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Passport control for foreign integrated DNAs

An unexpected checkpoint by class II HDAC4 revealed by amino acid starvation

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The endless battle between mammalian host cells and microbes has evolved mechanisms to shut down the expression of exogenous transcriptional units integrated into the genome with the goal of limiting their spreading. Recently, we observed that deprivation of essential amino acids leads to a selective, reversible upregulation of expression of exogenous transgenes, either carried by integrated plasmids or retroviral vectors, but not of their endogenous counterparts. This effect was dependent on epigenetic modifications and was mediated by the downregulation of the class II histone deacetylase-4 (HDAC4). Indeed, HDAC4 expression inversely correlated with that of the transgene and its inhibition or downregulation enhanced transgene expression. Could this be true also for "naturally" integrated proviruses? We investigated this question in the case of HIV-1, the etiological agent of AIDS and we observed that both amino acid starvation and HDAC4 inhibition triggered HIV-1 reactivation in chronically infected ACH-2 T lymphocytic cells (HDAC4$^+$), but not in similarly infected U1 promonocytic cells (HDAC4-negative). Thus, an HDAC4-dependent pathway may contribute to unleash virus expression by latently infected cells, which represent nowadays a major obstacle to HIV eradication. We discuss here the implications and open questions of these novel findings, as well as their serendipitous prelude.

Keywords: amino acid starvation, HDAC4, HIV latency, HIV eradication, transcription, epigenetics, ocular albinism

Abbreviations: For a full listing of abbreviations, see page 237.

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Commentary

Serendipity unveils a new pathway. Progress in science sometimes requires serendipity, i.e., "making discoveries by accident and sagacity of things not sought."[1] Serendipity often hits in a field unrelated to the one under investigation and requires an open mind toward unexplained or odd results, as well as a positive attitude when facing novel scenarios. The initial observation leading to our study derived from a typical case of scientific serendipity, since initial experiments on transgene expression upon amino acid starvation were actually performed while investigating a previous observation related to the ocular albinism type 1 protein (OA1).[2] In particular, Lopez and colleagues reported that tyrosine deprivation determined the upregulation of an OA1 transgene expressed by means of a plasmid vector in COS7 cells. In the authors' interpretation, this finding was attributed to the specific functional features of OA1, a G protein-coupled receptor.[3] Since the putative OA1 ligand, i.e., L-DOPA, is structurally similar to tyrosine, in the presence of this amino acid OA1 could be constitutively internalized and downregulated, while in its absence the receptor would recover its expression.[2]

Our laboratory has a long-lasting interest in OA1, and since previous studies by us and others on this protein had been performed in standard culture conditions, it was crucial for us to unequivocally establish the role of tyrosine in OA1 regulation: if tyrosine was really an agonist,

all studies thus far would have analyzed a constitutively downregulated receptor! Thus, we performed a number of controls, including the use of other essential amino acids (structurally unrelated to L-DOPA), and the analysis of endogenous OA1 and other exogenous and endogenous proteins. The results were puzzling: in the absence of tyrosine, but also of other critical amino acids such as methionine and cysteine, exogenously expressed OA1 was strongly upregulated. However, the upregulation concerned exclusively the exogenous protein, but not endogenous OA1, while other exogenous unrelated proteins, such as GFP and LAMP1, were upregulated as well. This effect was caused by transcriptional derepression of the integrated transgenes and was mediated, at least in part, by the specific downregulation of a class II histone deacetylase (HDAC), namely HDAC4.[4] HDACs represent a large family of enzymes, comprising four distinct structural/functional classes, and are responsible for removing acetyl groups from particular lysine residues of histones, although they may also play different roles.[5] Since histone acetylation is a common epigenetic mark, generally associated with a relaxed and transcriptionally active chromatin state, the function of HDACs is rather to promote chromatin compaction and transcriptional repression.[5] Thus, a serendipitous finding allowed us to jump from the biology of albinism to the mechanisms of epigenetic surveillance operated by mammalian cells to protect their DNA from the invasion of retroviruses and other parasitic nucleic acids.

