Essential Role of Cyclin D3 in dNTP Pool Control and HIV-1 Replication in Macrophages

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ABSTRACT

Background:

Cyclins control the activation of cyclin-dependent kinases (CDK), which in turn, control cell proliferation. We have shown that the virus restriction factor SAMHD1 is controlled by CDK6, a CDK controlling early G0 to G1 transition of the cell cycle. However, the cyclin controlling the process of SAMHD1 function has not been clearly identified in primary cells.

Methods:

Monocytes were isolated and transfected with small interfering RNA (siRNA) against different cyclins controlling the cell cycle. Transfected monocytes were differentiated to macrophages using M-CSF. RNA interference was assessed by quantitative PCR (qPCR) and Western blot. Primary monocyte-derived macrophages (MDM) were infected with a VSV-pseudotyped NL4-3 GFP-expressing virus or full-replicative R5 HIV-1 strain BaL. Total viral DNA formation was quantified by qPCR and HIV replication measured by flow cytometry. SAMHD1 phosphorylation and protein expression were analyzed by immunoblotting. dNTP intracellular levels in siRNA-treated MDM were determined using a polymerase-based method.

Results:

RNAi of eight distinct cyclins led to efficient and specific downregulation (up to 90% efficacy) of the corresponding cyclin as compared to mock-transfected cells or cells transfected with a non-targeting siRNA in MDM. Downregulation of the CDK6-associated cyclin D3 showed the strongest inhibitory effect (p=0.0002) of HIV-1 replication and total viral DNA formation in acutely infected MDM. Cyclins D1 and E2 (associated to CDK6 and CDK2, respectively) had a lower inhibitory effect. Cyclin A2, B1, B2 and D2 did not block HIV-1 replication. Alternative siRNA sequences targeting cyclin D3 confirmed the effect of cyclin D3 in HIV-1 infection and total viral DNA formation. Cyclin D3 downregulation led to a significant (p<0.01) reduction of SAMHD1 phosphorylation (at residue T592), CDK2 activation as measured by phosphorylation of T160 and decreased intracellular dNTP levels. The effect of cyclin D3 RNA interference was lost after degradation of SAMHD1 by HIV-2 Vpx.

Conclusions:

Our results show that knockdown of cyclin D3 has a major impact in SAMHD1 phosphorylation, dNTP levels and HIV-1 reverse transcription and replication. Cyclin D3 is the catalytic partner of CDK6. Thus, our results indicate a fundamental role of the CDK6-cyclin D3 complex in SAMHD1-mediated virus restriction during GO to G1 transition. Our results suggest that agents targeting cell proliferation may prevent new rounds of infection and hypothetically, might prevent the proliferation of persistently infected cells.

EVALUATION OF CYCLIN AND CDK PROMISCUITY AND OVERLAP IN MONOCYTE DERIVED MACROPHAGES

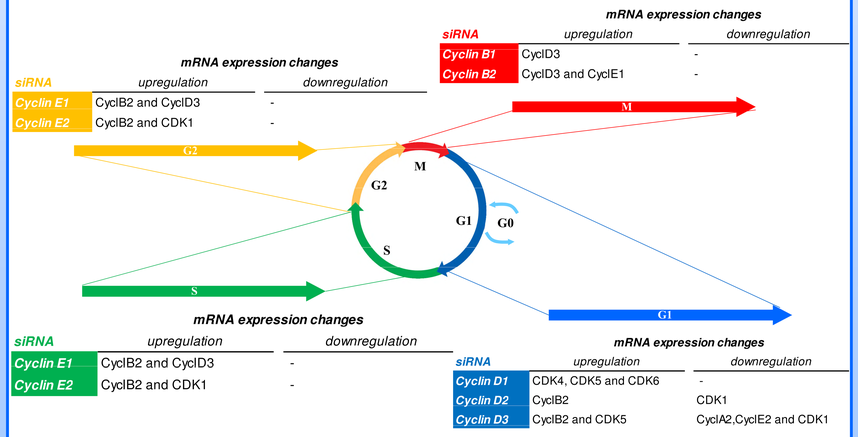


Figure 1. Cell cycle cyclin and CDK mRNA expression and their association to different stages of the cell cycle. Different phases of the cell cycle are driven by specific cyclins and their corresponding CDK. The figure depict the cyclins commonly associated to each phase of the cell cycle and the effect on cyclin or CDK expression of downregulating each cyclin through RNA interference .

