Ini1/hSNF5 is dispensable for retrovirus-induced cytoplasmic accumulation of PML and does not interfere with integration

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Abstract Retroviral infection triggers the cytoplasmic translocation of two Crm1-dependent shuttle factors, namely the Ini1 (integrase interactor 1, hSNF5) and the promyelocytic leukemia (PML) protein. Blocking nuclear export of shuttle factors by leptomycin B increases the efficiency of retroviral integration, suggesting that some may mediate antiviral activity. While PML was shown to counteract proviral establishment, it remained unclear whether Ini1, a protein implicated in various processes during human immunodeficiency virus replication, has the same potential. Employing RNA interference-mediated knockdown of Ini1, we show here that the simultaneous accumulation of both proteins in the cytoplasm likely reflects two non-interdependent phenomena. Furthermore, Ini1 does not interfere with retroviral integration, as cells lacking Ini1 show no increased infection susceptibility.

Keywords: Integrase interactor 1/hSNF5; Promyelocytic leukemia protein; Retroviral integration

1. Introduction

Leptomycin B (LMB) is a specific inhibitor of the Crm1/exportin1 protein and LMB-treatment of cells results in the nuclear accumulation of Crm1/exportin1-dependent cargo factors (reviewed in [1]). Retroviral infection implies trans-cytoplasmic migration and nuclear import of the viral genome and associated proteins. Surprisingly, LMB treatment at least doubles the number of cells infected with a lentiviral gene-transfer vector (our unpublished observation and [2]). This effect suggests that Crm1-dependent shuttle factors mediate a cellular anti-retroviral response and that this function is blocked in the presence of LMB. Interestingly, it was shown recently that two Crm1-dependent shuttle factors are exported to the cytoplasm shortly after infection with HIV-1: promyelocytic leukemia (PML) protein and integrase interactor 1 (Ini1) [2].

PML, first described because of its involvement in acute promyelocytic leukemia, is the organizing component of distinct intra-nuclear substructures known as promyelocytic oncogenic domains (PODs) [3,4]. A large number of alternatively spliced transcripts are synthesized from the PML gene, resulting in a variety of PML isoforms ranging in molecular weight from 48 to 97 kDa [5]. PML and PODs are involved in a variety of cellular processes, including gene expression, cell cycle and growth regulation, differentiation, and apoptosis [6]. While usually 10–30 PODs are present per nucleus, this speckled pattern is disrupted in cells either completely lacking PML or expressing a PML fusion protein arising from a chromosomal translocation [4,7–9]. Furthermore, a variety of viruses have been shown to interact with PODs and/or to modify their sub-nuclear distribution or their composition, amongst them is the human immunodeficiency virus 1 (HIV-1) [2,10].

Shortly after HIV-1 infection, PML translocates from the nucleus to the cytoplasm where the protein colocalizes with incoming viral preintegration complexes (PICs). This Crm1-mediated redistribution is independent of the presence of packaged viral RNA genomes, accessory gene products and the envelope protein used [2]. Treatment of cells with As_2O_3, a drug that induces the recruitment of nucleoplasmic PML into PODs and its degradation [11], rendered cells more susceptible to HIV-1 infection, a phenomenon that was counteracted by overexpression of PML. These observations suggest that cytoplasmic recruitment of PML may be part of an antiviral response against HIV-1 infection [2]. This conclusion was, however, subsequently challenged by the demonstration that As_2O_3 largely released the Ref1-dependent block to reverse transcription of N-tropic murine leukemia virus (MLV) and enhanced infectivity of vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-based vectors in murine embryonic fibroblasts (MEFs) from PML$^{-/-}$ and wild type (wt) mice at comparable levels [12]. Since As_2O_3 may also trigger apoptosis by disruption of the mitochondrial transmembrane potential, it was hypothesized that effects on mitochondria may account for its stimulatory effects on retroviral reverse transcription. Interestingly, arsenic trioxide may also mediate the specific degradation or deregulation of human TRIM5α, a Ref1 and Lv1 retroviral restriction factor that, like PML,

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Abbreviations: dsRNA, double-stranded RNA; EGFP, enhanced green fluorescence protein; FACS, fluorescence activated cell sorting; HIV, human immunodeficiency virus; Ini1, integrase interactor 1; LMB, leptomycin B; PBS, phosphate-buffered saline; PI, propidium iodide; PIC, preintegration complex; PML, promyelocytic leukemia protein; POD, PML oncogenic domain; RNAi, RNA interference; siRNA, small inhibitory RNA; VSV-G, vesicular stomatitis virus glycoprotein; wt, wild type

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belongs to the large family of proteins harboring a so-called RBCC/TRIM motif [5,13,14]. So far, it remains unclear if these observations denote independent phenomena or if they can be reconciled.

