รายงานวิจัยฉบับสมบูรณ์

สัญญาเลขที่ BRG5880001

การศึกษาเชิงลึกระดับโมเลกุลของแองไครินิน 1ดี4 เพื่อทราบกลไกการรบกวนการประกอบอนุภาคของเอชไอวี-1

นายชัชชัย ตะยาภิวัฒนา

Abstract

Ank^{GAG}1D4 is the designed repeat protein which recognizes capsid protein (CA) of HIV-1 and represent the antiviral activity to viral assembly process. Here we investigated the intensive mechanism of Ank^{GAG}1D4 with several pathways of HIV-1 life cycle. Using stimulated emission depletion microscopy (STED), We demonstrated that multimerization of Gag precursor protein was interrupted. In order to determine the encapsidation mechanisms of HIV-1, we determined the specificities of genomic RNA, spliced HIV mRNAs and different host RNAs. Ank^{GAG}1D4 interrupted the specificity of Gag precursor protein to select genomic RNA for encapsidation. Moreover, we also demonstrated that Ank^{GAG}1D4 decreased progeny virion without disturbing of Gag maturation, but Gag precursors accumulate inside cell instead.

บทคัดย่อ

โปรตีนแองไคริน (Ankyrin) Ank^{GAG}1D4 เป็นแองไครินที่มีความสามารถในการจับจำเพาะกับโปรตีนแคพสิด (Capsid) ของเชื้อไวรัสเอชไอวี-1 (HIV-1) ก่อให้เกิดการขัดขวางการประกอบอนุภาคใหม่ของเชื้อไวรัสเอชไอวี-1 ในการนี้ทางห้องปฏิบัติการได้ทำการสืบหากลไกต่าง ๆ ของโปรตีน Ank^{GAG}1D4 ในการยับยั้งการสร้างไวรัสตัวใหม่ของเชื้อไวรัสเอชไอวี-1 เมื่ออาศัยการวิเคราะห์ด้วยกล้องจุลทรรศน์ชนิด Stimulated emission depletion (STED) พบว่ากระบวนการเรียงตัวของโปรตีน Gag ของเชื้อไวรัสเอชไอวี-1 บนผิวเซลล์ถูกรบกวน อนึ่ง เพื่อที่จะวิเคราะห์กระบวนการ RNA-encapsidation ของเชื้อไวรัสเอชไอวี-1 ทางห้องปฏิบัติการได้ทำการศึกษาความจำเพาะของเชื้อไวรัสเอชไอวี-1 ในการเลือก RNA ชนิดต่าง ๆ เพื่อประกอบอนุภาคใหม่ของเชื้อไวรัสเอชไอวี-1 เช่น RNA ของเชื้อไวรัสเอชไอวี-1 (genomic RNA และ spliced RNA) และ RNA ของเซลล์เจ้าบ้าน ผลการทดลองพบว่าโปรตีน Ank^{GAG}1D4 มีผลต่อโปรตีน Gag ในการเลือก RNA ของเชื้อไวรัสเอชไอวี-1 (genomic RNA) ในการประกอบอนุภาคใหม่ของเชื้อไวรัสเอชไอวี-1 รวมไปถึงลดการสร้างไวรัสเอชไอวี-1 ตัวใหม่โดยที่ไม่รบกวนกระบวนการ Maturation ของโปรตีน Gag ในไวรัสตัวใหม่ แต่พบว่าโปรตีน Gag มีการสะสมอยู่มากภายในเซลล์เจ้าบ้าน

Introduction

Ank^{GAG}1D4, a trimodular repeat, was selected from a phage-displayed ankyrin library for its binding to the HIV-1 capsid (CA)(1). Ank^{GAG}1D4 showed the effect on inhibition of HIV-1 replication(1, 2). The viral progeny yield and viral genomic RNA (gRNA) were significant decreased in the HIV-1 infected SupT1 cells stably expressing Ank^{GAG}1D4(1). Moreover, Ank^{GAG}1D4 showed negatively interfere HIV-1 replication in primary $CD4^+$ cell(2).

The $Pr55^{Gag}$ precursor (Gag precursor) protein, the precursor of capsid protein which is the target of Ank^{GAG}1D4, is translated by unspliced RNA or gRNA and plays an important role in assembly process and RNA recruitment of HIV-1(3, 4). It composts of matrix (MA), capsid (CA), nucleocapsid (NC), and p6 domains(5).

Gag precursor recruits the dimer of gRNA, which occurs in the cytoplasm of HIV-1 infected cell(4), to initiate the multimerization of Gag polyprotein. This complex will traffic to the plasma membrane which is the assembly site. The thousands of Gag/Gag-Pol polyprotein molecules will be polymerized in this site(6-8). The accumulation of viral Env glycoproteins at this site and Gag polyprotein then recruits the cellular ESCRT machinery, which drives the membrane scission reaction for viral particle releasing(9).

After the synthesis of HIV-1 RNAs in nuclease, RNAs are transported to cytoplasm to undergo two process which are translation and packaging into new

virion particle(3, 9). The viral proteins are produced by genomic mRNA (gRNA) which is unspliced RNA or Full-length RNA (FL-RNA)(3). Gag precursor protein must select their gRNA from a variety of cellular and viral spliced RNAs Multi Spliced (MS) mRNAs and singly spliced mRNAs with env mRNA (env) as the major representative(10). Even viral spliced RNAs can be selected due to the presence of internal loop and lower part in SL1 (Stem loop 1), Gag precursor protein recognize gRNA with higher affinity than spliced RNAs(5).

It has been reported that retroviruses are able to package significant amounts of cellular RNA(11), even SL1 is not present. Cellular 7SL RNA, a component of signal recognition particles (SRPs), is involved in protein translocation across the endoplasmic reticulum, has shown to be package into HIV-1 viral particle(3, 12, 13) as well as U6 spliceosomal RNA(3, 13-15).

Here we studied the role of Ank^{GAG}1D4 for specific selection of gRNA by Gag polyprotein. We also investigated the effect of Ank^{GAG}1D4 on multimerization of Gag polyprotein and Gag maturation.

Material and Methods

1. Disturbing of HIV-1 Gag polymerization on plasma membrane using Ank^{GAG}1D4

Plasmid construction

To construct plasmid carrying fusion protein of Matrix-Capsid HIV-1 (MACA) protein and Halotag protein. We used pFC14K HaloTag® CMV Flexi vector (Promega) as an accepter plasmid. The MACA gene was amplified form pNL4-3 HIV-1 plasmid and then treated with *Sgf*I and *Eco*ICRI. The processed gene was then transferred into pFC14K HaloTag® CMV Flexi vector using *Sgf*I and *Eco*ICRI cloning site to get pFC14K-MACA-HaloTag® as shown in Figure 1.

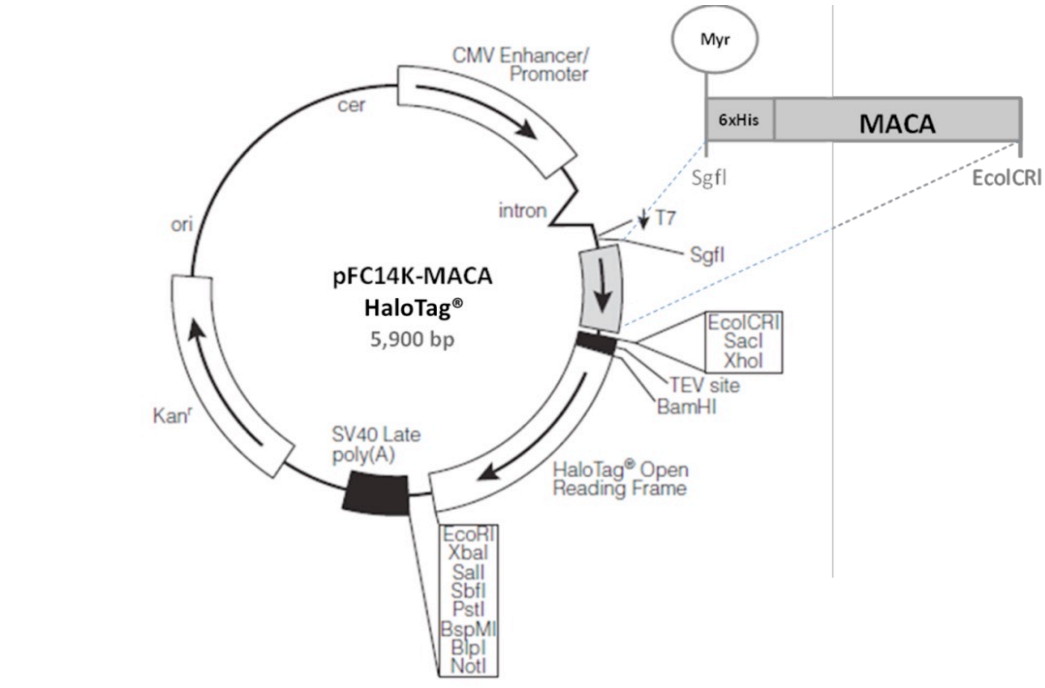

Figure 1 The plasmid map of pFC14K-MACA-HaloTag® used in this study.

Transfection

Monolayers of Hela cells at $1x10^5$ cells were transfected with 1 μg of DNA (pCEP4-Ankyrins as shown in Figure 2 and/or pFC14K-MACA HaloTag®) using transIT-X2 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer's protocol. The cells were cultured for 48 h at 37 °C 5 % CO_2 humidified incubator. MACA Halotag expressing cells were stained with HaloTag® TMRDirect™ ligand (Promega) at dilution 1:1000 for 16-18 h at 37 °C 5 % CO_2 humidified incubator. Membrane of non-MACA Halotag expressing cells were stained with 1X CellMask™ plasma membrane stain (Thermo Scientific, Rockford, IL) for 10 min at 37 °C 5 % CO_2 humidified incubator. Cells were then fixed with 4% paraformaldehyde and mount with ProLong® Diamond Antifade Mountants (Thermo Scientific). Fixed cells were performed using Leica TCS SP8 STED. Raw images were iteratively deconvolved with Huygens software (Scientific Volume Imaging, Netherlands).

