An antiviral process targeting HTLV-1: the complex relationship between HTLV-1 and nonsense-mediated mRNA decay

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Abstract

Before the adaptive immune response is established, retroviruses can be targeted by several cellular host factors at different stages of the viral replication cycle. This intrinsic immunity relies on a large diversity of antiviral processes. In the case of HTLV-1 infection, these active innate host defence mechanisms are debated. Among these mechanisms, we focused on a RNA decay pathway called nonsense-mediated mRNA decay (NMD), which can target multiple viral RNAs, including HTLV-1 unspliced RNA, as it has been recently demonstrated. NMD is a cotranslational process that depends on the RNA helicase UPF1 and regulates the expression of multiple types of host mRNAs. RNA sensitivity to NMD depends on mRNA organization and the ribonucleoprotein (mRNP) composition. HTLV-1 has evolved several means to evade the NMD threat, leading to NMD inhibition. In the early steps of infection, NMD inhibition favours the production of HTLV-1 infectious particles, which may contribute to the survival of the most fit clones despite genome instability; however, its direct long-term impact remains to be investigated.

Keywords : HTLV-1, RETROVIRUS, Antiviral process, Nonsense mRNA Decay, UPF1

1. Introduction

HTLV-1 is a delta-retrovirus infecting approximately 10 million people worldwide [1] Only 2%-

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5% of HTLV-1 carriers experience disease: either adult T cell leukaemia/lymphoma (ATLL), an aggressive form of leukaemia characterized by the proliferation of CD4+ T cells, or HAM/TSP, an inflammatory disease associated with demyelination of the spinal cord and the accumulation of HTLV-1-infected cells and CD8+ T cells directed against HTLV-1 in the central nervous system [2,3]). During infection, concomitantly with the initiation of the innate immune response and before the establishment of the adaptive response, retroviruses can be targeted by several cellular host factors at different stages of the viral replication cycle. This intrinsic immunity relies on a large and diverse set of antiviral processes involving restriction factors (RFs), generally described as type I IFN response-dependent [4,5]. First discovered and extensively studied for the case of HIV-1, there are fewer data available regarding how these antiviral factors can potentially restrict HTLV-1 replication and infectivity or how they can impact the latency of the virus. In fact, in the case of HTLV-1, involvement of most of the factors remains controversial, and the exact role of the innate host defence against HTLV-1 remains unclear [6]. In this review, we first describe what is known about how the antiviral process protects the host from HTLV-1 infection. Among these mechanisms, we focus on the nonsense-mediated mRNA decay (NMD) pathway, which has the capacity to target multiple viral RNAs, as has been recently demonstrated. In the second part of the review, we describe the NMD mechanism, its regulation and the features displayed by its RNA targets. In the final part of the review, we analyse how HTLV-1 protects itself against NMD and how the consequences of NMD inhibition converge with known outcomes of cell infection.

2. HTLV-1 infection cycle and host restriction factors (Figure 1)

HTLV-1 is a complex retrovirus that mainly infects CD4+ T cells, although it has the potential to infect a wide variety of other cells: CD8+ T cells, B lymphocytes, myeloid cells, endothelial cells, fibroblasts, neutrophils, monocytes, myeloid and plasmacytoid dendritic cells [7–10]. Cell-free HTLV-1 virions have poor infectivity, and the spreading of this virus depends mainly on two cell-to-cell

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transmission modes: viral biofilms and virological synapses [11,12]. After attachment to the cell membrane receptors GLUT1, HSPG or NRP-1 through the viral Env protein, a fusion process enables the release of the capsid core containing the viral genome and proteins into the cytoplasm [13].

2.1 TRIM family

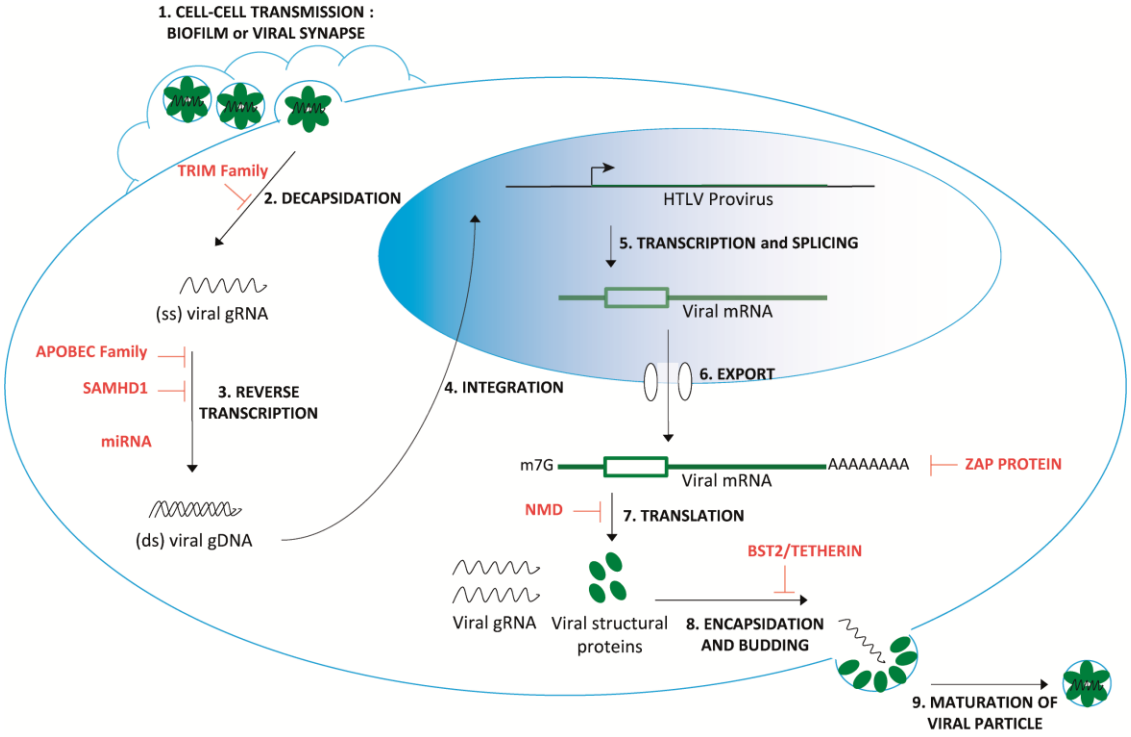

Figure 1. HTLV-1 infection cycle and host restriction factors (RFs)

In HIV-1 infection, shortly after viral entry and before reverse transcription, TRIM5α recognizes the capsid lattice and interferes with the disassembly of the viral particles. TRIM5α also exerts U3 ubiquitin ligase activity that triggers proteosomal degradation of the core components integrase and reverse transcriptase [5]. Although HTLV-1 has not yet been investigated at the mechanistic level, *Nozuma et al.* [14] recently described two rare TRIM5α variants that seem to correspond to differential proviral loads (PVLs), as indicated by the whole-exome sequencing analysis of a cohort of 113 HAM/TSP patients. Patients with the R136Q polymorphism, which is found at higher frequency in HIV-1-resistant individuals [15,16], had lower PVLs, whereas patients harbouring the H304L variant had higher PVLs [14]. Consistently, in a different HAM/TSP cohort, another study revealed a negative correlation

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between HTLV-1 virological parameters or clinical status and the expression of TRIM5α and TRIM22. Other members of the TRIM family, such as TRIM19/PML, were found to be negatively correlated with HTLV-1 Tax mRNA expression levels [17]. In line with previous work showing that TRIM19/PML interferes with HTLV-1 replication via Tax proteosomal degradation in ATL patients [18], all these data reinforce the potential involvement of the TRIM family in the early restriction of HTLV-1 replication.