Turelli et al. [2] also demonstrated that the human SWI/SNF-complex component Ini1 (or hSNF5) is exported jointly with PML. Ini1 is a cellular binding partner of the HIV-1 integrase and was hypothesized to be implicated in targeting HIV-1 PICs to favorable genomic sites [15]. Such an activity may be important in view of the fact that HIV-1 integrates preferentially into active genes [16]. The cytoplasmic delocalization of Ini1 seems not to depend on functional PML, as it also occurs after As_2O_3 treatment. Yet, it remains possible that Ini1, in turn, mediates the export of PML. The experimental proof of this hypothesis was hampered by the fact that PML is already mislocalized in Ini1-deficient cell lines [2]. Furthermore, it was not examined whether Ini1 may be implicated in an antiviral response mechanism, like it is suggested for PML. To improve and extend our understanding of cytoplasmic routing and fate of HIV-1 PICs, we sought to examine the effect of Ini1 knock-down during retroviral infection on the subcellular localization of PML and on the efficiency of retroviral integration.

2. Materials and methods

2.1. Plasmid construction

The vector pTRIP-LTR-EGFP is a derivative of the vector pTRIP-EF1α [17], an HIV-based gene-transfer vector that comprises two HIV-1 LTRs, the central triplex structure and a packaging signal, yet has no coding capacity for HIV proteins and solely expresses enhanced green flourescence protein (EGFP) from an internal EF1α promoter. pTRIP-LTR-EGFP lacks, however, the EF1α promoter and EGFP expression is consequently driven by the LTR. It was cloned in two steps: first, the vector pTRIP-PL was created by removal of the EF1α-promoter/EGFP cassette from pTRIP-EF1α via *Mlu*I and *Xho*I digestion and subsequent ligation of the annealed oligonucleotides 5′CGCGTCAGGATCC3′ and 5′TCGAGGATCCTGA3′ into the linearized vector, creating a new *Bam*HI site between the *Mlu*I and *Xho*I sites. Trip-PL was cut by *Bam*HI and *Xho*I and the *Bam*HI/*Xho*I-fragment from pTRIP-EF1α (see above) comprising the EGFP gene was inserted, giving rise to the pTRIP-LTR-EGFP vector.

2.2. Cell culture, immunofluorescence and Western blotting

Cells were cultured in DMEM supplemented with 10% foetal calf serum and Penicillin [50 U/ml]/Streptomycin [50 μg/ml] (Gibco). For immunofluorescence, HeLa cells (American Type Culture Collection, CCL-2) were fixed in 3% paraformaldehyde, permeabilized in Triton X-100 (0.5% in phosphate-buffered saline, PBS) and stained subsequently with a polyclonal rabbit antiserum against PML [18] in a 1:200 dilution in PBS. Hoechst 33258 (Sigma) was used to visualize the nuclear compartment. Immunofluorescence analysis was performed using a DMRX4 fluorescence microscope with filters A and TX2 (Leica) and images were taken with a Hamamatsu ORCAII-ER cooled CCD-camera and processed with Openlab® software (Improvision). Western blotting was done following standard protocols: protein extracts from a defined number of cells were separated in SDS–polyacrylamide gels, transferred to nitrocellulose membrane (Schleicher und Schuell), and incubated with the respective antibodies. As primary antibodies, a PML-specific polyclonal rabbit antiserum [18], an Ini1-specific polyclonal rabbit antiserum [19], a Tpr-specific mouse monoclonal antibody (Oncogene Research), a Ku80-specific polyclonal rabbit antiserum (Serotec), a HP1α-specific polyclonal rabbit antiserum (Upstate) or a α-tubulin-specific mouse monoclonal antibody (Sigma) were applied overnight after blocking of the membrane in 5% dried-milk (in PBS). After incubation with the respective horseradish peroxidase-coupled secondary antibody, the membrane was subjected to enhanced chemiluminiscence immunodetection (Pierce).