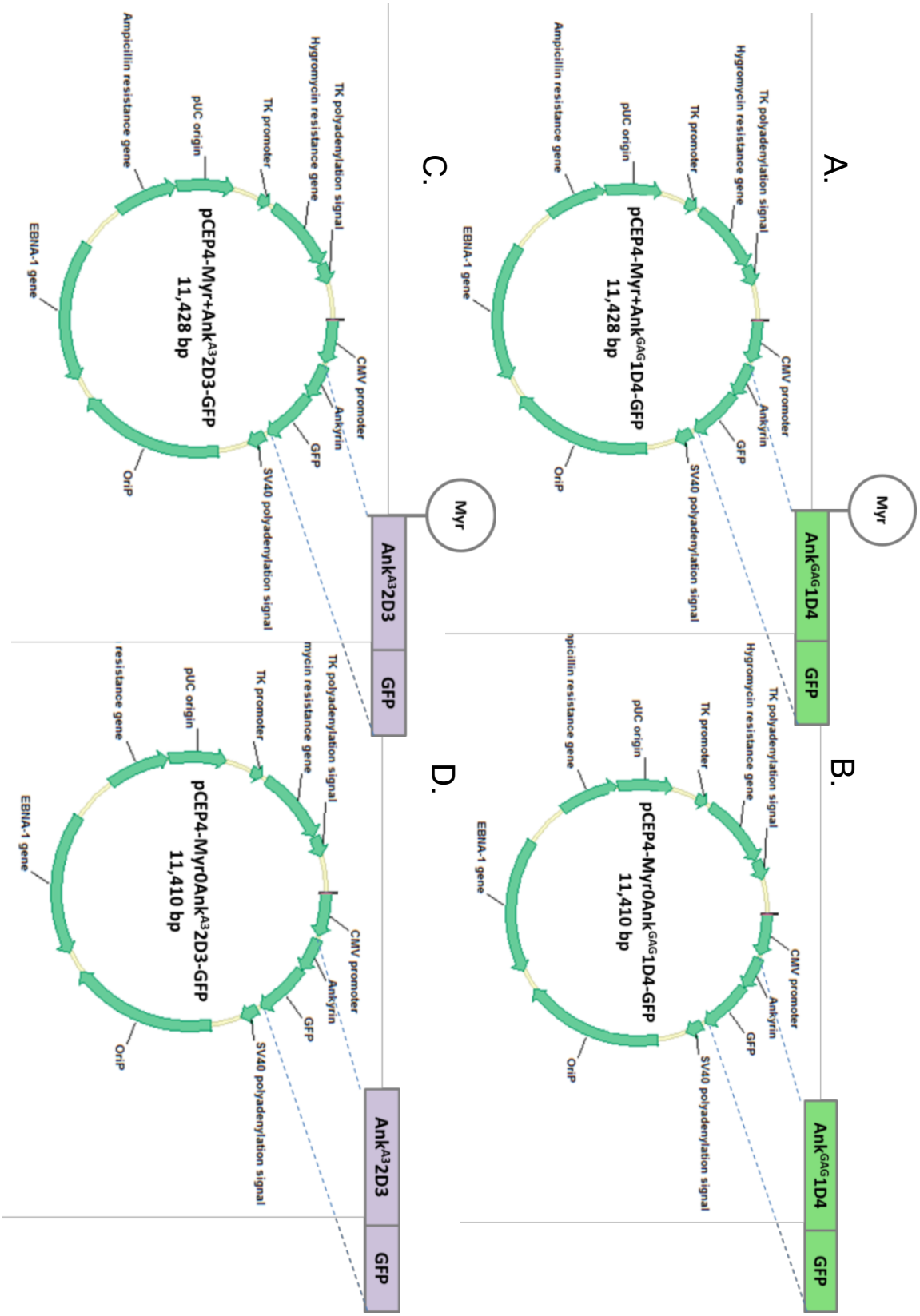

Figure 2 Plasmid map of Myr+Ank^{GAG}1D4-GFP (A), Myr0Ank^{GAG}1D4-GFP (B), Myr+Ank^{A3}2D3-GFP (C) and Myr0Ank^{A3}2D3-GFP (D)

2. Testing of the HIV-1 Gag maturation effect by Myr+Ank^{GAG}1D4

Transfection

$3x10^6$ cells of 293T cell were cultured in completed Dulbecco's Modified Eagle's medium (DMEM). 1 day after, Cells were transfected with 2 µg of pNL4-3Δenv along with DNA carrier (plasmid pSP72) with or without 1.6 µg of pCEP4-Myr+Ankyrins (Ank^{GAG}1D4 or Ank^{A3}2D3). 5 h after transfection, Cells were trypsinized and washed twice to remove the excess DNA plasmid. 24 h after transfection, Cells and culture supernatant were harvested for protein and RNA analysis.

Viral purification

The culture supernatant was centrifuged at 1,500 rpm for 10 mins and transferred to filter pore size 0.45 µm. Virions were purified by ultracentrifugation through 20% sucrose cushion at 30,000 rpm for 1 h 30 min at 4 °C. Viral pellets were resuspended in 160 µL of DMEM containing 8 U RQ1 RNase-Free DNase (Promega). 25 µL of virion sample was used for protein analysis.

Western immunoblotting

Half of cellular dried pellet was lysed to extract cellular protein using Complete lysis-M kit (Sigma-Aldrich, St. Louis, Missouri) with protease

inhibitor cocktail according to the manufacturer's instructions. Total amount of protein was calculated by Bradford assay. Proteins (100 µg of cell lysate or 15.6 % of viral lysate) were load on SDS-PAGE and transferred on nitrocellulose membrane. HIV-1 Gag was detected with mouse anti-capsid (CA) antibody (1/200, NIH AIDS Reagent Program, hybridoma H183). Ankyrin protein expression was determined with mouse anti-GFP (1/1000). Horseradish peroxidase-conjugated (HRP) anti-mouse IgG (diluted at 1/2000, Santa Cruz) was used as secondary antibody. Cellular actin was detected with mouse monoclonal anti-β-actin−peroxidase antibody produced (1/20000, clone AC-15, Sigma-Aldrich). ECL fluorescence was recorded by ChemiDoc™ Touch Imaging System (Bio-rad).

3. Quantification of RNAs

RNA Extraction

Half of cellular dried pellet was used to extract cellular RNA using TRI Reagent® (MRC, Cincinnati, OH) according to the manufacturer's instructions. The rest of virion sample that previously purify and resuspended was incubated at 37 °C for 45 min to eliminate the remained plasmid DNA. Then, nucleic acids in virion were extracted using phenol/chloroform precipitation in the presence of 20 µg tRNA carrier and 44 µL of TES 4X (200 mM Tris-HCl pH 7.5, 20 mM EDTA, 0.4% SDS). The RNA pellet was dissolved in ultra-pure water. All RNA samples were treated with treated with with RQ1 DNase (Promega) in presence

of RNasin® (Promega) for 25 min at 37 °C to eliminate the remaining DNA. RNA pellets were dissolved with 15 ml of ultra-pure water. RNAs were quantified by measuring optical absorption at 260 nm.

Cellular and Viral RNA Quantification

Reverse transcription was performed with the expand RT (Roche) with 0.5 µg of RNA sample for the quantification of viral RNAs, GAPDH RNA and the U6 (aU6-103 5′-TATGGAACGCTTCACGAATTTGCG) and 7SL (a7SL148 5′-CCCGGGAGGTCACCATATT) cellular RNAs. Oligo(dT) primer (3.75 µM) was used with viral RNAs and GAPDH cell RNA. Specific internal primers (3.75 µM) were used for and 7SL RNAs. After RT, RT product was diluted and performed with the LightCycler® FastStart DNA MasterPLUS SYBR Green I kit (Roche) and the RotorGene apparatus (Labgene). The RT products were specifically amplified by 35 cycles of PCR: 95°C for 15 s; 58°C for 12 s and 72°C for 20 s. 0.5 mM of each of the following primers were used: for

FL, sHIV-1306 5′-TCAGCATTATCAGAAGGAGCCACC and

aHIV-1541 5′-TCATCCATCCTATTTGTTCCTGAAG; for MS,

sHIV-5967 5′-CTATGGCAGGAAGAAGCGGAG and

aHIV-8527 5′-CAAGCGGTGGTAGCTGAAGAG; for env,

sHIV-729SD1A5 5′-GAGGGGCGGCGACTGGAAGAA and

aHIV-6134 5′-ACTATGGACCACACAACTATTGC; for GAPDH,

GA-721 5′-GCTCACTGGCATGGCCTTCCGTGT and

GA-931 5′-TGGAGGAGTGGGTGTCGCTGTTGA; for 7SL,

s7S-22 5′-CTGTAGTCCCAGCTACTCG and

a7S-148 5′-CCCGGGAGGTCACCATATT; and for U6,

sU6-3 5′-GCTCGCTTCGGCAGCACATATACT and

aU6-103 5′-TATGGAACGCTTCACGAATTTGCG.

The quantification was calculated using a standard curve of 10^2 to 10^6 copies of pNL4-3 plasmid.

Results

The disturbing of Gag polymerization by Ank^{GAG}1D4

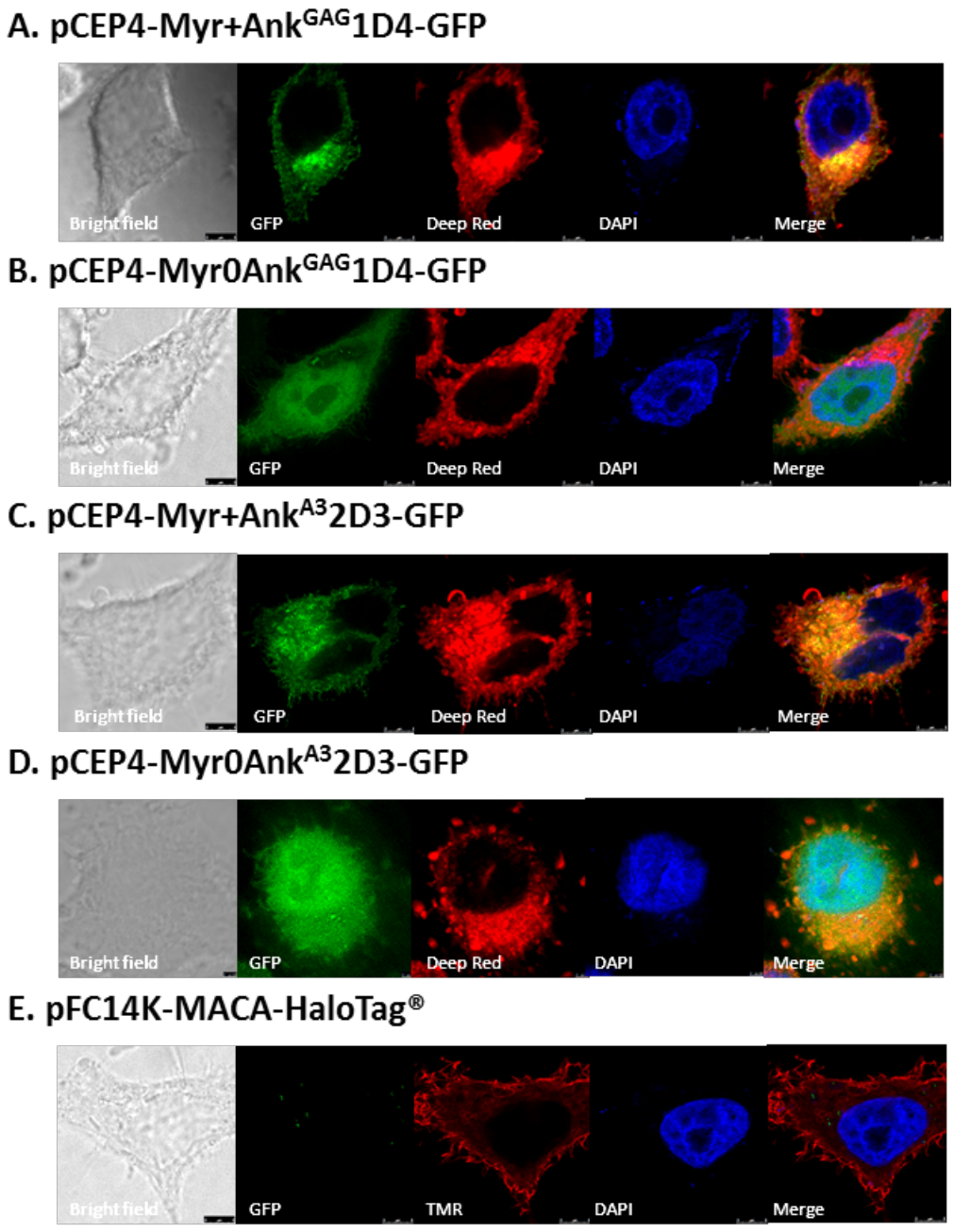

Figure 3 The expression of Ankyrins-GFP fusion protein and MACA-HaloTag® fusion protein with Hela cell as visualized by stimulated emission depletion microscopy (STED).