Following entry, the viral RNA genome is reverse transcribed into double-stranded DNA, and together with associated proteins, this transcribed DNA forms the reverse transcription complex, also called the pre-integration complex. It is still unclear if, as is thought for HIV-1, HTLV-1 needs an uncoating capsid step and/or integrase and reverse transcriptase interactions to drive reverse transcription. However, this reverse transcription step is thought to be targeted by several host restriction factors such as APOBEC3G and SAMHD1 and, as more recently indicated, the microRNA miR-28-3p [13,19].

2.2 APOBEC family

For HTLV-1, the potential implication of the APOBEC3 subfamily (APOBEC3A to H) is one of the most well documented. The APOBEC3 subfamily is an ensemble of dC->U editing enzymes that can induce the conversion of cytosine to uracil by incorporating a G-to-A mutation in the retroviral genome resulting in premature stop codons [20,21]. It was first hypothesized that similarly to HIV-1, APOBEC3G (h3AG) could be packaged into HTLV-1 virions. Then, during the next round of infection, h3AG could inhibit HTLV-1 replication early during retrotranscription by binding viral ssDNA or RNA, leading to a G-to-A mutation in the newly synthesized minus viral DNA strand or degradation of the dU-rich reverse transcript [22,23]. Although the first study demonstrating this incorporation of APOBEC3G used HTLV-1 virions [23] and although the HTLV-1 genome may be edited *in vivo* by h3AG, as well as other hA3 members (A, B, C, F, and H) [24,25]), HTLV-1 appears to be more resistant to APOBEC3G than HIV-1 due to a motif in the HTLV-1 Gag protein that considerably reduces the packaging of h3AG into HTLV-

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1 virions [26]. HTLV-1 mainly replicates by Tax-driven clonal expansion, rather than by reverse transcription, and consistently, G-to-A mutations were not detected in the proviruses from infected patients, supporting the finding indicating limited effects of the APOBEC family in HTLV-1 restriction [24]. Nevertheless, for ATL and asymptomatic carriers (ACs), it was hypothesized that h3AG generates nonsense mutations in the viral genome that enable HTLV-1-infected cells to escape from the host immune system. In a cohort of 60 ATL patients, in whom the authors observed loss-of-expression mutations in several viral genes, including Tax, the HBZ oncogene remained intact [27]. Consistently, *Leal et al*. [17] pointed to a large "antiviral cluster", a negative correlation between different members of the APOBEC3 family and Tax mRNA levels, as having greater impact than PVLs, clinical status or HAM/TSP parameters. Interestingly, an increase in h3AB, but not h3AG, was recently reported in HTLV-1-infected humanized mice exhibiting ATL-like features [28], a finding in line with that of another integrated molecular analysis showing increased h3AB expression in large ATL and AC cohorts [29]. On the other hand, similar levels of all APOBEC3 enzymes were found in HAM/TSP and healthy donor cohorts [28], and no significant differences were observed in the clinical status, PVLs or viral genome mutations regarding the APOBEC family member expression in the *Nozuma et al.* HAM/TSP cohort [14]. All these data indicate that the involvement of the APOBEC family in the restriction or pathogenesis of HTLV-1 infection remains unresolved.

2.3 SAMHD1

SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase that normally converts dNTPs to nucleosides in a single step. By decreasing the pool of available dNTPs to a level insufficient to support reverse transcription, SAMHD1 was able to restrict a broad range of retroviruses, including HIV-1, during the early reverse transcription step [30,31]. In addition, SAMHD1 might have the ability to modulate antiviral activity by inhibiting the NF-κB and interferon pathways [32]. Regarding HTLV-1, *Gramberg et al.* [33] demonstrated that, in macrophages and cycling CD4+ T cells, the virus was not blocked by SAMHD1 and that Tax was produced. On the other hand, HTLV-1 infection of human

primary monocytes triggered a SAMHD1-mediated apoptotic response through cellular recruitment of STING, a sensor of reverse transcription intermediates [34], consistent with the monocyte depletion observed in HTLV-1-infected patients [35,36]. *Leal et al* retrieved SAMHD1 in a large antiviral cluster, while no significant polymorphism of SAMHD1 seemed to be associated with the HAM/TSP in patients [14], emphasizing the need for further studies to assess the implication of SAMHD1 in HTLV-1 restriction and/or pathogenesis.

2.4 miRNAs

As previously demonstrated for HIV-1 [37,38], the role of miRNA in HTLV-1 infection has recently emerged [39]). An example is miR-28-3p, which was found to be an inhibitor of the Tax-independent post-entry step, likely by preventing the formation of the pre-integration complex. miR-28-3p targets a conserved sequence localized within the genomic viral mRNA of HTLV-1 strains [40].

2.5 BST2/tetherin

BST2/tetherin inhibits the release of enveloped viruses by tethering them to the cell surface after the budding process is complete. As described for HIV-1, the nascent virions of HTLV-1 can be tethered by BST2 to infected cells at the membrane, where they undergo endocytosis and degradation [41]. Indeed, in chronically HTLV-1-infected cells, BST2/tetherin is highly expressed, but its silencing negligibly impacts cell-cell transmission, which was demonstrated to be the main route of HTLV-1 transmission [12]. Interestingly, in the large antiviral cluster described in the *Leal et al.* study, BST2 was part of the most significantly anti-correlated targets in the HAM/TSP cohort. In contrast, the results from the whole-exome sequencing analysis of the HAM/TSP cohort in the *Nozuma et al.* study did not reveal any significant mutations/polymorphisms in tetherin, thereby eliminating BST2 from consideration as an effective restriction factor of HTLV-1 replication.

2.6 ZAP

The zinc-finger antiviral protein (ZAP) targets viral RNA at specific response elements (such as ZRE) and recruits cellular decay factors, including the PARN deadenylase, Dcp1 de-capping enzyme, and the 3'–5' exosome. Recently, Miyazato *et al*. demonstrated that HTLV-1 is susceptible to the host antiviral system, which depends on ZAP-mediated viral RNA processing [42] and might be triggered by the burst of HTLV-1 expression observed during early infection. The authors suggest that this post-transcriptional regulation of HTLV-1 transcripts could be leveraged to minimize viral antigen expression, a strategy through which HTLV-1 achieves persistent infection in the host [43].

