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ABSTRACT

In this study, we investigated how Staufen1 influences the HIV-1 production. The overexpression of Staufen1 increased virus production without any negative affect on the viral infectivity. This increase was not caused by transcriptional activation; but by influencing post-transcriptional steps. Using multiple Gag protein derivatives, we confirmed that the zinc-finger domains of the HIV-1 nucleocapsid (NC) are important for its interaction with Staufen1. We also found that Staufen1 colocalized in stress granules with the mature form of the HIV-1 NC protein.

INTRODUCTION

Staufen is a double-stranded RNA-binding protein. Mammals have two homologs, Staufen1 and Staufen2, both of which have similar functions (1). Two Staufen1 isoforms are produced by differential splicing, with molecular weights of 55 and 63 kDa.

Staufen1 is known to contribute to multiple cellular processes involving mRNAs. For example, human Staufen1 plays a role in mRNA localization and transportation on microtubules (2). This transport involves the formation of a ribonucleoprotein (RNP) complex generated by Staufen1 self-association (3). Staufen1 also contributes to the mRNA decay process in mammalian cells. Staufen1-mediated mRNA decay (SMD) involves interaction with a Staufen1 binding site (SBS) in the 3'-untranslated regions (UTRs) of target mRNAs, recruiting the ATP-dependent RNA helicase UPF1 (4). Studies have shown that SMD contributes to the differentiation of myoblasts (5), as well as to the regulation of adipogenesis, through competition with nonsense-mediated mRNA decay (6). Furthermore, in binding to the 5' ends of specific mRNAs, Staufen1 may also enhance translation (7). Post-transcriptional regulation has been shown to be involved in cell-cycle progression (8). During oxidative stress, Staufen1 may be recruited to stress granules, leading to their dissolution and the assembly and stabilization of polysomes (9).

Structurally, Staufen1 has tubulin-binding domains (TBDs) that associate with the cytoskeleton during mRNA transport (10). Staufen1 has been shown to undergo both homo- and heterodimerization during SMD (11). This process involves the Staufen1 swapping motif (SSM) which resides between the tubulin-binding domain (TBD) and dsRNA-binding domain 5 (RBD5). Recently, Staufen1 has been shown to contain flexibility linkers that make it possible for the protein to adopt an elongated conformation (12).

A series of studies have investigated the involvement of Staufen1 in viral replication and

interaction. Its association with non-structural protein 1 (NS1) of the influenza A virus was shown to reduce viral replication (13, 14). On the other hand, hepatitis C virus (HCV) protein and RNA levels were found to increase when Staufen1 levels rose. Staufen1 is also known to promote replication by inhibiting protein kinase R activity and by its involvement in the transport of HCV RNA (15, 16). The human endogenous retrovirus K (HERV-K) Rec protein interacts with Staufen1, promoting viral RNA transportation and virion production (17). In Ebola virus (EBOV) infection, Staufen1 has been shown to promote RNA synthesis by binding to the 3' and 5' extracistronic regions of the EBOV genome, as well as by interacting with viral components such as NP, VP30, and VP35, to form a viral RNP complex (18). The 5'-UTR region of enterovirus 71's RNA has a Staufen1 binding site, at which interaction facilitates and extends viral RNA stability for translation and replication (19). In summary, Staufen1 has been shown to interact with many viral proteins and RNAs, contributing to both RNA replication and virus production.

Staufen1 has been shown to play a role in HIV-1 replication. It is incorporated within HIV-1 particles, with overexpression of the protein enhancing selective HIV-1 genomic RNA encapsidation (20). It has also been reported that both the dsRBD3 and 88 N-terminal amino acids of Staufen1 are required to enhance Gag multimerization, encapsidation, and virus-like particle (VLP) release (20-23). A Staufen1-HIV-1-dependent ribonucleoprotein (SHRNP) complex has been reported that it is distinct from stress granules and processing bodies. This complex prevents degradation of viral RNA and enhances both Gag assembly and RNA packaging (24). It was recently reported that Staufen1 overexpression caused a reduction in NC protein-induced stress granules, resulting in the rescue of viral mRNA translation by relieving inhibition by eIF2 α phosphorylation, a downstream pathway of PRK that facilitates polysome stabilization (25). In a later study, HIV-1 RNA was mistrafficked to stress granules in Staufen1 knockout cells, resulting

in reduced virus production and impaired infectivity (26).