2.3. RNA interference

We selected small interfering RNAs (siRNAs) corresponding to a conserved sequence (nucleotides 925–947; Accession No. U04847) to target all published transcripts of the human *hSNF5/INI1* gene (annealed oligos GGAGAACUCACCAGAGAAGdTdT and CUUCUCUGGUGAGUUCUCCdTdT, Genset/Proligo). The conditions of the RNA interference (RNAi) protocol were essentially as described [20]. Briefly, HeLa cells were seeded in 24-well dishes to reach a confluency of about 50% at the time of transfection. The next day, 3 μl of Oligofectamine® (Invitrogen) was mixed (per well) with 12 μl of Opti-MEM® medium (Gibco) and incubated for 5 min at room temperature (RT). 3 μl of pre-annealed siRNAs (at a concentration of 20 μM) was diluted in 50 μl of OptiMEM® medium, mixed with the diluted Oligofectamine® and incubated for another 20 min. Before adding the mixture to the cells (in 500 μl of DMEM without antibiotics per well), the volume was adjusted to 100 μl to reach a final concentration of 100 nM RNA in the well. Forty-eight hours after transfection, protein levels were examined by Western blot analysis and cells were infected and subjected to immunofluorescence where applicable.

2.4. Virus preparation and infection

The VSV-G pseudotyped particles were produced as previously described [21] using pTRIP-LTR-EGFP as the genomic vector and the infectious titer of the stocks was determined by limiting dilution analysis of transduced HeLa cells using the vector-encoded EGFP protein as a marker. Cells were transduced by incubation with the concentrated virus stock in a small volume of medium at 37% for the indicated time span.

2.5. Cell fractionation

To isolate nuclei, cells were resuspended (4×10^7 cells/ml) in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 5 μg of aprotinin/ml, 5 μg of leupeptin/ml, 0.5 g of pepstatin A/ml, and 0.1 mM phenylmethylsulfonyl fluoride). Triton X-100 was added (at 0.1% final concentration) and the cells were incubated for 5 min on ice. Nuclei were collected in the pellet by low speed centrifugation (4 min, $1300 \times g$, 4 °C) and washed once in buffer A before resuspension in 1× sample buffer (2× volume of supernatant). The supernatant fraction was diluted 1:1 in 2× sample buffer. The samples were boiled for 8 min and equal volumes were loaded on the gel.

2.6. Annexin-V assay

Double staining for Annexin-V-Fluorescein and propidium iodide (PI) was performed using the Annexin-V-FLUOS staining kit from Roche according to the manufacturer's instructions. Cells that stained positive for PI (dead cells) were excluded from the analysis. Data were acquired on a FACSCALIBUR cytometer and analyzed using CellQuest software (both Becton–Dickinson, Pont de Claix, France).

2.7. Quantitative PCR

To avoid quantification of unintegrated vector DNA, we prepared genomic DNA from HeLa cells several weeks after infection [22,23] in the presence or absence of Ini1 protein. Quantitative PCR (qPCR) was carried out on an ABIPRISM® 7000 sequence detection system using SYBR® green (both Applied Biosystems). All primers were designed with the PrimerExpress 2.0® software (Applied Biosystems). Primer pairs PP1 and PP2 are mapping within the provirus (5′TGCTCTGGAAAACTCATTTGCA3′ (PP1 forward) and 5′CCAACTAGCATTCCAAGGCAC3′ (PP1 reverse) as well as 5′CCATTCGATTAGTGAACGGATCT3′ (PP2 forward) and 5′TGCCATTTGTGAATTCGGC3′ (PP2 reverse)). Primers amplifying β-actin (exonic) were used as cellular reference for normalization (5′TGTCCACCTTCCAGCAGATGT3′ (forward) and 5′CGGACTCGTCATACTCCTGCTT3′ (reverse)). All experiments were performed in triplicate.

3. Results and discussion

3.1. Cytoplasmic accumulation of PML after retroviral infection is independent of Ini1

We initiated our studies by setting up a RNAi protocol to specifically deplete Ini1. We selected siRNAs corresponding

to a conserved sequence, in order to target all published transcripts of the human *hSNF5/INI1* gene. Cellular Ini1 levels were reproducibly and specifically decreased to undetectable levels 48 h after transfection of Ini1 siRNAs (Fig. 1). This degree of depletion lasted for about two days before endogenous Ini1 levels rebounded to normal levels at days 5–6 (data not shown). Importantly, the expression of several unrelated proteins including PML was not affected (Fig. 1). Moreover, Ini1 expression levels remained unchanged after retroviral infection (Fig. 1A) and after transfection of siRNAs targeting unrelated proteins (Fig. 1B and unpublished data).