2.7 NMD

Another RNA decay process studied since the late 1970s, known as nonsense-mediated mRNA decay (NMD), was recently verified as an intrinsic cellular antiviral process and proposed to target HTLV-1. As described in detail herein, NMD is a cotranslational process that is highly regulated and organized around the RNA helicase UPF1 and its enzymatic activities. The initial suggestion that NMD is an antiviral pathway was based on studies of the retroviral Rous sarcoma virus [44–46] and was corroborated in multiple studies on RNA viruses: potato virus X (alfaflexiviridae) and turnip crinkle virus (Tombusviridae). Infections of *A. thaliana* and *N. benthamiana*, respectively, in which the *upf1* gene (the central factor of NMD) was mutated, led to higher levels of genomic viral RNA compared to those expressed by the *wild-type* (wt) *upf1* control plants [47,48]. These observations support an antiviral function, and this effect was often observed at the beginning of infection. Semliki Forest virus (Togaviridae) replication is increased (more viral proteins and virions are released) after UPF1 knockdown, similar to West Nile virus, Dengue virus and Zika virus (Flaviviridae) [49–51]. Finally, a recent study demonstrated that cytoplasmically synthesized mouse hepatitis virus (Coronaviridae) sub-genomic RNA was susceptible to NMD, and its stabilization by NMD factor silencing was also associated with enhanced replication [52]. Recently, the impact of NMD on HIV *gag-pol* RNA was also demonstrated: while UPF1 was necessary for the export, translation and reverse transcription of gag RNA, its silencing provoked a decrease in the level of this RNA [53,54]. However, the knockdown of 2

other NMD factors, UPF2 and SMG6, conversely led to an upregulation of gag RNA and GAG protein levels in Jurkat and monocyte-derived macrophages, revealing an antiviral function of NMD with respect to HIV [55,56]. The HTLV-1 was shown to be sensitive to NMD since RNA interference targeting key proteins (such as the RNA helicase UPF1) led to an upregulation in the steady state levels of all viral mRNAs, revealing a direct and/or indirect sensitivity to NMD [57]. A complementary study subsequently validated the direct involvement of NMD by measuring the stability of unspliced genomic RNA (gRNA), exposing an inverse correlation between UPF1 levels and viral gRNA stability. The knockdown of the NMD factor UPF2 was also associated with an increase in the GAG protein encoded by the full-length gRNA [58]). Altogether, these data indicate that NMD targets the RNAs of multiple viruses, including HTLV-1, thus revealing a function for NMD in host protection against pathogens.

In the following sections, we describe the NMD mechanism, focusing on its regulation in the cell and the definition of its RNA targets. Then, we discuss the ways in which HTLV-1 RNA matches these standards and how the cellular consequences of HTLV-1 protection against NMD converge with what is known about HTLV-1 biology.

3. NMD in the cell.

3.1 Mechanism

3.1.1 From translation termination to NMD initiation.

NMD is a cotranslational pathway conserved through evolution from yeast to mammals and leads to the degradation of mRNA. It is initiated when translation is terminated in a specific unfavourable environment. Translation termination is usually prompted when the ribosome reaches and reads the first stop codon leading to the recruitment of the release factor eRF1 to ribosome site A. Then, upon GTP hydrolysis, eRF3 simulates eRF1 to provoke peptide release [59]. Finally, the recruitment of the

ABCE1 factor leads to the dissociation of the ribosome subunits [60,61]. Translation termination is then a turning point where the ribosome dissociates or RNA undergoes decay induced by NMD.

The slowing of those terminating steps due to mRNA sequence, organization and/or mRNP composition greatly favours the retention of the NMD central factor UPF1 at the stop codon with the stalled ribosome, leading to NMD activation. For instance, converging studies demonstrated that the longer the distance between the stop codon and the poly(A) tail associated with PABPC, the higher is the rate of NMD initiation leading to RNA decay. PABPC binds eRF3 and plays an important role in mRNA circularization by binding eIF4G, facilitating translation termination and re-initiation on the same RNA strand, thereby opposing NMD initiation [62–64]. Competition between PABPC1 and UPF1 for the eRF3 binding supports these observations [65–68].

UPF1 is a helicase that binds RNA without sequence specificity. It appears to be displaced by the translating ribosome and subsequently accumulates on the mRNA 3'UTR [69,70]). It is not yet clear whether UPF1 plays a direct role in translation termination despite its association with eRF1 and eRF3, but it is an indispensable factor of NMD initiation. Recent studies have shown that another NMD factor, UPF3B, can inhibit translation termination (without UPF1) and favours post-termination complex dissociation *in vitro* [71]. UPF1 also interacts with UPF3B, mutually increasing the stability of them both at the stop codon. UPF3B is also associated with the exon junction complex (EJC), which marks splicing events and might shuttle from the nucleus to the translation site together with the RNA.

The interaction of UPF1 with the SMG1 PI3K kinase at the terminating ribosome completes the NMD initiating complex, which is named SURF (SMG1-UPF1-release factors) and is thought to also play a role in delaying translation termination. SMG1 phosphorylates UPF1 on the S/TQ residues and is regulated by two factors: SMG8 and SMG9 [72–74].

3.1.2 UPF1 triggers mRNA degradation.

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Next, UPF1 phosphorylation is stimulated by the recruitment of UPF2 and DHX34 [75–77]. UPF2 may be recruited to the vicinity of UPF1 through an interaction with UPF3B [78,79]. UFP2 binds UPF1, inducing the large conformational change necessary for triggering UPF1 ATPase and helicase activity [80]. UPF1 hyperphosphorylation at its Nter and Cter (CH and SQ domains, respectively) creates scaffolds to recruit degradation-promoting factors such as the endonuclease SMG6 [81]. SMG6 cleaves RNA at the vicinity of the stop codon, leading to unprotected 5' and 3' RNA fragments, which are then degraded by XRN1 and the exosome [82]. Phospho-UPF1 can also recruit the heterodimer SMG5-SMG7, which is directly linked to deadenylation (with CCR4-NOT) or de-capping (with DCP1a and DCP2) activity [83–85]. Well characterized *in vitro*, the *in vivo* steps necessary for UPF1 enzymatic activity have not yet been elucidated but could play a role in stripping proteins from the 3' cleavage products

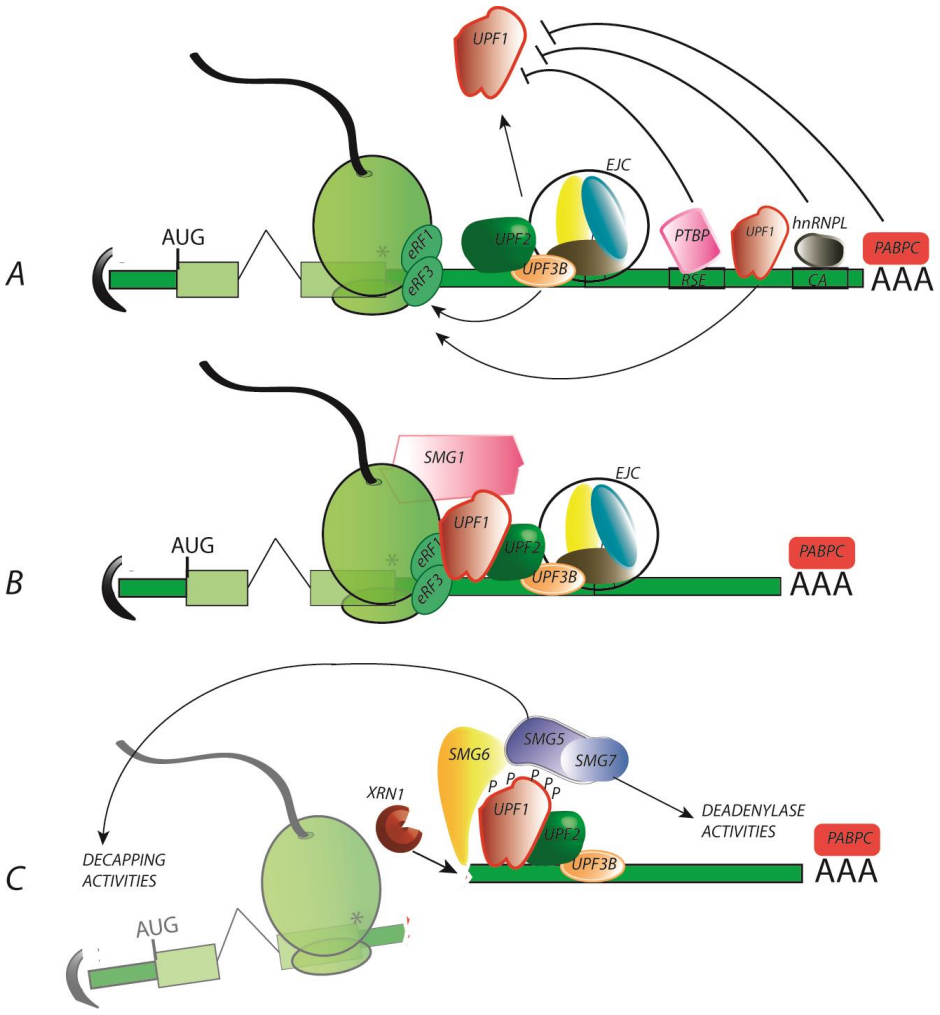

Figure 2 : The NMD mechanism in 3 key steps

to enable XRN1 exonuclease activity. UPF1 may also be involved in complex remodelling, controlling the necessary sequence of steps leading to decay [86]. In the last step, UPF1 is unphosphorylated by PP2A and dissociates from the target mRNP [87].

