Endogenous LINE-1 reverse transcriptase activity in platelets controls translational events through RNA-DNA hybrids

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Abstract

Objective—One source of endogenous reverse transcriptase (eRT) activity in nucleated cells is the Long Interspersed Element-1 (LINE-1/L1), a non-LTR retrotransposon that is implicated in the regulation of gene expression. Nevertheless, the presence and function of eRT activity and LINE-1 in human platelets, an anucleate cell, has not previously been determined.

Approach/Results—We demonstrate that human and murine platelets possess robust eRT activity and identify the source as being LINE-1 ribonucleoprotein-particles (RNPs). Inhibition of eRT *in vitro* in isolated platelets from healthy individuals or in persons with HIV treated with RT inhibitors enhanced global protein synthesis and platelet activation. If HIV patients were treated with reverse transcriptase inhibitor, we found that platelets from these patients had increased basal activation. We next discovered that eRT activity in platelets controlled the generation of RNA-DNA hybrids, which serve as translational repressors. Inhibition of platelet eRT lifted this RNA-DNA hybrid-induced translational block and was sufficient to increase protein expression of target RNAs identified by RNA-DNA hybrid immunoprecipitation.

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Conclusions—Thus, we provide the first evidence that platelets possess L1-encoded eRT activity. We also demonstrate that platelet eRT activity regulates platelet hyperreactivity and thrombosis and controls RNA-DNA hybrid formation, and identify that RNA-DNA hybrids function as a novel translational control mechanism in human platelets.

Keywords

blood platelets; retroelements; protein translation; LINE-1; translational regulation

Subject Code

Basic Science Research

Introduction

Megakaryocytes invest platelets with a complex transcriptome and requisite translational machinery including ribosomes, initiation and termination factors, microRNAs, and messenger RNAs (mRNAs)[1, 2]. Platelets use a variety of translational and posttranslational mechanisms to finely regulate their protein profile, although only some of these have been identified. For example, a subset of mRNAs are under the translational control of the mTOR pathway[3, 4], although this is thought to be mainly mRNAs possessing a 5′ terminal oligopyrimidine tract (5′ TOP)[5] or a pyrimidine-rich translational element (PRTE)[6]. An additional translational regulatory pathway in platelets is cytoplasmic, signal-dependent pre-mRNA splicing[7, 8]. Nevertheless, only a limited number of pre-mRNAs have been identified in human platelets[7–9]. Therefore, additional translational control mechanisms likely exist in human platelets. Because of the anucleate nature of platelets, they serve as an ideal model system to study translational regulatory mechanisms.

Due to their ability to insert themselves into various genomic locations, human transposable elements contribute importantly to a highly dynamic genome[10]. The only functional autonomous non-long terminal repeat retrotransposon in humans is LINE-1 (long interspersed nuclear element-1, L1)[11]. Almost 17% of the human genome consists of LINE-1 elements[12, 13]. Active, full-length L1 elements consist of a 5′-UTR with internal promoter activity, two open reading frames (ORFs) separated by a short intergenic region, a 3′-UTR that ends with an AATAAA polyadenylation signal, and a poly(A) tail[11, 14, 15]. L1 mRNAs are atypical of mammalian mRNAs because they are bicistronic[11]. The two human ORFs in L1 elements (ORF1 and ORF2p) are in frame, separated by a 63-bp non-coding spacer that contains stop codons[11]. Translation of ORF1 protein occurs via ribosomal initiation in the 5′-UTR[16] while translation of ORF2p occurs by an unconventional termination/reinitiation mechanism[17]. The COOH-terminal half of the 40-kDa ORF1 protein (p40) is involved in RNA binding and is well conserved across species[11, 18–20]. ORF2 encodes an ~150-kDa protein with three conserved domains: NH2-terminal endonuclease (EN) domain, central reverse transcriptase (RT) domain, and COOH-terminal zinc finger-like domain[11, 21–23]. Both L1-encoded proteins preferentially associate with their own encoding RNA (*cis* preference) to form a ribonucleoprotein (RNP) complex[24, 25]; a proposed retrotransposition intermediate[26, 27]. However, L1-encoded reverse transcriptase (RT) is also

known to reverse transcribe RNAs encoded by *Alu* elements[28], SVA elements[29], and Pol II mRNAs causing processed pseudogene formation (26) *in trans*. L1 RT can reverse transcribe mRNAs coding for actin, GAPDH, and nucleolin into cDNAs[30]. In addition to shaping the genome over evolutionary time, L1 retrotransposition events alter gene expression patterns[31]. The most notable gene altering process involves insertional mutagenesis, which can serve as a functional gene knock-out[32]. Retrotransposed L1 sequences can also function as alternative promoters that regulate gene transcription[33]. The insertion of L1 sequences into introns considerably reduces transcriptional elongation of target genes[34]. L1 retrotransposition events are responsible for diseases including hemophilia, β-thalassemia, and muscular dystrophy[35]. There is also evidence that endogenous RT activity regulates cellular proliferation, differentiation and gene expression[36–41].

Reverse transcription of RNA also generates RNA-DNA hybrid intermediates. The formation of RNA-DNA hybrids has been used in molecular biology strategies as an *in vitro* technique to identify specifically translated mRNAs[42, 43]. RNA-DNA hybrids also regulate transcriptional efficiency[44], and are required for efficient double-strand break repair[45].

Persons with HIV are typically prescribed RT-inhibitor based therapy. The introduction of anti-retroviral therapy (ART) has resulted in improved life expectancy[46]. However, persons with HIV are at increased risk of thrombotic disorders, including atherothrombosis[47–49]. The molecular mechanisms underpinning this increased thrombosis risk remain unclear. In addition, whether platelets, which promote thrombosis, are directly affected by RT-inhibitors has not been studied in detail. Accordingly, we sought to determine if human platelets possess RT activity and, if so, its functional effects.

Material and Methods

Material and Methods are available in the online-only Supplement Material.

Results

Endogenous RT activity is present in human platelets

Although in nucleated cells endogenous retrotransposon-encoded RT (eRT) is essential for the mobilization of transposable elements, eRT activity has not previously been examined in human platelets. Therefore, we first determined if human platelets possess eRT activity using a PCR-based *in vitro* assay[37]. An exogenous MS2 phage RNA is added to isolated platelet lysates and evidence of reverse transcription is detected by probing for the DNA product of MS2 phage. We found that eRT activity is present in platelets at baseline and is retained over time (Figure 1A). Stimulating platelets with thrombin (t=6 hours) did not visibly alter eRT activity (Figure 1A, lane 5). In parallel experiments, we also confirmed that platelets *in vitro* transcribe an exogenously added GFP RNA, thus excluding the possibility of contaminating bacteria which are used to produce MS2 phage RNA[50] in the PCR reagents (data not shown). Treating platelets with nevirapine, a clinically used RT inhibitor, blocked reverse transcription of exogenous MS2 phage in a dose-dependent manner (Figure 1B). Additional experiments of platelet lysates with 100kDa size-exclusion based spin columns

(Supplemental Figure S1), suggested that LINE-1 elements could account for most of the eRT activity (i.e. fully processed HERV-K RT has a MW of 66kDa).