DIFFERENCES IN HIV-1 INFECTION SUSCEPTIBILITY ARE MINIMIZED AFTER Vpx-MEDIATED DEGRADATION OF SAMHD1

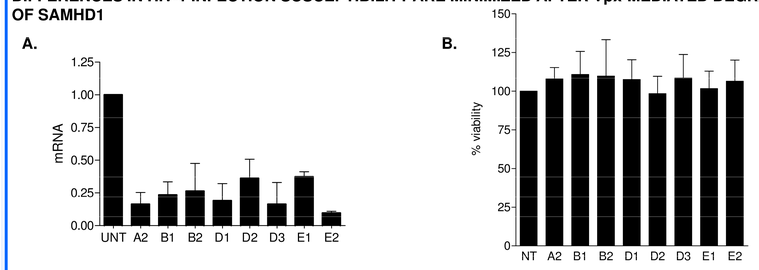


Figure 2. Effect of RNA interference of cyclins relevant to cell cycle on HIV replication. (A) A panel of siRNA were transfected in MDM and mRNA of the corresponding cyclin was measured by quantitative PCR and normalized to GAPDH expression. mRNA levels are shown compared to a non-transfected sample (Mock). **(B)** Primary cells were gated as living or dead, according to flow cytometry FSC and SSC parameters. Mean ± SD of four independent donors are shown.

RNA INTERFERENCE OF CYCLINS D1-3 AND CYCLIN E2 SIGNIFICANTLY IMPAIRED HIV-1 REPLICATION IN MACROPHAGES

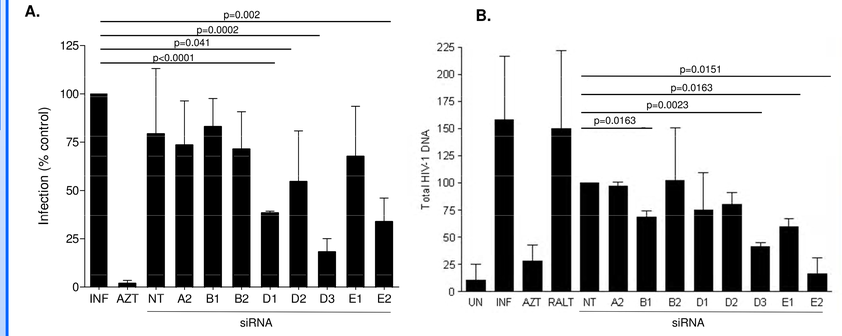


Figure 3. Figure 3. Inhibition of HIV-1 replication by cyclin knockdown. (A) Transfected MDM were infected with a VSV-pseudotyped, GFP-expressing HIV-1 and infection measured 72h later by flow cytometry and expressed as the percentage to non-transfected cells. **(B)** Total viral DNA formation after 16h infection with HIV-1 BaL of macrophages transfected with the indicated siRNA or treated with AZT (3 μM) or raltegravir (Ralt; 2 μM). Data are compared to the non-targeting siRNA. Mean ± SD of four independent donors are shown. Statistical significance was calculated by one-sample t test. p-values>0.05 are not shown. INF; infected, UN; uninfected, NT; non-targeting sequence.

CYCLIN D3 DOWNREGULATION REDUCED VIRAL DNA FORMATION

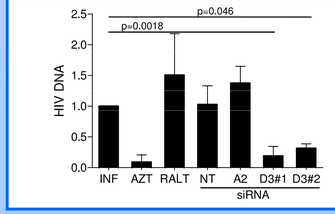


Figure 4. The effect of siRNA targeting cyclin D3 was confirmed by additional siRNA. Total viral DNA formation after 16h infection with HIV-1 BaL of macrophages transfected with two different siRNA targeting cyclin D3 (D3#1 and D3#2) or treated with AZT (3 μM) or raltegravir (Ralt; 2 μM), or the corresponding control siRNA. Data represent Mean±SD of at least two donors. Statistical significance was calculated by one-sample t test. p-values>0.05 are not shown. INF; infected, NT; non-targeting sequence.