3.2 NMD regulation in the host cell.

Closely related to translation termination, NMD efficiency is naturally also modulated by mRNA organization and mRNP composition. For example, a longer 3'UTR moves the poly(A) tail bound to PABPC1 away from the termination codon, decreasing the translation termination efficiency, favouring UPF1 accumulation and eliciting NMD [69,70,88,89]. However, the intramolecular secondary structures of RNA can also bring the poly(A) tail closer to antagonize NMD [65,67]. Polypyrimide track binding protein1 (PTBP1), when associated with RNA downstream of a stop codon, inhibits NMD by preventing UPF1 recruitment [90]. It binds a specific sequence known as the RNA stability element, which was first identified in the Rous sarcoma virus RNA and more recently found in many cellular 3'UTR sequences close to the termination codon. Similarly, mRNA bound with hnRNPL can evade NMD [91]. Additionally, the presence of an EJC is a critical parameter: when located downstream of the stop codon (at least at 50 nt to allow termination codon reading by the ribosome without being sterically removed), the EJC promotes the enrichment of the 3'UTR in NMD factors such as UPF3B and UPF2, leading to strong stimulation of NMD (reviewed in [92–94]). EJC was also described as an enhancer of SMG6-mediated endonucleolysis [95]. Moreover, different compositions of the EJC have been observed and associated with NMD modulation. For instance, the replacement of RNPS1 by MLN51/barentsz causes a reduction in NMD efficiency [96]. While the EJC can be considered the most important NMD stimulator, multiple mRNAs can, nevertheless, undergo NMD in an EJC-independent manner [96]. Finally, NMD displays great variability (although they all require UPF1), and although alternative NMD pathways without UPF2 or UPF3 have been described [97,98], all are organized around translation termination delay, namely, through UPF1 stabilization followed by its activation and the recruitment of degradation factors (Figure 2).

According to the above description, any stop codon, as soon as it is read by the ribosome, might be able to initiate NMD, the outcome being dependent on the equilibrium between the stimulators and the inhibitors at each step. The unique function of NMD is to degrade mRNA. However, depending on the origin of the stop codon initiating NMD, two main functions are usually associated with NMD: RNA quality control and gene expression regulation at the post-transcriptional level (for an exhaustive review, see [99]).

3.3 NMD targets and functions (Figure 3)

*3.3.1 NMD controls mRNA quality***.**

It is estimated that 5-30% of human transcripts have a premature termination codon (PTC), which may be the result of genomic mutations (nonsense and frameshift), faulty alternative splicing (approximately 30% of alternative splicing events) or translational errors. The truncated protein can be deleterious for the cell since it is non-functional, or it may even have a dominant negative effect. In reality, PTCs are present inside the ORF, (a) creating a longer 3'UTR, (b) are distant from the physiologic stop codon and the related translation termination regulatory environment and (c) may be upstream of exon-exon junctions marked by an EJC. As described above, these 3 parameters greatly favour NMD triggering and lead to the elimination of the PTC-harbouring mRNA.

3.3.2 NMD controls gene expression at the posttranscriptional level.

In addition to the quality control function, in which NMD prompts the cell to systematically degrade "aberrant" mRNA, NMD can also periodically regulate specific "non-aberrant" mRNA. This intermittent sensitivity to NMD is a programmed cellular protocol based on the local modification of an mRNA sequence, organization and/or mRNP composition. Thus, NMD regulates ~15% of normal physiologic mRNA. Six "families" of non-aberrant mRNA are subject to NMD-induced regulation:

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(a) mRNA with a uORF: a uORF, present in ~50% of human transcripts, can be translated depending on the ribosomal density [100,101]. The termination at the uORF stop codon triggers NMD due to the downstream EJC in the main ORF. CREB-2/ATF4 mRNA is a well-documented example [102–104].

(b) mRNA with a long 3'UTR and (c) mRNA with a 3'UTR EJC. The 3'UTR organization is finely controlled by alternative 3'end formations. The 3'UTR can be lengthened or shortened by intron retention or alternative polyadenylation, also modifying its size and secondary structure. The 3'UTR length and structure are major characteristics that trigger NMD, as mentioned above. Similarly, alternative

