs into SGs also drive A3F and A3G into these granules (Gallois-Montbrun et al., 2007; Kozak et al., 2006; Marin et al., 2008). Puromycin causes A3G release from the high molecular mass RNP in cytoplasmic extracts (Goila-Gaur et al., 2008). In addition, A3G and A3F bind small retrotransposon-related 7SL, Y, and Alu RNAs (Apolonia et al., 2015; Bach et al., 2008; Chiu et al., 2006; Gallois-Montbrun et al., 2008; Khan et al., 2007; Wang et al., 2008). Thus, although they bind many RNAs, substantial proportions of A3G and A3F directly or indirectly associate with mRNAs that traffic between polysomes and mRNA granules.

These results implied that these cytidine deaminases might cotraffic with gRNA in single cells. Moreover, it seemed possible that cytoplasmic gRNA might alter APOBEC3 traffic or *vice-versa*, causing them to locate specifically at sites of virion assembly or inhibiting such localization. However, despite extensive information about synthesis, splicing, and nuclear export of HIV-1 RNAs, the factors that control translation and cytoplasmic movements of gRNA and its incorporation into assembling virions remain uncertain. Cytoplasmic clusters of gRNA have been observed but their identities and potential roles have not been unambiguously established. Some clusters, which have been detected in the nuclear periphery in association with centrioles, slowly move as “RNA transport granules” on

cytoskeletal proteins (Cochrane et al., 2006; Kemler et al., 2010; Kimura et al., 1996; Levesque et al., 2006; Poole et al., 2005). In addition, two groups observed cytoplasmic colocalization HIV-1 mRNA granules with PB components including DDX6 in transfected cells (Chable-Bessia et al., 2009; Nathans et al., 2009), while gRNA localization in PBs was not detected by others (Abrahamyan et al., 2010; Phalora et al., 2012). Although PBs normally hold mRNAs transiently while they are degraded or returned to polysomes (Kedersha et al., 2005; Parker and Sheth, 2007; St Johnston, 2005), these studies did not determine whether the gRNA associated with PB markers dynamically moves between locales (Chable-Bessia et al., 2009; Nathans et al., 2009). This is important because these putative PBs could be modified in infected cells for virus-specific or cellular defense purposes, consistent with RNA granules involved in replication of retrotransposons and several viruses (Ariumi et al., 2011; Beckham et al., 2007; Beckham and Parker, 2008; Beliakova-Bethell et al., 2006; Borghese and Michiels, 2011; Khong and Jan, 2011; Lu et al., 2011a).

Although the factors that control gRNA traffic are not fully understood, dimerization is a prerequisite for its assembly into virions, whereas monomeric gRNA is translationally competent (D'Souza and Summers, 2005; Lu et al., 2011b). Monomeric and dimeric gRNA also have extensive double-stranded folded regions (dsRNA) that bind proteins containing dsRNA binding domains (dsRBDs) including TRBP, ADAR1, PACT, HIV-1 encoded Tat, and the interferon inducible dsRNA-dependent protein kinase PKR (Bannwarth and Gagnon, 2005; Clemens et al., 1994; Clerzius et al., 2009; Clerzius et al., 2011; Clerzius et al., 2013; Ong et al., 2005; Sanghvi and Steel, 2011; Siegfried et al., 2014; Singh et al., 2011). PKR is potently activated *in vitro* by the dimerized TAR region of gRNA (Cai et al., 2000; Daher et al., 2009; Heinicke et al., 2009; Nallagatla et al., 2011). In contrast, PKR is inhibited in extracts from HIV-1 infected or transfected cultures, at least in part by these other proteins with dsRBDs (Brand et al., 1997; Cai et al., 2000; Clerzius et al., 2009; Clerzius et al., 2011; Clerzius et al., 2013; Daher et al., 2009; Endo-Munoz et al., 2005; Ong et al., 2005; Sanghvi and Steel, 2011; Singh et al., 2011).

To analyze the cytoplasmic movements of gRNA and A3F and to identify the factors including PKR that might force gRNA into granules, we used fluorescence *in situ* hybridization and immunofluorescence deconvolution microscopy. We used protein synthesis inhibitors to drive gRNA between locations and identified proteins in these diverse clusters. Because previous studies of gRNA or APOBEC3 localization employed transfected cultures in conditions that minimized cytopathology, we used these methods initially, with subsequent analyses of infected cells. Our results complement previous results by revealing cellular and subcellular diversities within the cultures and establishing that gRNA and A3F cotraffic. Cells with cytoplasmic gRNA concentrations above a low threshold activate PKR, resulting in phosphorylation of eIF2 α and its sequestration with gRNA into SGs. We also confirmed recent evidence that PKR is normally activated by chromosome associated cellular dsRNAs after nuclear membranes disperse during mitotic prophase (Kim et al., 2014). By arresting cells in G2, HIV-1 suppresses this potent mechanism for PKR activation and protein synthesis suppression. Nuclear membrane defects that eventually form in interphase cells infected with a highly cytopathic HIV-1 isolate also enable PKR entry and

activation. These results identify PKR movements into different lines of cellular defense and potential HIV-1 evasion strategies.

2. Materials and Methods

2.1. Cells and viruses

HEK293T cells (ATCC) were maintained according to the supplier specifications. The A3F-HA expression vector pA3F-HA was a generous gift from Dr. B. Cullen (Wiegand et al., 2004). Expression vectors for β -galactosidase (pCS2 β gal), enhanced GFP (pEGFP), and myoD (pMyoD-Flag) were generously donated by Dr. L. Musil (OHSU). The pHIV-*gpt* wild-type (wt) or a derivative with a *vif* deletion (Madani and Kabat, 1998, 2000; Page et al., 1990) were used to express HIV-1 genomic RNA (gRNA). Pseudotyped infectious pHIV-*gpt* virions were obtained from the media of cells cotransfected with the latter plasmid plus the HIV-1 envelope plasmid pJRCSF*env* (Platt et al., 2001) using PolyFect transfection reagent (Qiagen). Thirty-six to forty hours post-transfection the virion-containing media were collected, filtered (0.45 μ m pore), and serial dilutions were incubated for 1h at 37°C with HeLa-CD4/CCR5 JC.53 cultures that had been pretreated with Polybrene to enhance virus adsorption (Platt et al., 2001). Infected JC.53 cell colonies were selected for *gpt* expression with mycophenolic acid (Sigma) and counted (Page et al., 1990; Platt et al., 2001). Where indicated, cultures producing infectious pseudotyped HIV-*gpt* virions were treated for different times with inhibitors before harvesting the virus-containing media. In that case, control media or media with inhibitors were added to cultures at time zero and were replaced at hourly intervals for 4h. The virions in each hourly harvest were removed from inhibitors by precipitation with calcium phosphate (Pham et al., 2001). These media were adjusted to 100mM CaCl₂ and incubated at 37°C for 30 min followed by microcentrifugation at 13,000 rpm for 5 min. The supernatants were discarded and the viral pellets were resuspended in growth medium lacking inhibitors for infectivity assays. Replication-competent HIV-1_{LAI} (provided by the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: from Dr. Jean-Marie Bechet and Dr. Luc Montagnier) was amplified and was titered by the focal infectivity assay and used to infect HeLa-CD4 (clone HI-J) cells (Platt et al., 2001) and H9 leukemic T cells at multiplicities of infection (moi) 2.0 or 0.2. H9 leukemic T cells were from the NIH AIDS Reagent program, Division of AIDS, NIAID, NIH donated by Dr. Robert Gallo.

