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Development And Validation Of A High Throughput

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DEVELOPMENT AND VALIDATION OF A HIGH THROUGHPUT SCREENING SYSTEM FOR INHIBITORS OF HIV INFECTION

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DEDICATION

DEVELOPMENT AND VALIDATION OF A HIGH THROUGHPUT SCREENING SYSTEM FOR INHIBITORS OF HIV INFECTION

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BACKGROUND

The HIV pandemic

In the last 23 years, the Human immunodeficiency virus (HIV) infection has reached pandemic levels with an estimate of 65 million infected people by 2006. Non-controlled HIV infection can result in the acquired immunodeficiency syndrome (AIDS) that has caused 25 million deaths by 2006. According to the Center of Disease Control, 1.1 million people are infected with HIV in the US. This number is predicted to considerable increase due to antiretroviral drug treatments continue to provide a larger expectancy of life to people with HIV.

HIV proteins are the main targets of the anti-retroviral treatment. The compounds used, block the HIV life cycle allowing the decrease of symptoms and suppression of the viral load. These compounds are classified in three categories: protease inhibitors, nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors. These inhibitors block the earlier step in viral infection and also arrest maturation of the virus and consequently block infection of vulnerable cells.

HIV has a high rate of mutation, thus currently available drugs targeting viral proteins frequently encounter drug-resistant viruses. A viable alternative to this limitation is to develop drugs that target cellular factors required for HIV infection but that are not essential to the cell. It is expected that the emergence of drug-resistant viruses will substantially decrease by using drugs that target these cellular co-factors. LEDGF/p75 is the first cellular factor to be identified that is required for HIV infection but is dispensable or normal cellular physiology [1]. Here we propose to develop a cellular-based screening system for the identification of small-molecules that disrupt the role of LEDGF/p75 in HIV infection.

HIV life cycle

HIV enters the target cell by fusion of the viral and cellular membranes mediated by the specific interaction of viral surface glycoproteins with cellular receptors (Fig.1). Immediately after entry, the viral RNA genome is reverse transcribed to a double-stranded DNA copy, which associates with viral and cellular proteins forming the preintegration complex (PIC). This complex is imported into the nucleus of the infected cell where the viral DNA is integrated into the host chromatin (Reviewed in [2]).

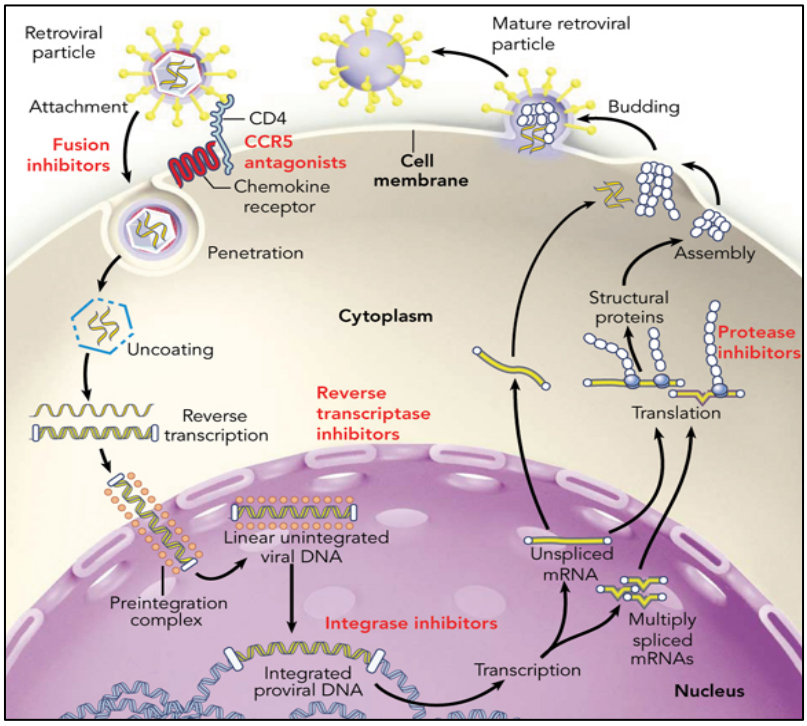

Figure 1. HIV viral life cycle*. After entry, the viral RNA genome is reverse transcribed and the resulting cDNA, in association with viral and cellular proteins, forms the pre-integration complex (PIC) that is later imported into the nucleus. The viral cDNA integrates into the host genome and the integrated provirus is efficiently transcribed and new viral progeny produced.*

Integration of the viral DNA into the host chromatin is then catalyzed by the viral enzyme integrase (IN), which performs two enzymatic activities essential for viral integration; 3' processing of the linear viral genome and DNA strand transfer of the viral genome into the host genome [2]. 3' processing occurs soon after reverse transcription when a dinucleotide from each HIV-1 genome end is cleaved off, leaving sticky ends on either end of the viral DNA [2]. IN's second enzymatic activity, DNA strand transfer, takes place after the PIC enters the nucleus and locates a suitable integration site. Then, IN cleaves the chromosomal DNA and integrates the viral genome. The resulting DNA recombination intermediate harbors single strand gaps that are then repaired by the host DNA repair machinery allowing for a complete provirus formation. It is only after integration, that proviral DNA is efficiently transcribed and the new viral progeny is generated, making integration an essential step in HIV infection [2].

The HIV integration process is not completely understood yet. It is though that integration requires the concerted action of both viral and cellular proteins [3,4]. Apparently, the integrase is the only viral protein essential for this process. Integrase interacts with the cellular protein LEDGF/p75 and this interaction promotes HIV-1 integration as well as influences the integration site distribution in the host chromatin [1,5,6].

Role of LEDGF/p75 in HIV-1 infection

LEDGF/p75 is a ubiquitously expressed chromatin bound protein that belongs to the hepatoma-derived growth factor family. Chromatin binding is mediated by the functional interaction of the PWWP domain and two AT hooks motifs, all located in the N-terminus of this protein (Fig. 2) [7,8]. In the C-terminal region of LEDGF/p75, an evolutionarily conserved region called the integrase binding domain (IBD) allows the interaction of LEDGF/p75 with cellular proteins and with the viral integrase [9,10,11,12]

Figure 2. LEDGF/p75 protein modular structure*. The chromatin-binding domain is formed by the functional interaction of the PWWP domain and two AT hooks motifs. The integrase binding domain mediates the interaction of LEDGF/p75 with HIV integrase and several cellular proteins.*

Cells severely depleted of LEDGF/p75 are resistant to HIV-1 infection because a defect in viral integration[1,6]. HIV-1 susceptibility is rescued in these cells upon re-expression of the LEDGF/p75 wild type protein. However, expression of LEDGF/p75 mutants lacking the chromatin binding capacity or the integrase binding domain did not rescue HIV-1 infectivity[1,6]. Based in these results, it was proposed that LEDGF/p75 acts as a molecular tether linking the HIV-1 integrase in the pre-integration complex to the host chromatin (Fig. 3) [1].

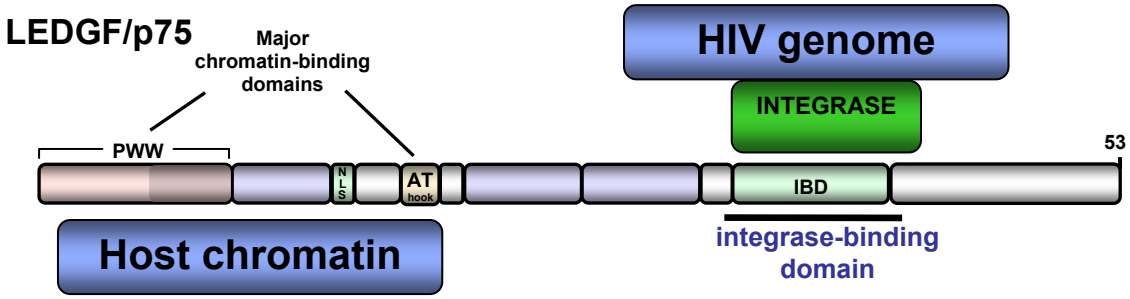

Figure 3. LEDGF/p75 tethering model*. Based on the study of the HIV co-factor activity of LEDGF/p75 mutants it is postulated that chromatin bound LEDGF/p75 tethers integrase linked to the pre-integration complex to the host chromatin facilitating viral integration.*

In addition to tethers integrase to the host chromatin, LEDGF/p75 protects the viral enzyme from proteasomal-mediated degradation [13]. The half-life of integrase in LEDGF/p75-deficient cells is importantly reduced; however, integrase mRNA levels are similar to the observed in control cells. Importantly, integrase degradation is prevented in these cells by re-expression of LEDGF/p75 wild type or by pharmacological inhibition of the proteasome [13]. LEDGF/p75 mutants lacking the IBD, however, did not protect integrase from proteasome degradation, indicating that this effect is dependent on the interaction of both proteins[13] (Figure 4).