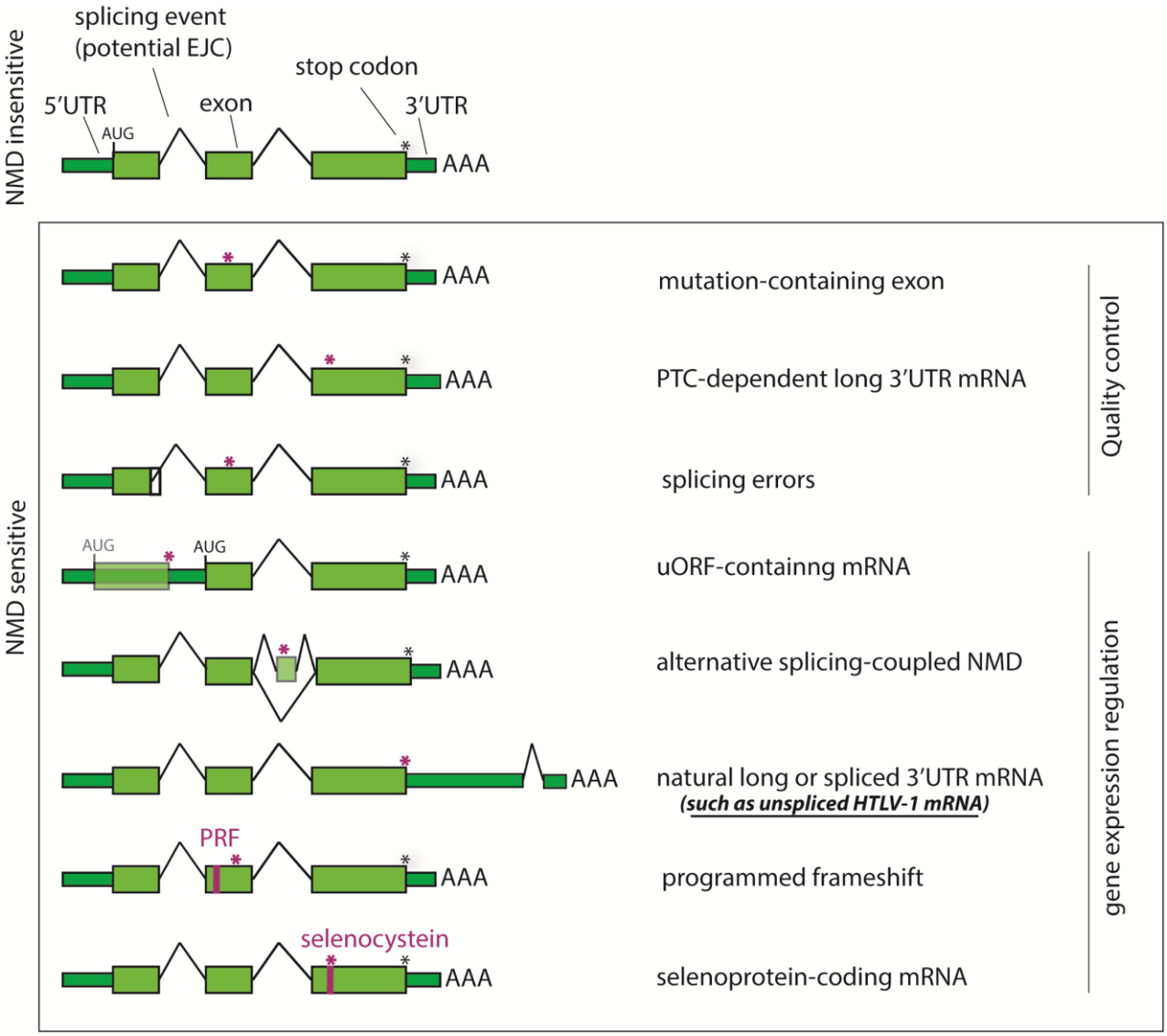

Figure 3: The architecture of NMD substrates

splicing events occurring downstream of the stop codon may lead to the deposition of an EJC in the 3'UTR, strongly stimulating NMD [89,105].

(d) mRNA isoforms generated from intron/exon skipping or retention. Alternative splicing (AS) and NMD are closely related: in addition to 3'end shaping, AS can also periodically modify the body of an ORF, producing alternative isoforms bearing a PTC. Multiple transcripts have been identified with high-throughput approaches and documented in databases. Several types of DNA repair or splicing factors, such as SR proteins, are regulated by this AS/NMD pathway, indicating an expanded indirect impact of NMD on post-transcriptional regulation [106–109].

(e) mRNA with programmed ribosomal frameshifts (PRFs): PRFs specifically reposition a ribosome from one reading frame to another with variable efficiency. In more than 95% of the cases, the new reading frame redirects the ribosome towards a PTC (compared to directing to the original ORF), leading to NMD. For instance, the translation of the CC chemokine receptor type 5 mRNA, as well as several interleukins, is subjected to PRF. The rate of frameshifting is greatly increased by miRNA binding and leads to PRF-induced NMD [110].

(f) mRNA coding selenocysteine. Selenocysteine is encoded by a UGA codon. Under low concentrations of selenocysteine, this codon is read as a stop codon, eventually leading to NMD [111].

4. When HTLV-1 confronts NMD

4.1 HTLV-1 viral mRNA exhibits NMD-initiating features.

HTLV-1 is a complex retrovirus with multiple proteins coded in a single genomic RNA. The overall sensitivity of HTLV-1 retroviral RNA to NMD has been known for a few years [57,58]. However, the molecular determinants or RNA features triggering NMD sensitivity have not yet been clearly defined [112]. Additional work is needed to identify which stop codons are the most likely to induce NMD.

Direct regulation: As described above, the half-life of unspliced HTLV-1 RNA is increased under NMD inhibition. How can this sensitivity be explained? Since the mRNA is unspliced, it should not have bound

an EJC, especially in the 3'UTR, which is expected to hamper its sensitivity to NMD. However, according to the above findings and our knowledge of HTLV-1 RNA organization, the 3'UTR size stands out as a possible factor. While the median human 3'UTR size is ~750 nt [113], the gag mRNA 3'UTR is ~4000 nt, making it a suitable target for NMD. For instance, Garcia *et al*. showed that 2 (+) RNA viruses had genomic and sub-genomic RNA NMD sensitivity because their 3'UTRs ranged from 1 kb to 2.5 kb [47]. In contrast, the secondary structure of RNA and its mRNP composition can also compete for the long 3'UTR to have an effect on sensitivity. For example, the unspliced RNA from the retroviral Rous sarcoma virus is protected from NMD due to a short sequence downstream from the GAG stop codon, favouring the recruitment of the PTBP1 protein that antagonizes UPF1 recruitment to the terminating ribosome, although UPF1 is enriched due to the long 3'UTR [90].

The unspliced mRNA encodes the GAG, GAG-PRO and GAG-PRO-Pol fusion proteins. Another noteworthy parameter of HTLV unspliced mRNA (and that of other retrovirus) is the -1 frameshift (-FS). HTLV-1 has 2 successive -1FS in the unspliced mRNA region, allowing for the synthesis of 3 different polyproteins (see above). The low frequency of these -1FSs maintains the correct ratio of GAG, GAG-PRO and GAG-PRO-POL proteins. By slowing ribosome reading and preventing translation termination, a FS can promote NMD. On the other hand, Hogg *et al*. suggested that retroviral readthroughs and frameshifting (even at a rate of approximately 1%), by redirecting the ribosome and avoiding the in-frame stop codon, destabilize UPF1 accumulation in the 3'UTR and impair NMD [88]. In the case of HTLV-1, the treatment of infected cells by okadaic acid (OA) led to increased levels of unspliced viral RNA associated with UPF1 [58]. OA prevented the dephosphorylation of active UPF1 molecules and suspended NMD in the latest steps of mRNA decay. These observations strongly suggest that UPF1 is not stripped from the unspliced viral RNA and that this RNA is subjected to active decay.

Indirect regulation: In cells transfected with an HTLV-1 molecular clone, the knockdown of UPF1 led to a 3-4-fold homogeneous upregulation of all viral RNAs in the steady state [57]. This homogeneity might suggest that the transcriptional activator(s) of HTLV-1 can be controlled by NMD, leading to the indirect

regulation of viral mRNA. Supporting this hypothesis, CREB-2/ATF4 has been shown to be involved in LTR transactivation, regulated by NMD and stabilized by Tax at the post-transcriptional level [57].

While the sensitivity of HTLV-1 to NMD is well documented, the underlying reasons for this sensitivity remain open questions. Moreover, to maintain its capacity to replicate, HTLV-1 had to evolve solutions to evade the NMD threat.

4.2 HTLV-1 protection against NMD.

The arms race involving HTLV-1 and NMD led to the evolution of two viral countermeasures. The viral proteins Tax and Rex were shown to target the NMD process in what is called “trans-inhibition”, incapacitating the decay of viral as well as cellular NMD targets (the opposite of “cis-inhibition” where the inhibition protects only the viral RNA (for example, the RSE of RSV)). This evidence emerged from observations that HTLV-1-infected lymphocytes were able to specifically stabilize globin mRNA with a PTC [57]. Nakano *et al*. also showed that, in HeLa cells co-cultivated with HTLV-1-infected cells, this inhibition was maintained as long as Tax/Rex mRNA was expressed[58].