2.2. *In situ* hybridization

The single-strand DNA probe was prepared by asymmetric-PCR using Pol2687 Reverse primer 5'-TTTCCCTTCCTTTTCCATCTCTG-3', Gag2250 Forward primer, 5'-TTCCCTCAGGTCACCTCTTGGC-3', pHIV-*gpt*(wt) plasmid as template and PCR-DIG Probe Synthesis Kit (Roche Diagnostics). Initially, these primers were used to make a stock of double-stranded dU-labeled DNA using dUTP instead of dTTP, which was then used as the template for asymmetric-PCR using the reverse primer alone. The dU-labeled template was degraded with uracil DNA glycosylase (Invitrogen) and heat before final purification of the single-stranded DIG-labeled DNA probe.

RNA fluorescent *in situ* hybridization (FISH) analyses were performed on HEK293T cells at 36h post-transfection or on HeLa-CD4 cells (clone HI-J) or H9 T cells that had been infected 72h earlier with HIV-1_{LAI}. All steps were performed using RNase free solutions containing 2 mM vanadyl ribonucleoside. Cells were washed once with phosphate buffered saline (PBS) (Invitrogen) and fixed with 5% formaldehyde and 2% sucrose in PBS for 20 min at room temperature (RT). After three washes with PBS, cells were permeabilized with 0.25% Triton-X-100 in PBS for 10 min at RT. The cells were next incubated with denaturated (75°C for 10 min) hybridization solution (5X SSC, 1X Denhardt's, 100 µg/ml of Salmon testes DNA, 50% formamide and 5 mM DTT) for 30 min at 45°C. The single-stranded DIG-labeled DNA probe (200 ng probe in 0.2 ml hybridization solution/well) was denaturated at 75°C for 10 min, and immediately incubated on ice for 3 min. Cells and probe were incubated overnight at 50°C in a humid chamber. Cells were next washed twice, 5 min each, with 45% formamide - 2X SSC at 45°C, once with 2X SSC at 45°C for 15 min, and once with 2X SSC at RT for 10 min. Cells were incubated with sheep anti-Digoxigenin-Flourescein Fab fragments 1:200 (Roche Diagnostics) in 4X SSC +1% BSA for 2h at RT or, where indicated with the IgG fraction of mouse monoclonal anti-DIG 1:200 (Jackson Immuno Research) followed by Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes, Inc.).

2.3 Immunocytochemistry

HEK293T cells were cultured in Permanox chamber slides (Nalge Nunc International) that had been pretreated with Poly-L-lysine (0.1 mg/ml) for 20 min at 37°C. They were transfected using FuGene 6 transfection reagent (Roche). At 36 h post-transfection, the cells were fixed in 5% formaldehyde and 2% sucrose in phosphate buffered saline (PBS) (Invitrogen) at room temperature for 20 min and then permeabilized with 0.25% Triton X-100 in PBS for 10 min. For stress response experiments, prior to fixation, the cells were treated with 0.5 mM sodium meta-arsenite (Ars) (J. T. Baker) for 1 h at 37°C. Some cultures were treated with 0.1 mg/ml cycloheximide (CHX) (Sigma) for 20 min at 37°C prior to and during the 1-h Ars treatment, while other cultures were treated for 1h with CHX alone prior to fixation. Where indicated, cultures were treated with 1-2µM of the PKR imidazole-oxindole inhibitor C16 (Sigma) for the times noted. Untreated cultures were used as negative controls. Primary antibodies were anti-HA monoclonal antibody HA-7 (1:1,000), anti-HA polyclonal antibody H6908 (1:1,000) (Sigma), mouse anti-PABP1 antibody 10E10 (1:300) (Abcam Inc.), rabbit anti-DDX6 (Rck/p54) (1:100) (Bethyl Laboratories), rabbit polyclonal antibody to EDC4 (1:100) (Abcam) and mouse anti-p24 Hybridoma 183 supernatant (1:2) (NIH AIDS Research and Reference Reagent Program, donated by Bruce Chesebro). Nucleoli were detected using mouse mAb to nucleolin [4E2] (Abcam). We also used the phospho-specific antibodies rabbit anti-phospho-eIF2α (1:100) (Sigma) and rabbit anti-phospho-PKR (1:200) (Sigma) and rabbit antibodies specific for the unphosphorylated proteins PKR (1:200) (Sigma) and eIF2α (1:100) (Sigma). Staining with anti-PABP1 was performed overnight at 4°C. Incubations with all other primary antibodies were performed for 2h at room temperature. Secondary fluorescent antibodies were Alexa Fluor 488 goat anti-rabbit immunoglobulin G (heavy plus light chains) [IgG(H+L)], Alexa Fluor 594 goat anti-mouse IgG(H+L), Alexa Fluor 350 goat anti-rabbit IgG(H+L) (1:500) (Molecular Probes Inc.). All secondary antibodies bound in a species-specific manner to appropriate

primary antibodies and were used in controlled conditions that excluded cross-reactivity. Secondary antibodies were used at a dilution of 1:1,000 unless otherwise indicated, and incubations were performed for 1 h at room temperature. For FISH/immunocytochemistry co-analyses the FISH was performed first as described above. Slides were mounted in FluoroGuard antifade reagent (Bio-Rad Laboratories) and observed with a Zeiss Axiovert 200M deconvolution microscope equipped with Chroma filters 41,001 (excitation 480 nm, emission 535 nm) and 41,004 (excitation 560, emission 645 nm). Stacks of images were acquired using a 40x objective lens with an AxioCam MRm Zeiss camera, and were analyzed using AxioVision Zeiss software. Relative fluorescent intensities of Gag were quantified using the Automatic Measurement Program provided by AxioVision Release 4.8.2 (Carl Zeiss Microscopy). The above procedures were also used to analyze HeLa-CD4 cells (clone HI-J) that had been infected at multiplicities of infection (moi) 2.0 or 0.20 with HIV-1_{LAI} for 72h. Consistent with random viral entry, at the higher moi, approximately 86% of the cells became infected as indicated by Gag staining ($\text{moi} = -\log P_0/0.44$, where P_0 is the fraction of uninfected cells) (Kozak and Kabat, 1990).