We tested the effect of starvation on different transgenes, carried by plasmids or retroviral vectors, under the control of either viral or human promoters. All of them were indeed de-repressed by amino acid depletion, suggesting the existence of a general response aimed at silencing exogenous non-native genetic material recently integrated (in evolutionary terms) into the host genome. How a transgene is recognized as "exogenous material" might depend on the specific chromatin remodeling subsequent to the insertion of new sequences into the genome and possibly on specific epigenetic signatures that are attributed to silenced foreign sequences.

Genome-wide epigenetic control of retrotransposable elements. In addition to recently integrated sequences, our genome also contains a huge amount of anciently integrated and silenced parasitic elements, including DNA transposons and retrotransposons, which are now "endogenized" and can play structural and functional roles, but can occasionally cause pathology by reactivation and insertional mutagenesis or by genomic rearrangements.[6,7] This raises the question of whether transposable elements are also responsive to amino acid restriction. Among them, retrotransposons are of particular importance for their contribution to human evolution since they constitute a large fraction of eukaryotic genomes, including about 45% of human genome.[8] Retrotransposons include two main groups: LTR retrotransposons and non-LTR retrotransposons. LTR retrotransposons (or endogenous retroviruses) are a vestige of past infections of germ cells by viruses that lost their infectivity and became entrapped into the genome. Non-LTR retrotransposons mainly include LINEs, SINEs, SVAs.[6] The retrotransposon abundance in the genome reflects the battle between their tendency to expand and the control by the host, which neutralizes their expression by defense mechanisms involving RNA interference and epigenetic silencing.[9] Only a small fraction of these genetic elements, such as LINE1, Alu and SVAs, are currently mobile moving into new genomic positions, eventually leading to mutations and disease.[6,8]

However, transposable elements are also considered as "dark energy"[10] for their impact on shaping and plasticity of the genome, since DNA rearrangements caused by their insertion give rise to a genetic variability important for cell adaptation and selection. Indeed, reactivation of silenced transposable elements has been associated with a number of stressful stimuli that could represent a challenge for the survival of eukaryotic cells, including temperature changes, UV irradiation, infections and exposure to DNA damaging agents.[9-11] It is tempting to speculate that the deprivation of essential amino acids triggers a genomic stress response, promoting activation of a pathway that favors genetic variability, not only because this kind of stress seriously compromises cell survival, but perhaps also because it directly affects the functioning and "readability" of the DNA code. If amino acid starvation and its pathway through HDAC4 only affects recently integrated elements or even more ancient mobile elements remains an important unsolved issue, with relevance for both potential genome-wide epigenetic effects of diets, anorexia or famine, and for the correct evaluation of the risk-benefit ratio related to the use of specific class II HDAC inhibitors (HDACi) in clinical applications, as discussed further.

Integrated HIV provirus is read as a transgene by the cell response to amino acid starvation. HIV-1, the etiological agent of the acquired immunodeficiency syndrome (AIDS), infects cells of the immune system expressing both CD4 and a chemokine receptor (usually CCR5 or CXCR4) on their cell surface, namely a subset of T lymphocytes (and their precursors), mononuclear phagocytes and myeloid dendritic cells. In vivo, the infection leads to a progressive depletion of $CD4^+$ T cells (but not of mononuclear phagocytes or dendritic cells) that represents the best marker (and partially a determinant) of the profound state of immunological deficiency that, in the absence of combination anti-retroviral therapy (cART), leads to death by opportunistic infections and tumors. The virus can replicate and kill the infected cells, but also establish a relatively small pool of latently infected cells, including resting memory $CD4^+$ T cells, which are responsible for rekindling the infection at therapy suspension. These cells (and others, such as tissue macrophages) are considered nowadays "public enemy number 1" preventing the achievement of either a sterilizing or a functional cure of HIV infection.[12]

The establishment and maintenance of HIV-1 latency involves several molecular and cellular factors. After HIV-1 DNA is integrated into the host genome, the chromatin conformation at the transcription start site can influence whether the provirus becomes transcriptionally active.[13] The presence of nucleosomes in certain areas of the 5'-LTR significantly restrains HIV-1 provirus transcriptional

activity[14] and makes histone modifications regulatory mechanisms of HIV-1 expression. Indeed, the constitutive deacetylated state of Nuc1 mediated by recruitment of HDACs contributes to keep the HIV-1 provirus latent. In this scenario, the role of HDACs in HIV-1 latency and the implication for the use of HDACi in strategies involved in provirus reactivation has inspired several studies aimed to understand how to eradicate HIV infection. Starting from Van Lint and colleagues,[15] several compounds were tested for HDAC inhibition and HIV purging (TSA, TPX, Na-Butyrate, Valproic Acid, Oxamflatin, SAHA, CG05-CG06, ITF2357)[16] demonstrating that these molecules can indeed reactivate HIV-1 transcription, with different concentration profiles and kinetics. Up to now, only class I HDACs have been reported to have a prominent role in HIV-1 latency by means of their direct binding the HIV LTR.[17,18]