The GFP expression in HeLa cell transfected with single pCEP4-Myr+Ank^{GAG}1D4-GFP or pCEP4-Myr+Ank^{A3}2D3-GFP, localized mainly in cytoplasm and partially at plasma membrane as shown in Figure 3A and 3C, respectively.

The GFP expression in HeLa cell transfected with single pCEP4-Myr0Ank^{GAG}1D4-GFP or pCEP4-Myr0Ank^{A3}2D3-GFP, distributed throughout cell as shown in Figure 3B and 3D, respectively.

The MACA-HaloTag® fusion protein was expressed obviously at the plasma membrane of transfected Hela cell after staining with HaloTag® TMRDirect™ ligand as shown in Figure 3E.

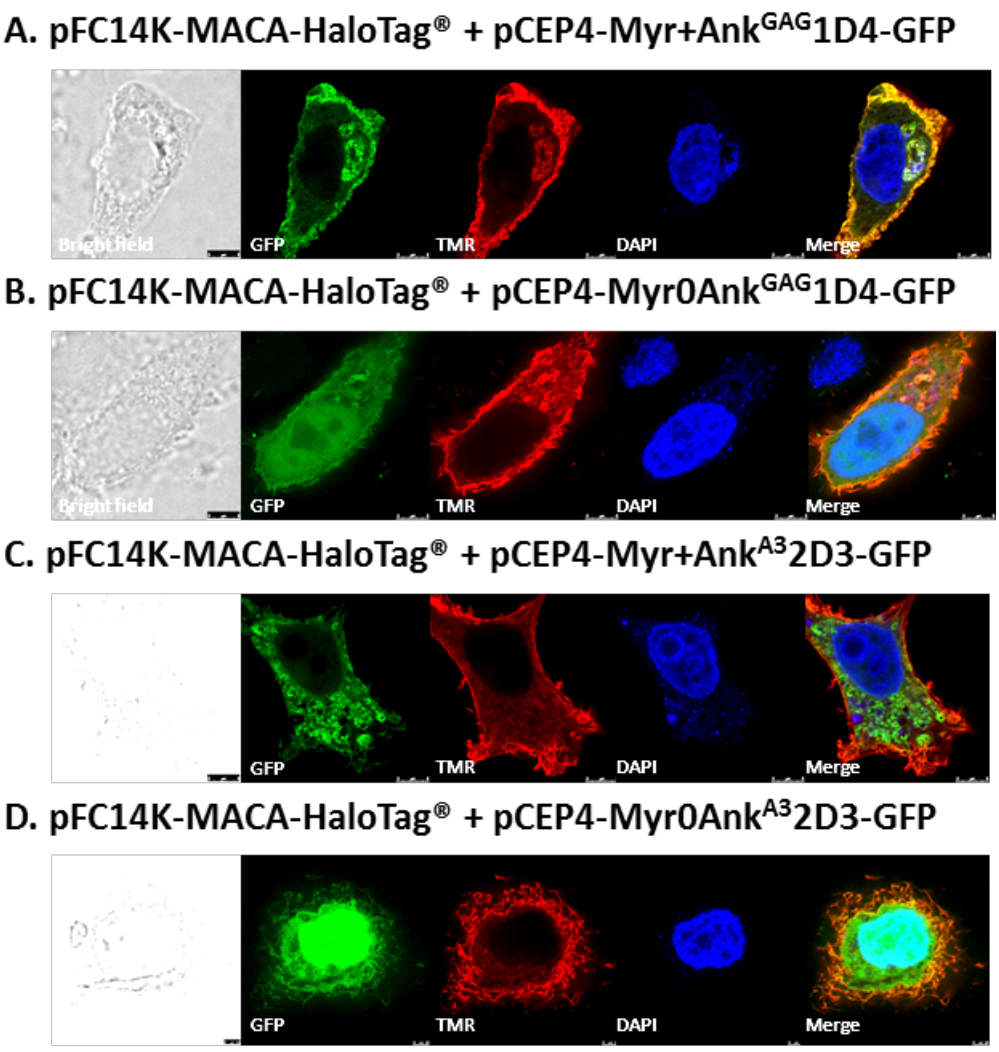

Figure 4 Co-expression of Ankyrins-GFP fusion protein and MACA-HaloTag® fusion protein in Hela transfected cell as visualized by stimulated emission depletion microscopy (STED).

Myr+Ank^{GAG}1D4-GFP fusion protein and MACA-HaloTag® fusion protein were expressed mainly at plasma membrane of transfected HeLa cell. Colocalization of Myr+Ank^{GAG}1D4-GFP fusion protein and MACA-HaloTag® fusion protein was also occurred at plasma membrane as shown in Figure 4A.

The Hela cell transfected with plasmid pFC14K- MACA-HaloTag® and pCEP4-Myr0Ank^{GAG}1D4-GFP, expressed the Myr0Ank^{GAG}1D4-GFP fusion protein throughout cell and MACA-HaloTag® fusion protein in cytoplasm and plasma membrane. The colocalization of Myr0Ank^{GAG}1D4-GFP fusion protein throughout cell and MACA-HaloTag® fusion protein occurred in cytoplasm and plasma membrane as shown in Figure 4B.

The Hela cell transfected with plasmid pFC14K- MACA-HaloTag® and pCEP4-Myr+Ank^{A2}2D3-GFP, expressed the Myr+Ank^{A2}2D3-GFP fusion protein within cytoplasm and MACA-HaloTag® fusion protein at plasma membrane. No colocalization of Myr+Ank^{A2}2D3-GFP fusion protein and MACA-HaloTag® fusion protein has been visualized as shown in Figure 4C.

The Hela cell transfected with plasmid pFC14K- MACA-HaloTag® and pCEP4-Myr0Ank^{A2}2D3-GFP, expressed the Myr0Ank^{A2}2D3-GFP fusion protein in nucleus and cytoplasm, and MACA-HaloTag® fusion protein at plasma membrane. No colocalization of Myr0Ank^{A2}2D3-GFP fusion protein and MACA-HaloTag® fusion protein has been visualized as shown in Figure 4D.

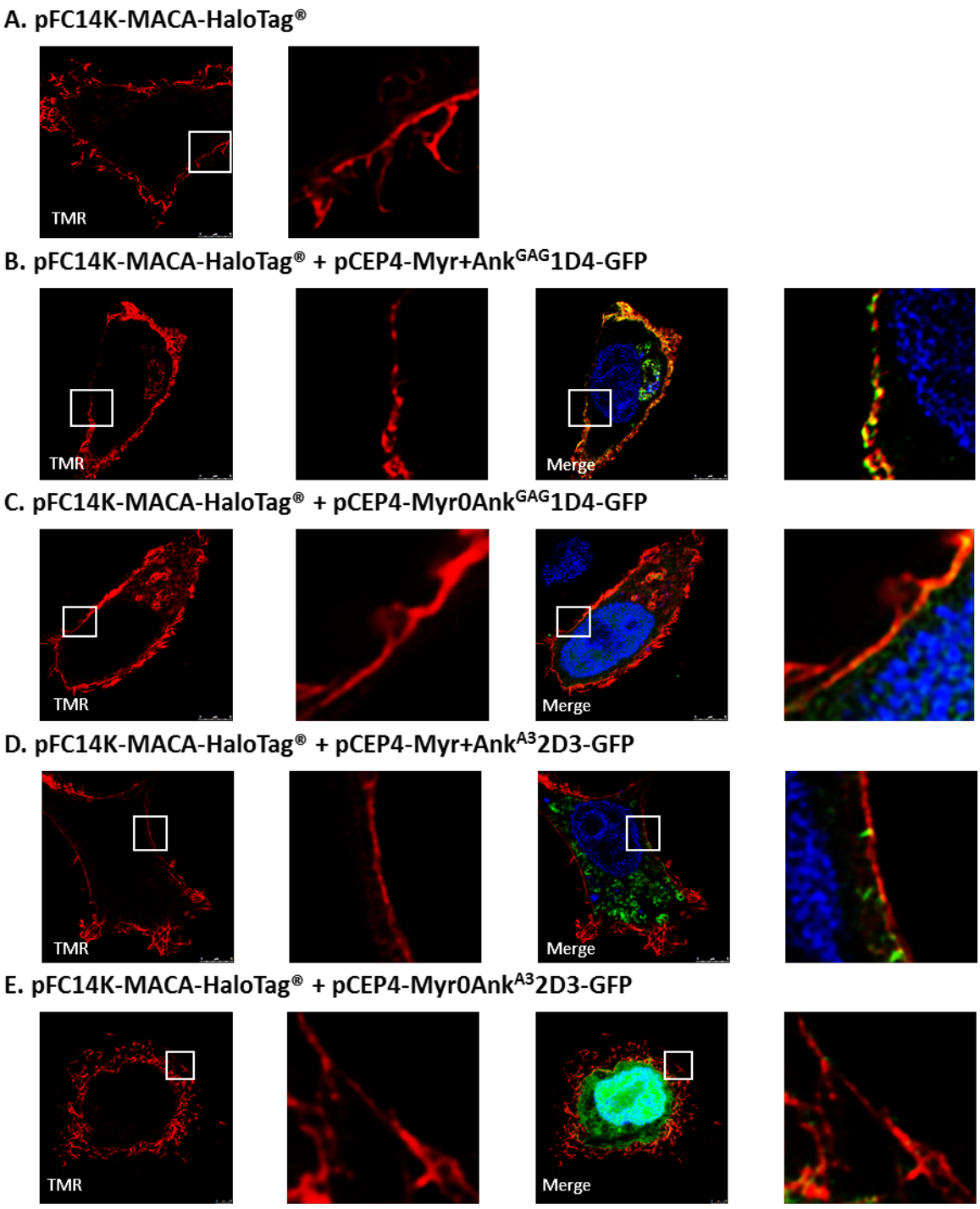

Figure 5 The zoomed-in view of expression of Ankyrin-GFP fusion proteins and MACA-HaloTag® at plasma membrane of HeLa transfected cell as visualized by stimulated emission depletion microscopy (STED).

The zoomed-in view of HeLa transfected cell showed that the expression of MACA-HaloTag® fusion protein was expressed continuously at plasma membrane in HeLa cell transfected with 1. pFC14K- MACA-HaloTag® single plasmid 2. pFC14K- MACA-HaloTag® and pCEP4-Myr0Ank^{GAG}1D4-GFP 3. pFC14K- MACA-HaloTag® and pCEP4-Myr+Ank^{A3}2D3-GFP 4. pFC14K- MACA-HaloTag® and pCEP4-Myr0Ank^{A3}2D3-GFP as shown in Figure 5A, 5C, 5D and 5D, respectively. Nevertheless, the HeLa cell co-transfected with pFC14K- MACA-HaloTag® and pCEP4-Myr+Ank^{GAG}1D4-GFP showed that the expression of MACA-HaloTag® fusion protein at plasma membrane was not continuous pattern as Figure 5B.