Human platelets express LINE-1 ORF1 and ORF2

Human platelets express mRNA encoding LINE-1 ORF1 and L1 ORF2 (Figures 1C and 1D). Activation of platelets did not visibly alter the expression of LINE-1 ORF1 or ORF2 (Figure 1C). We next performed *in situ* hybridization experiments using custom designed probes against LINE-1 (Figure 1E). The specificity of the *in situ* experiments is based on the ability to detect a visible signal only if multiple probes align with their target RNA sequentially (mismatches indicated in Figure 1D are most likely artifacts introduced during the sequencing process). Consistent with our findings by PCR and Sanger-sequencing (Figures 1C and 1D), we found that *LINE-1* mRNA is endogenously expressed *in situ* in the cytoplasm of unstimulated human platelets (Figure 1E, see also Figure 1F demonstrating binding sites of primer pairs and *in situ* probes). Laser capture microscopy (LCM) of individual human platelets further confirmed LINE-1 *ORF1* and *ORF2* expression (Supplemental Figure S2). LCM also excludes the possibility that signals detected in the PCR experiments are derived from LINE-1 mRNA or DNA introduced by contaminating nucleated cells.

We next investigated the localization of LINE-1 encoded ORF1 (ORF1p) and ORF2 proteins (ORF2p) in human platelets. Both ORF1p and ORF2p are robustly expressed basally in the cytoplasm of unstimulated platelets (Figure 2A). In activated platelets, LINE-1 proteins concentrated and accumulated in the middle of the cell bodies (Figure 2A, arrows), cellular regions rich in mRNAs and ribosomal constituents[7, 8]. LINE-1 proteins were also detected in megakaryocytes and proplatelet extensions (data not shown). When platelets were allowed to culture overnight (18 hours), ORF2p continued to be robustly expressed and was also identified in the cytoplasm of platelets with extended cell bodies[51, 52] (Supplemental Figure S3). Immunoblotting also demonstrated that ORF1p (Figure 2B) and ORF2p (Figure 2C) protein was present in platelets. Specificity of the anti-ORF2p antibody was confirmed by performing a quench experiment using the immunogenic peptide (Figure 2C).

Platelets contain ribonucleoprotein particles (RNPs) harboring L1 mRNA, and L1 ORF2p

We hypothesized that RNPs in platelets contain functional LINE-1 mRNA and LINE1-ORF2p harboring reverse transcriptase activity. Therefore, to further identify the localization and function of endogenous L1-encoded RT (eRT) in platelets, we focused on the subcellular localization of eRT. Ribonucleoprotein particles (RNPs) are intracellular complexes integral to the regulation of gene expression[53]. To examine whether the eRT expression and activity we identified (Figures 1–2) is contained within RNPs, we next determined whether RNPs isolated from platelets harbored reverse transcriptase activity. Purified RNPs from platelets were sufficient to reverse transcribe an exogenous MS2 phage RNA in an *in vitro* RT-activity assay (Figure 3A), indicating that platelet eRT activity is found within RNPs. We next investigated if the eRT activity is ORF2p (which contains the RT domain) dependent. RNP lysates used for RT-activity assays were depleted of LINE-1 ORF2p using the anti-ORF2p antibody characterized in Figure 2. Immunodepletion of LINE-1 ORF2p reduced RT activity by more than 60% (Figure 3B), indicating that the

LINE-1 ORF2p RT domain is the major source for platelet eRT. Consistent with the eRT activity associated with RNPs, RNA coding for LINE-1 ORF1 and ORF2 was identified within isolated platelet RNPs (Figure 3C). Furthermore, L1 ORF2p was confirmed in platelet derived RNPs (Figure 3D). To confirm that platelets are capable of forming L1 RNPs, we *in vitro* translated T7-tagged ORF1p using the plasmid pAD2TE1[27] as a template. Next, isolated total platelet RNA was incubated with the recombinant T7-tagged ORF1p and ORF1p-bound RNA was subsequently immunoprecipitated using the T7-tag. T7-tagged ORF1p specifically binds to platelet derived LINE-1 mRNA indicating that platelets are capable of forming LINE-1 RNPs (Figure 3E). In addition, the experiments confirmed that the RNPs isolated from platelets (Figure 3A) are composed of LINE-1 encoding mRNAs and encoded proteins. After identifying that the major source of eRT activity in platelets is the L1 ORF2p RT domain in isolated RNPs, we next sought to determine the function of eRT activity in platelets.

Endogenous RT in platelets alters cytoskeletal dynamics, promotes pro-thrombotic functional responses *in vivo*, and regulates global protein synthesis

Previous studies by our group and others[51, 52] demonstrated that cytoskeletal reorganization can promote the formation of extended platelets with 2 or more cell bodies, a process termed progeny formation that resembles how megakaryocytes form new platelets. In tumor cells, inhibition of eRT activity induces cellular differentiation[37, 40], a process that also regulates cytoskeletal events. Therefore, we hypothesized that platelet eRT activity regulates platelet progeny formation; morphological changes in human platelets orchestrated by cytoskeletal dynamics. When platelet eRT activity was inhibited with nevirapine (an RT-inhibitor used to treat persons with HIV), at doses similar to concentrations seen in treated patients, the number of newly-formed cell bodies that extended from cultured platelets significantly increased (Figure 4A).

Platelet cytoskeletal reorganization and progeny formation can result in platelet activation [51]. We next sought to determine if platelet eRT activity regulated functional responses and thrombosis. We first stimulated platelets pre-treated *in vitro* with nevirapine (or its vehicle control) with thrombin. Inhibition of platelet eRT activity with nevirapine increased activated integrin αIIbβ3 expression on the platelet surface (Figure 4B). To establish whether nevirapine regulated thrombosis *in vivo*, we first confirmed that similar to human platelets, murine platelets possess eRT activity (Supplemental Figure S4). In a well-established pulmonary thrombosis model[54], which is platelet dependent, nevirapine significantly accelerated thrombosis-dependent mortality (Figure 4C and D). To determine the clinical relevance of these findings, we examined activation of integrin αIIbβ3 in platelets isolated from persons with HIV treated with RT inhibitors clinically and matched healthy controls. As shown in Figure 4E, unstimulated platelets from persons with HIV had significantly increased basal activation of integrin αIIbβ3, thus recapitulating our *in vitro* findings (Figure 4B).

Based on these *in vitro* and *in vivo* results and observations[51] that platelet cytoskeletal events require protein synthesis, we next determined whether eRT activity regulates *de novo* protein synthesis. We performed metabolic labeling by incubating platelets with Click-IT

AHA (L-Azidohomoalanine[51]) in the presence or absence of nevirapine. Inhibiting eRT activity significantly increased the incorporation of labeled AHA into platelets (Figure 4F). The specificity of eRT-regulated translation in platelets was demonstrated by using puromycin (a translational inhibitor) as a control (Figure 4F). These data indicate that eRT activity in platelets regulates protein synthetic events.

To establish the relevance of platelet eRT activity in human disease, we examined the activity and function of eRT in platelets isolated from persons with HIV. We confirmed, similar our observations in platelets from healthy subjects, that platelets from persons with HIV (with undetectable viral load) possess eRT activity (data not shown). Next, we incubated platelets isolated from healthy donors or a person with HIV treated with an RT-inhibitor with [^{35}S]methionine and [^{35}S]cysteine. Compared to a healthy control subject, protein synthesis was increased in a person with HIV treated clinically with an RT-inhibitor (Supplemental Figure S5A). Increased protein synthesis was also observed when platelets from a healthy donor were treated with nevirapine *ex vivo* (Supplemental Figure S5B). Furthermore, platelets from persons with HIV on ART (antiretroviral therapy) demonstrated dramatically increased morphologic changes (Figure 4G) that require cytoskeletal reorganization.