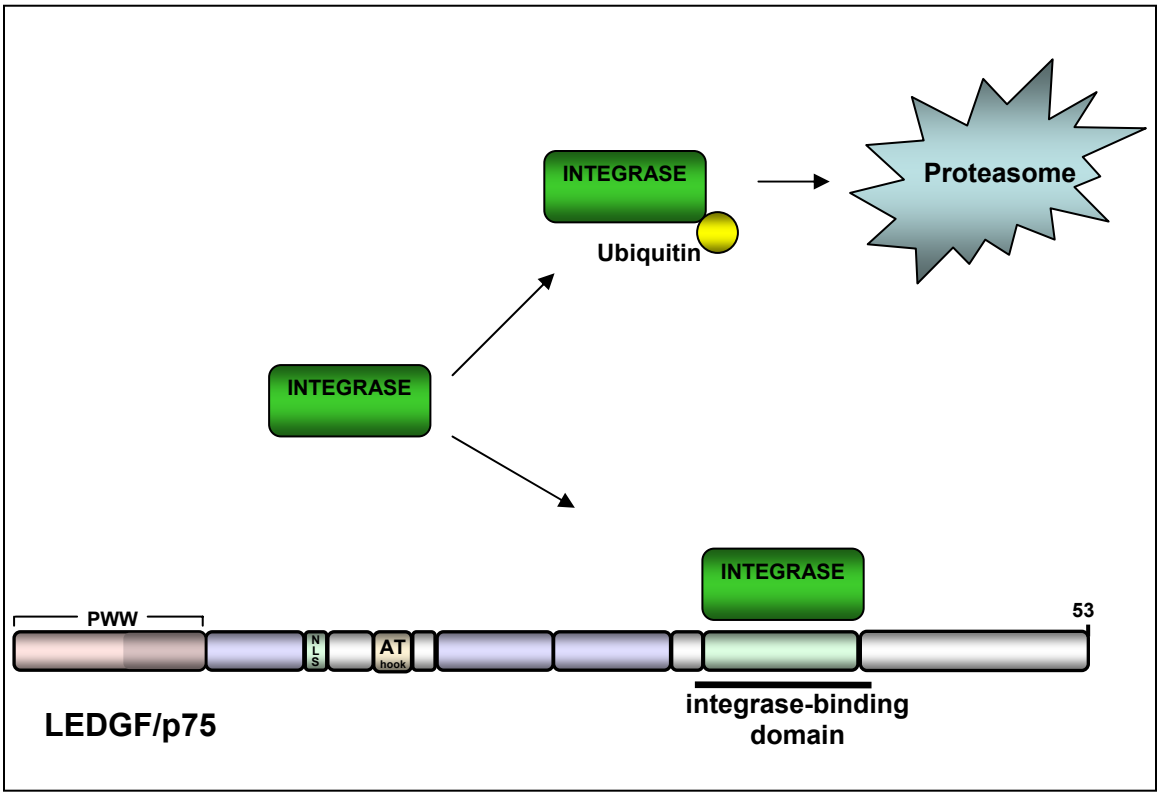

Figure 4. LEDGF/p75 protects HIV-1 integrase from proteasomal-mediated degradation*. In the absence of LEDGF/p75, integrase is ubiquitinated and degraded through the proteasome. The shielding effect of LEDGF/p75 requires binding to integrase.*

LEDGF/p75-Integrase interaction as an anti-HIV drug target

The interaction of LEDGF/p75 with HIV-1 integrase is a good candidate for anti-HIV drug development. LEDGF/p75, although essential for efficient HIV replication, is dispensable for the normal cellular physiology since cells lacking or severely deficient in LEDGF/p75 exhibited a normal phenotype in culture[1,6]. In addition, embryogenesis was not affected in knockout mice, although musculoskeletal defects were observed in the progeny[14]. These results strongly suggest that important host toxicity is not expected with drugs affecting the interaction of LEDGF/p75 with HIV-1 integrase.

The structural bases of the LEDGF/p75-integrase interaction indicate that this is a drugable interphase. Their surface of interaction is relatively small compared to other protein-protein surface of interaction and only three amino acids in LEDGF/p75 are required for the high binding affinity of integrase. More importantly, mutation of any of these residues to alanine completely abolishes this protein-protein interaction[15] indicating that small-compounds are suitable to interrupt the binding of LEDGF/p75 to HIV-1 integrase.

In summary, these results suggest that small-molecules could block the interaction of LEDGF/p75 with HIV-1 integrase and that host toxicity is not expected with the use of these compounds.

Small-compounds as inhibitors of the LEDGF/p75-integrase interaction.

Different approaches can be designed to inhibit the interaction LEDGF/p75-integrase interaction. These include the use of small-compounds, small peptides, RNAi or antibodies. However, there is a variety of advantages over using small-molecule compounds than the other

inhibitors. Antibodies have a quite large size that impede their intracellular distribution and in addition are complicated and costly to produce. Small peptides are another attractive method to use but they are unstable when used *in vivo*. Similarly, RNAi molecules are unstable *in vivo* and the antiviral effect of LEDGF/p75-depletion requires very stringent knockdowns [1]. These levels of depletion are difficult to be achieved *in vivo* with the actual technology. In addition, like antibodies, small-peptides and RNAi are not suitable for oral administration, only by injection. This particular feature is not convenient for HIV treatment since patients are more likely to take oral medication than injection. Small-molecule compounds, instead, are easier to use to target protein-protein interaction because of its small size, cellular permeability, in vivo stability and they can interfere with protein-protein interaction efficiently; in addition they are easy and cheap to produce [17].

High Through-put screening systems

Small-compounds inhibiting protein-protein interactions can be identified using High through-put screening (HTS) systems. This strategy has been an important tool for drug discovery.

HTS is a large-scale procedure in which numerous compounds are screened in a simple biological assay that can be based on processes occurring in cells or between purified reactants. This process is assessed in a precise and rapidly manner [18]. In order to find the best candidates for possible drug development several steps have to be performed. A primary screening using a biological assay and a large library of chemical compounds is performed, in which a group of candidates are selected, called ‘hits’. A secondary screening is carried out with previously selected hits to confirm possible candidates for a structure-activity relationship (SAR) and

medicinal chemistry [18]. Compounds passing secondary screenings are developed further to reduce toxicity and improve pharmacokinetic characteristics.

SIGNIFICANCE

HIV-1 infection has become a continuous challenge in the finding of therapeutic drugs to eradicate or at least decrease infection. The current anti-HIV therapy is based on the combination of drugs that target viral proteins involved in different steps of the viral life cycle. This treatment has substantially proven its effectiveness by extending the quality and length of life expectancy in HIV infected individuals. However, combination of multiple drugs is mandatory to reduce the development of drug-resistant viruses, a common outcome due to the high mutation rate of HIV proteins. Multi-drug therapies increase the cost and toxicity of the anti-HIV treatment, severely limiting its use worldwide and leading to the failure in preventing infection spreading.

A second generation of anti-HIV drugs targeting cellular cofactors dispensable for cells but required for HIV infection, such as LEDGF/p75, is currently in development. These drugs are intended to block the interaction of the cellular cofactor with its viral counterpart affecting HIV-1 infection. Due to the low rate of mutation of cellular proteins, it is unlikely the development of drug resistance mechanisms affecting the second generation of anti-HIV drugs. Therefore, mono-drug regimes are suitable alternatives for these drugs.

We describe here the development a high-throughput screening (HTS) strategy to identify small-molecules interfering with the interaction of HIV integrase - LEDGF/p75. These small-molecules may represent starting scaffolds for therapeutic drug development.

SPECIFIC AIMS

Here, we report the development and validation of a HTS for small compounds inhibitory of the HIV integrase-LEDGF/p75 interaction. The screening system is based in the ability of LEDGF/p75 to tether integrase to chromatin [16] and protect the viral enzyme from proteosomal-mediated degradation [13]. We generated a HEK293T-derived stable cell line expressing eGFP-tagged HIV integrase (referred here as reporter cell line or IN-eGFPc cells). Our rationale is that drugs inhibiting the LEDGF/p75 - integrase interaction will cause a redistribution of IN-eGFP and a proteasome-mediated degradation of this fusion protein. These effects were registered by measuring the subcellular distribution of IN-eGFP or the green fluorescence levels and in the treated cells.

In order to validate this reporter cell line, we depleted LEDGF/p75 in these cells by stable expression of a LEDGF/p75 specific shRNA. It is expected that fluorescence levels will be significantly reduced in knockdown cells and the nuclear localization of IN-eGFP will change. In addition, re-expression of LEDFG/p75 wild type or pharmacological inhibition of the proteasome will rescue fluorescence levels and nuclear localization of IN-eGFP in the LEDGF/p75-deficient cells.

The aims to be tested in this proposal are as follows:

Specific aim # 1: ***Generation a stable cell line expressing integrase-eGFP fusion protein (*****IN-eGFP cells)**

- Construction of a mammalian expression plasmid for the expression of HIV integrase-eGFP fusion protein

- Generation of stable cell lines expressing HIV IN-eGFP fusion protein in HEK293T cells.
- Characterization of IN-eGFP cells by FACS, confocal microscopy, and immunoblotting analyses.

Specific aim # 2: ***Development of a LEDGF/p75-deficient cell line in IN-eGFP cells ($2L_{KD}$-IN-eGFP cells)***

- Production of a lentiviral vector expressing a shRNA against LEDGF/p75
- Generation of LEDGF/p75-deficent cells in IN-eGFP cells.
- Rescue of integrase expression in $2L_{KD}$-IN-eGFP cells.

Specific aim # 3: ***Validation of the suitability of the developed reporter cell lines for drug screening in a HTS setting***

- Drug screening of a small-compound library using the IN-*eGFP reporter* cell line

MATERIALS AND METHODS

Specific aim # 1: ***Generation of a stable cell line expressing integrase-eGFP fusion protein (*IN-eGFP cells)**

Rationale: A reporter cell line stably expressing integrase fused to eGFP was generated. This cell line allowed determination in real time of the subcellular distribution and the levels of integrase proteins in cells. Drugs interfering with the LEDGF/p75-HIV integrase interaction are expected to trigger proteasome-mediated degradation of integrase and nuclear exclusion of integrase determining a decrease in fluorescence levels and a pancellular distribution of the fusion protein [13,16].

- **Construction of a mammalian expression plasmid for HIV integrase-eGFP fusion protein**

The HIV-1 integrase eGFP expression plasmid was constructed by cloning an internal ribosome entry site (IRES)-puromycin N-acetyltransferase (pac) expression cassette into a unique BglII site in pHINeGFP (Fig. 5) [16]. IRES pac was PCR amplified from the pEFIRESp plasmid. Correct sequence of the construct was verified by DNA sequencing.