The effect of Myr+Ank^{GAG}1D4 on Gag processing

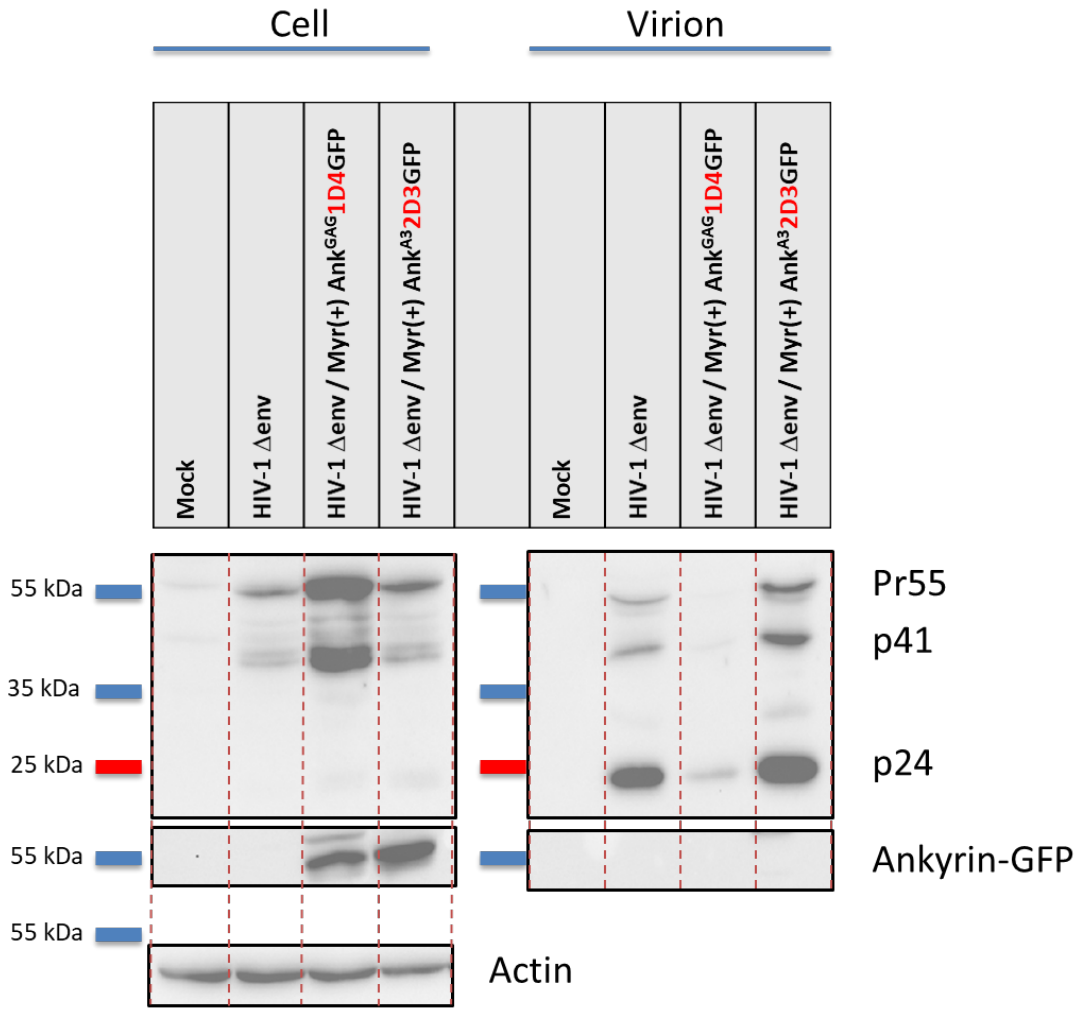

Figure 6 Representative Western blot images of Gag maturation in cells and virus from HIV-1 Δenv transfected 293T cell with and without Myr+Ank^{GAG}1D4 or Myr+Ank^{A3}2D3

HEK 293TCells were co-transfected with pNL4-3Δenv plasmid with and without pCep4-Myr+Ank^{GAG}1D4-GFP or pCep4-Myr+Ank^{A3}2D3-GFP for 24 h. Proteins were extracted from cells and pelleted virus and were analyzed by Western blotting with an anti-capsid (CA), anti-GFP and anti-β-actin−peroxidase

antibody. The results (Figure6) show that in the equal amount of protein as determined by actin expression, Myr+Ank^{GAG}1D4-GFP and Myr+Ank^{A3}2D3-GFP presented only inside transfected cell. The presence of both Myr+Ank^{GAG}1D4-GFP and Myr+Ank^{A3}2D3-GFP did not interfere the Gag processing pattern in both transfected cell and viral particle compared to 293T cell transfected with pNL4-3Δenv plasmid alone. However, 293T cell transfected with pNL4-3Δenv plasmid and pCep4-Myr+Ank^{GAG}1D4-GFP showed the strong accumulation of Pr55 precursor protein and Capsid precursor within cell. Moreover, inside the viral particle produced, Myr+Ank^{GAG}1D4-GFP totally decreased p24 capsid protein as shown in Figure 7 and all proteolytic Gag proteins.

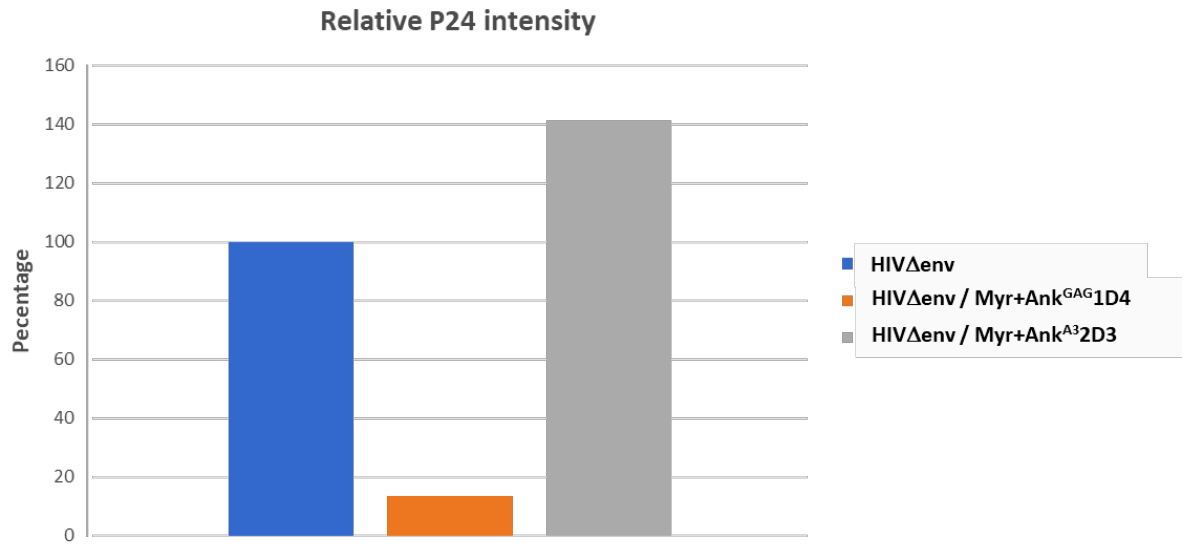

Figure 7 The percentage of relative P24 intensity determined by ImageJ software.

The effect of Myr+Ank^{GAG}1D4 RNA packaging ability

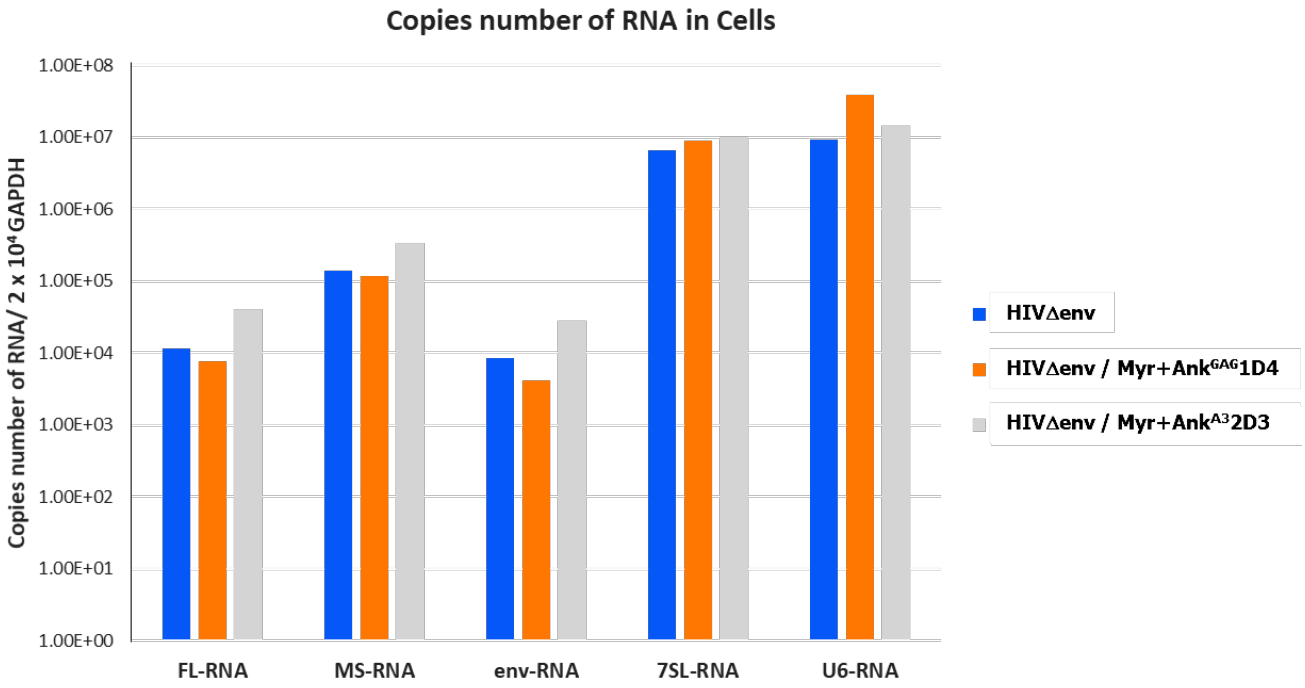

Figure 8 The number of viral RNA (FL, MS and env) and cellular RNA (U6 and 7SL) copies in cells was normalized to the cellular GAPDH gene.

After 24 h transfection, transfected cells were collected and performed RNA purification. The copies of extracted RNA were quantified using qPCR. As shown in Figure 8, the copies number of each RNA inside transfected cell did not have the same levels. Moreover, the levels of viral RNA (FL, MS and env) and cellular RNA (U6 and 7SL) in each transfected cell were not different.