RNA-DNA hybrids are present in human platelets

As our findings suggest that platelet eRT activity controls translational events, we hypothesized that one function of eRT in platelets is to generate RNA-DNA hybrids, resulting in a translational block (see graphic abstract). We identified RNA-DNA hybrids in human platelets *in situ* (Figure 5A and B). We next determined the mechanisms controlling RNA-DNA hybrid formation. In freshly isolated platelets from healthy control subjects, RNA-DNA hybrids were present in 6.6%±3.2% of the cells. When platelets were incubated overnight, the percentage of hybrid-positive cells increased to 20.0%±1.6% (Figure 5, $p<0.05$). This increase in hybrid formation was RT-dependent, since the treatment of platelets with nevirapine reduced hybrid-positive cells (11.1%±2.9%, Figure 5, $p<0.05$). To demonstrate the specificity of the antibody-based hybrid detection, we treated platelets with DNase and RNase A in a low concentration NaCl buffer, conditions favoring the digestion of DNA or RNA in RNA-DNA hybrids. As expected, both DNase and RNase A significantly reduced hybrid formation (Figure 5). In contrast, using a high concentration of NaCl in combination with RNase A (conditions under which hybrids are not digested) did not decrease hybrid formation.

Yoshida and colleagues[55] also used exogenous MS2 phage RNA to examine RT activity in washed platelet extracts from patients with myeloproliferative neoplasms. They speculated that RT activity may be found in mitochondria. While not a primary focus of our studies, in additional control experiments, we stained platelets with mitotracker to detect mitochondria, and the anti-RNA-DNA hybrid antibody in parallel, correlating potential intra-mitochondrial transcription events resulting in co-transcriptional RNA-DNA hybrid formation. While some co-staining could be detected in platelet mitochondria, about 50% of the hybrid signals were independent of mitochondrial staining (data not shown). Next, we compared the RNA-DNA hybrid expression levels between platelets isolated from persons with HIV before initiation

of ART, and platelets isolated from persons with HIV following treatment with RT-inhibitor based ART. Platelets from persons with HIV had reduced numbers of RNA-DNA hybrids, compared to platelets from persons with HIV not being treated with RT-inhibitor based ART (Figure 5C and D).

To identify candidates regulated by RNA-DNA hybrids, we immunoprecipitated hybrids from platelet lysates from healthy control subjects. RNA-DNA hybrids were isolated, a cDNA library of these RNAs was created, and sequencing was performed. Sequence analysis of the cDNA library revealed the enrichment of several target RNAs (see Supplemental Table 1). Three of the enriched identified RNAs coded for *MAP1LC3B* (Microtubule Associated Protein 1 Light Chain 3 Beta), *SELP* (P-selectin, which regulates platelet activation), and *RPL26* (ribosomal protein L26, which regulates gene expression) (Figure 6A). MAP1LC3B mediates autophagy[56, 57] and autophagy has been linked to platelet activation[58]. Accordingly, we focused on validating the presence of *MAP1LC3B* nucleotide sequences. Enrichment of *MAP1LC3B* sequences within RNA-DNA hybrids was confirmed in subsequent IP and qPCR experiments (Figure 6B). Thus, *MAP1LC3B* mRNA is expressed within RNA-DNA hybrids in platelets.

LINE-1 ORF1p interacts with *MAP1LC3B* RNA in *trans*

To determine if *MAP1LC3B* mRNA can be a substrate for LINE-1 ORF1p and ORF2p, we *in vitro* translated T7-tagged ORF1p from the expression plasmid pAD2TE1[27] as a template (Figure 3E). Platelet RNA was incubated with the recombinant T7-tagged ORF1p and immunoprecipitated using an anti-T7-tag antibody. ORF1p binds to platelet derived *MAP1LC3B* mRNA (Figure 6C) indicating that this mRNA is associated with LINE-1 RNPs and would therefore be accessible to the RT domain of LINE-1 ORF2p.

RNA-DNA hybrids regulate protein synthetic events in human platelets

We next *in vitro* transcribed a MAP1LC3B-myc-DDK-tag encoding plasmid, generating an mRNA template for a modified *in vitro* RT activity assay and allowing us to specifically detect the translated MAP1LC3B fusion protein. Some platelet lysates used in the RT activity assay as RT donor were treated with the RT-inhibitor nevirapine to block hybrid formation. In addition, select samples underwent a melting step, resulting in a separation of RNA-DNA hybrids and releasing the translational block. The cDNA/hybrids were *in vitro* translated and the resulting proteins were separated by SDS-PAGE using a myc-DDK tag antibody for detection of *in vitro* translated proteins. The bar graph (Figure 6D) demonstrates that inhibiting hybrid formation (either by nevirapine or by inducing melting of the RNA from the DNA) increased the synthesis of MAP1LC3B protein. As these data suggest that MAP1LC3B synthesis in platelets may be translationally controlled, at least in part, by RNA-DNA hybrids, we next examined MAP1LC3B expression in live platelets pre-treated with nevirapine. Nevirapine significantly increased total MAP1LC3B protein (LC3B I and LC3B II) in platelets (Figure 6E). Similar findings were observed with two other proteins: P-selectin and ribosomal protein L26 (Supplemental Figure S6A and S6B). P-selectin surface translocation to the platelet surface was also increased in persons with HIV treated with RT inhibitors (Supplemental Figure S6C). These data indicate that nevirapine

inhibits RNA-DNA hybrid formation and enhances protein synthesis by releasing a translational block.

Discussion

Platelets are anucleate and traditionally viewed as having a fixed molecular signature and associated functional responses. Nevertheless, platelets use an ever increasing, and often unexpected, repertoire of mechanisms to regulate their transcriptional and proteomic signature in health and disease[2]. Here, we provide the first evidence that human platelets possess LINE-1 derived eRT activity, regulating platelet morphology and protein synthesis, and leading to pro-thrombotic functional responses *in vitro* and *in vivo*. We also demonstrate that these findings are concordantly observed in persons with HIV clinically treated with RT inhibitors.

Both LINE-1 ORF1p (RNA binding) and ORF2p (endonuclease and RT domain) are encoded by the bicistronic LINE-1 and expressed in platelets. Concordant with the detected expression of both LINE-1 proteins, we discovered that platelets possess eRT activity (Figure 1). Previous studies have demonstrated the importance of LINE-1 ribonucleoprotein particles[27, 30, 59], intracellular complexes that are integral to the regulation of gene expression[53] and for retrotransposition in other cell types. Consistent with this, we report that the majority of platelet eRT activity is localized to LINE-1 RNPs (Figure 3). Depleting LINE-1 ORF2p from platelet RNPs demonstrates that LINE-1 RNPs are the major (e.g. ~60% of greater) contributor to platelet eRT activity. Furthermore, we show that exogenously expressed LINE-1 ORF1p containing an epitope tag (T7) at the carboxy terminus binds selectively to its endogenous encoding RNA (cis-preference[30]) in platelets. These findings indicate that human platelets contain functional LINE-1 RNPs. When platelets were incubated on immobilized fibrinogen and stimulated with thrombin, we observed trafficking of the LINE-1 proteins towards the central areas of platelets (Figure 2), subcellular regions known to be rich in mRNAs and ribosomal constituents[7, 8]. This shift in localization would allow LINE-1 proteins to interact with endogenous mRNAs that code for other cellular proteins (*trans*-acting LINE-1), a concept evaluated in previous studies[30].

Since LINE-1 ORF2p includes an RT domain, it is worth discussing the functions of RT enzymes. In biochemical *in vitro* assays, RTs are used to reverse transcribe mRNA into cDNA to enable the subsequent PCR amplification step. This reaction forms mRNA-cDNA hybrids. Here, we demonstrate that platelets dynamically express RNA-DNA hybrids. Hybrid expression was dependent on eRT (ORF2p) and hybrid formation was blocked by nevirapine at doses achieving concentrations relevant to patients treated with this drug.