Only a few studies have dealt with the roles of class II HDACs in HIV-1 infection and latency regulation. HDAC6 was shown to affect HIV-1 infection by suppressing the viral Env-mediated cell fusion process through deacetylation of α-tubulin, inhibiting HIV-1 entry into cells.[19] Later, HDAC6 was shown to deacetylate the HIV regulatory protein Tat, affecting its transactivation activity on the provirus.[20] HDAC4 was reported to associate with HIV-1-based lentiviral vector DNA in infected HeLa cells and to form foci at the site of integration, with a likely involvement in the post-integration repair system, without excluding a role in the regulation of transcription.[21]

In this scenario, our findings argue for a clear contribution of HDAC4, but not HDAC6, in the regulation of HIV-1 proviral expression. Indeed, we studied the role of this enzyme in two well-established models of HIV-1 post-integration latency, the T-lymphocytic ACH-2 and the promonocytic U1 cell lines. These cell lines were originated by infecting their parental CD4$^+$ cells, the CEM T cell line and U937 promonocytic cell line, respectively, with the CXCR4-dependent HIV-1 strain LAI/IIIB.[22,23] They both share a basal state of relative latency (i.e., little or no virus is produced by unstimulated cells) that can be promptly reverted to full virus production by phorbol esters or tumor necrosis factor- α (TNF-α), or several other cytokines (in the case of the U1 cells), as reviewed in ref. 24. Further studies have highlighted that both cell lines carry proviruses defective in the Tat/TAR trans-activation elements,[25,26] which are responsible for promoting transcriptional elongation, although other factors of cellular origin can mediate this process. Therefore, these cell lines can also serve as models of Tat-independent regulation of HIV expression at transcriptional and post-transcriptional level. Crucial for this study, while ACH-2 express HDAC4, U1 cells do not, therefore providing an excellent internal control for addressing the potential role of HDAC4 in HIV-1 latency.

Consistently, amino acid limitation enhanced HIV-1 expression only in ACH-2 cells. Moreover, class II HDACi (MC1568 or MC1575) significantly increased the levels of H3 acetylation at the HIV promoter, followed by enhanced HIV-1 transcription and viral protein expression in ACH-2 cells, without affecting the state of viral latency in U1 cells. In addition, specific pharmacological inhibition of HDAC6 by MC2726 did not influence HIV-1 expression in ACH-2 or U1 cells, both expressing the enzyme, therefore excluding a role for this class II HDAC in the control of provirus reactivation, at least in these cellular models.[4]

HDAC4 is highly expressed in the brain, chondrocytes, heart and skeletal muscle[27] and to a minor extent in other tissues, although not ubiquitously. With regard to HIV-1 target cells, the enzyme might indeed be more expressed in T cells compared with myeloid cells, as suggested by our preliminary observations (**Fig. 1** and unpublished results), conferring more specificity to the action of class II HDACi. This could represent a favorable circumstance, considering that the best characterized, and perhaps most important, latently infected cells are resting memory CD4$^+$ T cells; however, tissue macrophages as well as circulating monocytes have been also identified as relevant candidate reservoirs. Thus, future studies are clearly required to better characterize the expression of HDAC4 in primary T lymphocytic and myeloid cell subsets of both HIV-1-infected and -uninfected individuals, in order to properly evaluate the efficacy, as well as potential side effects, of HDACi.