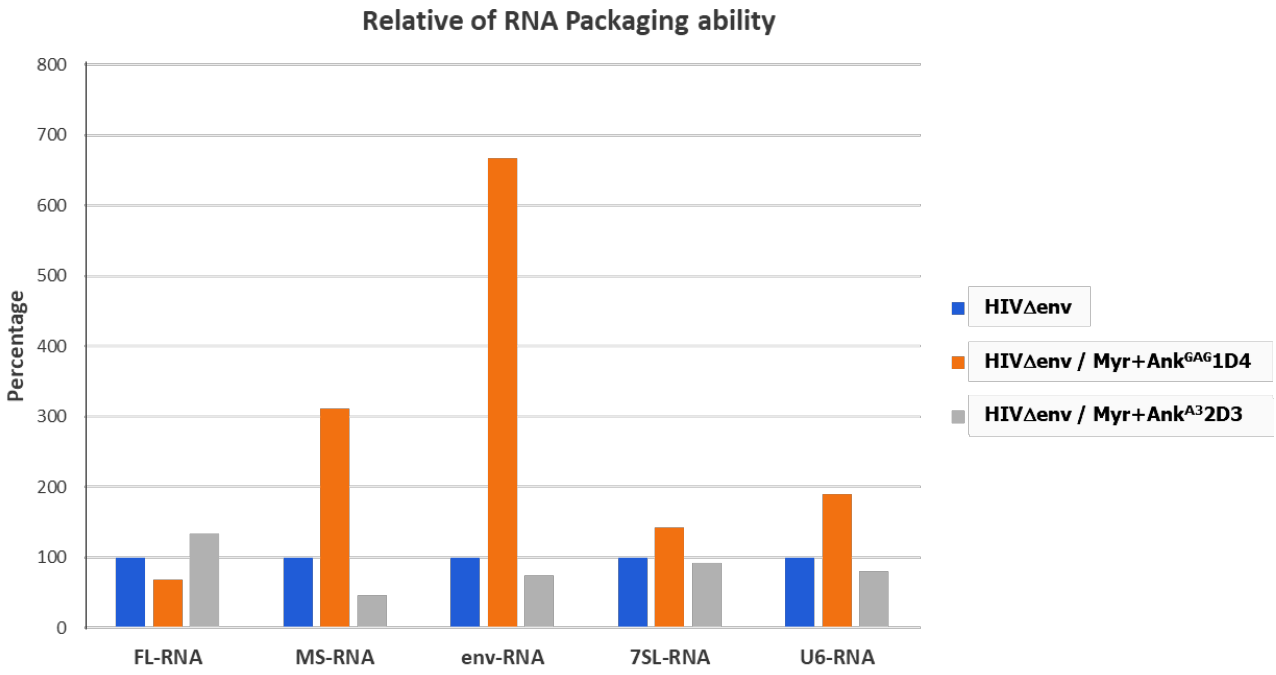

Figure 9 The relative packaging viral RNA (FL, MS and env) and cellular RNA (U6 and 7SL) ability of viral particle.

To determine the RNA Packaging ability, at equal number of P24, the result showed that FL RNA was packaged into viral particle released from 293T cell co-transfected with pNL4-3Δenv and pCep4-Myr+Ank^{GAG}1D4-GFP less than viral particle released from 293T cell transfected with pNL4-3Δenv alone and co-transfected with pNL4-3Δenv and pCep4-Myr+Ank^{A3}2D3-GFP. Interestingly, viral particle released from 293T cell co-transfected with pNL4-3Δenv and pCep4-Myr+Ank^{GAG}1D4-GFP packaged others viral RNA (MS-RNA and env-RNA) and cellular RNA (U6 and 7SL) more than viral particle released from 293T cell transfected with pNL4-3Δenv and co-transfected with pNL4-3Δenv and pCep4-Myr+Ank^{A3}2D3-GFP as shown in Figure 9.

4. Effects of Myr(+)Ank^{GAG}1D4 to genomic RNA distribution in the 8E5 cell and HeLa cell

Construction of 8E5 cell stably expressing Myr(+)Ank^{GAG}1D4 using lentivirus vector

The 8E5 cells were transduced with CGW-Myr(+)Ank^{GAG}1D4-EGFP lentivirus. After 16 h post-transduction, the cells were washed and fresh growth medium was replaced following incubated at 37ºC, 5% CO_2. Once the cells healthily growth, they were collected to test the expression of Myr(+)Ank^{GAG}1D4-EGFP in an 8E5 cell by flow cytometry.

HIV-1 specific probe design

The 8E5 that we used in this study is an HIV-1 LAV-infected cell. Therefore, the DNA probes were designed as 20 nucleotides long specific to the genomic RNA of this HIV-1 strain. Each of them conjugated with one molecule of fluorescein dye at its 3' end. The 40 DNA probes were obtained by Stellaris® RNA FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) using the gRNA of an HIV-1 LAV as a template. For probe designing please go to www.biosearchtech.com/stellarisdesigner.

The distribution of HIV-1 gRNA in 8E5-Myr(+)Ank^{GAG}1D4-EGFP

The stock of DNA probe was prepared by dissolving the lyophilized form of the DNA probe with nuclease-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to make the final concentration at 12.5 μM.

The experiment started with coating 0.01% Poly-L-Lysine on the coverslip for 5 min. Then the coverslip was washed with dH_2O before adding the suspension of 8E5-Myr(+)Ank^{GAG}14D-EGFP cells onto it and further incubated at 37ºC, 5% CO_2 for 1 h. Unattached cells were removed by 2 times washing with PBS followed by adding the fixation buffer (3.7% formaldehyde in PBS) for 10 min at room temperature (RT). The fixed cells were then washed with PBS and undergo permeabilization step by adding 70% ethanol, incubated overnight at 4ºC. Next, the fixed-permeabilized cells were washed with washing buffer (10% formamide in 2X SSC) for 5 min at RT. Consequently, the HIV-1 probe or human TFRC probe was added at final concentration 125nM in hybridization buffer (100 mg/mL dextran sulfate and 10% formamide in 2X SSC), incubated at 37ºC in a moist chamber and kept in dark for 16 h. The cells were washed again with warm (37ºC) washing buffer, incubated in dark for 30 min then added DAPI at final concentration 5 ng/mL, incubated in dark for 30 min more. Final washed with 2X SSC at RT. Removed the excess washing buffer and applied the anti-fade (Vectasheild) to the coverslip, immediately image by fluorescence microscope (Axio Imager Z.1, Carl Zeiss, Jena, Germany). The data obtained were then

processed by AxioVision version 4.8.2. The summary of all steps as shown in Figure 10.

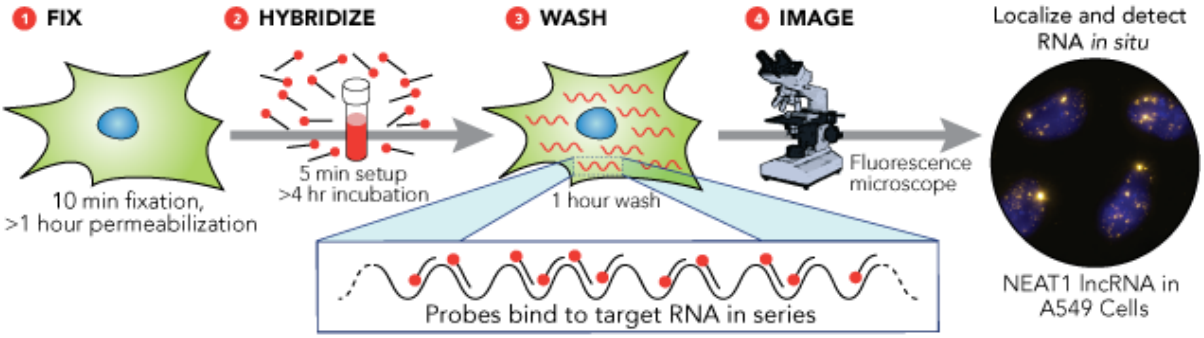

Figure 10 The steps for detecting the HIV-1 genomic RNA in 8E5-Myr(+)Ank^{GAG}14D-EGFP cells by Fluorescence *In Situ* Hybridization (FISH). (1) Fixed cells with 3.7% formaldehyde for 10 min and permeabilized with 70% ethanol, left overnight (2) Hybridized with a specific probe for overnight (3) Removed the excess probe by washing (4) Analyzed by fluorescence microscope

Transfection of HeLa cell with pNL4-3

Seed HeLa cells 1X10^6 cells/well into 24 cell culture plate, incubated at 37ºC, 5% CO_2, overnight. Cells should be 70-80% confluent. Transfected cells with 1 µg of pNL4-3 plasmid/well using TransIT-X2 transfection reagent as the protocol directed. Transfected cells were then incubated for 48-72 h before fixation and permeabilization. Investigated the transfection efficiency by intracellular staining of HIV-1 Gag protein using monoclonal antibody clone G18, the mAb specific to the p24 domain of Gag polyprotein.

Determination of p24 level in the culture supernatant of pNL4-3 transfected HeLa cells

To determine the presence of HIV-1 p24 from transfected cells, the culture supernatant was collected and performed 10-fold dilution in I-DMEM. The presence of HIV-1 p24 was tested using the Genscreen™ ULTRA Ag-Ab (BIO-RAD), the enzyme immunoassay (EIA) for detection of HIV-1 p24 antigen and antibodies to HIV-1 (groups M and O) and HIV-2 in human serum or plasma as the protocol directed.

Detection of HIV-1 gRNA in pNL4-3 transfected HeLa cells by FISH

Seed HeLa cells $1X10^6$ cells/well in 24 well plate in complete growth medium, incubated at 37ºC, 5% CO_2, overnight. Cells should be 70-80% confluent. Transfected cells with 1 μg of pNL4-3 plasmid/well using TransIT-X2 transfection reagent as the protocol directed. The transfected cells were then fixed and permeabilized after 48-72 h post-transfection. Performed the hybridization as briefly explained above.

Results

Construction of 8E5 cell stably expressing Myr(+)Ank^{GAG}1D4

The flow cytometry analysis was performed after 8E5-Myr(+)Ank^{GAG}1D4-EGFP cells get recovered from the lentiviral transduction. The expression of the green fluorescent protein was used as the indicator to see the expression of Myr(+)Ank^{GAG}1D4-EGFP in the cells. The result showed that 8E5 cells transduced with CGW-Myr(+)Ank^{GAG}1D4-EGFP lentivirus gave 99.14% positive EGFP (Figure 11).

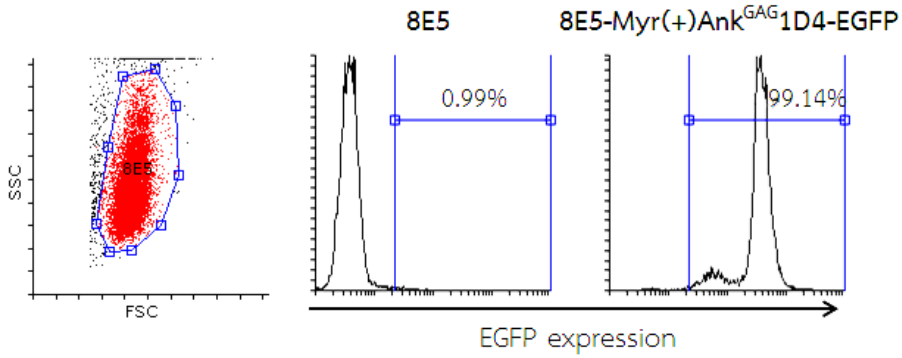

Figure 11 Flow cytometry analysis of Myr(+)Ank^{GAG}14D-EGFP expression in 8E5 cells

The HIV-1 specific probe design

Using Stellaris® RNA FISH Probe Designer, we obtained 40 oligonucleotide probes which specific to the genome of the HIV-1. At the 3'-end of the oligonucleotide probes was designed to conjugate with the Quasar® 570

(maximum excitation wavelength = 548 nm and maximum emission wavelength = 566 nm). The sequences of the oligonucleotide probes were shown in **Table 1**.