One study reported that the overexpression of Staufen1 reduced viral infectivity 6.7-fold (20), while later Staufen1 knockdown data suggested that it is required for the generation of infectious virus (23). More recently, Staufen1(-/-) cells have been used to show that depletion decreases both virus production and infectivity (26). These results show similarities to the aforementioned case involving Staufen1 overexpression. This raises questions regarding the exact nature of the relationship between Staufen1 and virus infectivity.

The present study aims to increase our understanding of the role Staufen1 in HIV-1 production. We found that Staufen1 overexpression can increase the production of viruses and their associated proteins, while not generating a negative impact on the viral infectivity. Moreover, the enhancement of virus production by Staufen1 is not due to transcriptional activation, but rather to post-transcriptional process. We also found that Staufen1 can be co-localized in stress granules with a mature form of the HIV-1 NC protein.

RESULTS and DISCUSSION

Generation of A high-expression Staufen1 construct

To investigate the role of Staufen1 in HIV-1 replication and production, we first employed a Staufen1 expression vector obtained from Dr. Andrew J Mouland's laboratory (20). However, the expression level of Staufen1 was deemed to be too low for our analyses. In this plasmid, expression is driven by the respiratory syncytial virus (RSV) promoter. To meet the requirements of our study, we constructed a new expression vector by replacing the RSV promoter with a cytomegalovirus (CMV) promoter, as shown in Figure 1A. Then, 293T cells were transfected with either the resulting CMV-Staufen1 vector or the original RSV-Staufen1 vector for comparison; we observed that Staufen1 expression levels in the former had increased greatly 5-fold or more when compared to the latter (Figure 1 B, C). Thus, this new construct was utilized in subsequent experiments.

Staufen1 overexpression increases HIV-1 production

Previous studies have shown that Staufen1 increases HIV-1 VLP release (22). To examine the effect Staufen1 overexpression has on protein and virus production levels, 293T cells were co-transfected with CMV-Staufen1 and the proviral vector pNL4.3-GFP. A red fluorescent protein (RFP) expression vector was used as an internal control in each transfection assay, with transfection efficiency confirmed by fluorescence microscopy (Figure 2A). After 24 h, we harvested the cell lysate and supernatant for analysis. Western blotting and enzyme-linked immunosorbent assays (ELISAs) were used to assess protein and virus levels. We found that Gag increased in a dose-dependent manner with Staufen1 overexpression. Furthermore, we noted that green fluorescent protein (GFP) levels were not significantly changed (Figure 2B), indicating that only Gag level had increased. ELISA showed that when the Staufen1 levels increased, the amounts

of capsid protein in the viral supernatant increased approximately 3.5-fold over the control (Figure 2C). As shown in Figure 2A-C, overexpression of Staufen1 markedly increased cellular levels of Gag, resulting in an associated increase in virus production.

Previous studies have shown that both overexpression and depletion of Staufen1 can decrease somehow the infectivity of HIV-1 (20, 23, 26). Therefore, we tested the infectivity of virus produced from 293T cells with and without Staufen1 overexpression by using culture supernatants (Figure 2C) to infect MT4 cells. Infection using equal amounts of virus showed clearly that infectivity increased with Staufen1 overexpression. The intensity of GFP fluorescence in the western blot and ELISA data (figure 2D) also supported this observation. These results highlight that Staufen1 increased the production of HIV-1 without an associated any negative effect on the infectivity. To confirm this, we subjected fresh MT4 cells to reinfection with identical amounts of virus from cells with and without Staufen1 overexpression (Figure 2E). Subsequent fluorescence microscopy and FACS analysis showed no detectable difference between these two groups, again indicating that Staufen1 overexpression did not negatively affect the infectivity of HIV-1 generated with Staufen1 overexpression.

Staufen1 does not influence HIV-1 LTR promoter activity

To examine whether the increase in virus production was due to Staufen1 transcriptional activity, we examined the effect this protein had on the HIV-1 LTR promoter-driven reporter gene. HEK293T cells were transfected with pGL/U3RU5psi/EGFP at increasing concentrations of Staufen1. As shown in Figure 3, the transfected cells showed similar fluorescence in both the transfection efficiency control and in the presence of increased Staufen1 levels. Western blotting also demonstrated no significant change (Figure 3C). These results indicate that even as Staufen1 expression levels increased, the HIV-1 LTR promoter retained normal activity levels.

We also conducted another reporter gene assay using the firefly luciferase (Fluc) gene. We transfected pGL/U3RU5psi/Fluc and pCDNA4_TO_EGFP (control) with increasing amounts of the Staufen1 expression vector. We normalized luciferase activity values to the internal control for GFP intensity, which was found to be similar to the intensity of the enhanced GFP (EGFP) reporter gene (Figure 3D). Western blotting also showed no increase in Fluc protein level when Staufen1 was overexpressed (Figure 3E).