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Tax was first described as the main viral transactivator [114]. It has the capacity to bind multiple host factors in the nucleus and in the cytoplasm, leading to dysfunctions promoting cell transformation [115,116]. Tax-dependent NMD inhibition was initially investigated due to its interaction with the translation initiation factor eiF3E/INT6 [117], known to interact with UPF2 and to be involved in NMD [118]. In addition to this interaction, INT6 was observed to be delocalized. This study also revealed contact between Tax and several NMD factors and a direct interaction between Tax and the RNA helicase UPF1. A complementary study introduced interesting details on Tax: first, Tax can bind to the helicase domain of UPF1 at the exit of the RNA binding channel, preventing UPF1 loading onto its target. Second, when UPF1 is already bound to RNA (due to its action in NMD), Tax binding blocks ATP hydrolysis and helicase activity, freezing UPF1 on RNA. These observations suggest a broad effect on UPF1 with the capacity to impact NMD at different steps [119]. When analysing viral mRNA, it is difficult to dissociate the transactivation role of Tax on the viral promoter from its post-transcriptional effect via NMD. Therefore, a mutant form of Tax specific for NMD interference must be engineered.

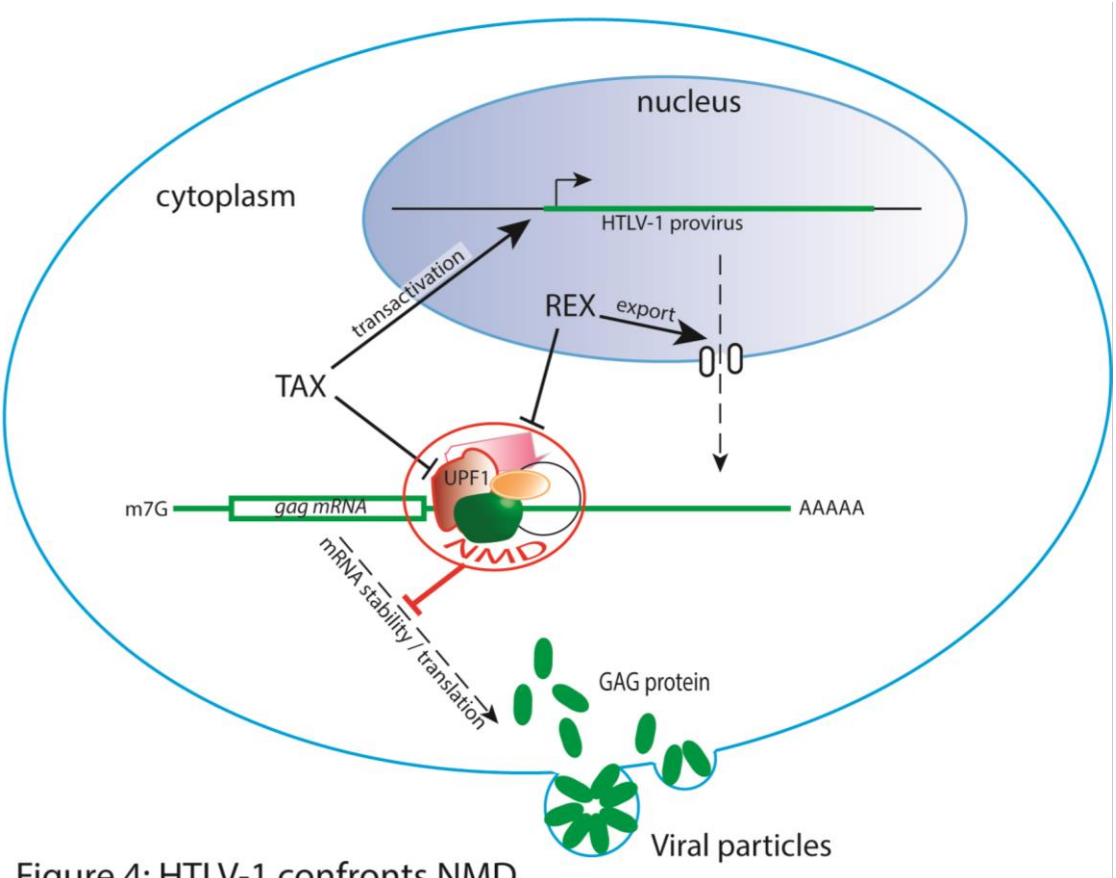

Figure 4: HTLV-1 confronts NMD .

Nevertheless, when Tax is expressed alone or from a provirus (with other viral proteins), the half-lives of host mRNAs, such as CREB-2/ATF4, GADD45A, and SMG5, are stabilized as a consequence of NMD trans-inhibition.

The Rex protein was also shown to inhibit NMD. Similarly to Tax, several host mRNAs known to be NMD sensitive had increased half-lives upon Rex expression. Rex is known to bind viral RNA at the RxRE motif. Upon binding to RxRE, Rex controls viral mRNA splicing. It also contacts the CRM1 export system to ensure the nucleo-cytoplasmic shuttling of the unspliced viral mRNA [120–122] To date, the mechanism of NMD inhibition by Rex has not been described.

It has not yet been investigated whether the HTLV-1 RNA secondary structure provides a first line of defence against NMD (Figure 4).

4.3 When does NMD inhibition occur during HTLV-1 infection?

During infection, HTLV-1 is spread in two different ways: viral propagation is initially dependent on cell-to-cell transmission and evolves towards oligoclonal and monoclonal expansion (reviewed elsewhere [13]). Cell-to-cell infection depends on virion production. These proteins are composed of structural proteins translated from singly spliced mRNA (ENV) and non-spliced viral mRNA (GAG). Tax, as the viral transactivator, is indispensable for the production of this mRNA. Additionally, the modulation of splicing, leading to the stabilization of viral non-spliced mRNA, as well as their nuclear export, depends on Rex. By targeting the *gag* non-spliced mRNA, NMD prevents virion formation. Supporting this supposition, knockdown of UPF2 was associated with increased levels of the p24 and p19 GAG protein [58]. Interestingly, HIV *gag* mRNA and GAG protein expression were also shown to be affected by UPF2 and SMG6 expression in the context of virus reactivation [56]. Hence, it is understandable that NMD, which does not require induction by type I interferon and thus acts as a cell-intrinsic antiviral barrier, plays a role in the early steps of HTLV-1 infection. The results of an analysis of viral mRNA kinetics showed a clear separation between the early phase when Tax and Rex

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(p21 and p27) are produced and the later phases characterized by the increase in other mRNAs, including *gag* mRNA [123]. This finding suggests that the stabilization of *gag* mRNA and the production of the gag protein necessitate the formation of a favourable environment: NMD inhibition might contribute to this initial condition. Notably, the results from kinetics experiments performed with RNA(+) virus infections suggest that the impact of NMD inhibition on viral RNA is greater in the early steps of the infection [47,52]. Finally, it is striking how the same factors, Tax and Rex, are involved in both the production and the protection of viral particles.