Based on our findings, we propose a previously unrecognized cellular mechanism. In our model, eRT in platelets generates RNA-DNA hybrids that block translation by partially reverse transcribing mRNA (see graphic abstract). The cellular concept of intentionally stalling translation was used in molecular biology strategies as an *in vitro* technique to identify specifically translated mRNAs[42, 43], but so far has not been identified as an actively used translational control mechanism in primary human cells. In contrast, it is a well-accepted concept that the stability of co-transcriptional RNA-DNA hybrids affects

transcriptional efficiency[44], and even that stability differs between coding and non-coding regions[60]. However, this transcriptional mechanism is not based on eRT utilization. Nevertheless, in 2013, Sciamanna et al[61] suggested that the regulation of tumor cell biology by LINE-1[36, 37, 39, 62], an area of intense research, could be induced by the formation of RNA-DNA hybrids. Using CsCl density gradients, they selectively identified *Alu* and LINE-1 containing RNA-DNA hybrid molecules in cancer but not in normal cells. They concluded that LINE-1 mediated RNA-DNA hybrids would interfere with the formation of dsRNA and therefore, the synthesis of regulatory small RNAs[61].

Our findings demonstrate that RNA-DNA hybrid formation regulates synthesis of specific candidate RNAs by the induction of a translational block. This is supported directly by protein *de novo* synthesis assays, but also indirectly by the formation of platelet progeny, a process strictly dependent on protein synthetic activity[51]. *MAP1LC3B* was identified as one of the target mRNAs of *trans*-acting L1 ORF1p. MAP1LC3B plays an important role in autophagic processes[56, 57] and serves as a good autophagy marker[63]. In addition, this catabolic process maintaining cellular hemostasis, adaptation to starvation, development and pathogen elimination needs to be tightly regulated[64]. Therefore, the translational control of one of the major players in autophagy by RNA-DNA hybrid formation is an attractive focus of future research. Two other identified hybrids also underscore the importance of this novel regulatory pathway. RPL26 is important for ribosomal hemostasis and regulation of gene expression, while SELP (P-selectin) is one of the major α-granular constituents being translocated to the surface of activated platelets upon stimulation[65].

This novel mechanism for translational control has clinical implications. As part of ART (antiretroviral therapy), patients with HIV are typically prescribed RT-inhibitor based therapy. In persons with HIV, the introduction of ART has dramatically improved life expectancy[46]. As a result, chronic and often accelerated disorders, including atherothrombosis[47–49], are replacing opportunistic infections as important causes of morbidity and mortality[66]. Furthermore, in patients with HIV under ART, and despite undetectable virus levels, a 30-fold increase in mortality when compared to uninfected controls could be demonstrated. The increased mortality was attributed to ongoing innate immune responses and activation of coagulation factors [67–69].

We demonstrated that platelet eRT activity regulates integration activation *in vitro* and *in vivo*. Similar findings of platelet integrin activation were observed in persons with HIV prescribed antiretroviral therapies that include RT inhibitors. We postulate that inhibition of platelet eRT activity may contribute to the increased risk of thrombosis observed in persons with HIV[70]. Furthermore, since we detected LINE-1 expression in other primary human blood cells, including megakaryocytes and T-cells (Supplemental Figure S8), our findings may have implications not only in the megakaryocyte-platelet axis, but also in other cell lineages.

Platelets isolated from persons with HIV were more synthetically active (Supplemental Figure S5) and had altered morphology resembling dumbbells (Figure 4C, as seen *in vitro* with nevirapine treated platelets), suggesting deregulated translational control mechanisms. Consistent with these observations, hybrids were higher in platelets from patients with HIV

not treated with ART than compared to healthy individuals or persons with HIV treated with ART. Our findings suggest that administration of ART to persons with HIV alters platelet protein synthesis by lifting the RNA-DNA hybrid induced translational block; although we cannot exclude the possibility that differences in RNA-DNA hybrids in persons with HIV was also induced by HIV encoded RT. In this context, it is also interesting to note that platelets contain dengue virus DNA when infected with dengue virus, a (+)ssRNA virus (Supplemental Figure S7). Dengue virus DNA is not part of the virus life cycle and these data suggest a reverse transcription event. Furthermore, when eRT was inhibited, dengue DNA could not be detected. One might speculate if inhibiting dengue DNA formation removes a RNA-DNA hybrid induced translational block, leading to increased dengue virus replication.

While our findings confirm and extend prior studies in murine cells and tumor lines[37, 39, 71], we recognize that several reports did not confirm that nevirapine blocks LINE-1 RT[72]. There are several potential explanations for these seemingly contradictory findings. First, we used higher concentrations of nevirapine (750μM), specifically chosen to recapitulate systemic concentrations of nevirapine achieved clinically. Second, we did not use tumor cell lines, but primary human cells (e.g. platelets). Finally, we examined RNA-DNA hybrid formation, rather than retrotransposition events. We also identified that another RT inhibitor, delavirdine, similarly blocked eRT activity (data not shown), indicating that our findings are not confined to nevirapine alone. While size-exclusion experiments (Supplemental Figure S1) did not completely exclude the possibility of eRT activity deriving from proviral insertions of human endogenous retroviruses (HERV), the fully processed HERV-K RT has a molecular weight of 66kDa, making this unlikely.

In conclusion, our data identify a previously unrecognized function for LINE-1 encoded proteins. We propose a model whereby endogenous LINE-1 mediate RNA-DNA hybrid formation controls protein expression for select mRNAs (please see graphical abstract). Because LINE-1 is classically considered to be involved in disease inducing *de novo* disruption of gene expression by retrotransposition events[35], or tumor genesis[73, 74], our findings also demonstrate that in human platelets, LINE-1 functions in a newly described constructive and potentially regulated fashion. Finally, our results provide evidence for a new translational regulatory mechanism that is present in platelets and which regulates integrin activation and thrombosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Nonstandard Abbreviations and Acronyms

eRT	endogenous reverse transcriptase
LINE-1	Long Interspersed Element-1
RNP	ribonucleoprotein-particles
PRTE	pyrimidine-rich translational element
UTR	untranslated region
ORF	open reading frame
ART	anti-retroviral therapy
HERV	human endogenous retrovirus
LCM	laser capture microscopy
AHA	L-Azidohomoalanine

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Highlights

- LINE-1 elements are one source of endogenous RT-activity (eRT).

- Presence and function of eRT activity and LINE-1 in human platelets has not previously been determined.

- LINE-1 retrotransposon's RT-activity reverse transcribes select mRNAs, inducing RNA-DNA hybrids.