2.4. Protein analyses

Postnuclear extracts of HEK293T cells cotransfected with pHIV-*gpt* and pJRCSF_{env} were prepared in RIPA buffer (50 mM Tris-Cl, 1% Nonidet P40, 0.1% sodium deoxycholate and 150 mM NaCl, pH7.4) with complete protease inhibitors (Roche) followed by centrifugation at 1,500 g for 5 min. These extracts were adjusted to equivalent protein concentrations using Bradford reagent (BioRad Laboratories), and equal amounts were analyzed. For assays using polyacrylamide gel electrophoresis, Western immunoblotting with antiserum for α -tubulin (Sigma) was used for the equal loading control. Cotransfected cultures were metabolically labeled using Tran ³⁵S label (MP Biomedicals) diluted with unlabeled L-cysteine and L-methionine to a final specific activity of 25 Ci/mmol in the absence of inhibitors or in the presence of Act D (1 μ g/ml), Ars (100 μ M), puromycin (25 μ g/ml) or CHX (100 μ g/ml) for various times (0.5, 1, 2, and 4h total) before harvesting. The labeled cultures were washed with cold PBS and stored frozen at -80 C. The frozen cultures were later lysed in RIPA, normalized for protein concentration and aliquots were used for trichloroacetic acid (TCA) precipitation of the proteins, following a protocol from a Membrane Protein Expression Kit (Invitrogen). Briefly, duplicate GF/C filters (Milipore) were spotted with 25 μ l of the cell lysates, allowed to dry, batch washed in cold 10% TCA for 10 min, washed twice with 5% trichloroacetic acid for 5 min, and rinsed in methanol to facilitate drying. The filters were then placed in a vial with scintillation fluid and counted in a scintillation counter. In addition, the labeled cell lysates were used for immunoprecipitation using anti-p24 #6521 (NIH AIDS Research and Reference Reagent Program, HIV-1 p24 Gag Monoclonal (#24-4) from Dr. Michael H. Malim.) and Protein A-Sepharose beads (Sigma), electrophoresed in SDS-polyacrylamide gels, transferred to MagnaGraph nylon membranes (0.45 micron) (GE Water & Process Technologies), and exposed to film for autoradiography.

2.5. Statistical analyses

Categories of granules and cell types were counted in multiple photographed images of cell fields in N independent assays and the relevant mean percentage values $\pm$ SEMs are shown, with N values in legends. Radioactivity measurements are averages $\pm$ ranges of duplicate

assays. Virus titers are means of duplicate assays done using multiple serial dilutions. The effect of HIV-1 on mitotic progression was analyzed using the binomial equation..

3. Results and Discussion

3.1. Unspliced HIV-1 genomic RNA moves between polysomes and SGs

We analyzed the intracellular localizations of HIV-1 gRNA in transiently transfected HEK293T cultures by *in situ* hybridization using a DIG-labeled antisense ssDNA probe for *gag-pol* sequences that are absent in subgenomic HIV-1 mRNAs. By 36h post-transfection, approximately 30-50% of the cells contained cytoplasmic gRNA and its Gag translation product. However, cytoplasmic gRNA levels and localizations were highly diverse, being substantially dispersed in cells that had small amounts and clustered into large granules in cells with greater amounts. Although approximately 80% of the cytoplasmic gRNA granules colocalized with DDX6 (Fig 1A and B), they were generally larger than the DDX6-positive PBs that occurred in cells lacking gRNA and in control cultures transfected with an empty pcDNA3.1 vector.

To analyze the functional dynamics of these gRNA clusters, we treated the cultures with CHX, which inhibits translational elongation and causes mRNAs to emigrate from disintegrating PBs and SGs and to accumulate in polysomes (Kedersha and Anderson, 2002; Kedersha et al., 2000; Kozak et al., 2006; Marin et al., 2008; Parker and Sheth, 2007). CHX dispersed all cytoplasmic gRNA clusters and DDX6-containing cytoplasmic granules (Fig 1A), indicating that this clustered gRNA can be rapidly recruited into polysomes.

In conditions of stress typified by Ars treatment, 5'-7mG cap-dependent mRNA translation is blocked at the initiation step, and the resulting ribosome-free mRNAs accumulate in SGs that contain PABP but lack DDX6 (Kedersha and Anderson, 2002; Kedersha et al., 2000; Kozak et al., 2006; Novoa et al., 2003; Parker and Sheth, 2007). Accordingly, Ars caused the rapid separation of DDX6-containing clusters from gRNA granules (Fig 1A, third row). Moreover, the resulting DDX6-positive granules were substantially smaller than the residual gRNA clusters. Consistent with these mechanistic interpretations, CHX overrides the Ars-dependent breakdown of polysomes and formation of SGs, thereby eliminating these gRNA clusters and the small DDX6-containing granules (Fig 1A, lower row). These results suggest that a substantial cytoplasmic pool of translationally competent gRNA dynamically moves between polysomes and granules that have diverse compositions.

Many PB components including DDX6 have been reported in SGs in some stress conditions (Kedersha et al., 2005). To determine whether the gRNA granules in transfected cells might be DDX6-positive SGs rather than PBs, we analyzed their colocalization with the specific SG component PABP. Previous evidence suggests that PABPs bind to poly(A)-containing mRNAs and to initiation factors bound to mRNA 5'-7mG cap sites, thereby causing mRNA circularization and activating mRNA translation by the ribosome scanning mechanism (Derry et al., 2006; Gallie, 1998; Martineau et al., 2008). In contrast, removal of PABP and deadenylation of mRNAs (resulting in linearization of the mRNAs) is a prerequisite for mRNA transfer into PBs (Eulalio et al., 2007; Zheng et al., 2008). As shown by the representative field in Fig 1C (top row), the cytoplasmic gRNA clusters in transfected cells

all colocalized with PABP, whereas PABP was dispersed in cells in the same cultures that lacked clustered gRNA. As expected, Ars caused SG formation as indicated by clustering of PABP in all cells, including cells lacking gRNA. CHX overrode Ars and eliminated all the gRNA and PABP clusters (Fig 1C). Similarly, transfected HeLa cells with abundant cytoplasmic gRNA formed SGs that contained gRNA, DDX6, and PABP, indicating that DDX6-positive SG formation does not depend on presence of SV40 T antigens (Fig 1D). SGs also formed in other cells that had abundant gRNA (results not shown).

For biosafety reasons, most of our experiments used pHIV-*gpt*, which lacks an *env* gene and is replication defective. However, abundant gRNA-positive SGs were also induced by transfecting plasmids encoding full length HIV-1s including HIV-1_{NL4-3}, HIV-1_{IIIB}, and the distantly related Zaire clade D isolate HIV-1_{ELI} (Fig 2). Thus, gRNA-containing SGs were induced independently of *gpt* by all tested plasmids that encode full length infectious HIV-1s of different clades. In contrast, cells transfected with the pcDNA3.1 vector (see below) or vectors encoding other highly expressed proteins (MyoD, EGF-P, and β -Galactosidase) lacked SGs (results not shown).

These results suggest that the large cytoplasmic gRNA granules in transfected cells are SGs that contain DDX6, in contrast to SGs induced by Ars. Similarly, these gRNA granules contain EDC4, an enhancer of decapping that also occurs in PBs (Fig 3A) but not in Ars-induced SGs (see below).