CYCLIN D3 DOWNREGULATION REDUCED SAMHD1 PHOSPHORYLATION AND CDK2 ACTIVATION

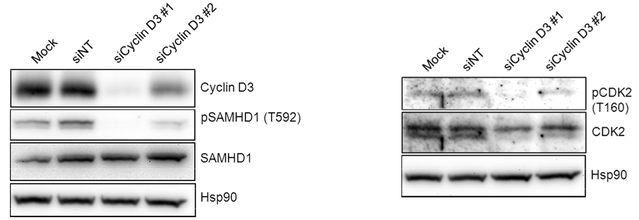


Figure 5. RNA interference of Cyclin D3 blocks SAMHD1 phosphorylation and CDK2 activation. Western blot of lysates from untreated macrophages (Mock) or transfected with siRNA targeting cyclin D3 or a non-targeting sequence (siNT) showing SAMHD1 (left) and CDK2 (right) expression and phodphorylation (left) . Representative blots of one of three independent donors are shown. Hsp90 was used as loading control.

KNOCKDOWN OF CYCLIN D3 RESTRICTED HIV-1 INFECTION IN A SAMHD1-DEPENDENT MANNER

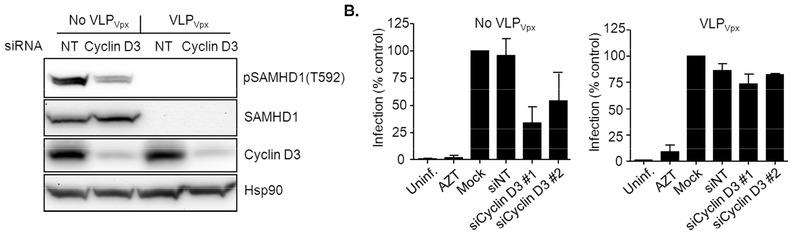


Figure 6. Cyclin D3 effect on HIV replication is dependent on SAMHD1 expression. (A) Macrophages transfected with a non-targeting siRNA (siNT) or a siRNA targeting cyclin D3 were treated (VLPVpx) or not (No VLPVpx) with viral-like particles carrying Vpx. Cells were lysed 24h after the treatment and samples were subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies. Representative blots of one out of two independent experiments are shown. **(B)** siRNA-transfected macrophages were treated (right panel) or not (left panel) with viral-like particles carrying Vpx and then infected with GFP-NL4-3. Infection was measured 72h later by flow cytometry and expressed as the percentage to non-transfected cells. Mean ± SD of three independent donors are shown. Statistical significance was calculated by one-sample t test. p-values>0.05 are not shown.

KNOCKDOWN OF CYCLIN D3 DECREASED THE INTRACELLULAR dNTP POOL

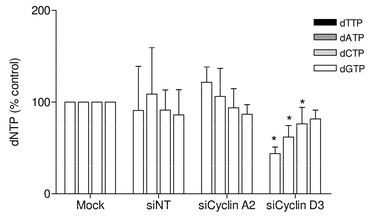


Figure 7. dNTP pool of siRNA–treated MDM. dNTP pool of MDM transfected or not (Mock) with siRNA targeting cyclin A2, cyclin D3 or a non-targeting sequence (siNT) was measured using a polymerase-based method. Data represent mean and SD of three independent donors. Statistical significance was calculated using the unpaired, two-tailed Student's t test.* p<0.05

DISCUSSION

- Knockdown of each specific cyclin led to up or down regulation of the expression of other cyclins or CDK, adding complexity to the understanding of the control of SAMHD1 function during cell cycle progression.
- Cyclin D3 inhibition correlated with decreased activation of CDK2, confirming the role of the CDK6-cyclin D3 pair in controlling SAMHD1 phosphorylation and dNTP pool and pinpointing its potential role as antiviral factor.