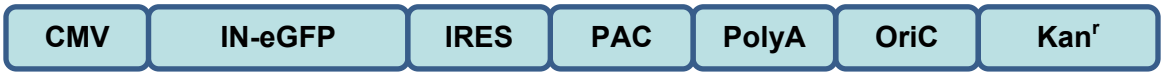

Figure 5. HIV IN-eGFP expression plasmid used to develop the IN-eGFP cell line*. The expression of IN-eGFP is driven by the CMV immediate early promoter and linked through an internal ribosome entry site (IRES) to the puromycin N-acetyltransferase (PAC) gen.*

- **Generation of stable cell lines expressing HIV IN-eGFP fusion protein in HEK293T cells.**

HEK293T cells were used for the stable expression of HIVIN-eGFP-IRES-pac. The new constructed plasmid was transfected by the calcium phosphate method as described in [16]. Briefly, HEK293T cells were plated at $3X10^6$ cells in a T75 flask in 12 ml of culture medium. The expression plasmid was linearized at the prokaryotic backbone with a restriction enzyme. Transfection media with 20ug of linearized DNA was added to the cells and incubated at 37^0C in the presence of 5% CO2 and 95% humidity. After 24 hrs, culture media was changed for fresh media with 3ug/ml puromycin antibiotic. Cells were grown in this selection medium until a resistant culture emerges.

- **Characterization of IN-eGFP cells by FACS, confocal microscopy, and immunoblotting analyses**

Expression of IN-eGFP was evaluated in the puromycin resistant cells by fluorescence activated cell sorting (FACS) analysis. In addition, the correct size of the fusion protein was verified by immunoblotting with an anti-eGFP monoclonal antibody (Mab). Finally, subcellular distribution of IN-eGFP was determined by confocal microscopy analysis.

For FACS analysis cells were harvested by trypsin treatment, washed in PBS and samples analyzed in the flow cytometer of the Cell Culture and High throughput Screening Core Facility. As a negative control, parental HEK293T cells were used.

For immunoblotting analysis procedures described in [16] were followed. Briefly, 10^6 IN-eGFP cells were lysed in 300ul of RIPA buffer (150mM Tris-HCl, pH 8.0, 150mM NaCl, 0.5% DOC, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors (final concentration:

leupeptine 2ug/ml, aprotinin 5ug/ul, PMSF1mM, pepstatin A 1ug/ml) and centrifuged at 22, 000g for five mins at 4°C. Cellular lysates, 10ul, were resolved by SDS-PAGE and transfered overnight to PDVF membranes at 100 mAmp at 4°C. Membrane was blocked in TBS containing 10% milk for one hour and then incubated with primary antibodies diluted in TBS-5% milk-0.05% Tween-20 (antibody dilution buffer). IN-eGFP was detected with anti-eGFP monoclonal antibody (Mab) diluted 1/4000. As a loading control, anti-GAPDH Mab was used (1/2000). Membranes were incubated for two hrs at room temperature with, anti-eGFP, and anti-GAPDH Mabs and then membranes were washed three times for five minutes in TBS-0.1% Tween-20 (washing buffer). Bound antibodies were detected with goat anti-mouse Igs-HRP diluted 1/2000 in antibody dilution buffer followed by chemoluminescence detection.

For confocal microscopy analysis procedures described in [16] were followed. Briefly, cells were plated at 0.2 $X10^6$ cells in LabTek II chambered coverglasses. After 24 hrs incubation for attachment, cells were washed 3X with PBS, fixed with 4% formaldehyde in PBS for 10 mins at 37°C then washed once with PBS and stained with DAPI. Then, cells were analyzed for subcellular distribution of HIV-1 IN-eGFP under the confocal microscope.

Specific aim # 2: ***Development of a LEDGF/p75 deficient cell line in*** **IN-eGFP cells ($2L_{KD}$-IN-eGFP cells)**

Rationale: It is expected that drugs interfering with the LEDGF/p75-integrase interaction will change the subcellular localization of IN-eGFP as well as induce its degradation by the proteasome [13,16]. In order to evaluate this hypothesis, we will deplete cellular LEDGF/p75 in IN-eGFP cells and analyze them by FACS and confocal microscopy to determine the levels of IN-eGFP and its subcellular distribution, respectively. Depletion of LEDGF/p75 mimic the lack

of LEDGF/p75 available for interaction encountered in the presence of a drug that interferes with the interaction of this protein with integrase.

- **Production of a lentiviral vector expressing a shRNA against LEDGF/p75**

LEDGF/p75 depletion of IN-eGFP was achieved by lentiviral transduction of a shRNA specific against LEDGF/p75. This lentiviral vector integrates into the host genome a module containing in cis an U6 small nuclear RNA promoter-driven shRNA expression cassette and a CMV-driven mCherry fluorescent protein expression cassette. This expression system allows selection of LEDGF/p75 knockdown cells based on their mCherry fluorescence levels[1]. Procedures described in [1] were followed for the production of the retroviral vector. Briefly, HIV-derived vectors expressing anti-LEDGF/p75 shRNA were produced by calcium-phosphate co-transfection of HEK293T with 15ug of pTSINcherry/p75, 15ug of pCMVΔR8.91 and 5ug of the Vesicular Stomatitis Virus glycoprotein G (VSV-G) expression plasmid, pMD.G. Forty-eight hours after transfection, viral supernatants were harvested and concentrated by ultracentrifugation at 124,750g for two hours on a 20% sucrose cushion. Concentrated vectors were used for transduction of IN-eGFP cells.

- **Generation of LEDGF/p75-deficent cells in IN-eGFP cells ($2L_{KD}$-IN-eGFP cells).**

IN-eGFP cells were transduced with the lentiviral vector expressing anti-LEDGF/p75 shRNA at a multiplicity of infection 300, as described before [1]. Twenty-four hrs later, the input viral vector was washed and cells were single-cell cloned by limiting dilution cultures. Clones expressing the highest levels of red fluorescence were selected for further analysis.

Levels of red fluorescence were determined under the fluorescence microscope. Knockdown levels of LEDGF/p75 were verified by immunoblotting with an anti-LEDGF Mab.

- **Rescue of integrase expression and nuclear localization in $2L_{KD}$-IN-eGFP cells.**

It is expected that the eGFP fluorescence levels of IN-eGFP cells will decrease and nuclear localization of this fusion protein will be altered following LEDGF/p75 depletion. In other to validate further that the change in levels and subcellular distribution of IN-eGFP are due to the lack of LEDGF/p75 and subsequently proteasome-mediated degradation of HIV integrase, rescue experiments was performed. We expect that re-expression of LEDGF/p75 wild type or pharmacological inhibition of the proteasome will cause a re-bound in the green fluorescence levels of $2L_{KD}$-IN-eGFP cells associated with a nuclear re-localization of IN-eGFP.

$4x10^5$ $2L_{KD}$-IN-eGFP cells were transfected with 2ug of the expression plasmid pLEDGF/p75 WT-flag using the calcium phosphate method. Transfection medium was removed the next day and cells were analyzed twenty-four hrs later for green fluorescence levels by FACS and for subcellular distribution of IN-eGFP by fluorescence microscopy. Additionally, integrase levels were also determined by immunoblotting with anti-eGFP Mab. As control, a LEDGF/p75 mutant lacking the IBD was expressed in $2L_{KD}$-IN-eGFP cells. Because this mutant does not interact with integrase, it is expected that the levels of green fluorescence will not increase in the transfected $2L_{KD}$-IN-eGFP cells.

The effect on the green fluorescence levels of $2L_{KD}$-IN-eGFP cells of several inhibitors targeting lysosomal function, proteasome and calpains were evaluated by FACS analysis 24 hrs after treatment. The lysosome inhibitors include drugs that destabilize the lysosomal membrane (ciprofloxacin, 300uM) or that increase the lysosomal pH such as Bafilomycin A1 (200nM), a

specific inhibitor of H+-ATPases of the vacuolar type, and lysosomotropic amines ammonium chloride (20mM) and chloroquine (100uM). Two proteasome inhibitors were used, ALLN (34.6uM), N-acetylleu-leu-norleucinal and MG132 (10uM), z-leu-leu-leucinal, both are peptide aldehydes that bind to he catalytic threonine residue of the proteasome inhibiting its chymotrypsin-like activity. In addition, PD150606 (99.4uM) that is a very specific inhibitor of calpains 1 and 2, was used.

Specific aim # 3: ***Validation of the suitability of the developed reporter cell lines for drug screening in a HTS setting***.

Rationale: The suitability of IN-eGFPc cells for high throughput screening was evaluated using a panel of eighty-eight different small molecules. In this screening, we evaluated if cells are useful in a high throughput screening setting. End-points in this screening were sensitivity of the reporter cell line to DMSO 1% and to the potential toxic effect of drugs used at uM concentration. These conditions emulated the general screening conditions of a larger small-compound library in a HTS setting. In addition the robustness of the assay was determined calculating the statistical parameter Z' for IN-eGFPc and $2L_{KD}$-IN-eGFP reporter cell lines.

- **Drug screening of a compound library**

The effects of eighty-eight different small-compounds in the Dimmock library (Table 1) on IN-eGFPc cells were analyzed using the BD Pathway high-content bio-imager. End-points in the analysis were cell morphology, cell distribution, fluorescence level, and number of live cells. Because there are not small-compounds inhibitory of the LEDGF/p75-integrase interaction

commercially available, hydrogen peroxide (500μM) was used as positive control. Negative control was DMSO 1% because this is a very common vehicle in small-compound libraries and regular culture medium. It is relevant for the robustness of the screening system that drugs at uM concentration or 1% DMSO do not alter the adhesion of the reporter cell line to the culture plate or their cellular morphology.