Downmodulation of DDX6 is widely used to disperse PBs, consistent with other evidence that it is essential for their assembly (Chu and Rana, 2006; Ernoult-Lange et al., 2012; Eulalio et al., 2007; Izumi et al., 2013; Nathans et al., 2009; Serman et al., 2007). In addition, DDX6 binds extensively onto mRNAs and has been reported to chaperone gRNA-Gag associations (Ernoult-Lange et al., 2012; Reed et al., 2012; Robinson et al., 2014; Yu et al., 2011). Moreover, EDC4 functions as a binding platform that recruits important PB proteins including the decapping enzymes Dcp1 and 2, thereby enhancing their activities (Chang et al., 2014). Consequently, the abundant recruitment of DDX6 and EDC4 into gRNA-containing SGs implied that PBs in these cells might lose important components and disperse. Accordingly, cells lacking gRNA contained small DDX6 and EDC4 presumptive PBs, whereas these small granules were relatively rare or difficult to discern in cells that contained gRNA-positive SGs (e.g., see Fig 3A). Moreover, cells without SGs with gRNA dispersed throughout their cytoplasm also appeared to have fewer PBs than adjacent cells (e.g., see Fig 3B). To examine this quantitatively, we scanned through sectioned deconvoluted images of many cells and added the EDC4-positive small granules that were resolved in all sections. As summarized in Fig 3C, the total numbers of these small granules were reduced in cells that contained gRNA. These results support the hypothesis that DDX6 binding to gRNA destabilizes PBs, consistent with previous evidence for PB dispersal in cells containing HIV-1 (Abrahamyan et al., 2010).

A corollary of the above results is that DDX6 must separate from gRNA-containing SGs when cultures are treated with Ars (see Fig 1). Accordingly, many cells in Ars-treated cultures contained gRNA granules closely paired with nearby DDX6 foci that appeared to be emergent (e.g., see Fig 3D). Our impression is that the newly emergent DDX6 clusters

gradually fragment to form smaller PBs. These results are consistent with evidence for close interactions of SGs and PBs (Anderson and Kedersha, 2008, 2009; Chahar et al., 2013; Kedersha and Anderson, 2007; Kedersha et al., 2005; Wilczynska et al., 2005) and for dispersal of PBs by several viruses (Ariumi et al., 2011; Chahar et al., 2013; Garaigorta et al., 2012; Khong and Jan, 2011; Pager et al., 2013; Piotrowska et al., 2010; Simpson-Holley et al., 2011).

3.2. APOBEC3F and gRNA colocalize and move between polysomes and SGs depending on metabolic conditions

To learn how A3F and gRNA might influence each other, we investigated their localizations within single cells. We used A3F because it is substantially resistant to Vif in the conditions of our experiments, which enabled us to use the same wild-type HIV-*gpt* plasmid for all our analyses. As shown in Fig 4A (untreated control row), the large cytoplasmic gRNA granules in HIV-1-positive cells contained A3F and most but not all of these colocalized with DDX6, consistent with Fig 1B. Treatment with CHX eliminated PBs and A3F- and gRNA-positive granules, suggesting that the gRNA and A3F in these granules can be recruited into polysomes. We previously found that A3G and A3F move from DDX6-positive cytoplasmic granules and polysomes into SGs lacking DDX6 in response to Ars (Kozak et al., 2006; Marin et al., 2008). Similarly, Ars caused DDX6 to emigrate from SGs that contained both A3F and gRNA (Fig 4A). Consistent with results above, the gRNA- and A3F-positive granules also contain colocalized PABP, suggesting that they are SGs (Fig 4B). A3F, gRNA, and PABP also remained together when the cells were treated with Ars. Consistent with data above, CHX overrode Ars and eliminated gRNA-A3F-PABP granules. Considered together with Figs 1 and 3, these results suggest that a substantial proportion of A3F is associated with PABP-containing gRNA and/or cellular mRNAs that move between SGs and polysomes, and that A3F does not noticeably redirect the cytoplasmic movements of gRNA.

3.3. Gag partially colocalizes with translationally suppressed gRNA in SGs

Fig 5A shows representative fields of cells in cultures transfected with pHIV*gpt* and subsequently used to localize gRNA and the p24 Gag antigen. Generally in the conditions of our assays, ~20-30% of the Gag-positive cells have gRNA-containing SGs. Moreover, as indicated in these representative fields, Gag substantially colocalizes with gRNA in these granules (Fig 5A). In contrast, as is also evident in these fields, Gag antigens were always dispersed in cells that contained trace amounts of unclustered cytoplasmic gRNA. Moreover, in agreement with additional evidence below, it is striking that cells with barely detectable trace amounts of dispersed cytoplasmic gRNA often contain much more p24 Gag antigen than adjacent cells with abundant clustered gRNA. For example in Fig 5A (lower row) quantitative analysis suggested that relative Gag amounts in the four cells with SGs were 9, 14, 12, and 5, whereas the cell lacking SGs in the upper right corner of the field had a relative Gag value of 78. Fig 5B shows a quantitative summary of Gag quantities in many cells that contain or lack gRNA-positive SGs. Although it is unsurprising that some cells with only traces of dispersed cytoplasmic gRNA also contain little Gag, many of these cells have large amounts. In contrast, cells with SGs lack detectable amounts of dispersed gRNA

and contain relatively little Gag. Consistent with evidence below, these results suggest that SGs may be induced by gRNA abundance rather than by its Gag translation product.

It is important to note that the above conclusions are based on cytoplasmic gRNA and Gag abundances rather than on inferences concerning gRNA translation. We emphasize this distinction because gRNA and Gag amounts are affected not only by synthesis but also by degradation and virion release from the cells. Nevertheless, the latter results (Fig 5A and B), the cytoplasmic movements of gRNA induced by CHX and Ars (Figs 1, 3, and 4), and previous analyses of SGs (Kedersha and Anderson, 2002, 2007) strongly suggest that gRNA in SGs is translationally silent whereas dispersed cytoplasmic gRNA is polysome-associated and translationally active. Evidence consistent with this interpretation was obtained by analyzing incorporation of L-[³⁵S] amino acids into total cellular proteins and into Gag polyproteins (Fig 5C). Whereas CHX and puromycin act directly on translating ribosomes and rapidly inhibited synthesis of all proteins, Ars acted more slowly and strongly inhibited total incorporation and Gag polyprotein synthesis after a lag of ~30-40 min, coincident with Ars-induced movement of mRNAs from polysomes into SGs (Kozak et al., 2006). Moreover, Ars and the other protein synthesis inhibitors all reduced infectious virion release from cultures cotransfected with pHIVgpt and the pJRCSFenv vector within several hours (Fig 5D), consistent with evidence that newly synthesized Gag polyproteins are required for assembly of infectious HIV-1 virions (Klein et al., 2007). In contrast, older Gag enters a more stagnant pool. Treatments with the transcription inhibitor actinomycin D for 4h did not significantly curtail synthesis of total cellular proteins or Gag (Fig 5C), suggesting that intracellular Gag mRNA was not rapidly turning over. Control studies demonstrated that actinomycin D was active in the conditions of these experiments (results not shown).