Next, we aimed to reproduce the cytoplasmic movement of PML after retroviral infection. Immunofluorescence analysis of untreated HeLa cells using a PML-specific antiserum [18] revealed the expected pattern of nuclear speckles in a slight nucleoplasmic background staining of soluble PML. Only rarely, we detected a cytoplasmic accumulation of the protein in speckled structures (Fig. 2A and B). We then employed VSV-G-pseudotyped, HIV-1-based TRIP-LTR-EGFP vector particles [24] to investigate the effect of retroviral infection on PML localization. In sharp contrast to untreated cells, we observed a marked increase in cytoplasmic PML staining accumulating in the appearance of speckled structures in the cytoplasm of HeLa cells, which had been exposed for 2 h to vector particles equivalent to a MOI of 2.5 (Fig. 2A and B). Determination of the number of nuclear vs. cytoplasmic dots in more than 200 randomly chosen, morphologically intact cells from three independent experiments revealed a roughly 10-fold increase in the average number of cytoplasmic speckles per cell shortly after retroviral infection (Fig. 2B). Dilution of the infection dose to 1/10th (m.o.i. of 0.25) resulted in a roughly 50% decrease in the average number of cytoplasmic speckles, indicating a dose-dependence (data not shown). This rapid response was not accompanied by detectable changes in the total amount of PML as shown by Western blot analysis (Fig. 1A) and hence most likely reflects a signal-mediated reorganization of preexisting PML molecules in the cytoplasm. In agreement with previous reports [2,12,25], we could not observe a significant change in the average number or integrity of nuclear PML bodies (Fig. 2B). It remained therefore formally possible that the cytoplasmic speckles originated from PML molecules already present in the cytoplasm [26]. To this end, we prepared nuclear and cytoplasmatic extracts from cells exposed to vector particles for 2 h along with mock-treated control cells and subjected them to Western blot analysis. The short exposure to retroviral particles led to a significant increase in the amount of several PML isoforms in the cytoplasm barely detectable in untreated cells. This was accompanied by the simultaneous decrease in the nuclear fraction (Fig. 2E), while the total amount of PML remained unchanged after infection (Fig. 1A). As expected, neither nuclear nor cytoplasmatic localization of HP1-α or α-tubulin, respectively, were affected by short-term exposure to retroviral particles. Finally, no increase in the proportion of apoptotic cells could be detected after infection by Annexin-V staining (Fig. 2F). Since phosphatidylserine translocation precedes oligo-nucleosomal DNA-fragmentation, after which nuclear membrane leakage occurs [27], it is evident that early apoptotic events are not involved in the nucleo-cytoplasmatic translocation of PML. In summary, these results substantiate the observation that incoming HIV-1 PICs trigger the accumulation of nuclear PML in speckled cytoplasmic structures, as previously shown [2]. Since number and integrity of nuclear PML bodies were not perturbed (Fig. 2B), it appears likely that the PML molecules accumulating in the cytoplasm are recruited from soluble, nuclear pools.

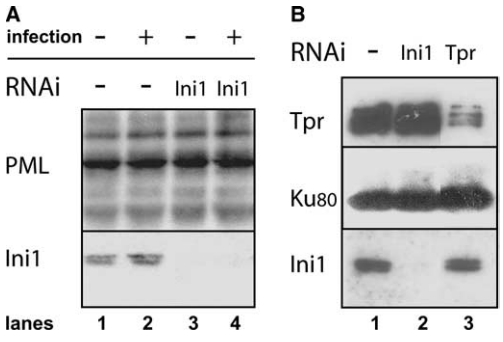

Fig. 1. Influence of RNAi and viral infection on cellular levels of different proteins. Western blot analysis 48 h after RNAi treatment on cellular extracts (total protein of 4×10^4 cells/lane). Antibodies are described in the main text. (A) PML and Ini1 levels are shown 48 h after Ini1-specific (Ini1) or mock RNAi treatment (−) followed by 2 h of retroviral (+) or mock-infection (−), respectively. (B) Tpr, Ku80 and Ini1 protein levels were detected 48 h after application of the dsRNAs as indicated (RNAi): mock transfection (−), Ini1-siRNAs or siRNAs against the human Tpr protein.