In addition, IN-eGFPc and $2L_{KD}$-IN-eGFP treated with selected compounds were analyzed for green fluorescence levels by FACS. Commercially available proteasome inhibitors were used as positive control with $2L_{KD}$-IN-eGFP cells, while negative controls were 1% DMSO and several small-compounds inhibitory of different non-proteasomal protein degradation pathways.

IN-eGFPc cells were plated in a BD 96-well flat bottom plate at a density of $5X10^3$ cells per well in a total volume of 100 μl of culture media and treated the next day. Cells were analyzed before the treatment and 3, 6 and 24hrs later using the BD Pathway high-content bio-imager. Compounds were used at 1:10 dilution from the stock plate (master plate #2), with a final concentration of 1:100 in the experimental plate. For FACS analysis, cells were plated at 1.0 X 10^5 cells per well in 500 μl of culture media in regular 24-wells tissue culture plates.

The robustness of these HTS systems were determined by calculating the Z' factor. The HTS system reported here can be analyzed using FACS or the BD Pathway high-content bio-imager, then, we estimated the Z' factor for these reporter systems using both methods of analysis. Z' was calculated according to the following equation:

$$\text{Zfactor} = 1 - \frac{3 \times (\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

Where σ is the standard deviation and μ is the mean of the green fluorescence levels of cells treated with the positive (p) and the negative (n) compound. HTS systems with Z' factors between 0.5 and 1.0 are considered excellent assays while between 0.5 and 0 are marginal assays.

Table 1.Dimmock library table

ID No.	ID	STOCK Conc. [mM]	1:10 DIL Conc. [mM]
1	NC1043	5.0	0.5
2	NC1113	10.0	1
3	NC1114	50.0	5
4	NC1153	25.0	2.5
5	NC1267	50.0	5
6	NC1831	25.0	2.5
7	NC1876	5.0	0.5
8	NC1910	50.0	5
9	NC1949	5.0	0.5
10	NC2066	50.0	5
11	NC2067	10.0	1
12	NC2071	2.5	0.25
13	NC2072	1.0	0.1
14	NC2081	50.0	5
15	NC2085	25.0	2.5
16	NC2089	25.0	2.5
17	NC2090	10.0	1
18	NC2091	50.0	5
19	NC2094	5.0	0.5
20	NC2095	10.0	1
21	NC2099	5.0	0.5
22	NC2102	50.0	5
23	NC2106	50.0	5
24	NC2107	50.0	5
25	NC2140	2.5	0.25
26	NC2154	50.0	5
27	NC2162	25.0	2.5
28	NC2175	25.0	2.5
29	NC2215	50.0	5
30	NC2216	50.0	5
31	NC2217	50.0	5
32	NC2311	50.0	5
33	NC2312	10.0	1
34	NC2313	10.0	1
35	NC2315	5.0	0.5
36	NC2316	25.0	2.5
37	NC2317	50.0	5
38	NC2320	50.0	5
39	NC2321	50.0	5
40	NC2322	50.0	5
41	NC2324	50.0	5
42	NC2325	25.0	2.5
43	NC2326	50.0	5
44	NC2329	50.0	5

ID No.	ID	STOCK Conc. [mM]	1:10 DIL Conc. [mM]
45	NC2361	10.0	1
46	NC2362	5.0	0.5
47	NC2365	10.0	1
48	NC2366	50.0	5
49	NC2384	50.0	5
50	NC2385	50.0	5
51	NC2386	50.0	5
52	NC2387	50.0	5
53	NC2388	50.0	5
54	NC2389	50.0	5
55	NC2390	50.0	5
56	NC2391	50.0	5
57	NC2392	50.0	5
58	NC2393	1.0	0.1
59	NC2394	50.0	5
60	NC2395	10.0	1
61	NC2396	50.0	5
62	NC2397	10.0	1
63	NC2398	10.0	1
64	NC2399	50.0	5
65	NC2400	50.0	5
66	NC2401	50.0	5
67	NC2402	10.0	1
68	NC2403	10.0	1
69	NC2404	25.0	2.5
70	NC2405	2.5	0.25
71	NC2406	50.0	5
72	NC2407	10.0	1
73	NC2408	0.5	0.05
74	NC2409	1.0	0.1
75	NC2410	50.0	5
76	NC2411	0.5	0.05
77	NC2412	0.5	0.05
78	NC2413	2.5	0.25
79	NC2414	10.0	1
80	NC2415	2.5	0.25
81	NC2416	2.5	0.25
82	NC2417	10.0	1
83	NC2418	5.0	0.5
84	NC2419	2.5	0.25
85	NC2420	0.1	0.01
86	NC2421	0.1	0.01
87	NC2422	1.0	0.1
88	NC2423	50.0	5

RESULTS

Specific aim # 1: ***Generation of a stable cell line expressing integrase-eGFP fusion protein (*IN-eGFP cells)**

IN-eGFP cell line was successfully generated by stable transfection of the pIN-eGFP-IRES-pac expression plasmid. A robust polyclonal cell population was obtained after two weeks of selection in puromycin. High levels of green fluorescence were detected by FACS analysis of these cells (Fig.6) and, as expected[16], integrase-eGFP was detected in the nuclear compartment of the cells by confocal microscopy analysis (Fig.7).

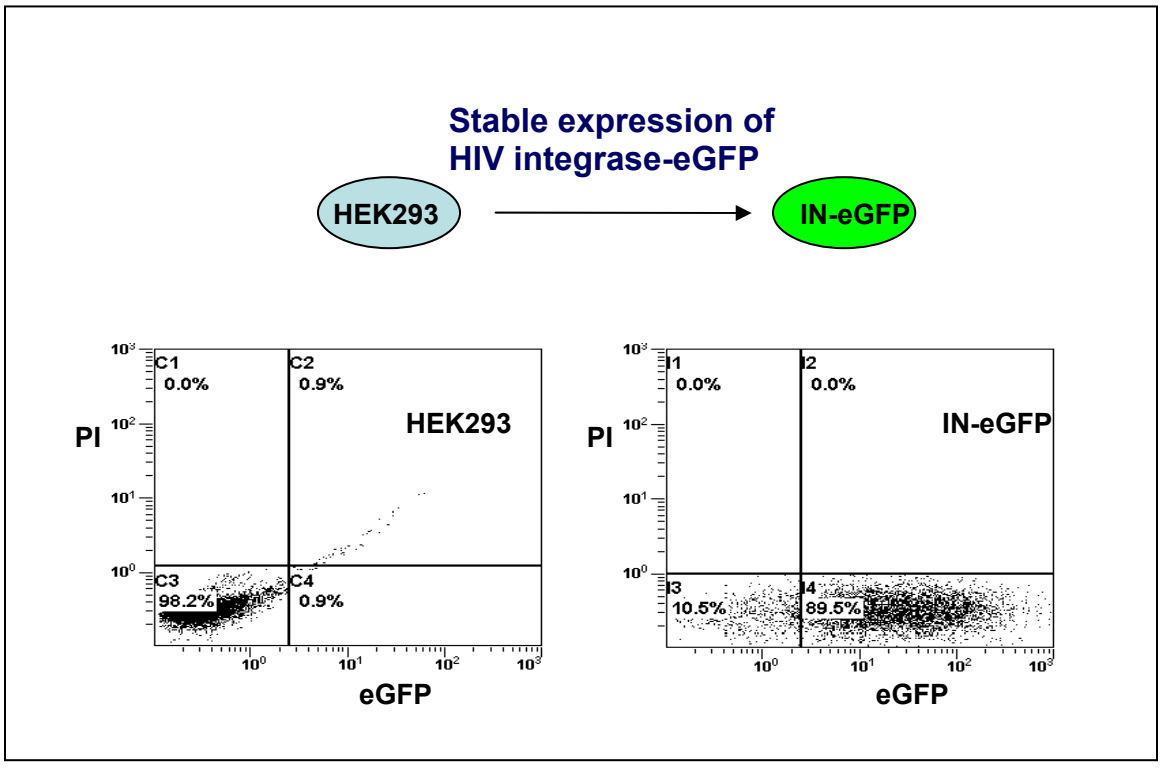

Figure 6. FACS analysis of IN-eGFP cells*. High levels of green fluorescence were detected in cells stably transfected with the expression plasmid encoding IN-eGFP-IRES-PAC (Fig. 5). Parental HEK293T cells were sued as a negative control.*