Table 1 The oligonucleotide sequences of the 40 HIV-1 DNA probe designed by Stellaris® RNA FISH Probe Designer

tgaagggatggttgtagctg	atgtcctgcttgatattcac	tgttatttgacccttcagta	ctggctacatgaactgctac
tactacttttacccatgcat	ttattaatgctgctagtgcc	atctcttgttaatagcagcc	tggtacaagcagttttaggc
tggtagggctatacattctt	aaagctctagtgtccattca	ccaggtcatgttattccaaa	tagaaaaattcccctccaca
cgcattttggaccaacaagg	gtcttctgctctttctatta	cccagataagtgctaaggat	tactccaactagcattccaa
tggaatattgctggtgatcc	gactgtgacccacaattttt	ccacaatcctcgttacaatc	actacttttgaccacttgc
atgttttttgtctggtgtgg	acctctgtatcatatgcttt	caccaatatttgagggcttc	attgctacttgtgattgctc
catgtttccttttgtatggg	tctgttctaccatgtcattt	cttctaggtatgtggcgaat	gtcttaaaggtacctgaggt
atagtactttcctgattcca	cctgtgtaatgactgaggtg	atcctatttgttcctgaagg	gagtgaattagcccttccag
tggctactatttcttttgct	gctgacatttgtacatggtc	tctttccccatattactatg	ggtagatccacagatcaagg
tggtgaaattgctgccattg	tcttgtattgttgttgggtc	ttgttcatttcctccaattc	caatcagggaagtagccttg

The distribution of HIV-1 genomic RNA in 8E5-Myr(+)Ank^{GAG}1D4-EGFP

The distribution of HIV-1 genomic RNA in 8E5-Myr(+)Ank^{GAG}1D4-EGFP cells were imaged by optical sectioning (Z-stack) mode of the fluorescence microscope and further processed by the deconvolution function to eliminate the noise signal from the out of focus layers. The result showed that the positive signal from the human transferrin receptor 1 mRNA (Stellaris® FISH Probes,

Human TFRC with Quasar® 570 Dye) was found in nucleus and cytoplasm of both 8E5 and 8E5-Myr(+)Ank^{GAG}1D4-EGFP cells as shown in Figure 12 and 13. Meanwhile, using an HIV-1 gRNA specific probe showed the weak signal in 8E5 cell as same as 8E5-Myr(+)Ank^{GAG}1D4-EGFP cells (Figure 14 and 15).

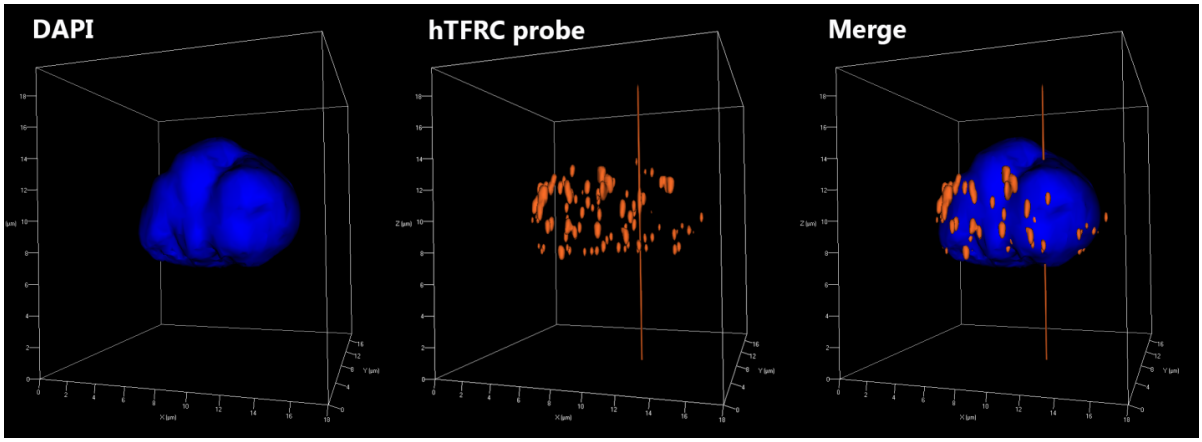

Figure 12 The hybridization signal of human transferrin receptor 1 mRNA (hTFRC) in the 8E5 cell which found in both nucleus and cytoplasm. (*left panel*) The nucleus was stained by DAPI. (*middle panel*) The red signal represented the presence of hTFRC mRNA. (*right panel*) Merge photo.

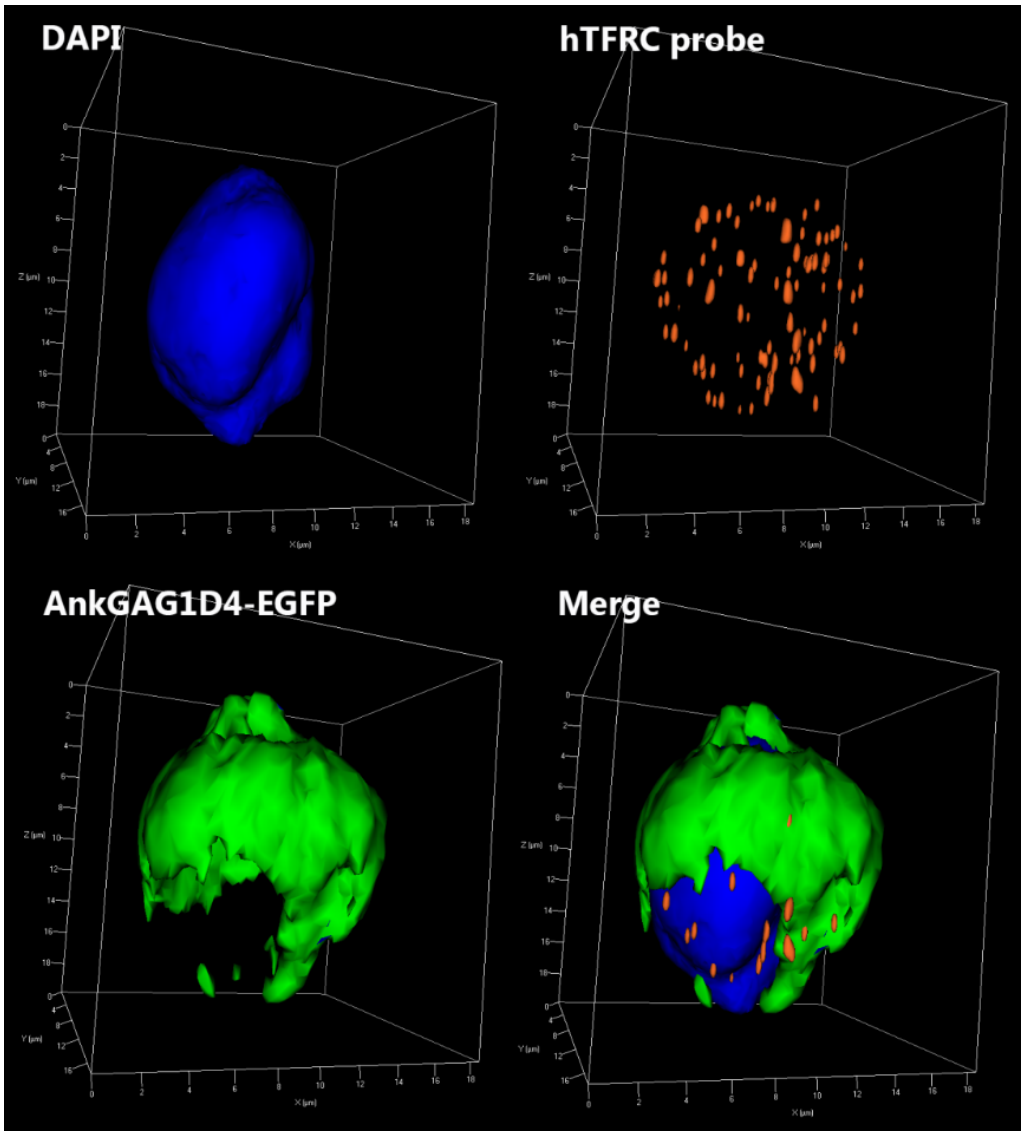

Figure 13 The hybridization signal of human transferrin receptor 1 mRNA (hTFRC) in the 8E5-Myr(+)Ank^{GAG}1D4-EGFP cell. (*top right*) The signal was found in both nucleus and cytoplasm. (*bottom left and right*) The Myr(+)Ank^{GAG}1D4-EGFP was expressed and mainly addressed in the cytoplasm.

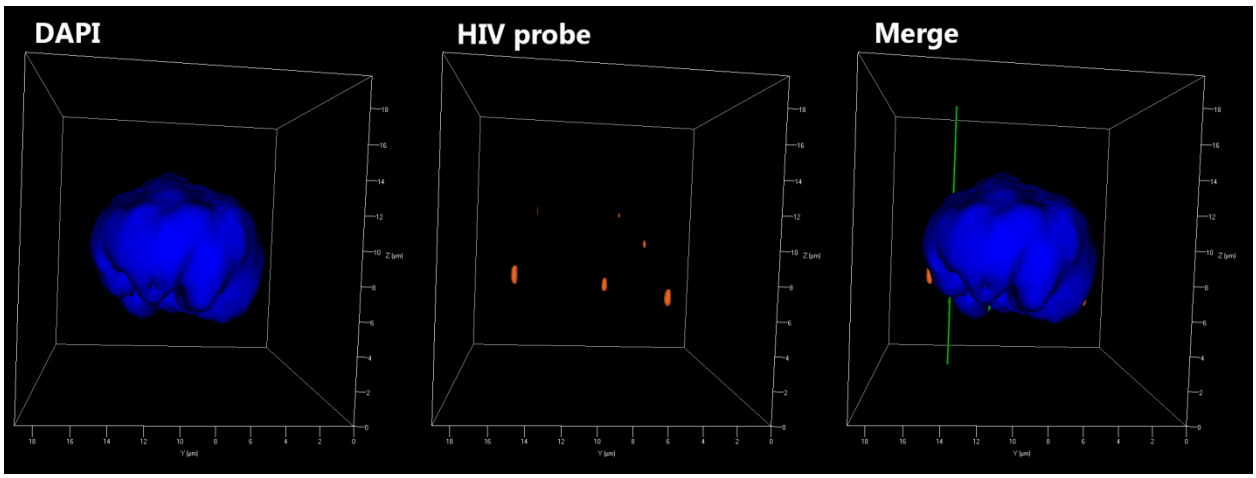

Figure 14 The hybridization signal of HIV-1 genomic RNA in the 8E5 cell. (*left panel*) The nucleus was stained by DAPI. (*middle panel*) The red signal represented the presence of HIV-1 genomic RNA. (*right panel*) Merge photo.