We next applied our specific RNAi scheme to determine the influence of transient Ini1 depletion on subcellular PML redistribution upon retroviral infection. To this end, HeLa cells were seeded in 6-well dishes containing small coverslips and subjected to siRNA treatment the next day as described above. After further culture for 48 h, cells were either mock infected or exposed to pseudotyped HIV-1 particles for two hours at 37 °C. Then, the coverslips were removed and the cells were fixed and stained (see above). In parallel, protein extracts were prepared from the remaining cells in the wells in order to control for efficient depletion of Ini1 by Western blot analysis. The simultaneous examination of PML and Ini1 by immunofluorescence was precluded since none of the α-Ini1 antibodies we tested was functional in this assay. Similar to untreated cells, PML was predominantly present in nuclear dots of cells either mock transfected or treated with Ini1-specific siRNAs, the accumulation of PML in speckled structures in the cytoplasm being only occasionally detectable. Although this never reached statistical significance, the average number of nuclear PML bodies was slightly increased in siRNA-treated cells compared to untreated controls (compare Figs. 2B and D). This could indicate an interferon-mediated response to the treatment of cells with double-stranded RNA (dsRNA) [28]. PML has been implicated in interferon-mediated processes and number and size of PODs increase in response to interferon [10]. However, neither the transfection procedure per se nor the cellular uptake of small, dsRNA or the depletion of Ini1 profoundly influenced the subcellular location of endogenous PML. Yet, upon short exposure to VSV-G pseudotyped, HIV-based TRIP-LTR-EGFP vector particles, a strong accumulation of speckled PML structures occurred in the cytoplasm. Importantly, this occurred irrespective of the presence of Ini1. Comparable to what is observed in cells expressing normal Ini1 levels (Fig. 2B), an increase in the average number of cytoplasmic speckles per cell from 0.7 (no infection) to 5.2 (after infection) accompanied by a diffuse cytoplasmic staining following infection was noted in Ini1-knock-down cells (Fig. 2D). This effect was reliably reproduced in several independent experiments (the slight