Our study becomes now part of an interesting scenario in which HDACi are credited as major players in the search for an HIV-1 eradication regimen.[12] Indeed, recently vorinostat (VOR), a class I HDACi already used for cancer therapy, was reported to be a promising drug for reactivation of latently infected resting CD4$^+$ T cells in HIV-1$^+$ patients under cART and with levels of plasma HIV RNA below 50 copies per millilitre.[28] A single oral administration of VOR (400 mg) induced the upregulation of HIV RNA expression in the resting CD4$^+$ T cells isolated from the tested patients 4–7 h later, although no "blips" of HIV RNA levels in blood (viremia) were observed during the course of this experimental therapy.[28] Based on the low cytotoxic effects observed with our class II HDACi and on the efficacy of class I HDACi, the synergistic use of these compounds (VOR and MC1568) represents an interesting hypothesis to be tested.[4,28]

Our results shed new light on another important aspect of HIV-1 infection that, together with malaria and tuberculosis, is grouped in the so-called "poverty-related diseases." In fact, malnutrition increases the rate of HIV-1 disease progression, since poor dietary proteins and microbial translocation from the gut mucosa promote inappropriate/exaggerated activation of both T lymphocytes and innate immune cells.[29-31] Accordingly, cART is severely impaired in HIV-1$^+$ adults with advanced malnutrition and immunosuppression, as underlined in a study performed in Zambia.[32] Underfeeding and high viremia levels in HIV-1$^+$ individuals represent important predictors for acquisition of *Mycobacterium tuberculosis* and for a reduced capacity to mount adaptive immune responses.[33] Unfortunately, only a few prospective studies have been published on nutritional support in HIV disease, as reviewed in reference 34, and none of these focused on the role of essential amino acids in regulating viral replication, which therefore represents an important area of future investigation.

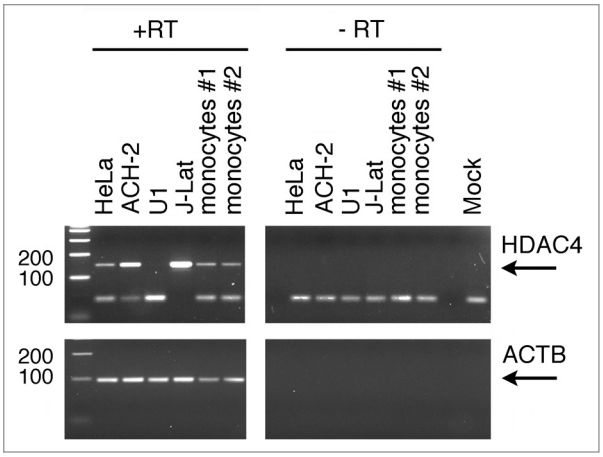

Figure 1. HDAC4 expression in different cell lines and in primary monocytes. PCR amplification of HDAC4 and b-actin (ACTB) transcripts in different cell lines and in primary monocytes from two different healthy donors (#1–2). Mock, amplification in the absence of template; RT, reverse transcriptase.

Epigenetic control by HDAC4: open issues. Many viruses, such as herpesviruses, remain episomal and can undergo silencing processes as part of their infective life cycle. An open question is whether HDAC4 regulates their latency as well. In this context, latency was correlated, among different mechanisms, with the chromatinization of the viral DNA after its entry into the nucleus, and with post-translational modifications of histones.[35] Interestingly, a role for class II HDACs in maintaining the latency of episomal herpesviruses has been proposed. In particular, HDAC4 and/or 5 have been found recruited on the promoters of different immediate early genes of Epstein Barr virus and murine γ-herpesvirus 68, acting as viral repressors.[36,37] Moreover, down-regulation of HDAC4 in murine macrophages infected with γ-herpesviruses was sufficient to activate the viral replication.[36] On the other end, viral proteins, such as Epstein Barr nuclear antigen 2 leader protein (EBNALP) and the Herpes simplex 1 protein ICP0 are known to interact with class II HDACs and relieve their repression.[38,39] Since herpesviruses can be reactivated by different signals, it remains to be investigated whether they can be de-repressed by amino acid starvation and/or HDAC4 inhibition.