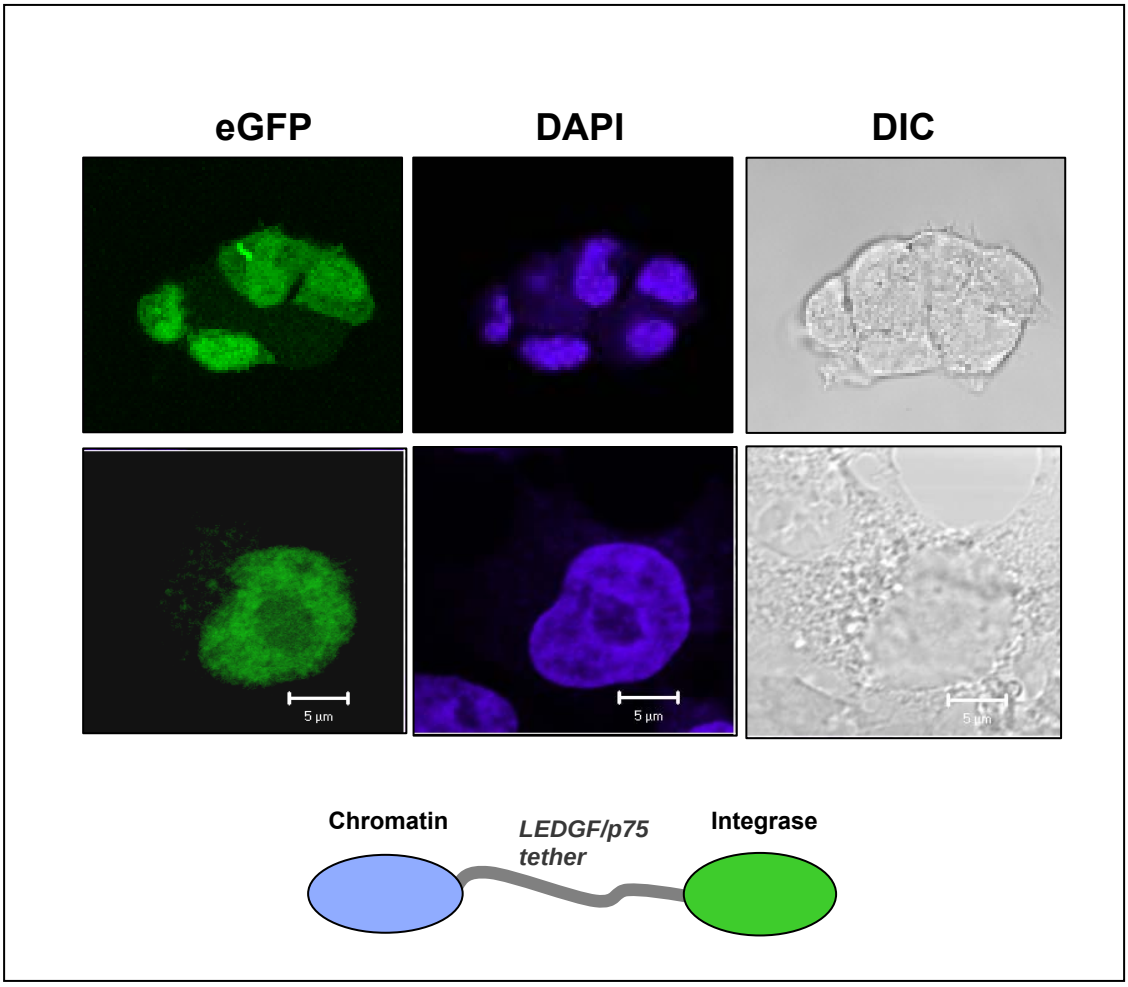

Figure 7. Confocal microscopy analysis of IN-eGFP cells*. IN-eGFP was localized to the nuclear compartment of these cells.*

Specific aim # 2: ***Development of a LEDGF/p75 deficient cell line in IN-eGFP cells (2L$_{KD}$-IN-eGFP cells)***

The screening strategies that we were planning for the identification of inhibitors of the LEDGF/p75-integrase interaction are based on the capacity of LEDGF/p75 to tether integrase to

the chromatin and to shield it from proteasomal degradation. Our rational is that drugs interfering with this protein-protein interaction will determine a pancellular redistribution of the fusion protein and trigger its degradation causing a decrease in the green fluorescence levels of the IN-eGFP cells. Because small-compounds inhibitory of the LEDGF/p75-integrase interaction are not commercially available, we used RNAi methodology to validate our screening system. LEDGF/p75-specific and control shRNAs were delivered in IN-eGFP cells by retroviral transduction. The retroviral-delivered shRNA expression cassette also contains *in cis* a transcription unit expressing monomeric cherry fluorescence protein (mCherry). Therefore, IN-eGFP transduced cells also expressed mCherry. In contrast with the green fluorescence protein that is linked to integrase and its total levels directly depend on LEDGF/p75 availability, mCherry levels are absolutely LEDGF/p75-independent and can inform about the cellular homeostasis. Then, we propose to detect cherry fluorescence by FACS or confocal microscopy analysis during HTS of small-compounds as a counter-screening method to detect compounds causing a global decrease in protein synthesis; i.e. toxic compounds. The advantage of this strategy is that the counter screening does not require additional manipulation and can be done concomitantly with the primary screening.

Generation of $2L_{KD}$-IN-eGFP cells

$2L_{KD}$-IN-eGFP cells were efficiently generated by lentiviral transduction of IN-eGFP cells. Several single-cell clones expressing high levels of cherry fluorescence were isolated and analyzed further for LEDGF/p75 and green fluorescence levels.

LEDGF/p75 was undetectable in $2L_{KD}$-IN-eGFP cells by sensitive immunoblots (Fig. 8) and this correlated with a decrease in green fluorescence levels as compared to IN-eGFP cells expressing wild type levels of LEDGF/p75 (Fig. 9).

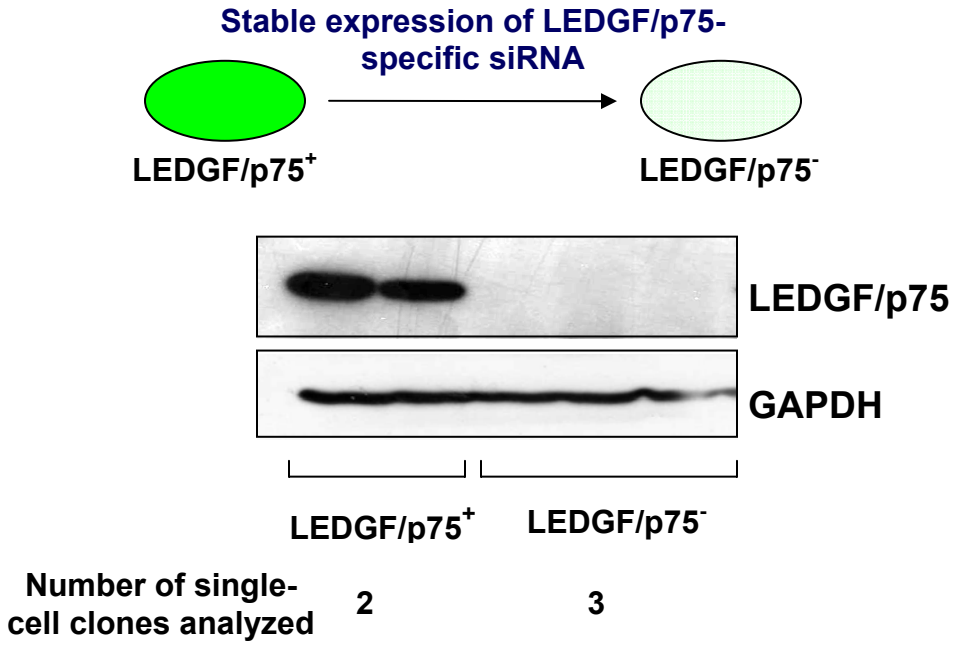

Figure 8. LEDGF/p75 levels in $2L_{KD}$-IN-eGFP and IN-eGFP cells*. LEDGF/p75 levels were analyzed by immunoblotting in three different single-cell clones derived from IN-eGFP cells transduced with a lentivirus expressing a shRNA specific to LEDGF/p75 ($2L_{KD}$-IN-eGFPcells) or two different single-cell clones derived from control IN-eGFP cells. GAPDH was determined in the same immunoblot membrane as a loading control.*

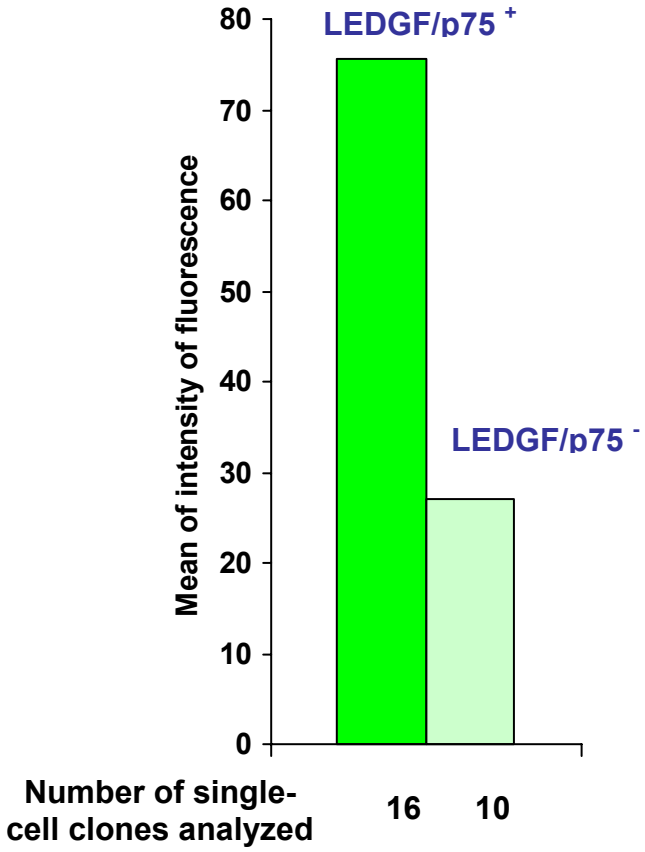

Figure 9. FACS analysis of IN-eGFP- and 2L$_{KD}$-IN-eGFP-derived single-cell clones*. Green fluorescence levels were routinely higher in IN-eGFP-derived (LEDGF/p75+) than in 2L$_{KD}$-IN-eGFP-derived single-cell clones (LEDGF/p75-).*

Characterization of 2L$_{KD}$-IN-eGFP cells

In addition to a reduction in the green fluorescence levels of IN-eGFP cells, LEDGF/p75-depletion caused a redistribution of the subcelular localization of IN-eGFP. This fusion protein localized pancellularly in 2L$_{KD}$-IN-eGFP (Fig. 10) in marked contrast with the exclusively nuclear localization observed in the parental IN-eGFP cells (Fig. 7 and 10).