3.4. Roles of PKR in gRNA-dependent SG induction and in mitosis

Our hypothesis that cytoplasmic gRNA abundance rather than Gag induces SGs (Fig 5), is consistent with evidence that cells transfected with specific synthetic RNAs that cannot be translated form SGs by a PKR-dependent mechanism (Witteveldt et al., 2013), although other RNAs inhibit this kinase (Samuel, 1992). Moreover, monomeric and dimeric gRNA contain large dsRNA regions including the 5'TAR stem-loop that activates PKR *in vitro* (Siegfried et al., 2014). Alternatively, SGs can be induced by mechanisms not involving PKR (Dang et al., 2006) and by specific proteins (Anderson and Kedersha, 2008; Kedersha and Anderson, 2007).

Based on these considerations, we investigated the potential role of PKR in gRNA-dependent SG induction. In addition, we treated cultures for 4h with low partially inhibiting 1-2μM concentrations of the specific PKR inhibitor C16 (Jammi et al., 2003). In accordance with results above (Figs 1-5), in the absence of C16 all cells with abundant gRNA sequestered it efficiently into SGs and lacked detectable dispersed cytoplasmic gRNA. In striking contrast, cultures treated for 4h with C16 lacked gRNA-positive SGs and contained many cells with abundant cytoplasmic gRNA that was substantially dispersed (Fig 6A).

It was recently reported that PKR, which is normally cytoplasmic, is strongly activated by chromosome-associated inverted repeat Alu and LINE dsRNA regions after nuclear membranes disperse during prophase to metaphase stages of the mitotic cycle, and that its

activated autophosphorylated derivative PKR-P plays an essential role in mitosis (Kim et al., 2014). Accordingly, we detected strong specific PKR-P localization on condensed mitotic chromosomes (Fig 6B). Cells in metaphase contained aligned chromosomes surrounded closely by PABP (Fig 6B, upper row). In contrast, interphase cells contain rounded nuclei with intact membranes that exclude PABP (see Figs 1-5). Thus, as documented more fully below, PABP localization formed a negative image indicative of aligned metaphase plates. Unlike PKR-P, unphosphorylated PKR was dispersed throughout the cytoplasm and was not detected on condensed chromosomes (e.g., Fig 6D). Moreover, 4h treatments with C16 eliminated PKR-P from all cells (Fig 6E), strongly supporting the specificity of this antiserum. Interestingly, whereas metaphase plates identified by negative staining with PABP were frequently seen in control cultures, they were absent after 4h treatments with C16, consistent with evidence that PKR-P is essential for mitosis (Kim et al., 2014).

Importantly, in cultures transfected with pHIV*gpt*, the mitotic cells that contained chromosome-associated PKR-P all lacked detectable p24 Gag antigen (e.g., see the representative images in Fig 6B, lower two rows). The bottom row shows a low magnification field with many cells that supports this conclusion. We analyzed this quantitatively by counting large numbers of cells in a pHIV*gpt* transfected culture. In that experiment 8.8% of the cells (66 of 748 cells) were in mitotic M-phase as indicated by PKR-P staining and 13.5% (101 cells) contained detectable Gag. However, no costained cells were detected in this or other assays, inconsistent with the hypothesis that chromosome-associated PKR-P and Gag were randomly distributed ($P < 0.01$ by the binomial equation). Although this result would be compatible with the hypothesis that HIV-1 inactivates PKR, evidence below excludes that possibility. In contrast, this striking dichotomy is consistent with abundant evidence that nuclear membrane breakdown and cell cycle progression are blocked in cells containing Gag and other HIV-1 proteins that are coexpressed late in the infection cycle in a Rev-dependent manner. These late proteins include Vpr and Vif, which cause cell cycle arrest in the G2 stage (Izumi et al., 2010; Malim and Emerman, 2008; Roshal et al., 2003). This evidence strongly suggests that HIV-1 reduces PKR activation and eludes PKR-P in part by causing G2 arrest.

Importantly, PKR-P was present not only on mitotic chromosomes of cells lacking HIV*gpt*, but was strongly and specifically induced in the cytoplasm of all transfected cells that contained SGs but not in other interphase cells in the same culture, and it colocalized with PABP in these SGs (Fig 6C, top row). Although cells with SGs generally contain only small amounts of Gag compared to cells with dispersed cytoplasmic gRNA (Fig 5A and B), this Gag was also present in the PKR-positive SGs (Fig 6C, bottom row). Similarly, PKR-P has been detected in SGs in other systems (Okonski and Samuel, 2013; Onomoto et al., 2012). Supporting the specificity of our phospho-specific antibody, PKR-P labeling was substantially eliminated in cells treated with C16 (Fig 6E).

Consistent with the substrate specificity of PKR-P and the specificities of our antibodies, phosphorylated eIF2 α (eIF2 α -P) was present in all HIV-*gpt*-induced SGs but not in interphase cells that lacked SGs (Fig 7A). Indeed, eIF2 α -P colocalized with PABP (Fig 7A, upper rows) and p24 Gag antigen (lower rows) in these SGs. In contrast, unphosphorylated eIF2 α did not congregate into PABP- and Gag-containing cytoplasmic granules and was

dispersed in all cells (Fig 7B). Similarly, PABP-positive SGs were absent in cultures transfected with the pcDNA3.1 control vector, and eIF2 α was dispersed in the cytoplasm of these cells (Fig 7C).

Strikingly, although PKR-P and eIF2 α -P were present in SG-positive cells that contained small amounts of colocalized p24 Gag antigen, they were absent or present at undetectable levels in cells lacking SGs that contained much larger amounts of dispersed Gag (Fig 6C lower row and 7A lower two rows). This supports the evidence in Fig 5 A and B, and strongly suggests that abundant gRNA activates PKR-P dependent eIF2 α phosphorylation, resulting in SG formation and suppression of Gag synthesis. In contrast, interphase cells with trace amounts of gRNA lack SGs and fail to activate PKR or phosphorylate eIF2 α , but frequently contain relatively large amounts of Gag (Figs 5A and B, 6C and 7A). Thus, abundant cytoplasmic gRNA induces a signaling cascade that phosphorylates PKR and eIF2 α , and causes gRNA release from polysomes and its sequestration in SGs. When the latter cells are treated with C16, the SGs disintegrate and release abundant gRNA into the cytoplasm (Fig 6A).