- Inhibition of RT activity regulates platelet activation, thrombosis, and RNA-DNA hybrid formation which is a new mechanism of translational control.
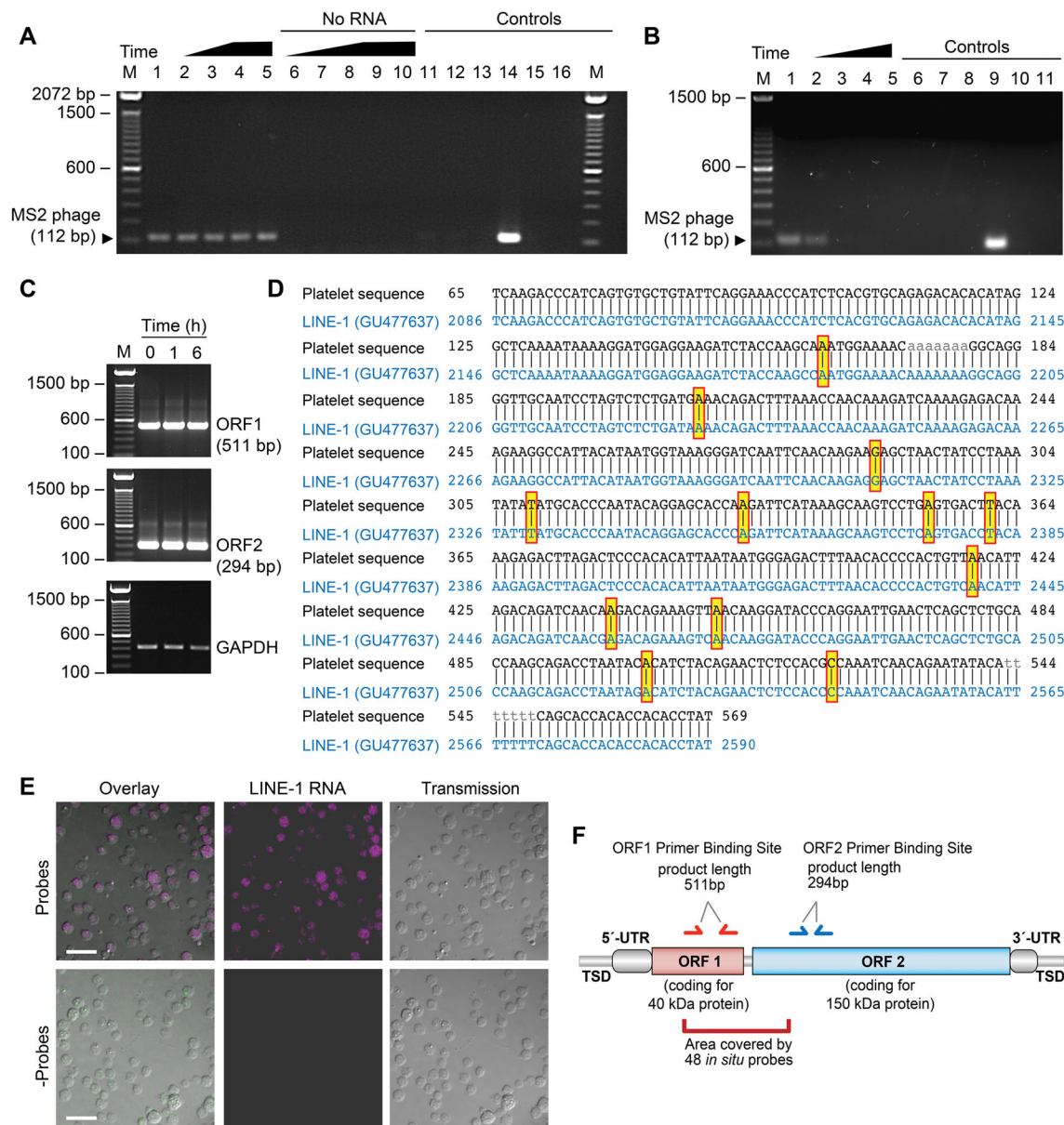
Figure 1. Human platelets possess endogenous RT activity and express LINE-1 *ORF1* and *ORF2* mRNA

(A) Platelet lysates were incubated with (**lanes 1–5**) or without (**lanes 6–10**) exogenous MS2 phage RNA. **Lanes 11–16** represent controls. The following conditions apply: **M** - marker; **lanes 1,6** - freshly-isolated platelets; **lanes 2,7** - platelets stored for 2 hours; **lanes 3,8** - platelets stored for 4 hours; **lanes 4,9** - platelets stored for 6 hours; **lanes 5,10** - platelets activated with thrombin (0.1 U/ml) for 6 hours; **lane 11** - platelet lysate replaced with the lysis buffer only; **lane 12** - omission of the platelet lysate; **lane 13** - omission of the MS2 phage RNA; **lane 14** - platelet lysate replaced with commercial RT; **lane 15** - omission of the reverse MS2 phage primer; **lane 16** - negative PCR. This figure is representative of n>10 independent experiments. **(B)** Platelets were treated with DMSO (**Veh, lane 1**) or increasing concentrations of nevirapine ([26 μg/ml], [130 μg/ml], and [195 μg/ml], **lanes 2–4**

respectively) and reverse transcription of exogenous MS2 phage RNA was measured. Controls include: **lane 5** - no RNA; **lane 6** - platelet lysate replaced with the lysis buffer only; **lane 7** - omission of the platelet lysate; **lane 8** - omission of the platelet lysate and MS2 phage RNA; **lane 9** - platelet lysate replaced with commercial RT; **lane 10** - omission of the reverse MS2 phage primer; **lane 11** - negative PCR. This figure is representative of 5 independent experiments. **(C)** Freshly isolated platelets were examined at baseline (t=0 h) or stimulated with thrombin (0.1 U/ml) for 1 or 6 hours. RNA from the platelets was isolated and *ORF1* and *ORF2* mRNA expression was evaluated by PCR. The bottom panel shows *GAPDH* as a loading control. This figure is representative of 3 independent experiments. **(D)** PCR products for LINE-1 were excised and sequenced using Sanger-Sequencing. The resulting sequence was aligned with a reference sequence. Yellow boxes highlight mismatches. **(E)** Human platelets were fixed in suspension and Stellaris FISH probes specific for LINE-1 (magenta, top panels) were hybridized to detect *LINE-1* mRNA *in situ*. The bottom panels show platelets where the probes were omitted (- Probes) as a control (scale bars = 10 μm). These images are representative of 4 independent experiments. **(F)** Schematic representation of the structural organization of active L1 retroelements. The boundaries of a retroelement are defined by the presence of tandem site duplications (TSDs). L1 elements are composed of a 5′UTR that contains an internal RNA polymerase II promoter, followed by two open reading frames (ORF1 and ORF2) and a 3′UTR containing a polyadenylation signal (pA). Primer binding sites for ORF1 (red) and ORF2 (blue), and the area of in situ probe binding (dark red) are indicated.
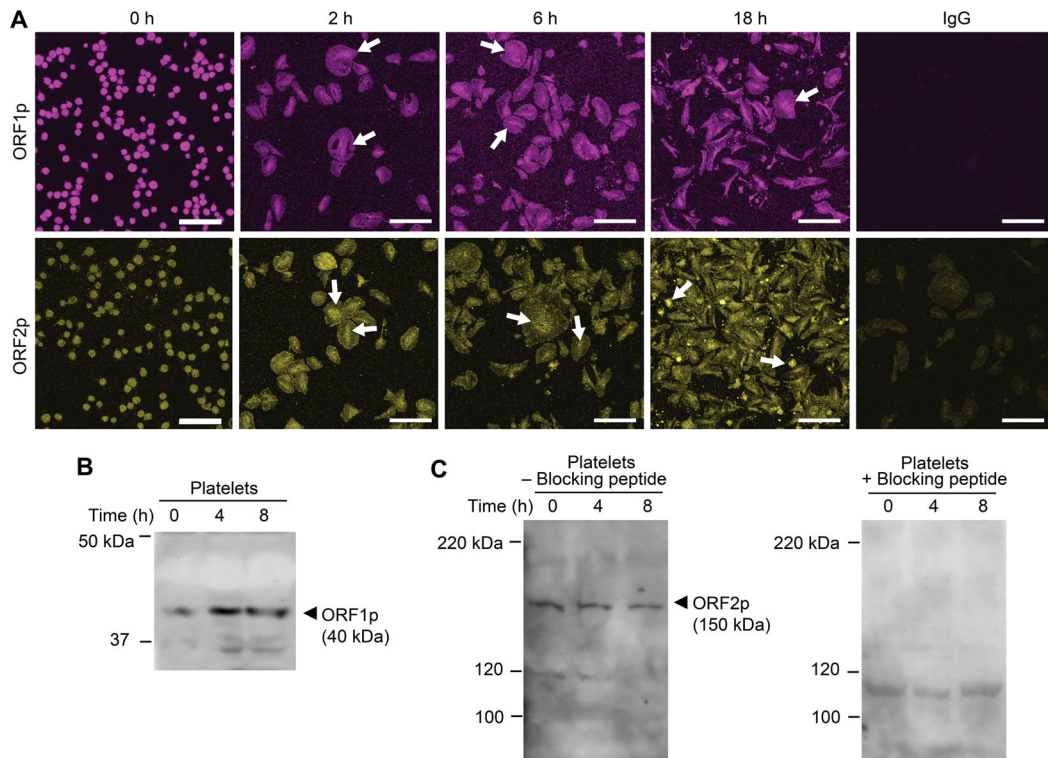
Figure 2. Human platelets express ORF1 and ORF2 protein