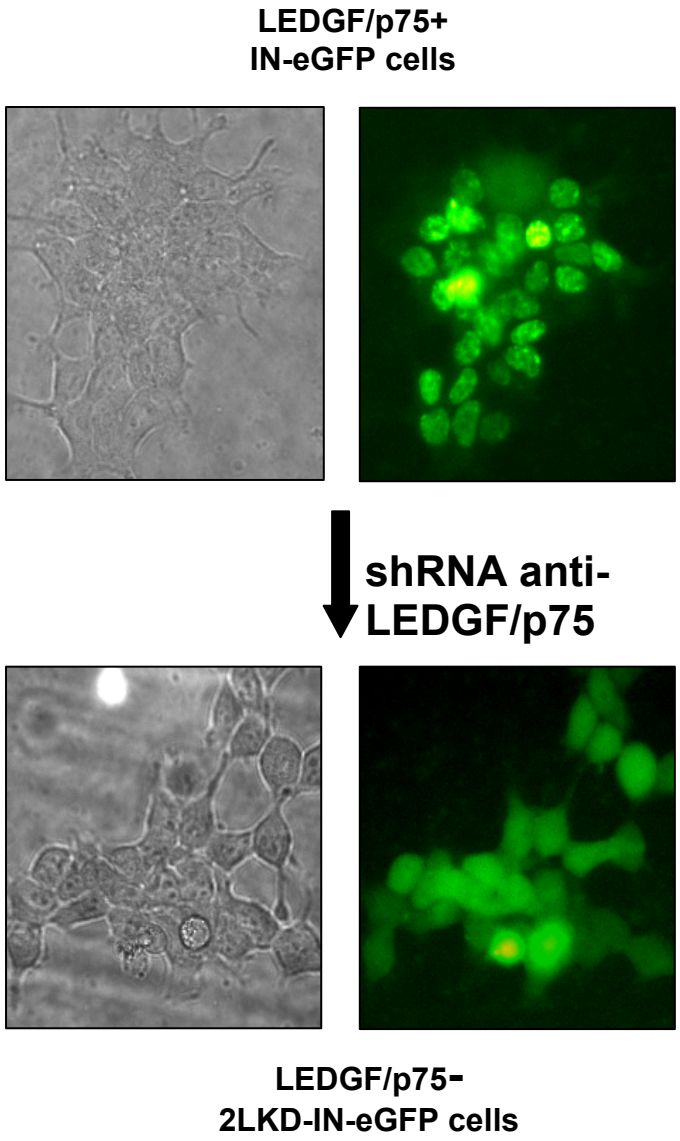

Figure 10. Sub-cellular distribution of eGFP tagged integrase in IN-eGFP and $2L_{KD}$-IN-eGFP cell lines. *The nuclear localization of the fusion protein in the LEDGF/p75+ cells IN-eGFP cells was lost after efficient knockdown of LEDGF/p75 ($2L_{KD}$-IN-eGFP cells).*

Transient expression of LEDGF/p75 wild type rescued the green fluorescence levels in $2L_{KD}$-IN-eGFP cells (Fig.12 and 13) and redistribute IN-eGFP to the nuclear compartment of cells in interphase and to chromosomes in cells undergoing mitosis (Fig. 11-ii). However, a LEDGF/p75 mutant lacking the IBD failed to produce any of these effects indicating that LEDGF/p75-integrase interaction was required (Figs.11-iii, 12 and 13).

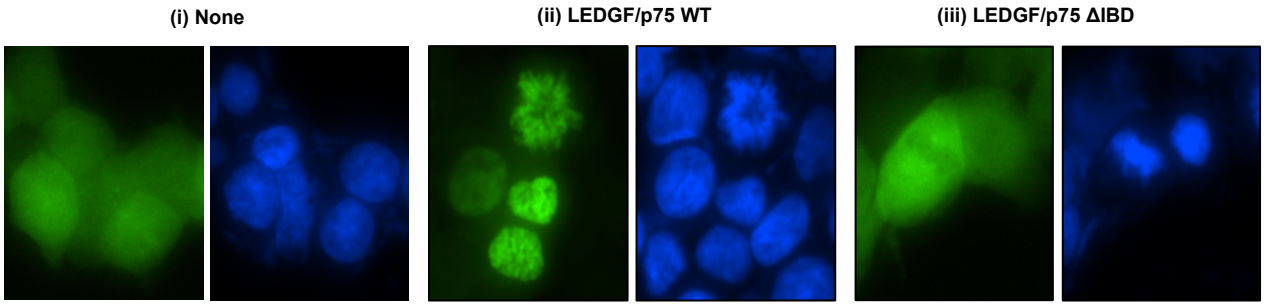

Figure 11. Effect of LEDGF/p75 expression in the localization of IN-eGFP in $2L_{KD}$-IN-eGFPcells*. Wild type LEDGF/p75, but not the ΔIBD mutant, can rescue nuclear and chromosomal localization of IN-eGFP in $2L_{KD}$-IN-eGFP cells, as determined by confocal microscopy analysis.*

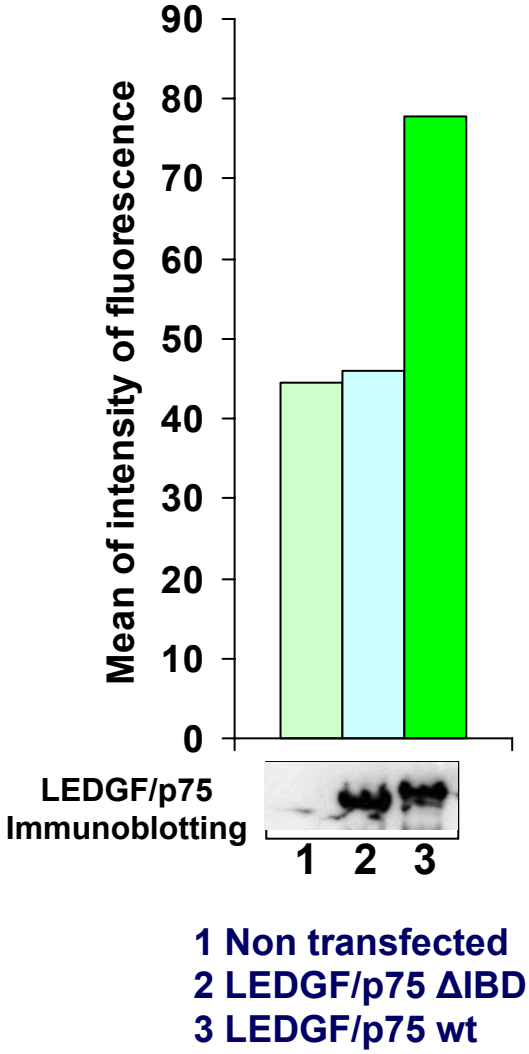

Figure 12. Green fluorescence levels of $2L_{KD}$-IN-eGFP cells after re-expression of LEDGF/p75 WT and ΔIBD mutant. *Wild type LEDGF/p75, but not the ΔIBD mutant, can increase the green fluorescence levels of $2L_{KD}$-IN-eGFP cells, as determined by FACS analysis. Levels of re-expressed LEDGF proteins were verified by immunoblotting.*

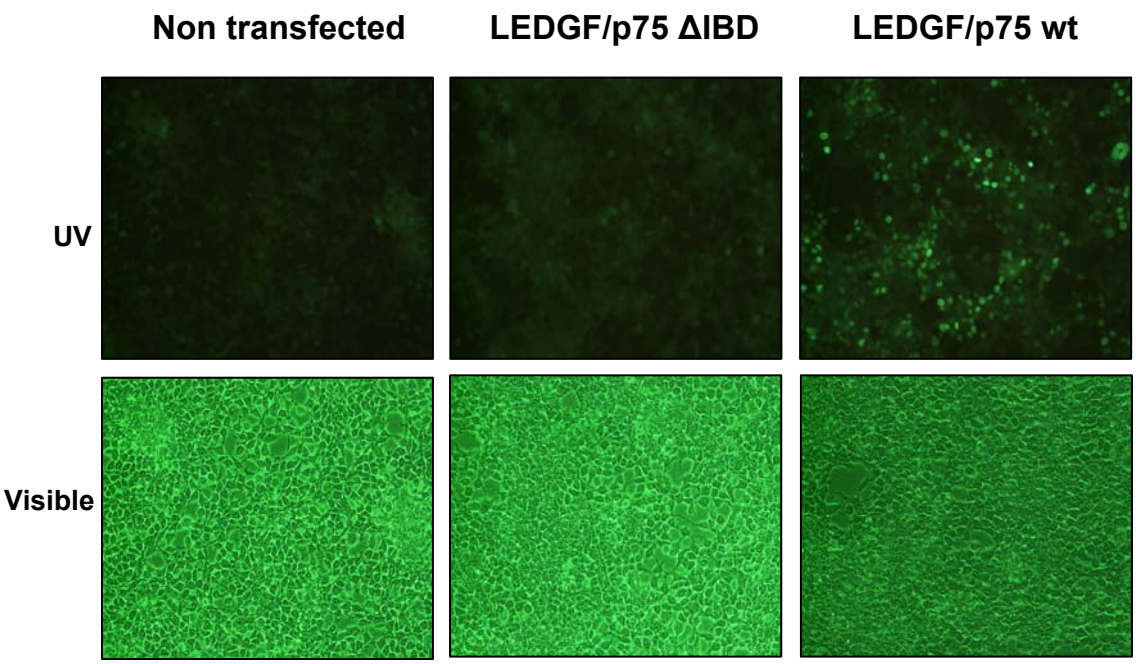

Figure 13. Green fluorescence levels of $2L_{KD}$-IN-eGFP cells after re-expression of LEDGF/p75 WT and ΔIBD mutant*. Wild type LEDGF/p75, but not the ΔIBD mutant, can increase the green fluorescence levels of $2L_{KD}$-IN-eGFP cells, as determined by fluorescence microscopy.*

Effect of proteasome inhibitors on the green fluorescence levels of $2L_{KD}$-IN-eGFP cells

Inhibition of the proteasome using MG132 or ALLN prevent IN degradation in the absence of LEDGF/p75 in $2L_{KD}$-IN-eGFP cells (Fig. 14 and 16). An increase of 2-fold was recorded using 50uM, 25 uM and 10 uM concentration of MG132. However, $2L_{KD}$-IN-eGFP cells treated with four different lysosome inhibitors, Ciproflaxacin, Ammonium Chloride,

Bafilomycin and Chloroquine or with the calpains 1 and 2 inhibitor PD150606 did not rescue the expression of IN-eGFP (Fig. 14 and 16).