3.5. Analyses of HIV-1 infected cultures reveal intranuclear PKR activation

We used these same methods to analyze gRNA localization in CD4 and CXCR4-positive HeLa-CD4 (clone HI-J) cells infected with replication-competent HIV-1_{LAI}, and we also attempted similar studies in H9 leukemic T cells as described below. Although such analyses are necessary for understanding pathogenesis, they are difficult because infected cultures coexpressing HIV-1 Env glycoproteins, CD4, and coreceptors become extremely diverse due to extensive cytopathology involving syncytia formation, destabilization of intracellular membranes and nuclear pores, bystander cell killing, and apoptosis (Jakobsen et al., 2015; Koga et al., 1990; LaBonte et al., 2003). Despite this complexity and consistent with our transfection results, many infected cells with normal adherent morphologies formed cytoplasmic gRNA granules containing colocalized PABP and DDX6 (Fig 8A). Also in agreement with our transfection results, Ars caused DDX6- and EDC4-positive PBs to segregate from PABP-, Gag- and gRNA-containing SGs (Fig 8B).

Consistent with our analyses of 293T cells (Figs 6 and 7) and previous studies of HeLa cells (Kim et al., 2014), PKR-P and eIF2 α -P were present in control uninfected HeLa-CD4 (clone HI-J) cultures only in rounded cells with reduced adherence that were in prophase or metaphase stages of mitosis (Fig 9A), although small amounts of eIF2 α -P persisted into anaphase before separation of the smaller daughter cells (Fig 9A, lower row). Moreover, PKR-P was substantially associated with the condensed mitotic chromosomes, whereas eIF2 α -P and PABP surrounded the aligned metaphase chromosomes in a negative staining pattern. The fact that cells with condensed mitotic chromosomes specifically contained both PKR-P and eIF2 α -P, supports the specificities of these antibodies and is consistent with evidence that PKR-P dependent phosphorylation of eIF2 α contributes to the protein synthesis suppression that occurs during mitosis in uninfected cells (Kim et al., 2014).

A major difference in PKR regulation was reproducibly seen (N=3) in HIV-1_{LAI} infected cells. Most strikingly, PKR-P was present in a large proportion (c.a., ~70%) of nuclei in adherent unrounded gRNA-positive single cells (Fig 9B) and in multinucleated syncytia (Fig

9B, middle row) and there was a corresponding large increase in eIF2 α -P expression (Fig 8C). In this experiment, approximately 94% of the cells stained for Gag at the time of fixation (results not shown), consistent with the moi of 2.0 used for the infection (see Materials and Methods, section 2.3). However, the same pattern of intranuclear PKR-P and eIF2 α -P localization occurred in infected cells when the moi was 0.2 (results not shown). Thus, whereas chromosome-associated PKR-P occurred in uninfected HI-J cells only after nuclear membranes dispersed during mitosis (Fig 9A), HI-J cells infected in the same experiment with HIV-1_{LAI} stopped dividing and contained PKR-P within the rounded nuclei of adherent nonmitotic cells. Based on evidence for nuclear membrane damage and pore leakage in HIV-1 infected interphase cells (de Noronha et al., 2001; Hatch and Hetzer, 2014; Jakobsen et al., 2015; Koga et al., 1990; LaBonte et al., 2003; Monette et al., 2011) and for cytopathic HIV-1 Env- and CD4-dependent damage to intracellular membranes (Cao et al., 1996; Etemad-Moghadam et al., 2002; Jakobsen et al., 2015; LaBonte et al., 2003; Madani et al., 2007), we propose that this enables nuclear entry of PKR and eIF2 α , with resulting activation of PKR and consequent eIF2 α -P formation and dispersal. Our preliminary evidence suggested that the intranuclear PKR-P may be principally situated in nucleoli (results not shown).

Additional evidence supported and clarified these interpretations. Specifically, PKR-P was not detected in interphase nuclei of uninfected HI-J cells (Fig 9A, top row), in HIV-*gpt* transfected cells (Fig 6B and C), or in CD4- and coreceptor-positive HeLa (clone JC.53) cells infected with pseudotyped HIV-*gpt* virions that lack an *env* gene (results not shown). In addition, as shown above, intranuclear PKR-P occurs in HIV-1_{LAI} infected single cells rather than only in syncytia. In another experiment we added the membrane impermeable peptide fusion inhibitor T-20 to culture media 6h after HIV-1_{LAI} was added to the cultures. This eliminated virus spread and syncytia formation, but allowed intranuclear PKR activation in the infected cells (results not shown). Considered together, these results support the hypothesis that intranuclear PKR activation in the conditions of our experiments might require Env- and CD4-mediated fusion or hemifusion of intracellular membranes. Despite this evidence, other viral and cellular proteins including Vpr can initiate nuclear pore defects (de Noronha et al., 2001; Hatch and Hetzer, 2014; Roshal et al., 2003), and nuclear pore breachments occur in proapoptotic cells (Lindenboim et al., 2010; Lindenboim et al., 2014) and in cells infected by other viruses (Chang et al., 2015; Gustin, 2003; Hatch and Hetzer, 2014; Yarbrough et al., 2014; Younessi et al., 2012). Based on these considerations, we propose that membrane damage induced by HIV-1_{LAI} may enable or accelerate additional viral and cellular processes including proapoptosis that contribute to the nuclear breachment implied by our data.

Results obtained using HIV-1_{LAI} infected H9 leukemic T cells were similar to infected HI-J cells, although the small size and cytoplasmic volume of T cells, their lack of adhesion to culture dishes, and the abundance of syncytia and other cytopathic changes including cellular debris made these cultures less useful for analyzing the compositions and movements of gRNA-containing cytoplasmic complexes (results not shown). Biohazard considerations also prevented cytocentrifugation of infected H9 cells onto microscope slides.

3.6 Additional considerations

Our conclusion that SG formation is PKR dependent and requires a suprathreshold concentration of cytoplasmic gRNA is compatible with evidence that cells contain endogenous PKR inhibitors including proteins with dsRBDs and inhibitory RNAs (Bannwarth and Gagnon, 2005; Brand et al., 1997; Cai et al., 2000; Clemens et al., 1994; Clerzius et al., 2009; Clerzius et al., 2011; Clerzius et al., 2013; Daher et al., 2009; Endo-Munoz et al., 2005; Ong et al., 2005; Samuel, 1992; Sanghvi and Steel, 2011; Singh et al., 2011). Those studies principally analyzed levels of PKR-P and other proteins in post-nuclear cytoplasmic extracts from whole cell cultures. Our microscopic results complement these previous investigations by revealing cellular and subcellular diversities in PKR-P levels and locations. Specifically, our results suggest that intracellular PKR inhibitors can be overcome by suprathreshold amounts of cytoplasmic gRNA and by chromosome-associated cellular dsRNAs that are exposed during mitosis and in cytopathically compromised HIV-1 infected cells. The presence of PKR inhibitors including Tat presumably prevents premature PKR activation and enables low amounts of gRNA to be efficiently translated. Our results are also consistent with evidence that gRNA dimerizes in a concentration-dependent manner and that dimerization increases the length of dsRNA regions and is necessary for PKR activation (Heinicke et al., 2009; Lu et al., 2011b).