(A) Freshly isolated platelets were fixed in suspension immediately (baseline, 0 h) or adhered to immobilized fibrinogen in the presence of thrombin for 2, 6, or 18 hours. Immunofluorescence staining with an anti-L1 ORF1p (top row, magenta stain), and anti-L1 ORF2p (bottom row, yellow stain) antibody, respectively, demonstrates expression of both L1-encoded proteins ORF1p and ORF2p in platelets, irrespective of presence or absence of thrombin stimulation. White arrows indicate the time-dependent concentration of L1 proteins in central cell areas, rich in mRNA and ribosomes. The isotype specific IgG control is shown on the far right (scale bars = 10 μm). This figure is representative of 3 independent experiments. **(B)** Immunoblot analysis of platelet lysates using the anti-L1 ORF1p antibody. Freshly isolated platelets were immediately fixed in suspension (t=0 h) or allowed to adhere to immobilized fibrinogen in the presence of thrombin for 4 or 8 hours. The cells were then lysed and proteins separated by SDS-PAGE electrophoresis. **(C)** Immunoblot analysis of platelet lysates using the anti-L1 ORF2p antibody. Platelets were treated as in B, however the samples were split and processed in parallel in the absence (left panel) or presence (right panel) of an ORF2p quenching peptide. Presence of the blocking peptide prevents the detection of L1 ORF2p with the anti-L1 ORF2p antibody. Panels B and C are representative of 4 and 5 independent experiments, respectively.
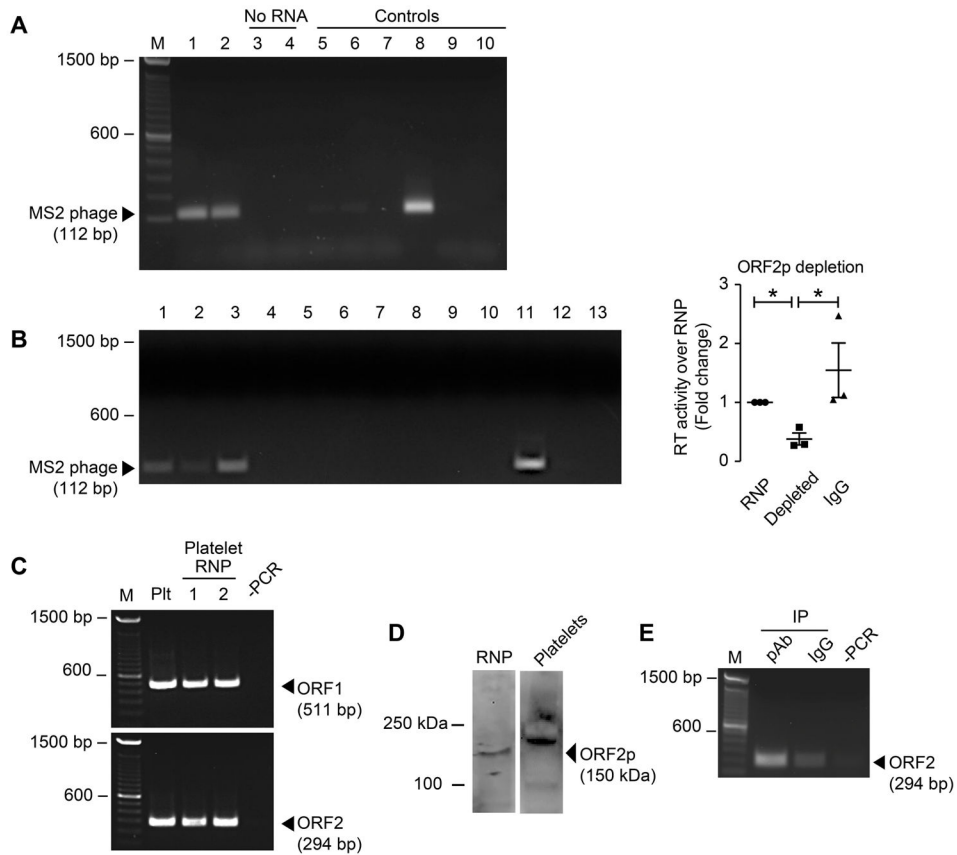
Figure 3. Platelet RNPs contain L1 RNA and are a source of eRT activity
(A) RT assays with platelet-derived RNPs. RNPs were isolated as described in the Methods section and incubated with **(lanes 1 and 2)** or without **(lanes 3 and 4)** MS2 phage RNA. Controls include: **lane 5** - platelet lysate replaced with the lysis buffer only; **lane 6** - omission of the platelet lysate; **lane 7** - omission of the platelet lysate and MS2 phage RNA; **lane 8** - platelet lysate replaced with commercial RT; **lane 9** - omission of the reverse MS2 phage primer; **lane 10** - negative PCR. This figure is representative of 5 independent experiments. **(B)** Depletion of L1 ORF2p from platelet-derived RNPs. RNPs were isolated as described in the Materials and Methods section, and L1 ORF2p was depleted using immunoprecipitation strategies. Non-depleted **(lane 1)**, depleted **(lane 2)**, or IgG-depleted platelet RNPs **(lane 3)** were incubated with **(lanes 1–3)** or without **(lanes 4–6)** MS2 phage RNA. Controls include: **lane 7** - platelet lysate without MS2 phage RNA ; **lane 8** - RNP replaced with the lysis buffer only; **lane 9** - omission of the RNP; **lane 10** - omission of the RNP and MS2 phage RNA; **lane 11** - platelet lysate replaced with commercial RT; **lane 12** - omission of the reverse MS2 phage primer; **lane 13** - negative PCR. The dot blot on the right side represents the densitometric analysis of the non-depleted (RNP), depleted, or IgG-depleted platelet RNPs (IgG), indicating a Å60% reduction in RT activity (mean±SEM). This figure is representative of 3 independent experiments. Single asterisk: p<0.05 **(C)** RT-PCR on RNA isolated from platelet RNPs. RNA from RNPs was isolated and probed for LINE-1 *ORF1* (top panel) and *ORF2* (bottom panel). RNA in two different RNP samples [1, 2] was analyzed using PCR and specific primer sets. An unfractionated platelet lysate