Together, these results indicate that Staufen1 did not affect HIV-1 LTR promoter activity. This confirms that the enhancement of virus production by Staufen1 has nothing to do with transcription, but instead function(s) post-transcriptionally.

Staufen1 and HIV-1 NC protein interaction and colocalization

Previously reported was that Staufen1 influences the multimerization of Gag, which results in an increase in VLP release (22). It has been reported that NC domain of Gag is required for this interaction. This was shown using a fusion NC protein tagged with a yellow fluorescent protein for BRET and coimmunoprecipitation assays. A mutation in the zinc finger of the NC domain resulted in decreased Staufen1/NC interaction (21-23). Therefore, it appears that the interaction of Staufen1 and the NC domain of Gag may have a significant effect on HIV-1 replication and production.

To further address further the specific interaction between Staufen1 and Gag, we employed and investigated a mature form of the NC protein without the presence of any fusion proteins. This allowed us to rule out the involvement of other protein structures. We transfected 293T cells with a Staufen1 expression vector along with multiple expression plasmids including pLP-Gag, pLP-D2 (P2-NC-P1-P6), pLP-NC, and MA_{G2A}CA (a myristylation site mutant; MA glycine-to-alanine point mutation). Staufen1 was found to interact with Gag, D2, and NC when pulled down with

anti-NC, but not with MA_{G2A}CA (Figure 4A). When pulling down with Staufen1, Gag, D2, and NC co-immunoprecipitated (Figure 4B). These data demonstrate the interaction between Staufen1 and NC, as well as with proteins containing an NC domain. Furthermore, we tested the interaction of Staufen1 with Gag proteins containing a mutation in the zinc finger of the NC domain. HEK293T cells were cotransfected with a Staufen1 expression plasmid and either pLP-Gag or pLP-Gag⁶¹², which has point mutations (cysteine to serine) in two zinc finger motifs of the NC domain. As shown in figure 4C, only wild-type Gag proteins precipitated with Staufen1. These results confirm affirmatively that the zinc-finger of the NC domain is critical for interaction between Staufen1 and Gag.

Next, to further investigate the interaction of Staufen1 and NC at the subcellular level, we transfected HeLa cells with Staufen1 and NC-EGFP or EGFP (Figure 4D). When Staufen1 was expressed alone, it localized to the cytoplasm. However, when Staufen1 was coexpressed with NC, both proteins colocalized in punctuated forms in the perinuclear area of the cell (Yellow arrows, Figure 4D). Previous studies highlighted an association between NC and stress-granule components through immunoprecipitation and immunohistochemistry analyses (25, 26). We then also examined the Y-box binding protein 1 (YB-1) in transfected HeLa cells, as this is a known stress-granule marker (27, 28). YB-1 was detected along with Staufen1 and NC in the perinuclear area (white arrows, Figure 4D), colocalized with stress granules. These results show that there is indeed an interaction between NC and Staufen1 that requires the NC zinc-finger motif. Moreover, this specific interaction could influence Staufen1 localization, resulting in NC and colocalization in stress granules.

MATERIALS and METHODS

Cell culture and transfection

HeLa and HEK293T cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (TCB, USA), penicillin, and streptomycin (GIBCO, Carlsbad, CA, USA). MT4 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing an identical serum and antibiotic composition. The cells were incubated in 5% CO₂ at 37 °C. HEK293T and HeLa cells were transfected with plasmid DNA using pMAX (Aptabio Co., South korea), following the manufacturer's protocol.

Plasmids

The RSV-Staufen1-HA plasmid DNA was kindly provided by Dr. Mouland. We cloned the CMV-Staufen1-HA plasmid using the DNA restriction enzymes *Bgl*II and *Hind*III to isolate the RSV-Staufen1-HA promoter fragment. The CMV promoter was inserted from the pCDNA3-control plasmid using T4 DNA ligase (Elpis Biotech Corp., South korea). Lastly, pDS-RedExpression-N1 was used as the RFP expression vector.