The intranuclear activation of PKR in interphase cells infected with HIV-1_{LAI} (Fig 9) might initially appear difficult to reconcile with our finding that HIV-1 evades chromosome-associated PKR-P by arresting cells in G2 prior to nuclear membrane dissolution during M-phase (Fig 6B, lower rows). However, it is possible that these processes occur at different times, and that G2 arrest prolongs HIV-1 protein synthesis and virion production by delaying PKR activation before the arrested cells subsequently undergo proapoptosis and nuclear membrane breachment. Indeed, prolonged cell cycle arrest can lead to later apoptosis (Andersen et al., 2008). In addition, it should be understood that HIV-1_{LAI} is an exceptionally fusogenic syncytium-inducing laboratory-adapted X4 isolate and that nonsyncytium-inducing nonfusogenic HIV-1s with low affinities for CD4 predominate *in vivo*, especially during the chronic phase of disease (Fenyo et al., 1989; Goudsmit, 1995). Indeed, reversion to a less fusogenic phenotype occurred in a laboratory worker accidentally infected with HIV-1_{LAI} (Beaumont et al., 2001). Conversely, patient viruses generally replicate poorly and indolently with minimal cytopathology in cell cultures (Fenyo et al., 1989) and are rapidly outgrown by laboratory-adapted fusogenic variants with increased CD4 affinities (Kabat et al., 1994; Kozak et al., 1997; Platt et al., 2000). These considerations imply that nonfusogenic noncytopathic patient HIV-1 isolates might cause less or slower intranuclear PKR activation and eIF2 α phosphorylation than HIV-1_{LAI}. Patient HIV-1s that are weakly expressed would also be more likely to avoid PKR-dependent SG formation. Additional investigations using diverse X4 and R5 HIV-1 isolates, culture conditions, and cells will be needed to evaluate these issues.

Many viruses have evolved mechanisms to suppress SGs by limiting synthesis and nuclear export of viral mRNAs and/or by inactivating factors needed for SG assembly or stability (Abrahamyan et al., 2010; Bannwarth and Gagnon, 2005; Brand et al., 1997; Cai et al., 2000; Clemens et al., 1994; Clerzius et al., 2011; Courtney et al., 2012; Emara and Brinton,

2007; Fros et al., 2012; Gordon et al., 2013; Kedersha and Anderson, 2007; McInerney et al., 2005; Montero et al., 2008; Nallagatla et al., 2011; Sanghvi and Steel, 2011; Simpson-Holley et al., 2011; White and Lloyd, 2012). Consequently, it has been proposed that SGs function in part as antiviral defense granules to inhibit translation, block mitosis, and prime cells for apoptosis in response to cytoplasmic surges of viral factors, and that extant viruses have necessarily evolved mechanisms to evade this innate intracellular defense. Our results suggest that suprathreshold cytoplasmic amounts of gRNA are required for PKR-dependent SG induction, that these SGs contain DDX6, and that they occur in HIV-1 infected cells. However, HIV-1 infected cultures often exhibit profound degenerative cytopathic changes including syncytia formation, disruption of intracellular membranes, bystander cell killing, apoptosis, and cell signaling changes that complicate their analysis and can cause PKR activation in interphase nuclei (Fig 9). Although this mechanism of PKR activation would be expected to counteract the suppression of PKR caused by virus-dependent cell cycle arrest in G2 (Fig 6B), we believe that the outcome would probably depend on the timing of these processes, on the fusogenicities and coreceptor dependencies of different HIV-1 isolates, and on the cells and culture conditions used. Further investigations using biochemical, virological, and microscopic methods will be needed to test these interpretations and to thoroughly understand the effects of PKR on HIV-1 infections.

4. Conclusions

Anti-HIV-1 APOBEC3 cytidine deaminases and HIV-1 genomic RNA (gRNA) occur in virions, polysomes, and cytoplasmic granules, but were not previously tracked together. Moreover, gRNA traffic is important, but the factors or antiviral defenses that move it into granules were unknown. Our results clarify these issues, show that gRNA is driven into SGs by PKR, and reveal previously unknown intracellular PKR movements that enhance its antiviral capability. Specifically, the cytoplasmic gRNA granules we detect are SGs rather than PBs. APOBEC3F cotraffics with gRNA without altering its movements or locations. Cells with little cytoplasmic gRNA translate it and lack SGs, whereas suprathreshold amounts activate PKR causing eIF2 α phosphorylation, protein synthesis suppression, and SG formation. Nuclear membrane dispersal during cell cycle M-phase enables PKR contact and activation by chromosome-associated cellular dsRNAs. By arresting cells in G2, HIV-1 delays or prevents this mechanism of PKR activation. Infection of cells containing CD4 and coreceptors with laboratory-adapted HIV-1_{LAI} causes extensive membrane damage and cytopathology that enables PKR entry and activation in interphase nuclei. This occurs at a late stage of infection in irreversibly damaged cells. We discuss evidence that patient HIV-1s may be selected to maximally elude PKR.

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Highlights

- Cytoplasmic gRNA granules are DDX6-positive stress granules rather than P-bodies.
- Cytoplasmic APOBEC3F cotraffics with gRNA without altering its movements.
- Suprathreshold gRNA concentrations activate PKR, driving gRNA into stress granules.
- By arresting cells in G2, HIV-1 blocks PKR activation by chromosomal dsRNAs.
- Membrane damage in HIV-1 infected cells enables intranuclear PKR activation.

Fig 1. Cytoplasmic gRNA in cultures transfected with pHIVgpt coalesces into DDX6-positive stress granules

(Panel A) Effects of CHX and Ars on cytoplasmic localizations of gRNA and DDX6 (Rck/p54) in HEK293T cultures transfected with pHIV-gpt. (Panel B) Counting analyses suggest that most but not all cytoplasmic gRNA clusters in untreated transfected cells contain DDX6. The percentages of gRNA granules containing DDX6 were counted in independent experiments (N=3) and are shown $\pm$ SEMs. (Panel C) All gRNA granules in transfected cells contain colocalized PABP, indicating that they are SGs. Ars induces PABP aggregation into SGs in all cells, and CHX drives gRNA and cellular mRNAs into polysomes. (Panel D) Genomic RNA in transfected HeLa cells also coalesces with DDX6 and PABP into SGs.

Fig 2. Cultures transfected with plasmids encoding diverse full-length HIV-1s efficiently form gRNA-positive SGs

The methods were identical to Fig 1. The plasmids tested were cloned isolate IIIIB derived from clade B HIV-1_{LAI}, clade B clone HIV-1_{NL4-3}, and Zaire clade C clone HIV-1_{ELI}. All gRNA granules contained PABP, indicating they were SGs.

Fig 3. PBs disperse in cells containing cytoplasmic gRNA, and PB components coalesce with gRNA into SGs

(Panel A) The gRNA-containing SGs incorporate EDC4 and DDX6, whereas nearby cells lacking gRNA contain small DDX6- and EDC4-positive PBs. (Panel B) Cells with dispersed cytoplasmic gRNA also contain relatively few PBs. Thus, the PBs (small red clusters) occur in neighboring cells but are absent in the gRNA-containing cytoplasm (green stain) of that cell. (Panel C) Quantitation of EDC4-positive PBs in cells that contained or lacked detectable cytoplasmic gRNA. PBs seen in deconvolved cell sections were added to obtain the total number. Approximately 70 cells in each category were analyzed. (Panel D) Ars induces DDX6 release from gRNA-containing SGs, enabling PB reformation. The emergence of DDX6 from gRNA-containing SGs is suggested by the frequent close apposition of SGs with newly forming P-bodies at early times after Ars treatments, although the P-bodies eventually fragment.