sample (Plt) was analyzed in parallel. This figure is representative of n=5 independent experiments. **(D)** Immunoblot analysis of platelet-derived RNP (left panel), and platelet lysates (right panel) using the anti-L1 ORF2p antibody. Panels are representative of 3 independent experiments. **(E)** RT-PCR detecting platelet-derived endogenous L1 RNA/ ORF1p interaction. L1 ORF1p was *in vitro* translated from the expression plasmid **pAD2TE1**, resulting in a T7 tagged ORF1p. Platelet RNA was isolated and incubated with ORF1p-T7 tag, then immunoprecipitated and analyzed for bound LINE-1 RNA. pAb – IP using anti-T7 antibody, IgG – isotype control, -PCR – control. This figure is representative of 3 independent experiments.
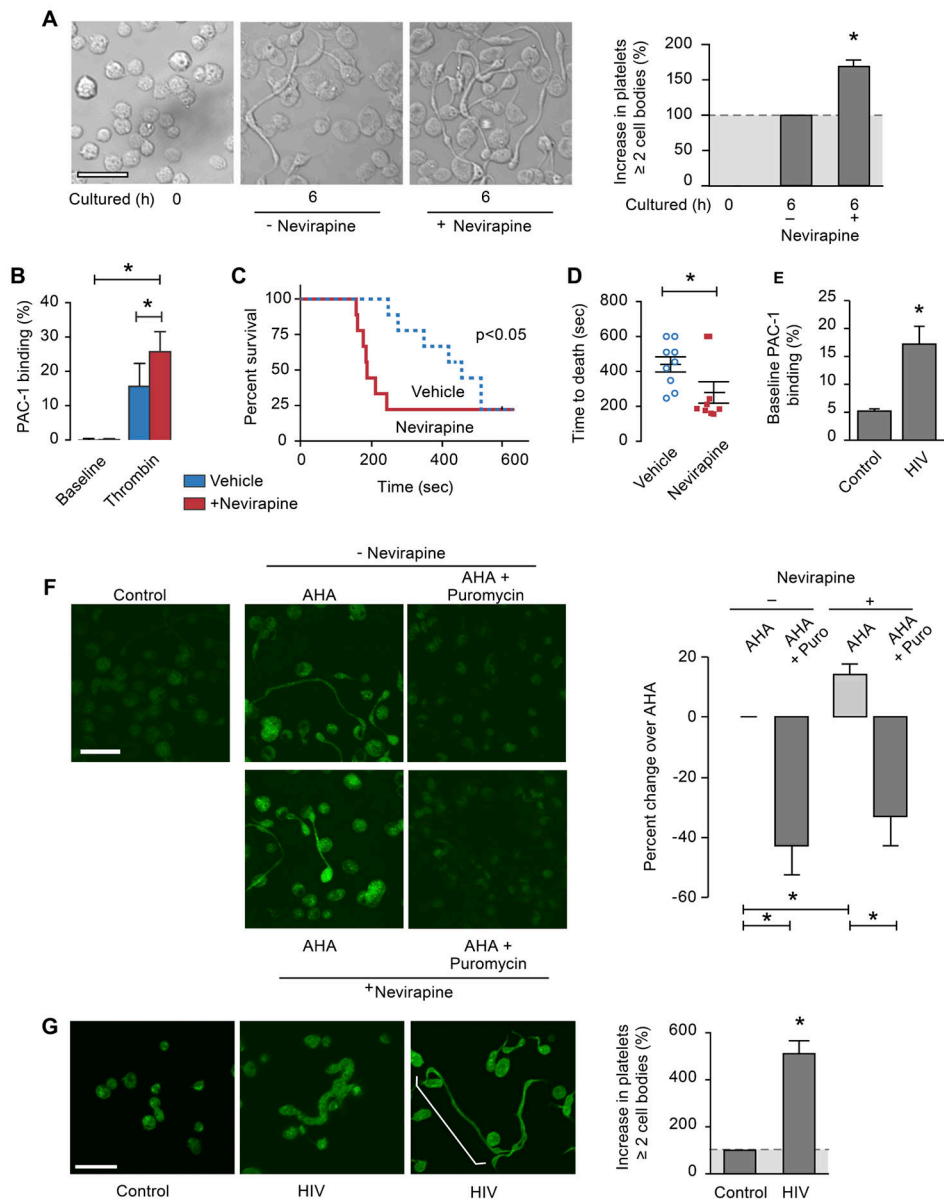
Figure 4. Inhibition of eRT activity in platelets alters actin cytoskeletal events, promotes pro-thrombotic functional responses, and induces global protein synthesis

(A) Platelets from healthy individuals were isolated and fixed in suspension (0 hr), or treated with nevirapine (750 **μ**M) or vehicle (control, DMSO) and subsequently incubated for 6 hours. The microscopy panels display representative examples of cultured platelets in the absence (-nevirapine) or presence of nevirapine (scale bar = 10 **μ**m). The bar graph displays the percent increase in the number of platelets with at least two cell bodies in treated versus untreated platelets (mean±SEM; n=8). Single asterisk: p<0.05. **(B)** Platelets from healthy individuals were pre-treated with nevirapine for 6 hr (750**μ**M, red) or its vehicle control (DMSO, blue). PAC-1 binding to activated integrin αIIbβ3 on the platelet surface was evaluated by flow-cytometry at baseline or after incubation with thrombin (0.1U/mL for 10 min.). The bar graph depicts the mean±SEM. Single asterisk: p<0.05. **(C)** Wild type mice

were treated *in vivo* with either nevirapine (red) or its vehicle (DMSO, blue) by daily gavage for four days. Pulmonary embolism was induced by retro-orbital injection of collagen/epinephrine as described in the methods. Mortality from respiratory failure was measured to the stopping point of 600 seconds (10 min.). The Kaplan-Meier plot depicts the percent of mice surviving over time. The x-axis shows time in seconds till cessation of breathing (n=8–12 mice/group). **(D)** Summary blot for the time to death (seconds) for the individual animals from the experiment shown in (C). Single asterisk: p<0.05. **(E)** Human platelets from healthy individuals or persons with HIV were isolated. PAC-1 binding to activated integrin αIIbβ3 on the platelet surface was evaluated by flow-cytometry without additional, exogenous stimulation (i.e. baseline). The bar graph depicts the mean±SEM. Single asterisk: p<0.05. **(F)** Analysis of *de novo* protein synthesis in platelets. Freshly isolated platelets were cultured in the presence of an azido-amino acid analog of alanine (AHA), without (top) or with (bottom) nevirapine. In addition, select samples were pre-incubated with puromycin (AHA + Puromycin) or its vehicle (AHA) for two hours. The platelets were fixed after 6 hours and incorporation of the amino acid analog into protein was visualized (green stain). Unlabeled platelets fixed at baseline (left panel) were used to control for background immunofluorescence (scale bar = 10 μm). The bar graph displays the Cell Profiler analysis of the staining intensity for AHA (green) and is expressed as % change over AHA treated cells (±SEM; n=3). Single asterisk: p<0.05. This figure is representative of n=3 independent experiments. **(G)** Platelets isolated from healthy individuals (control) and persons with HIV were fixed in suspension. The microscopy panels display representative examples of platelets isolated from the two experimental groups, including representative panels from two persons with HIV (actin stain in green, scale bar = 10 μm). The bar graph displays the percent increase in the number of platelets with at least two cell bodies (as shown by the white bracket in the far right panel) in persons with HIV versus healthy individuals (control) (mean±SEM; n=3). Single asterisk: p<0.05.
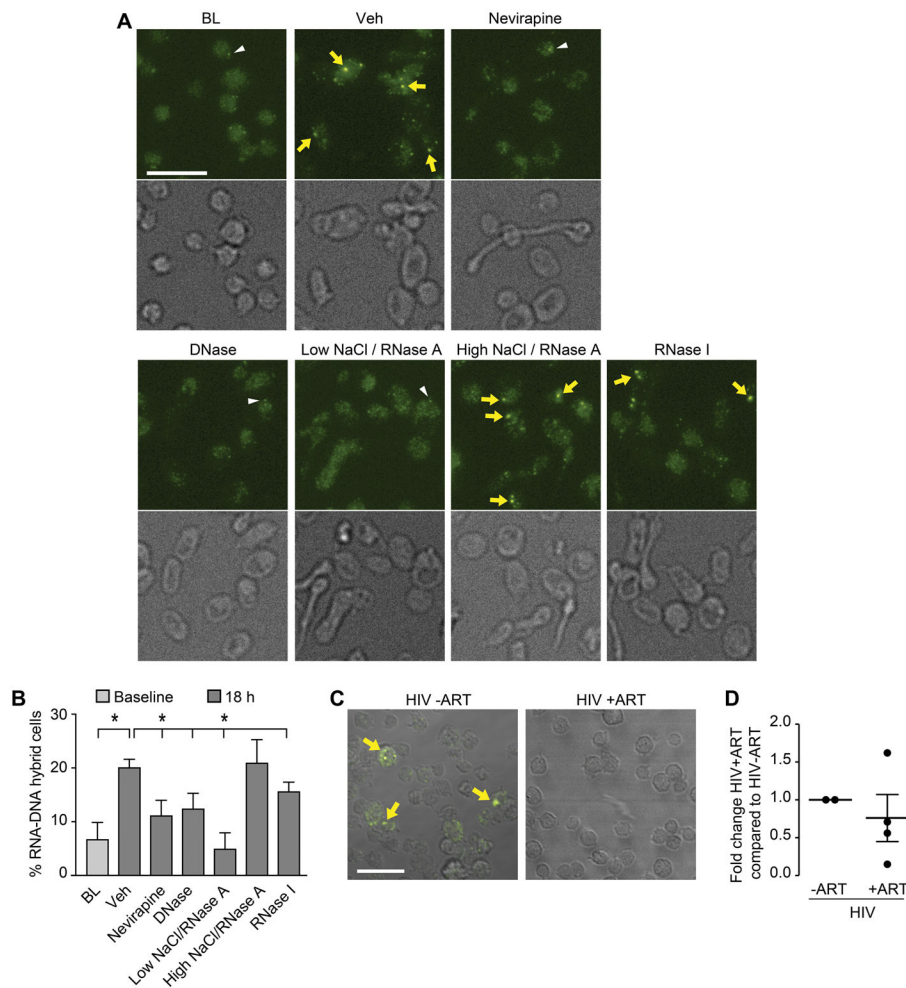
Figure 5. RNA-DNA hybrids are present in human platelets and increased in persons with HIV not treated with an RT inhibitor