The link between starvation and HDAC4 expression and regulation needs to be further explored. Amino acid starvation induced a progressive downregulation of HDAC4 expression that could be promptly reverted by the reintroduction of depleted amino acids in the culture medium. How is this regulation achieved? It is known that the activity of HDAC4 at the protein level is modulated in response to different conditions, such as serum starvation, muscle denervation[40] and cell differentiation,[41] by post-transcriptional and post-translational mechanisms involving miRNA production,[41] HDAC4 poly-ubiquitination and degradation[42] and HDAC4 proteolytic processing.[43] In contrast, little is known about the mechanisms regulating HDAC4 expression at the mRNA level except that Sp1 and Sp3 transcription factors are involved.[44] Our microarray analysis did not reveal any significant fluctuation in the mRNA levels of Sp1, Sp3 or other Sp transcription factors in amino acid starvation condition, but we cannot exclude that these proteins could be regulated by post-translational modifications. Thus, the investigation of the signaling cascade leading to regulation of HDAC4 transcription in response of amino acid restriction might reveal novel regulatory mechanisms.

The expression of the transgene and HDAC4 during starvation and recovery are strongly inversely correlated. While HDAC4 expression is progressively reduced during starvation concomitantly with the upregulation of the transgene, its expression raises gradually again in the recovery phase, when the transgene becomes repressed. However, the two kinetics are not perfectly mirror-like. In addition, pharmacologic inhibition or knockdown of HDAC4 reactivates the transgene expression, but to a minor extent compared with that induced by amino acid deprivation. These observations suggest that the enzyme might not be the only player, and/or a role in the process is also mediated by mechanisms modulating the function of HDAC4 in addition to its expression. Indeed, beyond the conserved catalytic domain, class II HDACs have a long N-terminal extension, containing a set of conserved serine residues whose phosphorylation creates binding sites for the cytosolic 14–3-3 chaperone protein, eventually resulting in cytoplasmic accumulation and allowing an efficient coupling between HDAC activity and cell necessity.[45]

How does HDAC4 work in the maintenance of transgene silencing? Our study shows that the enzyme is recruited on the CMV promoter of the transgene encoding vector and that its inhibition by MC1568 results in increased acetylation of histone H3 at the LTR promoter of HIV-1. It is known that class IIa HDACs have a weak enzymatic activity and they generally work acting as scaffolds for complexes containing catalytically active class I HDACs.[46] However, the transgene de-repression obtained by enzymatic inhibition of class II HDACs or by downregulation of HDAC4, but not by that of single class I HDACs, suggest that HDAC4 might maintain transgene silencing directly by means of its own deacetylase activity. Nevertheless, we cannot exclude the possibility that the pharmacological inhibitor might provoke the displacement of other putative interactors or that it might affect the levels of HDAC4 expression, as previously described.[47] The use of deacetylase mutants of HDAC4[48] would be an important discriminating proof to test the requirement of an enzymatic activity for the role of HDAC4 in transgene silencing.

How HDAC4 binds exogenous promoters remains to be investigated. Interestingly, HDAC4 has been reported to inhibit gene expression in a Sp1-dependent way.[49] Since Sp1 is a constitutively expressed host transcription factor playing a crucial role in the synthesis of

HIV-1 RNA and in Tat-driven transcription,[50] it might represent a potential candidate for HDAC4 recruitment, at least in the case of the HIV-1 provirus. Independently on the specific mechanism, the role of HDAC4 in the maintenance and perhaps establishment of transgene silencing remains restricted to the very cells in which the enzyme is expressed, given that HDAC4 is not ubiquitous and silencing can also be achieved and broken in cells that do not express it.

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Abbreviations

AIDS, acquired immunodeficiency syndrome; cART, combination antiretroviral therapy; CCR5, C-C chemokine receptor 5; CD4, cluster difference 4; CXCR4, CXC chemokine receptor 4; CMV, cytomegalovirus; EBNALP, Epstein Barr nuclear antigen 2 leader protein; Env, envelope protein; GFP, green fluorescent protein; HDAC, histone deacetylase; HDACi, HDAC inhibitor/s; HIV, human immunodeficiency virus; ICP0, infected cell protein 0; LAMP1, lysosome-associated membrane protein-1; L-DOPA, L-3,4-dihydroxyphenylalanine; LINEs, long interspersed elements; LTR, long terminal repeat; Nuc1, positioned nucleosome; OA1, ocular albinism type 1 protein; Sp, specificity protein; SAHA, suberoylanilide hydroxamic acid; SINEs, short interspersed elements; SVA, SINE-VNTR-Alu; Tar, TAT responsive region; TAT, transactivator of transcription; TNF-α, tumor necrosis factor- α; TPX, trapoxin; TSA, trichostatin A; VOR, vorinostat

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