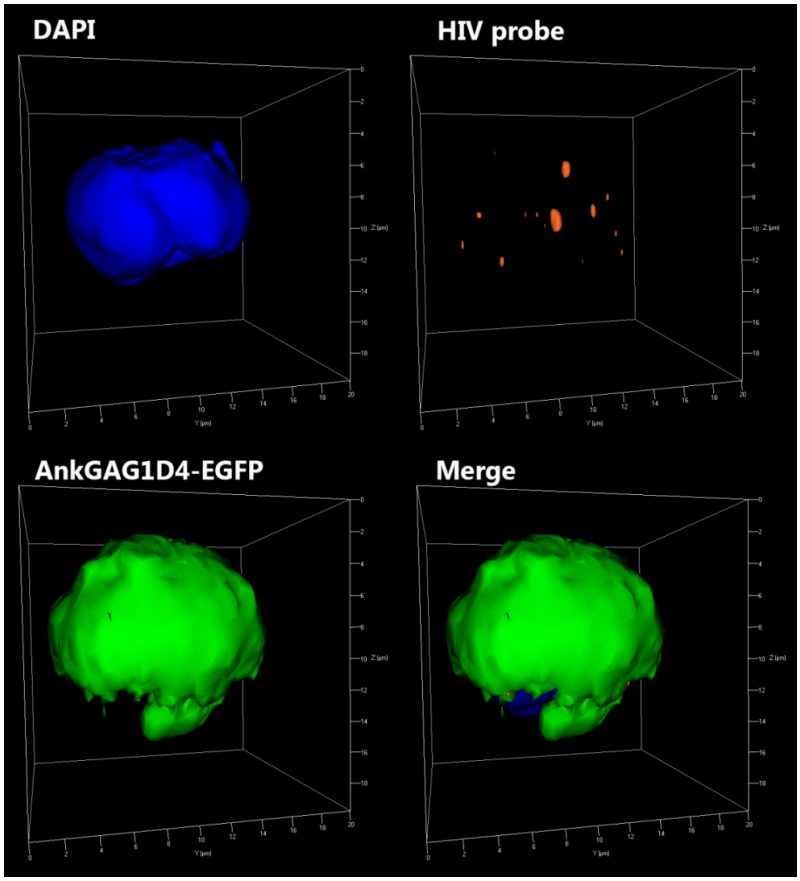

Figure 15 The hybridization signal of HIV-1 genomic RNA in the 8E5-Myr(+)Ank^{GAG}1D4-EGFP cell. (*top right*) The weak signal was found in nucleus and cytoplasm. (*bottom left and right*) The expression of Myr(+)Ank^{GAG}1D4-EGFP.

The expression of HIV-1$_{NL4-3}$ components in HeLa cell

The optical sectioning (Z-stack) of the pNL4-3 transfected HeLa cells were performed to detect the expression of the HIV-1 components. Intracellular staining using monoclonal anti-p24 antibody and goat-anti mouse conjugated Alexa Fluor 568 allowed us to monitor the expression of the HIV-1 p24, a domain

of Gag polyprotein. As shown in Figure 16, p24 protein was found at the membrane and cytoplasm of the transfected cells. Meanwhile, none of them was found in non-transfected HeLa cells (Figure 17).

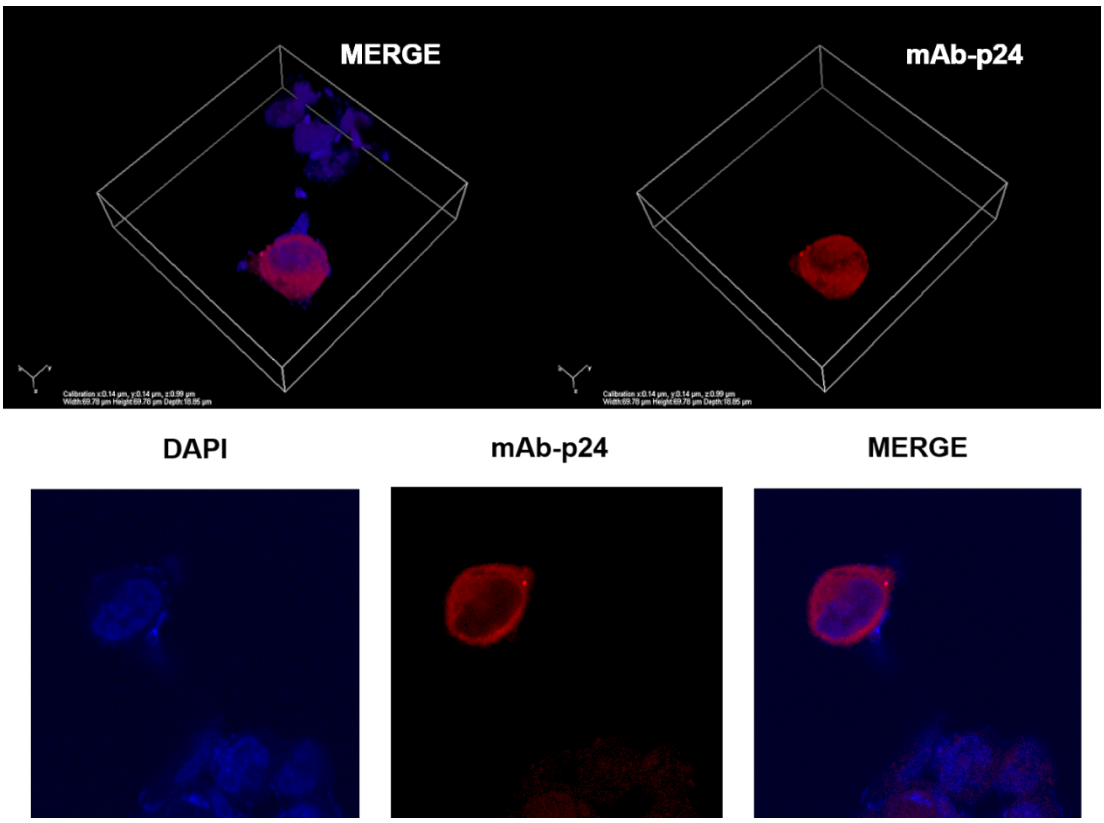

Figure 16 Fluorescence microscopy analysis of HIV-1 p24 expression in pNL4-3 transfected HeLa cells. (*top panel*) The optical sectioning (Z-stack) of the pNL4-3 transfected HeLa cells. (*bottom panel*) The expression of HIV-1 p24 visualized by confocal microscopy.

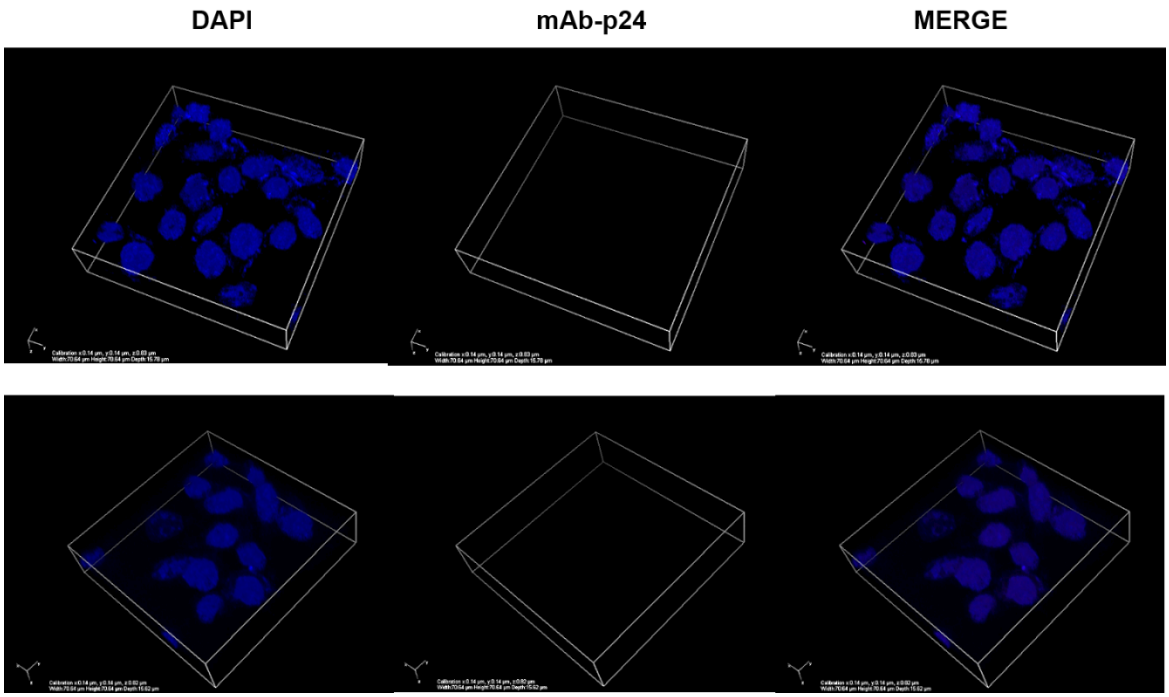

Figure 17 Fluorescence microscopy analysis of HIV-1 p24 expression in none transfected HeLa cells.

Determination of p24 level in the culture supernatant of pNL4-3 transfected HeLa cells

Regarding the previous experiment, to ensure that pNL4-3 transfected HeLa cells were able to produce the HIV-1 virions into the culture supernatant, the culture medium was collected. The 10-fold dilution was performed followed by determining the presence of p24 using Genscreen™ ULTRA Ag-Ab (BIO-RAD), the enzyme immunoassay (EIA) for detection of HIV-1 p24 antigen. The result indicated that at dilution 1:10 gave the higher signal than other dilution and negative control as showed in Figure 18. This suggested that pNL4-3 transfected HeLa cells were able to produce the p24 which is the indicator for the HIV-1

virion production. No signal was found in the culture medium of HeLa cell control.

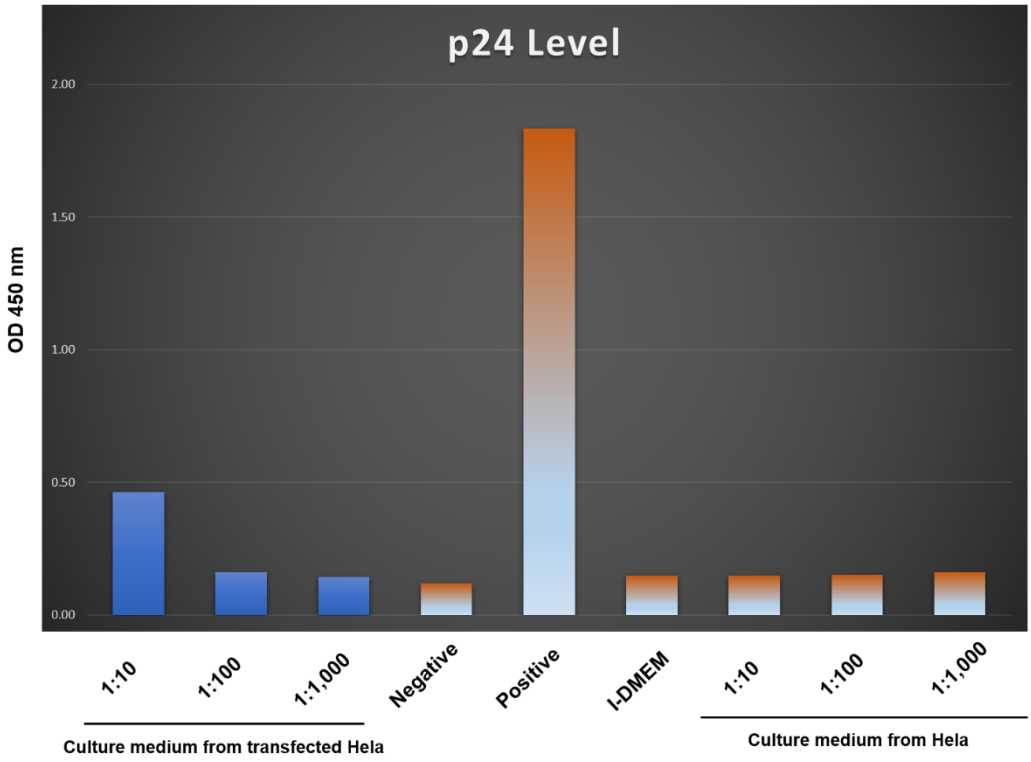

Figure 18 The bar graph represented the presence of p24 from pNL4-3 transfected HeLa cells and HeLa cell control.