The second mode of provirus amplification is clonal expansion, which depends on the proliferation of a few select clones. This oncogenic behaviour emerges from the modulation of multiple cellular processes by non-structural viral proteins; notably, Tax plays an essential role in this cellular transformation by generating instability and bypassing checkpoints: it inhibits DNA repair, disrupts cell cycle progression, and affects autophagy. It also modulates transcription through the modification of the epigenetic landscape and transcription complex composition and deregulates signalling pathways, including NF-κB (inducing its constitutive activation) and innate immune pathways (with an

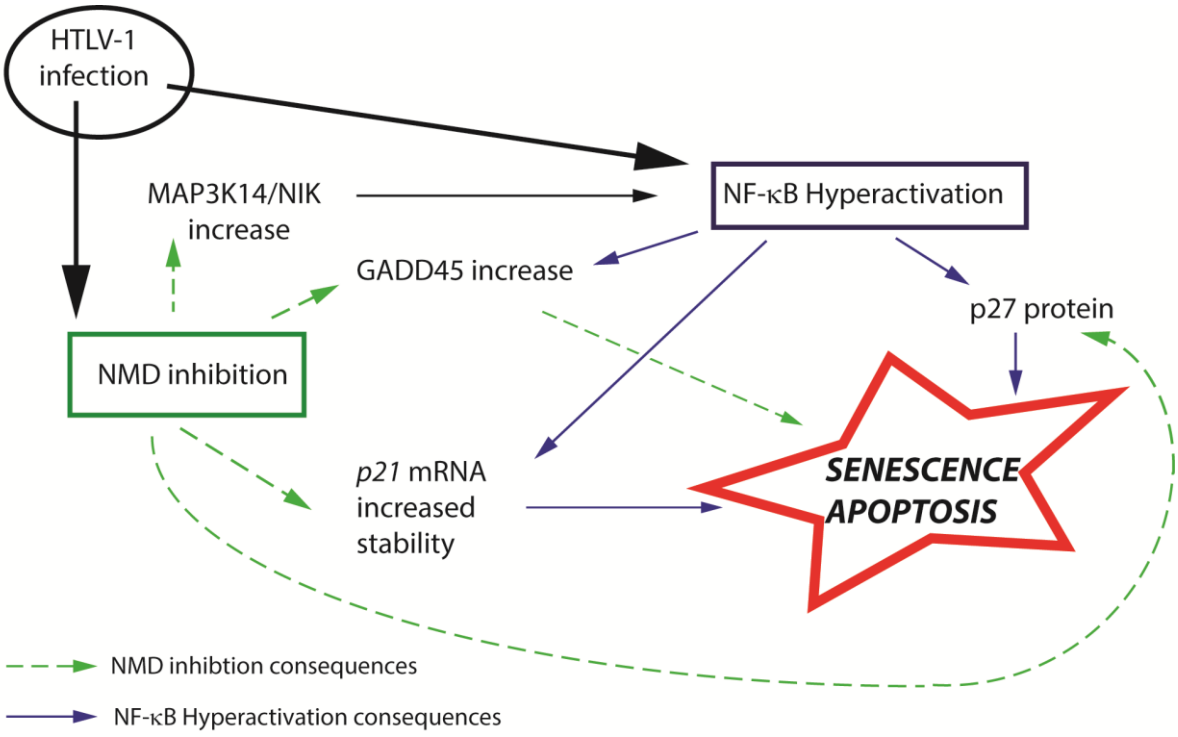

Figure 5: Convergence of NMD inhibition and NF-κB Hyperactivation.

immunosuppressive effect). While Tax expression is progressively inhibited or in occasional bursts [124], HBZ is constitutively expressed in infected cells and plays important roles in viral latency and the proliferation of infected cells. Interestingly, *hbz* RNA appears to play specific roles in T cell proliferation, in contrast to the HBZ protein (reviewed in [125]).

However, although it is widely accepted that HTLV-1 infection leads to cell proliferation, it has been frequently observed to cause apoptosis and senescence of lymphoid and non-lymphoid cells in a Tax-dependent manner. It has been proposed that cells responding by senescence or apoptosis have very high levels of expressed Tax and Rex, are associated with robust replication of the virus and are subjected to great oncogenic pressure. This viral hyperactivity might be dependent on the site of integration, stimulating LTR transactivation and thus may be specific to each clone. Senescence induction has been shown to be linked with the stabilization of the p27 protein and $p21^{CIP/WAF1}$ mRNA because of NF-κB hyperactivation ([126–128]; reviewed in [129]). NF-κB activation is a hallmark of Tax-expression and HTLV-1-infected cells, and multiple steps in the NF-κB pathway were shown to be regulated directly or indirectly by Tax. For example, NIK (MAP3K14), the NF-κB inducing kinase that activates IKKα, is highly expressed in HTLV-1-infected cells. Moreover, p21, p27 and NIK transcripts are known to be NMD targets. Their mRNA levels are tightly controlled, and NMD inhibition experiments induced their stabilization [104,130,131]. In lung inflammatory myofibroblast tumours, decreased NMD leads to the increased expression of the transcript for the NIK protein kinase, which activates the NF-κB pathway and promotes cytokine expression and inflammation [132]. Supporting this observation, the stabilization of the NIK mRNA half-life was observed in a separate Tax- and a Rex-dependent manner [58,119]. Additionally, it is worth noting that NMD is involved in the apoptotic response induced by high stress levels; more precisely, it has been demonstrated that maintained NMD inhibition induces cell death [133,134]. Growth arrest and DNA damage-inducible 45 (Gadd45) isoforms promote apoptosis by upregulating the mitogen-activated protein kinase (MAPK) signalling

pathway upon the activation of diverse forms of stress, including that from TNFα and DNA damage. Gadd45α and β transcripts are dependent on canonical as well as non-canonical NF-κB pathway activation and are upregulated in Tax- and HTLV-1-expressing lymphocytes [135]. Gadd45α and β mRNA are also sensitive to NMD due to their respective 3'UTRs. This regulation of Gadd45 by NMD is evolutionarily conserved from flies to mammals. It was recently shown that the upregulation of Gadd45 isoforms due to NMD inhibition is a major contributor to NMD-associated programmed cell death [136]. Altogether, these observations suggest that, in the context of HTLV-1 infection, high levels of Tax and Rex expression, viral-induced senescence/apoptosis and NMD inhibition might be correlated. However, NMD inhibition *in vivo* does not always lead to the activation of apoptosis; for example, the absence of the key NMD factors UPF1 and SMG1 induces embryonic cell death, but UPF3-null mice are viable. Similarly, during haematopoiesis, NMD inhibition by UPF2 knockdown prevents haematopoietic stem cell and progenitor survival, whereas mature cells are only mildly affected [137–140]. Thus, whether NMD inhibition can activate apoptosis might be dependent on the degree of the NMD inhibition, the cell type and +differentiation state. While in the early steps of infection, HTLV-1 directly benefits from NMD inhibition to produce infectious particles, it is conceivable that, in a second step, this NMD trans-inhibition also participates in the selection of a clone with attenuated expression of Tax and Rex that is more suited to avoiding the immune response and thus maintain the infection (Figure 5). This outcome would be in line with the recently observed expression of plus strand viral RNA by short bursts of transcription [124].

4.4 HTLV-1-associated pathologies. How can NMD inhibition impact the host in the long term?

As introduced above, HTLV-1 is the aetiological agent of ATL and HAM/TSP. ATL is a malignant lymphoproliferative syndrome established after decades of latency and characterized by genetic instability combined with checkpoint adaptation. A favourable environment enabling malignant proliferation is dependent on the establishment of an immunosuppressive state. The viral protein Tax plays a major role in these steps. HAM/TSP is associated with the accumulation of HTLV-1-specific CD8+

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T cells and infected CD4+ T cells in cerebrospinal fluid and neural tissues. It is characterized by a chronic inflammatory state due to elevated cytokine expression and production (reviewed in [13,141]). We wondered whether NMD downregulation, induced for the early infective stage of HTLV-1, may play a role in the later steps of the infection and could converge with ATL or HAM/TSP onset.

In 2015, genomic sequencing of 400 ATL samples showed that the mutation rate for ATL was relatively high compared to other haematologic malignancies, with an average of 2.3 mutations per megabase in coding regions [29,142]. Notably, GATA3, which is required for multiple steps of T-cell differentiation in both developing thymocytes and mature T cells, is commonly affected by nonsense and frameshift mutations. The authors suggest that these mutants confer altered protein function (possibly dominant negative functions) rather than GATA3 haploinsufficiency. Most CCR4 and CCR7 mutations in ATL-related proteins cause truncation of the cytoplasmic domain with gain of function. Moreover, more than half of ATL cases have either nonsense or frameshift mutations in the components of the MHC class 1 molecule. Although the NMD sensitivity of mRNA resulting from these hotspot mutations has not yet been analysed, these mutations seem to be promising targets, and NMD inhibition by Tax and Rex might be an important parameter to consider.