Reporter gene assays

HEK293T cells were transfected with U3RU5psi-EGFP and an RFP expression vector. They were then incubated for 48 h. Cells were harvested and lysed with radioimmunoprecipitation assay (RIPA) buffer. EGFP and RFP levels were measured using a fluorometer (Tecan Corp., Switzerland). EGFP levels were normalized to RFP levels. For the luciferase assay, HEK293T cells were transfected with U3RU5psi-Fluc and pCDNA4-TO-EGFP, then lysed using passive lysis buffer (Promega Corp., USA) 24 h after transfection. Luciferase activity was normalized to GFP levels.

Western blotting

Cells were lysed using RIPA or passive lysis buffers (Promega Corp., USA). Lysates were boiled in SDS sample buffer for approximately 5 min. Each sample was separated on 12 or 13.5% SDS-PAGE gels. The gels were then transferred to a PVDF membrane (Merck Millipore Corp. Billerica, MA, USA) and blocked with Tris-buffered saline containing 0.1 % Tween 20 (TBST) and 5% skim milk for approximately 1 h at room temperature. The membrane was incubated with the appropriate primary antibody overnight at 4 °C, for 2 h at room temperature, followed by further incubation with the appropriate secondary antibodies. The protein band signal was developed using the enhanced chemiluminescence system LAS4000.

Immunofluorescence (IF) analysis

We seeded HeLa cells on coverslips at 4×10^4 /well in 24-well tissue culture plates. These cells were transfected with CMV/Staufen1-HA, EGFP, or NC-EGFP plasmid DNA using jetPEI. After 24 h, the cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized for 20 min, blocked for 30 min in 0.1% Triton X-100, 2% bovine serum albumin, and 5 % normal horse serum, and incubated with the primary antibody for 2 h at room temperature. Next, cells were washed three times and incubated with fluorescent-conjugated secondary antibody for 1 h while under light protection. After a second round of washing, cells were counterstained with DAPI (1 μ g/ml in PBS-T) for the detection of nuclei. Finally, coverslips were mounted on glass slides using mounting medium and sealed with nail polish.

HIV-1 p24 ELISA

Cells were transfected with the indicated plasmid DNAs and pNL4-3_EGFP, then supernatants were harvested after 24 h. The amount of p24 in each supernatant was measured using a HIV-1 p24 ELISA kit (Xpressbio, Frederick MD, USA) according to the manufacturer's protocol. This

value was normalized to RFP levels.

Coimmunoprecipitation

For the Staufen1 and NC protein interaction study, HEK293T cells were transfected with the indicated plasmid DNAs and harvested after a 24 h incubation. Cells were lysed using NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, 0.5 % Nonidet P-40, 1 mM EGTA). The same volumes of samples were incubated with a specific antibody for 1 h at 4 °C. Then, the sample and antibody mixtures were incubated with A/G agarose beads (Santa Cruz) for 1 h (or overnight) at 4 °C. The beads were washed three times with PBS and boiled in SDS sample buffer. Protein detection was performed using western blotting.

Antibodies

Anti-HA monoclonal antibody (sigma Aldrich CO.), Anti-p24 monoclonal antibody (Santacruz), Anti-GFP polyclonal antibody (Santacruz), Anti-RFP polyclonal antibody (TaKaRa bio Inc.), Anti-Firefly antibody monoclonal (Calbiochem), Anti-NC antibody polyclonal (custom antibody), Anti-His monoclonal antibody (Santacruz).

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Conflicts of Interest:

The authors have no conflicting interests.

FIGURE LEGENDS

Figure 1. Construction of a Staufen1 expression plasmid.

(A) Schematics of the control, RSV-Staufen1 and CMV-Staufen1 plasmids. (B) HEK293T cells were transfected RSV-Staufen1 or CMV-Staufen1 with RFP. Cells were harvested after transfection for 24 h. The cell lysate was used for western blotting detection of Staufen1. (C) Measurements of band densities using the Image J program.

Figure 2. Increase in virus production due to Staufen1 overexpression

(A-C) HEK293T cells were seeded 2.5×10^5 and cotransfected with proviral DNA pNL4-3_{GFP} (200 ng) with CMV-Staufen1 followed by different level using the jetPEI. Incubation for 24 h after transfection. (A) Detection of cell fluorescence using by inverted fluorescence microscopy. Scale bar 2.0 mm. (B) Western blot analysis. (C) Effect of different concentrations of CMV-Staufen1 on p24 ELISA, normalized to RFP levels. Fold activity compared with control. (D-E) MT4 cells were infected with identical volumes of virus supernatant or same particle of virus for 4 days. Scale bar 2.0 mm (D) Inverted fluorescence micrograph (left panel) and GFP-positive cells quantified by FACS analysis (right panel). (E) Inverted fluorescence micrograph (left panel) and GFP-positive cells quantified by FACS analysis (right panel). Results are shown as means \pm SD for three independent experiments. Statistical significance was determined using one-way ANOVA (****P < 0.0001, **P = 0.0029, ns = not significant).