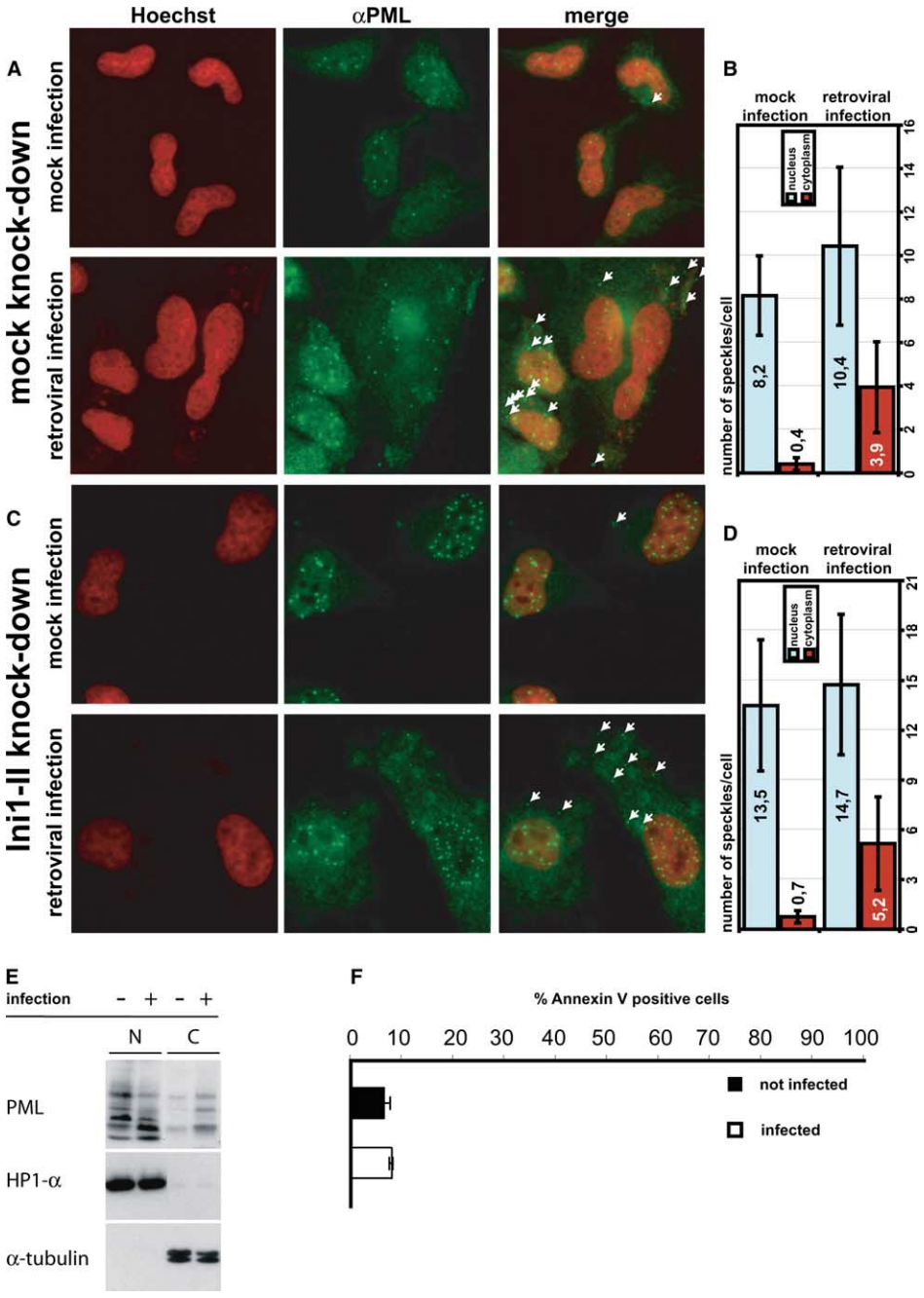

Fig. 2. Retroviral infection of HeLa cells induces the appearance of cytoplasmic PML foci irrespective of the presence of Ini1. (A) HeLa cells were mock-infected (upper panel) or infected with pseudotyped retroviral particles (lower panel). Hoechst 33258 staining was done to define the nuclear compartment (left panel, pseudocolored in red); Immunofluorescence (PML-specific rabbit antibody (see text) and Cy3-coupled anti-rabbit secondary antibody from Jackson Immuno Research Laboratories, pseudocolored in green) revealed intracellular distribution of PML protein (middle panel). Merged images allow for the identification of cytoplasmic speckles (see arrows). (B) The number of nuclear (defined as colocalizing with the DNA staining) and cytoplasmic PML speckles was determined for more than 200 randomly chosen, morphologically intact cells from three independent experiments. Average values are indicated and standard deviations are represented by error bars. (C) HeLa cells were mock-infected (upper panel) or infected (lower panel) on the day of apparent Ini1-depletion (see text). Hoechst 33258 staining and immunofluorescence as in (B). (D) Quantification of the number of nuclear (as evidenced by colocalization with Hoechst 33258 staining) and cytoplasmic PML speckles was determined for more than 200 randomly chosen, morphologically intact cells from three independent experiments. Average values are indicated and standard deviations are represented by error bars. (E) Cellular fractionation of equal numbers of cells was performed 2 h after infection (+) or mock infection (−) of Hela cells, to test for the pre- and postinfection subcellular distribution of PML and marker proteins in Western blot analysis. Purity of the fractions (N=nuclear, C=cytoplasmic fraction) was confirmed using α-HP1α (non-histone chromosomal protein, nuclear) and anti-α-tubulin (microtubule component, cytoplasmic) staining. (F) FACS analysis of Annexin-V-Fluorescein/PI double-stained HeLa cells 2 h after infection (white bar) or mock-infection (black bar). Indicated is the percentage of Annexin-V positive, PI-negative cells in the population. Error bars represent the standard deviation from two independent experiments.

increase in the average number of cytoplasmic PML speckles of siRNA treated versus control cells was statistically not significant, compare Fig. 2B and D). This further corroborated our conclusion that Ini1 is dispensable for retrovirus-mediated cytoplasmic accumulation of PML. Even if minute amounts of Ini1, not detectable by our Western blot analysis, were present in the cells after siRNA treatment, at least a notable reduction of the phenomenon would be expected. Recapitulating, we confirmed that the nuclear body constituent PML efficiently accumulates in the cytoplasm in response to retroviral infection. Moreover, employing a RNAi-scheme for the transient depletion of Ini1, we conclusively show that this phenomenon occurs independently of Ini1. Consequently, the SWI/SNF component Ini1 is not required for this process, which has been suggested to be part of a cellular innate immune response to retroviral infection.