Fig 4. Colocalization of APOBEC3F with cytoplasmic gRNA, and their coordinated movements between polysomes and SGs

(Panel A) Triple color immunofluorescence microscopy of gRNA, A3F-HA, and DDX6. Untreated control cells showing colocalization of cytoplasmic gRNA clusters with A3F-HA and confirming that most but not all of these gRNA clusters colocalize with DDX6. Ars causes gRNA and A3F-HA to colocalize in DDX6-negative SGs. CHX drives stored mRNAs into polysomes, causing all cytoplasmic gRNA, A3F-HA and DDX6 granules to disperse. (Panel B) Triple color staining of gRNA, A3F-HA, and PABP. A3F-HA colocalizes with gRNA and PABP in SGs. Ars induced SGs contain colocalized gRNA, A3F-HA, and PABP. CHX overrides Ars and disperses these granules.

Fig 5. Gag colocalizes with translationally silenced gRNA in SGs

(Panel A) Cells with relatively abundant cytoplasmic gRNA form SGs that incorporate Gag, whereas cells with barely detectable trace amounts of dispersed cytoplasmic gRNA often accumulate large amounts of dispersed Gag. (Panel B) Scatter plot of Gag amounts in cells containing or lacking SGs. (Panel C) Effects of actinomycin D, puromycin, Ars, and CHX on incorporation of L-[³⁵S] amino acids into total cellular proteins (upper graph) and on incorporation into Gag proteins immunoprecipitated from cell culture extracts with anti-p24 antibody and analyzed by electrophoresis and autoradiography (lower autoradiogram). The TCA precipitable radioactivity in the cell extracts are averages $\pm$ range (N=2). A tubulin loading control Western blot is also shown. Because puromycin and CHX rapidly and completely blocked synthesis of all proteins, Gag immunoprecipitates from those cultures lacked radioactivity and were not further analyzed. (Panel D) Effects of protein synthesis inhibitors on infectious HIV-gpt virion release from cell cultures that had been cotransfected with pHIV-gpt plus pJRCSFenv. Culture media containing the pseudotyped virions were harvested at hourly intervals and were precipitated with CaCl₂, resuspended in fresh media, and diluted before focal infectivity assays. Controls demonstrated that residual inhibitors had no effect on the assays..

Fig 6. Critical role of activated PKR in gRNA-dependent SG formation and PKR activation by mitotic chromosomes

(Panel A) gRNA-dependent SGs in transfected cultures are substantially dispersed by the PKR inhibitor C16. (Panel B) Activated phosphorylated PKR-P strongly labels condensed mitotic chromosomes independently of whether the cultures were transfected with pHIV-*gpt* or the empty pcDNA3.1 vector. Whereas PABP occurs in the cytoplasm of interphase cells, nuclear membrane dissolution in mitotic cells enables it to intimately surround the condensed chromosomes in a negative staining pattern, as is most clearly evident in cells with aligned metaphase plates (upper row). However, as shown in the lower two rows, cells expressing HIV-1 encoded Gag segregate from cells that contain chromosome-associated PKR-P. The cells with HIV-1 are arrested in G2 and do not enter M-phase. The bottom row is a low magnification field containing many cells, showing the absence of Gag in PKR-P positive mitotic cells. (Panel C) Cytoplasmic PKR-P also occurs specifically in all cells containing gRNA-induced SGs, but not in other interphase cells, and it colocalizes with PABP in these SGs. The lower row shows a representative field of cells stained for PKR-P and p24 Gag, confirming that PKR-P only occurs in cells having SGs, and that cells with SGs contain much less Gag than adjacent cells lacking SGs. (Panel D) Control culture showing inactive unphosphorylated PKR dispersed in the cytoplasm of all cells. (Panel E) Bar graph showing the percentage of cells containing PKR-P in a control culture transfected with pHIV-*gpt* and after 4h exposure to 2 μ M C16 or to the DMSO vehicle alone. Fields of photographed cells were examined and the average percentages containing PKR-P $\pm$ SD are shown. Approximately 3500 cells were examined for each treatment.

Fig 7. Phosphorylated eIF2α-P occurs specifically in gRNA-induced SGs and is absent in transfected cells lacking SGs

(Panel A) Representative fields showing eIF2α-P colocalized with PABP (top two rows) or Gag (lower two rows) in gRNA-induced SGs but absent in adjacent cells that lack SGs. Cells with SGs contain much less Gag than nearby cells lacking SGs. (Panel B) Unphosphorylated eIF2α is active in translation and is dispersed in all cells, including cells with PABP- or Gag-containing SG granules. (Panel C) Cells transfected with the empty pcDNA3.1 vector all lack SGs and contain uniformly dispersed eIF2α and PABP.

Fig 8. HeLa-CD4 (clone HI-J) cells infected with HIV-1_{LAI} form gRNA- and DDX6-positive SGs (Panel A) Some unfused HI-J cells infected with HIV-1_{LAI} retain adherent morphologies and form gRNA-positive SGs with colocalized PABP (second row) and DDX6 (lower two rows). The top row shows cells with dispersed gRNA, and one (upper left) uninfected cell lacking gRNA but containing PABP. (Panel B) ArS treated infected culture costained for gRNA and PABP, DDX6, EDC4, or Gag. The gRNA-positive SGs in this culture colocalize with PABP and Gag, but not with DDX6 or EDC4-containing PBs, consistent with results in transfected cultures. These data were from a culture infected at moi 2.0.

Fig 9. Disregulated PKR and eIF2 α phosphorylation in HeLa-CD4 (clone HI-J) cells infected with replication-competent HIV-1_{LAI}

(Panel A) Control uninfected HI-J cells stained for PKR-P or eIF2 α -P and PABP. Consistent with 293T cells and previous analysis of uninfected HeLa cells, PKR-P is specifically present in rounded mitotic cells, and is associated with condensed chromosomes. eIF2 α -P also is specifically present in these mitotic cells, and diminishes throughout anaphase (small paired cells on left). Although both fields contain many cells, rounding of the mitotic cells causes the PABP staining to appear stronger. Note that PABP and eIF2 α -P surround the aligned metaphase plates to form negative images of the condensed chromosomes. (Panel B) In striking contrast to uninfected cells, infected adherent interphase cells contain abundant PKR-P within rounded nuclei. The middle row shows intranuclear PKR-P in a multinucleated syncytium. (Panel C) Adherent infected cells also contain correspondingly elevated amounts of eIF2 α -P that is partially dispersed into the cytoplasm (lower row). The upper row shows a large multinucleate syncytium. These data were from a culture infected at moi 2.0.