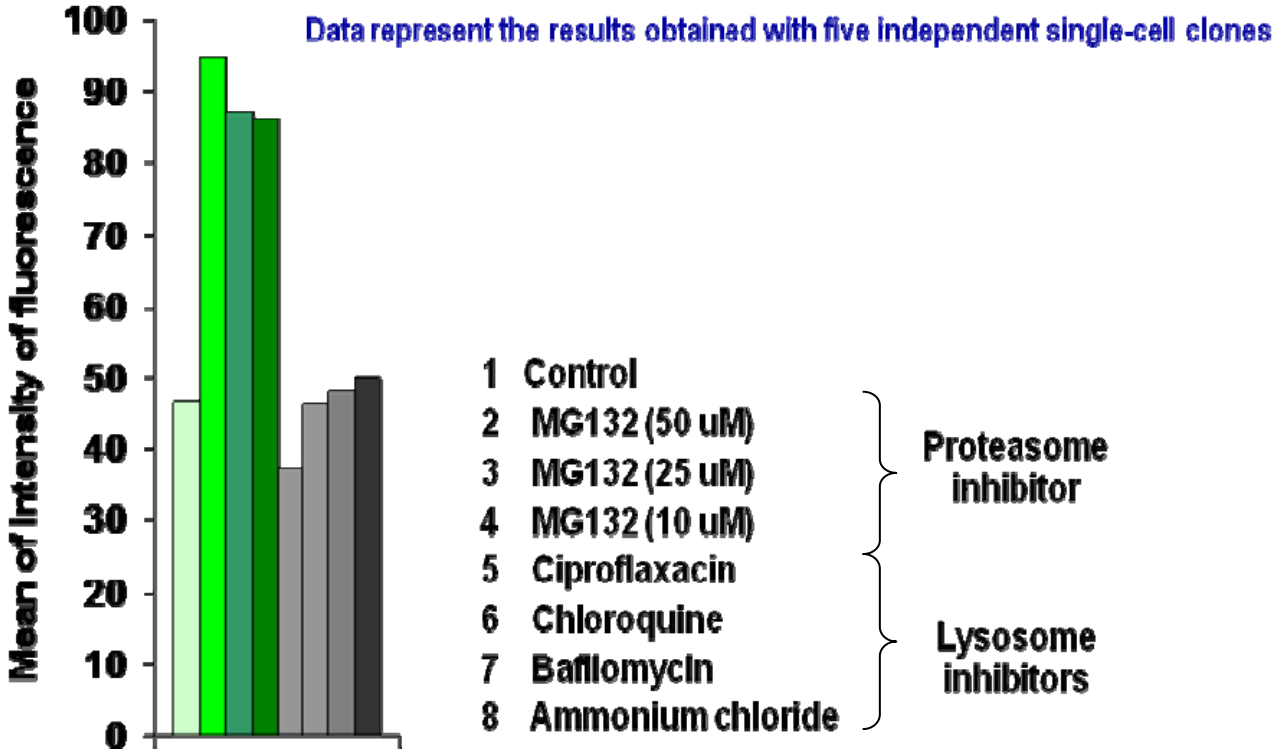

Figure 14. Effect of proteasome and lysosome inhibitors on green fluorescence levels of 2L$_{KD}$-IN-eGFP cells*. A two-fold increase in fluorescence levels was detected in 2L$_{KD}$-IN-eGFP cells treated with the proteasome inhibitor MG132. On the other hand, the fluorescence levels of 2L$_{KD}$-IN-eGFP cells did not vary after treatment with several lysosome inhibitors.*

Specific aim # 3: ***Validation of the suitability of the developed reporter cell lines for drug screening in a HTS setting***.

Z'-factors.

In order to validate the robustness of these two assays in a HTS setting we calculated the Z'-factor having as read-outs green fluorescence levels using the BD Pathway confocal microscope and the FACS.

To calculate the Z'-factor of the HTS system developed for identification of inhibitors of the LEDGF/p75-integrase interaction based in IN-eGFPc cells we used as positive control hydrogen peroxide. A small-compound affecting this protein-protein interaction is not commercially available yet. Hydrogen peroxide is highly cytotoxic and green fluorescence levels are expected to be importantly diminished in treated cells. As negative control we used DMSO 1% since compounds in chemical libraries used for HTS are commonly dissolved in DMSO. Cells treated with these compounds were evaluated after 3 and 6 hrs by confocal microscopy in the BD Pathway (Table 2).

Using the formula described in Material and Methods, we calculated that the Z'-factor for this HTS system using the BD pathway confocal microscope was 0.36. This Z'-factor value is indicative of a marginal HTS system. The main reason for these results was that the treated cells were partially detached from the assay plate and formed aggregates. A typical result is presented in figure 15 were cells are shown before and 3hrs after treatment with hydrogen peroxide, DMSO and compound B08. Although the cells were spread and attached to the plate before treatment, 3hrs after treatment these cells detached and aggregated. Confocal microscopic examination of the treated cells using BD pathway indicated that hydrogen peroxide treatment markedly reduced green fluorescence levels of IN-eGFPc cells as compared to DMSO treatment. However, because the BD Pathway software used for calculation is based on cellular

morphology, data processing showed only a modest 1.78 fold decrease. In addition, standard deviation of this analysis was between 4.4% and 8.8 % the mean green fluorescence value reducing the robustness of the assay. Therefore we decided not to continue this read-out strategy and instead use FACS analysis.

Then, the effect of hydrogen peroxide and DMSO treatment on the green fluorescence levels of IN-eGFP cells was analyzed by FACS. Using this read-out a Z'-factor indicative of an exceptional assay for HTS was obtained.

Based on these results with the reporter cell line IN-eGFPc we decided to use FACS analysis to calculate the Z'-factor of the HTS proposed for the identification of proteasome inhibitors. As positive controls two proteasome inhibitors, MG132 and ALLN, were used while DMSO was used as negative control. $2L_{KD}$-IN-eGFP cells were treated with the drugs described above and 24 hrs later green fluorescence levels were determined by FACS (Fig. 16). Similar to the HTS described above, the Z'-factor of this assay was 0.62 and 0.57 for the MG132/DMSO and ALLN/DMSO pairs, respectively. These data indicate this assay as a very robust screening system.

Screening of a small chemical library

A preliminary high-throughput screening of 88 compounds was conducted with IN-eGFP cells using a drugs in the uM range of concentration. Readouts were performed with the the BD Pathway confocal microscope 3 hrs, 6 hrs and 24 hrs after treatment. Screening of this chemical library with the BD pathway confocal microscope encounter the technical limitations described above when the Z'-factor was calculated; therefore we further concluded that the BD Pathway is not the optimal read-out system for this HTS.

Table 2. *Mean and standard deviation of green fluorescence levels of IN-eGFP cells treated with hydrogen peroxide and DMSO.*

Time points	Hydrogen peroxide Positive control	DMSO Negative control
3 hours	881.901	1630.677
6 hours	999.874	1735.428
Mean	940.887	1683.053
Standard deviation	83.42	74.07

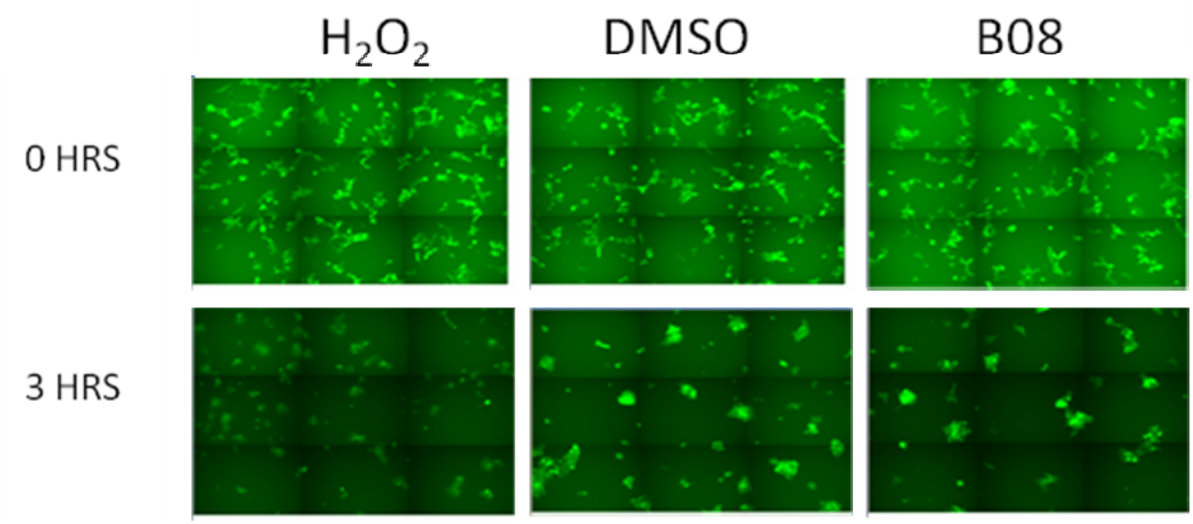

Figure 15. BD pathway confocal microscopy analysis of IN-eGFP cells. *Note that cells detach and aggregate after treatment.*