3.2. Ini1 depletion does not influence integration efficiency

Although Ini1 is not affecting PML de-localization, we wanted to know whether it potentially affects HIV-1 proviral establishment via another pathway. To test for this possibility, we determined the number of HeLa cells that were successfully transduced with the EGFP-carrying vector in the presence or absence of Ini1 using fluorescence activated cell sorting (FACS) analysis. As can be seen in Fig. 3, we did not observe a significant change in the transduction rate after knock-down of Ini1. Irrespective of the presence of Ini1, comparable percentages of GFP positive cells were detected at the first data point analyzed (day two post transfection) and remained constant for prolonged time of culture (more than 10 days; data not shown). Therefore, it appears rather unlikely that integration in the absence of Ini1 was delayed during depletion and occurred efficiently only after reappearance of Ini1, since Ini1 depletion lasted for 2–3 days after infection. Moreover, such a scenario would require a stable, integration-competent viral DNA form that is able to persist for at least two days. Yet, viral DNA entering the nucleus either rapidly integrates or will be circularized due to non-homologous DNA end joining mechanisms, thus eliminating substrates for delayed integration events [29]. A linear viral DNA implicated in pre-integration latency can persist in the cytoplasm of resting CD4+ T cells but has been shown to be rapidly degraded in dividing cells as used in this study [30]. Altogether, we assume that a significant stimulatory effect of Ini1 on integration could not have been simply masked and that retroviral integration was not merely delayed in Ini1-depleted cells.

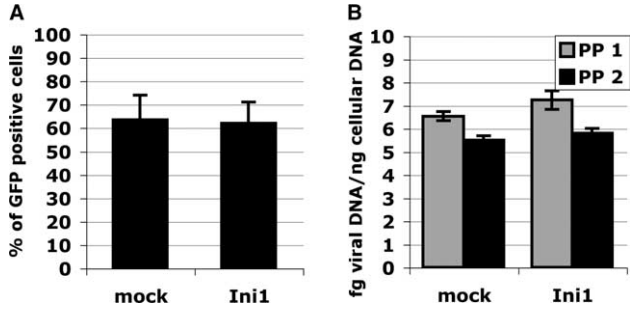

Fig. 3. Proviral establishment is not increased in cells devoid of Ini1. (A) The percentage of EGFP-positive cells was determined by FACS analysis after infection with a MOI of 0.6 of cells under mock- (mock) or Ini1-knock-down (Ini1). Error bars represent the standard deviation of 11 independent experiments. (B) qPCR analysis of DNA derived from a representative experiment shown in (A). Results obtained with two independent viral primer pairs (PP1 and PP2) are shown as fg viral DNA/ng cellular DNA (determined with β-actin primers). Error bars represent the standard deviation of triplicate experiments.

To confirm the FACS-based observation, we performed qPCR analysis. We extracted DNA from cells that were infected during Ini1- or mock knock-down after prolonged culture (more than three weeks) to avoid PCR-amplification of unintegrated viral DNA species [22,23]. Using two different primer pairs mapping within the TRIP-LTR-EGFP provirus and β-actin as a cellular reference, we could clearly confirm that the number of successful integration events per cell (Fig. 3B) is not significantly altered after Ini1 knock-down. Likewise, when transducing HeLa and MON cells (a tumor cell-line showing bi-allelic deletion of the *hSNF5/INI1* gene [31]) in parallel, we could not detect an increased susceptibility of the Ini1-deficient MON cells towards infection using the same approaches (data not shown).

Together, these results provide evidence that Ini1 is not central to an early cellular antiretroviral response. Moreover, Ini1 apparently does not quantitatively stimulate HIV integration in the context of viral infection, which contrasts the in vitro finding of Ini1 stimulating the HIV-1 integration process [15]. These observations should, however, not distract from a possible implication of Ini1 in targeting HIV-1 to preferred genomic sites or chromatin environments [15,16,32,33] with potential consequences for both, efficient viral replication and establishment of latent reservoirs.

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