Of course, due to its quality control function, NMD defects contribute to genetic instability: in combination with alternative splicing, NMD tunes the level of many DNA repair factors [108,143,144]. Pancreatic squamous carcinoma cells have mutations in the *Upf1* gene, allowing the synthesis of mutated dominant negative p53, functionally correlating NMD inhibition with cancer[145]. Loss of function or overexpression of NMD proteins is also associated with several other cancer types, including colorectal cancer, hepatocellular carcinoma and neuroblastoma [146–148]. NMD is also involved in the adaptation to stress response [149,150]. Hypoxia, amino acid depravation and reactive oxygen species production downregulate NMD, which leads to the stabilization of transcripts such as ATF4, ATF3, ATF6, CHOP and TRAF2, which re-establish homeostasis by the integrated stress response (ISR). It is now acknowledged that tumour cells must adapt to micro-environment stresses such as

these to proliferate. In this context, it has been shown that NMD downregulation plays a role in this adaptation, promoting tumorigenesis [151]. Moreover, it was also recently proposed that NMD tunes the immune response. Impaired NMD in mice with forebrain-specific UPF2KO triggers immune response activation and results in exacerbated neuroinflammation. The latter symptom was partially reversed upon UPF2 restoration [152]. In Arabidopsis, NMD downregulation due to bacterial infection has been shown to control the turnover frequency of numerous TIR domain-containing, nucleotide-binding, leucine-rich repeat (TNL) receptor mRNAs, inducing innate immunity. However, maintained NMD inhibition by silencing NMD components deregulates homeostasis, leading to an autoimmunity phenotype characterized by stunting, spontaneous formation of necrotic lesions, and elevated salicylic acid levels [153]. The role of NMD in cancer and immunity likely depends on the tissues implicated and the associated genomic stress.

However, to clarify whether there is an effective link between NMD and HTLV-1-associated pathologies, the status of NMD in the later stages of the infection and in patient cells (ATL and HAM/TSP) has yet to be analysed. The absence of plus strand transcription in most ATL cells due to epigenetic repression and genomic alterations raises the following questions: Is the burst of plus strand expression sufficient? HBZ, the protein expressed from the HTLV-1 minus strand, is a critical component of cell proliferation and tumorigenesis and maintains constant expression during infection, in contrast to Tax and Rex; could it also be involved in NMD inhibition? NMD is also sensitive to bivalent cation concentrations [154], and interestingly, ATL patients show hypercalcemia; does this suggest that NMD can be constitutively inhibited? Without additional experimental data, these questions remain unanswered.

5. Concluding Remarks

NMD initiation is determined by the conditions of translation termination. It targets host mRNA as well as exogenous mRNA. In an attempt to reduce its genome size, a virus, including HTLV-1, depends on a polycistronic RNA, which tends to have a long 3'UTR and is a critical NMD determinant. To protect its

mRNA, HTLV-1 evolved at least three independent mechanisms to trans-inhibit NMD through two viral proteins: Tax and Rex. The strength of these countermeasures shows the size of the threat that NMD constitutes for viral replication and the extent to which it delineates part of the cellular antiviral function.

In addition, we wonder which partner truly benefits from NMD inhibition during infection. The virus, in the early steps, because it is able to produce viral particles? The host, since NMD inhibition establishes the immune response? Is the apoptosis induced from NMD inhibition contributing to the clonal selection involved in leukaemia emergence? Similarly, does NMD inhibition contribute to the genetic instability and cell adaptation associated with HTLV-1, as observed in NMD-related cancers? All these questions have yet to be addressed in detail.

Finally, multiple “NMD-like” processes have been recently discovered, and they depend on the RNA helicase UPF1 and factors recognizing specific RNA features. In addition to NMD, UPF1 is involved in replication-dependent histone mRNA decay, miRNA decay, glucocorticoid receptor-mediated mRNA decay and regnase1-mediated mRNA decay (RMD) (reviewed in [155]). Since evidence has shown that Tax inhibits UPF1 enzymatic activity by preventing its association with RNA [119], we can hypothesize that other RNA decay pathways that depend on UPF1 are also likely altered, extending the impact of UPF1 on HTLV-1 deregulation. Notably, RMD plays a critical role in immunity and inflammation with the downregulation of multiple transcripts, such as IL-6, IL-2, IL-1b, TNFR2, CD44, and c-Rel, which are also often characterized in HTLV-1 infection [141,156].

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Figure Legends

Figure 1: **HTLV-1 infection cycle and host restriction factors (RFs).** During HTLV-1 infection, the retroviral cycle is targeted by several host factors that restrict different replication steps. Immediately after cell entry (1), the TRIM Family could limit the de-capsidation step by recognizing determinants of the capsid (2). Then, reverse transcription (3) could be restricted early by SAMHD1, decreasing the available pool of cellular dNTPs, by APOBEC members that can misedit the HTLV-1 genome by binding to retro-transcription intermediates (RTIs), and as recently suggested, by miRNA that likely prevents the formation of the pre-integration complex. To date, no host RFs have been fully characterized regarding the integration (4), transcription and splicing (5) or RNA export (6) steps. However, viral mRNA can be targeted at the post-transcriptional level by the ZAP protein and at the translational level by the NMD process (7). Finally, BST2 can tether nascent virions at the budding step (8) before the release and maturation of the viral particle (9).

Figure 2: **The NMD mechanism in 3 key steps.** The NMD mechanism is organized into approximately 3 key steps. A) Translation termination delay: NMD is initiated when translation termination is delayed.

Multiple factors can be involved, notably by preventing/promoting the recruitment of the RNA helicase UPF1 to the ribosome. B) UPF1 recruitment and stabilization favours the assembly of an active NMD complex. The EJC is a very potent stimulator of NMD initiation, bringing UPF2 and UPF3 in close vicinity to UPF1. Although EJC-independent NMD events occur, the details of the mechanism continue to be debated. C) UPF1 phosphorylation by SMG1 initiates the decay step with the recruitment of the endonuclease SMG6 or/and the SMG5/SMG7 complex. SMG6 cleavage is followed by XRN1 degradation, while SMG5 and SMG7 initiate de-capping and deadenylating activities, respectively.

Figure 3: **The architecture of NMD substrates.** Representation of NMD substrate organization. The black "*" is the natural stop codon. The purple "*" is the stop codon triggering NMD. Upon triggering of a stop codon, the NMD associated functions are initiated: RNA quality control for aberrant RNA or gene expression regulation. NMD substrates are also classified on the basis of whether an EJC is downstream of the triggering stop codon (EJC-dependent or EJC-independent NMD).

Figure 4: **HTLV-1 confronts NMD.** NMD is able to target viral gag mRNA, preventing further formation of viral particles. However, the viral proteins Tax and Rex, which are involved in viral transactivation and unspliced viral mRNA nuclear export, respectively, have been shown to inhibit NMD. The Rex mechanism of action has yet to be deciphered, while several approaches have revealed that Tax was shown to target UPF1.

Figure 5: **Convergence of NMD inhibition and NF-κB hyperactivation.** HTLV-1-induced NMD inhibition and NF-κB hyperactivation might induce apoptosis and senescence via the stabilization/stimulation of the same host factors.

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