(A) Immunofluorescence analysis of platelets stained with an anti-hybrid antibody. Platelets were fixed in suspension (BL), or treated with DMSO (Veh) or nevirapine (750 μM) and subsequently incubated for 18 hr. RNA-cDNA hybrids were detected using an anti-hybrid antibody (yellow arrows and white arrowheads). Post-fixation, platelets were treated with Turbo DNase (digests double strand DNA and DNA in hybrids), RNase A low (NaCl concentration at 70mM, digests single strand RNA, double strand RNA, and RNA in RNA-DNA hybrids), RNase A high (NaCl concentration at 500 mM, digests only single strand RNA and leave hybrids intact), or RNase I (which digests single and double stranded RNA). Scale bar = 10 μm. **(B)** The bar graph displays the percent of RNA-DNA hybrid positive cells (mean±SEM; n=3). Single asterisk: p<0.05, Veh compared to BL, nevirapine treated, low NaCl/RNase A, and RNase I treated cells. **(C)** Confocal images of platelets immunostained with an anti-RNA-DNA hybrid antibody (yellow, arrows). Platelets from persons with HIV before the initiation of treatment (HIV -ART) and persons with HIV on treatment (HIV +ART) were isolated and fixed in suspension. Scale bar = 10 μm. **(D)** The bar graph displays the fold change of RNA-DNA hybrid positive cells in samples from HIV –ART vs. HIV +ART (mean±SEM; n=2–4).
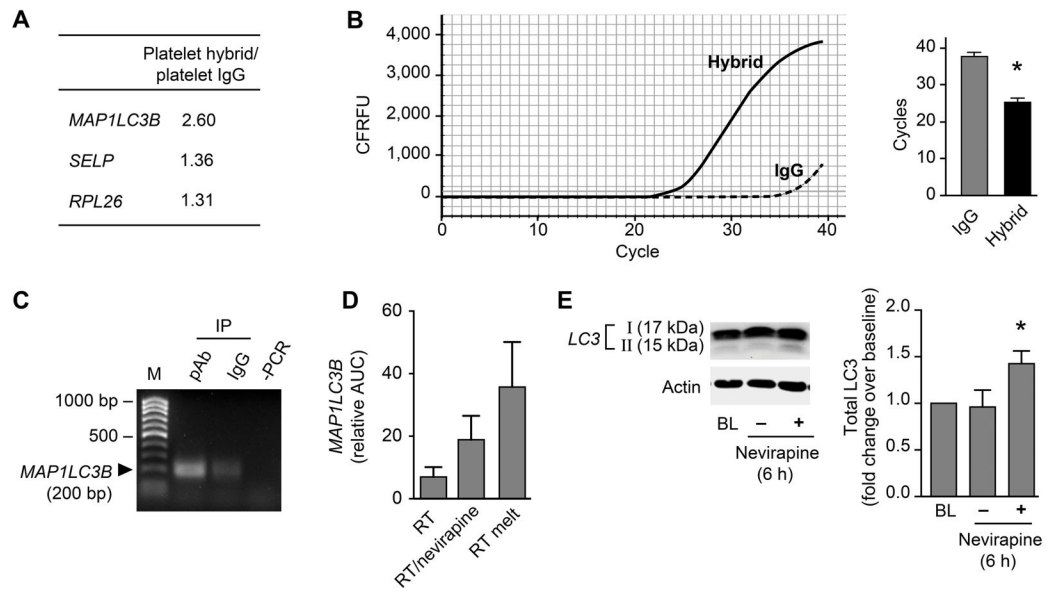
Figure 6. RNA-DNA hybrids serve as translational repressors in human platelets
(A) Fold enrichment of co-immunoprecipitated RNA-DNA hybrids that were sequenced with targets identified (mRNAs coding for *MAP1LC3B*, *SELP* – P-selectin, and *RPL26* – ribosomal protein L26) when comparing IgG vs. specific anti-hybrid antibody. **(B)** *MAP1LC3B* was verified using qPCR. An example of the real-time tracing is depicted on the left. The bar graph shows the change in cycle number threshold (mean±SEM; n=3, single asterisk: p<0.05). **(C)** RT-PCR analysis to demonstrate the presence of *MAP1LC3B* RNA in L1 ORF1p-T7 immunoprecipitates. T7-tagged L1 ORF1p was *in vitro* translated from the **pAD2TE1** expression plasmid. Platelet RNA was isolated and incubated with ORF1p-T7 tag, immunoprecipitated, and analyzed for bound *MAP1LC3B* RNA. pAb – IP using anti-T7 antibody, IgG – isotype control, -PCR – control for contaminants. This figure is representative of 3 independent experiments. **(D)** MAP1LC3B was *in vitro* transcribed, and the mRNA reverse transcribed using an RT-activity assay, however, with shortened extension time (15 min, RT). Select samples were pre-incubated with nevirapine (500µM, 45 min, 37°C, RT+nevirapine), or processed using a 2 min melting step (95°C, RT melt). The mRNA or RNA-DNA hybrids were subsequently introduced into an *in vitro* translation assay, and resulting MAP1LC3B protein was separated by SDS-PAGE and detected using an anti-myc-DDK tag antibody. The bar graph of the densitometric analysis is shown. **(E)** Platelets were lysed at baseline (0 hr), or treated with DMSO control or nevirapine (750 µM) and subsequently incubated for 6 hr. Total MAP1LC3B (LC3B) was detected by means of Western blotting. LC3 isoforms I and II were detected and analyzed using ImageJ. A representative Western Blot for LC3 and the corresponding control actin blot are depicted (left). The bar graph (right) displays the fold change of total LC3 of the 6 hr samples over baseline. Single asterisk: p<0.05. This figure is representative of n=4 independent experiments.