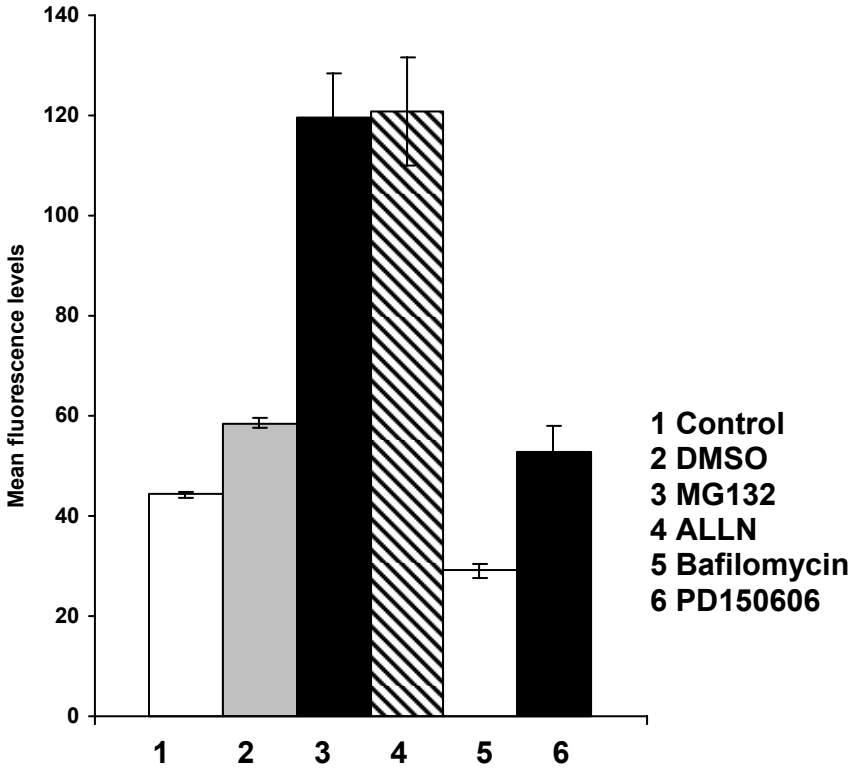

Figure 16. Green fluorescence levels in 2LKD-IN-eGFP cells treated with different compounds. *Cells were treated with the indicated compounds and 24 hrs later their green fluorescence was analyzed by FACS.*

DISCUSSION

Here we report the development and validation of two different HTS system. These are homogeneous cell-based assays and the read-outs are green and cherry fluorescence levels. Our results indicated that these systems will be useful to identify small-compounds antagonist the LEDGF/p75-HIV integrase interaction or compounds inhibiting the proteasome.

The cells used in these reporter systems are derived from HEK293T cells. They were engineered to stably express HIV-1 integrase C-terminally tagged with eGFP as well as the monomeric cherry fluorescence protein (mCherry). The use of these spectroscopically different fluorescence proteins allows to concomitantly measure in real time the levels of HIV-1 integrase (green fluorescence) as well as the healthy of the reporter cells (cherry fluorescence).

The screening system proposed for the identification of inhibitors of the LEDGF/p75-integrase interaction use IN-eGFP cells. Based in reported observations, we expect that drugs blocking the interaction of LEDGF/p75 with integrase will lead to integrase degradation and a change in its subcellular distribution. Hence, it is predictable that these drugs will reduce the green fluorescence levels in IN-eGFP cells. However, toxic drugs, that globally affect the cellular homeostasis, will also lead to an unspecific reduction of the green fluorescence in these cells. To exclude these toxic drugs earlier in the HTS campaign we propose to determine during the primary screening mCherry levels in the treated IN-eGFP cells. Only drugs that reduce the green fluorescence but that do not affect cherry fluorescence levels will be considered specific hits that will be selected for secondary screenings.

To validate this screening system we demonstrate that disruption of LEDGF/p75-integrase interaction lead to a specific decrease in green fluorescence levels in IN-eGFP cells. Because small compounds inhibitory of this protein-protein interaction are not commercially

available yet, we stably depleted LEDGF/p75 in IN-eGFP cells using a specific shRNA generating the 2LKD-IN-eGFP reporter cells. LEDGF/p75 depletion was achieved by lentiviral transduction of IN-eGFP cells with a vector that inserted in the host genome a bi-cistronic cassette containing in *cis* a LEDGF/p75-specific or control shRNA-expression unit and an immediately early CMV promoter-driven mCherry cassette. Using this strategy, we demonstrated that LEDGF/p75 depletion decreased the green fluorescence levels in IN-eGFP cells and that the IN-eGFP protein redistributed from an exclusively nuclear localization to a pancellular distribution. To further demonstrate the specificity of these effects, both green fluorescence levels and nuclear localization of IN-eGFP protein were rescued by transient transfection of LEDGF/p75 in $2L_{KD}$-IN-eGFP cells. Importantly, transient expression of a LEDGF/p75 mutant lacking IBD did not exhibit this rescue capability, further indicating that LEDGF/p75-integrase interaction was required.

Finally, we demonstrated the robustness of this HTS system when FACS analysis was used to detect green fluorescence levels. Instead, a high content image analysis was not suitable for screening showing poor reliability.

The HTS system that we have developed for the identification of inhibitors of the LEDGF/p75-integrase interaction offers several advantages compared to other reported HTS systems. Reported assays are alpha-screens that are homogeneous, cell-free systems that employ recombinant proteins. Different from these other screening systems, our assay will select only cellular permeable compounds that can interfere with the LEDGF/p75-integrase interaction in the intracellular environment. These are very important qualities that allow prioritizing at the primary screening only physiologically relevant hits. In addition, our reporter incorporates a counter-screening assay to exclude toxic compounds that is absent in the reported non-cell based

HTS systems. This counter-assay can be run concomitantly with the primary screening without extra manipulation. These are desired characteristics of HTS systems that diminish time and cost of the assay. Therefore, our system represents a clear advantage over other currently used HTS assays for the identification of inhibitors of the LEDGF/p75-integrase interaction.

The second HTS system developed is useful for the identification of small-compounds inhibitory of the proteasome pathway. In the absence of LEDGF/p75, integrase is ubiquitinylated and degraded through the proteasome. This phenomenon was observed in IN-eGFP-derived cells were LEDGF/p75 was severely depleted, $2L_{KD}$-IN-eGFP cells. It is expected that drugs interfering with the proteasome will increase the green fluorescence levels in $2L_{KD}$-IN-eGFP cells without altering mCherry levels. In order to validate this screening system we treated $2L_{KD}$-IN-eGFP cells with different drugs inhibitory of the proteasome, the lysosome and the calpains. Importantly, only proteasome inhibitors markedly increased green fluorescence levels in these cells, demonstrating the specificity of this reporter system. Robustness of this HTS system was also determined to be excellent (Z'-factor ~0.6) when cells were subject to FACS analysis.

CONCLUSION

We have developed and validated two robust HTS assays. The first assay is useful for identification of small-compounds antagonist of the LEDGF/p75-HIV integrase interaction while the second assay can identify inhibitors of the proteasome pathway. The most important features of these assays are:

- Cell-based. Different from *in vitro* screening systems, cell-based assays allow selection of compounds that are cell permeable, that reach the target intracellular compartments and that can disrupt physiological interaction occurring in the intracellular microenvironment. Therefore, using cell-based assays pharmacologically relevant compounds are selected reducing the number of unnecessary hits entering the secondary screening.

- Counter-screen build up in the system and using the same read-out that the primary screening. The Counter-screening will exclude toxic compounds. The fact that the developed HTS systems allow to conduct the counter-screening at the same time that the primary screening and without further manipulation facilitates the selection of relevant hits reducing time and price of the screening.

- Homogeneous assay. The developed HTS systems are mix-and-read assays that facilitate the screening campaign by reducing manipulation and time.

- Robust assays. Z'-factor calculated for these assays are above 0.5 indicating an excellent signal-to-noise ratio.

Further work is necessary now to develop secondary and tertiary assays for these primary HTS systems.

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CURRICULUM VITA

Elisa Morales was born in Mexico D.F. but raised in Chihuahua, Chih. The first daughter of Juan M. Morales Dajlala and Perla Chavez Amparan, graduated from Insituto la Salle de Chihuahua in spring 2002 and following her professional goals, Elisa entered The University of Texas at el Paso in the fall 2002. After 2 years of study, she was awarded with the Chihuahua Government Scholarship that led her to accomplish her bachelor's degree in Microbiology with a minor in Chemistry. While pursuing her bachelor's degree she was accepted at the Biology Undergraduate Research Scholars Program at UTEP where she gained research experience under Dr. Llano's supervision. She graduated with her Bachelors of Science in May 2007, the same year she started the Master's of Science program in biology. During her 2-years of study she participated in oral presentation in Society for the Advancement of Chicanos and Native Americans in Science National conference and American Society of Microbiology Regional Meeting. She also collaborated with her lab members on projects that were presented in The 3RD International Retroviral Conference. She was awarded with the Pan American Round Table of Texas scholarship that helped her to finish her Master's degree in May 2009. She is grateful, with the help Dr. Llano has provided her. In the near future, Elisa Morales would like to pursue a Ph.D in Virology in order to continue with her professional education. Meanwhile she is going to start working at the Texas Tech University Health Sciences Center in El Paso Texas, as a Medical Lead Technician, performing research on breast cancer, under Dr. Imagawa's mentorship.