Detection of HIV-1 gRNA in pNL4-3 transfected HeLa cells by FISH

After we confirmed that the pNL4-3 transfected HeLa cells are able to produce the HIV-1 components and new virions, the HIV-1 genomic RNA was detected by FISH. The optical sectioning (Z-stack) of the pNL4-3 transfected HeLa cells reviewed that the genomic RNA was observed mainly in the nucleus of the cells (Figure 19). However, the signal was not presented in HeLa cell control (Figure 20).

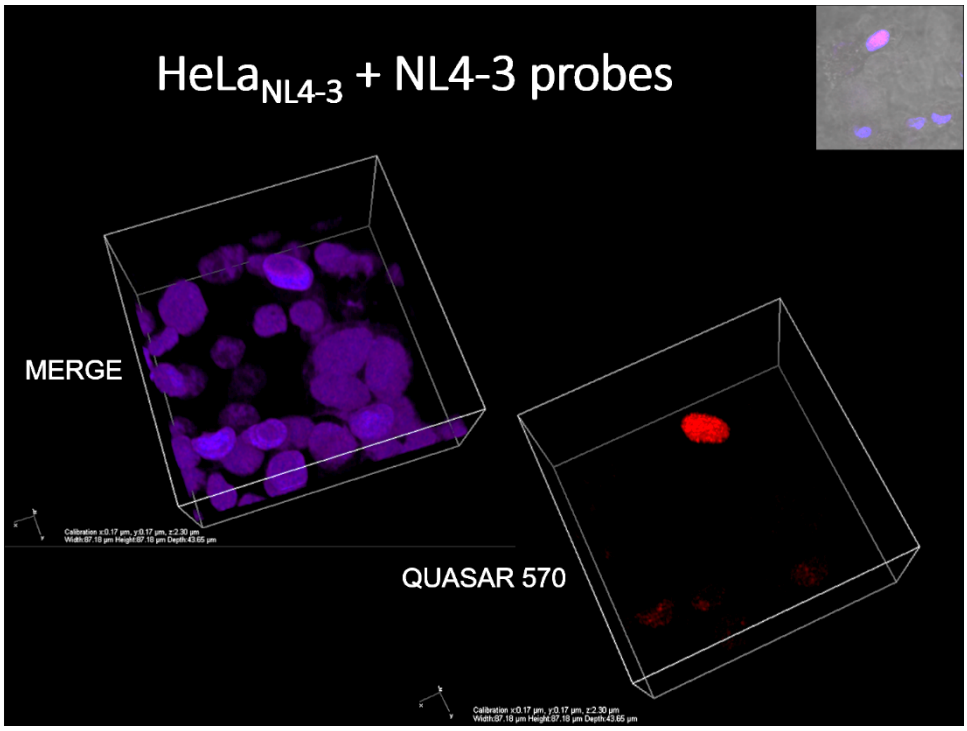

Figure 19 The optical sectioning (Z-stack) of the pNL4-3 transfected HeLa cells. (*left panel*) Merge photo, (*right panel*) the signal of Quasar 570 which refer to the presence of genomic RNA which mainly found in the nucleus. The nucleus was stained with DAPI.

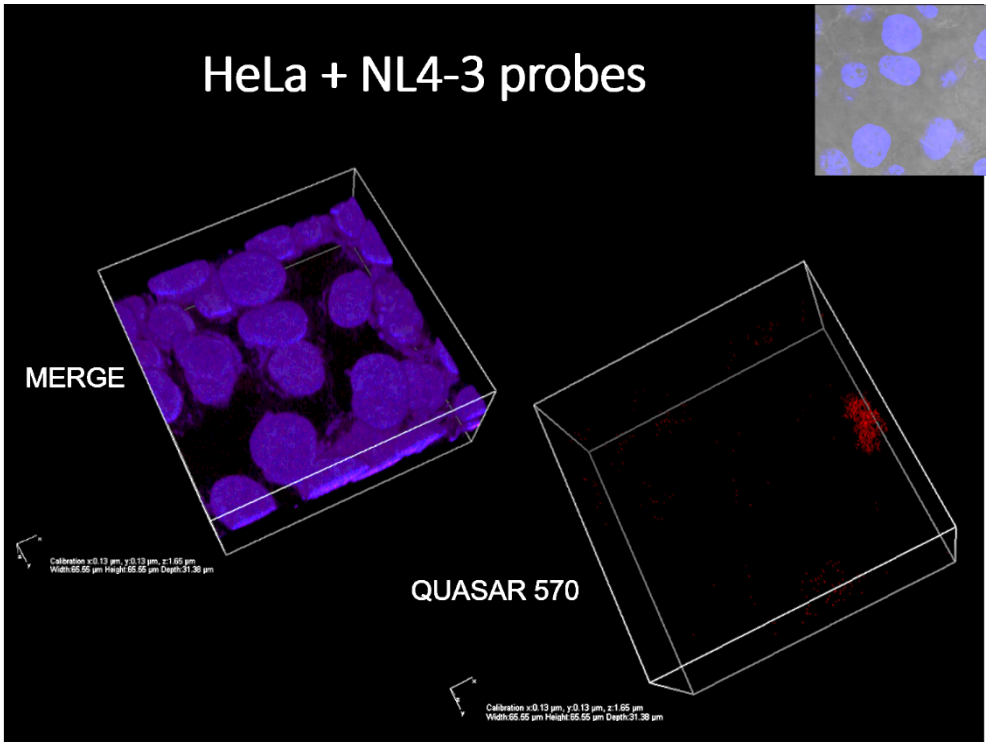

Figure 20 The optical sectioning (Z-stack) of HeLa cell control. (*left panel*) Merge photo, (*right panel*) the signal of Quasar 570 for detecting the HIV-1 genomic RNA. The nucleus was stained with DAPI.

Discussions

Ank^{GAG}1D4 is a designed ankyrin repeat which is able to recognize the capsid protein (CA) of the human immunodeficiency virus type 1 (HIV-1) and exhibits the intracellular antiviral activity on the viral assembly process (1, 2, 16). The critical amino acids which were used for the interaction between Ank^{GAG}1D4 and CA were illustrated (16, 17).

In this study, we aim to investigate the additional mechanism of Ank^{GAG}1D4 in the inhibitory process of HIV-1 production at the late stage of viral life cycle. First, we studied the effect of Ank^{GAG}1D4 to Gag multimerization process. This process first occurred when dimeric gRNA are recruited by Gag precursor protein, the complex of gRNA-Gag will initiate the multimerization of Gag precursor protein to reach to assembly site(9). We found that at the plasma membrane of transfected cell which is the assembly site of HIV-1, the recombinant MACA-Halotag® fusion protein is presented continuously at the assembly site. Anyhow, the continuously pattern of recombinant MACA-Halotag® fusion protein at plasma membrane was disturbed when Myr+Ank^{GAG}1D4 was expressed. It indicated that Myr+Ank^{GAG}1D4 might interrupt the polymerization of Gag polyprotein at the plasma membrane of transfected cell. Therefore, the assembly process of HIV-1 life cycle may be lost.

Then, we further investigated the effect of Ank^{GAG}1D4 on Gag processing or maturation process. After HIV-1 virion particle was released, the HIV-1

protease will process the Gag precursor to mature Gag proteins(18). We found that in the presence of Myr+Ank^{GAG}1D, the Gag precursor protein and Capsid precursor protein accumulated inside the HIV-1 transfected cell. Even Myr+Ank^{GAG}1D4 was efficient to inhibit viral production, we could observe some viral particle released. It seemed that, in viral particle released, Myr+Ank^{GAG}1D4 might not interrupt the maturation process of HIV-1 life cycle in which we could observe some capsid protein in virion released which is the crucial indicator for final product of Gag mature protein. Interestingly, we found that Myr+Ank^{GAG}1D4 and Myr+Ank^{A3}2D3 only presented inside the HIV-1 transfected cell, and it could not be carried into viral particle. This indicated that the role of Myr+Ank^{GAG}1D4 was successful inside the cell.

Next, we investigated the role of Myr+Ank^{GAG}1D4 on the specificity of RNA packaging into viral particle. Our data revealed that the specificity of Gag precursor protein to select and incorporate HIV-1 gRNA into virion was lost when Myr+Ank^{GAG}1D4 was present. After all, spliced RNA (MS and env RNA) were replaced. Both HIV-1 gRNA and spliced RNA are able to be packaged due to the presence of internal loop and lower part of SL1 in RNA. However, gRNA is selected by Gag precursor with higher affinity than spliced RNA due to the counter balance regulation domain in downstream of SL4 to upstream SL1 and the specific region between nucleotides 355–400 which is not present in spliced RNA(5, 19). Many studies showed that HIV-1 viral particle can package cellular

RNA(3, 12, 13, 20). Our data also reveal that loss of specificity on gRNA selection, cellular RNA (U6 and 7SL) were increasingly incorporated.

The accumulation of Gag precursor and capsid precursor inside HIV-1 transfected cell when Myr+Ank^{GAG}1D4 was present, would be caused by the defect of polymerization of Gag polyprotein on the assembly process since the level of HIV-1 gRNA inside HIV-1 transfected cell was not different.

Finally, we also explored the effect of Myr+Ank^{GAG}1D4 on the subcellular distribution of the HIV-1 gRNA in the HIV-1 infected cells. The distribution of HIV-1 gRNA was tracked by specific DNA probe using the FISH technique. The result showed that gRNA was weakly presented in the cytoplasm of both 8E5-Myr+Ank^{GAG}1D4 and 8E5 control. This led us to the inference that single copy of HIV-1 provirus DNA in 8E5 cells was not able to produce enough viral components for detecting the effect of Myr+Ank^{GAG}1D4 on it.

Therefore, the experiment design was changed, HIV-1 expressing HeLa cells were used in order to increase the viral components production. We first confirmed the expression of the Gag polyprotein in HIV-1 expressing HeLa by intracellularly stained the cells with mAb anti-p24 (G18). The data presented that the Gag polyprotein was located in the cytoplasm and cell membrane but not in the nucleus. The p24 in the culture supernatant of HIV-1 expressing HeLa was assayed and found presented, this confirmed that this cell was able to release the HIV-1 particles. After that, we investigated the distribution of HIV-1 gRNA

inside this cell. The HIV-1 specific DNA probed were used in FISH technique as same as what we performed with 8E5 cells. The result demonstrated that the gRNA was clearly visualized, mainly in the nucleus of HIV-1 expressing HeLa cells. None of FISH signal was found in HeLa cell control. Take it together, this new experiment design has been proved, the following step will be the introducing Myr+Ank^{GAG}1D4 into the HIV-1 expressing HeLa cell and determine the effect of Myr+Ank^{GAG}1D4 on the gRNA distribution in HeLa cell model.

These valuable results bring us to realize more intensive mechanism of Ank^{GAG}1D4 in HIV-1 antiviral activity. And we will apply these knowledge to study on HIV-1 gene therapy further.

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- **รางวัลผลงานวิจัย**

ผลงานวิจัยดีเด่น เรื่อง การพัฒนาต้นแบบการรักษาผู้ติดเชื้อ HIV อย่างยั่งยืน ด้วยการฝากยีนต่อต้านการติดเชื้อ HIV ชนิดพิเศษ ในเซลล์ต้นกำเนิดเม็ดเลือด **จากสำนักงานกองทุนสนับสนุนการวิจัย** ปี พ.ศ. 2559