Figure 3. Lack of Staufen1 effect on HIV-1 LTR promoter activity.

(A-C) HEK293T cells were cotransfected with the reporter gene U3RU5psi-EGFP (200 ng) and CMV-Staufen1 followed by different level using the jetPEI. (A) Inverted fluorescence micrograph.

Scale bar 2.0 μm (B) GFP and RFP intensities of cell lysates measured by fluorometry. (C) Western blot results. (D-E) HEK293T cells were cotransfected with reporter gene DNA, U3RU5psi-Fluc (200 ng), and CMV-Staufen1 at different levels using jetPEI analysis. (D) Fluc intensity of cell lysate measured by fluorometer. (E) Western blot results. Data are shown as means \pm SD for three independent experiments. Statistical significance was tested by one-way ANOVA (ns; not significant).

Figure 4. Interaction and colocalization of Staufen1 with the NC domain.

(A-B) HEK293T cells were transfected pLP1-Gag, and derivatives with CMV-Staufen1, and incubation for 24 hr. Coimmunoprecipitation (co-IP) of Gag, derivatives and CMV-Staufen1. (A) Co-IP performed with rabbit anti-NC antibody. Western blotting using anti-p55 antibody (empty represents the control plasmid). (B) IP performed with mouse anti-HA antibody (CMV-Staufen1 contains the HA tag). Western blotting using anti-p55 antibody. (C) HEK293T cells were transfected with CMV-staufen-1 and pLP-Gag or pLPGag⁶¹². Cell lysates subjected to pulldowns with anti-His (negative control) or anti-p24 antibodies. (D) HeLa cells transfected with CMV-Staufen1 and NC-EGFP or EGFP. Scale bar 20 μm .

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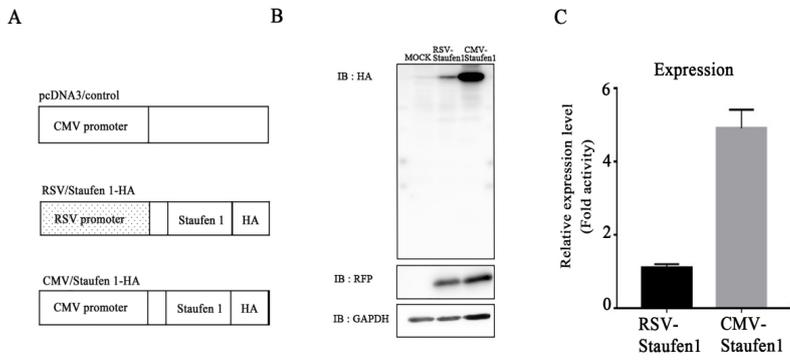


Fig. 1.

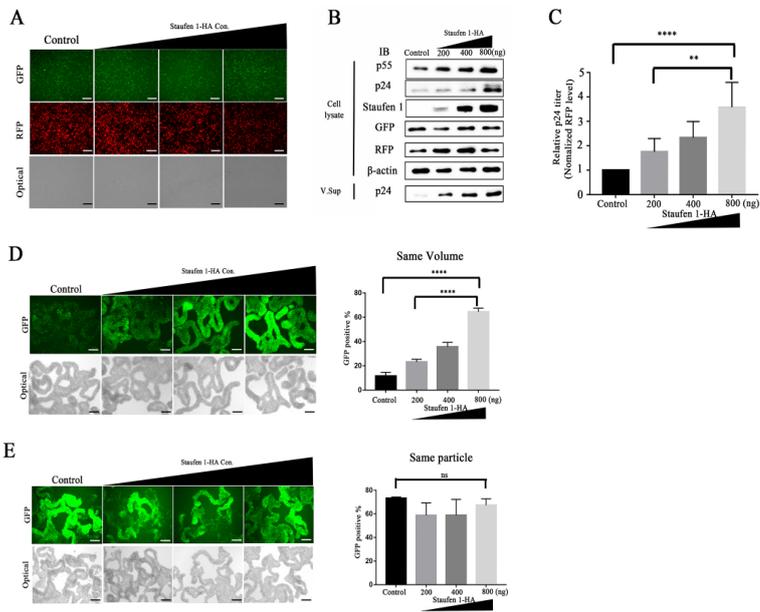


Fig. 2.

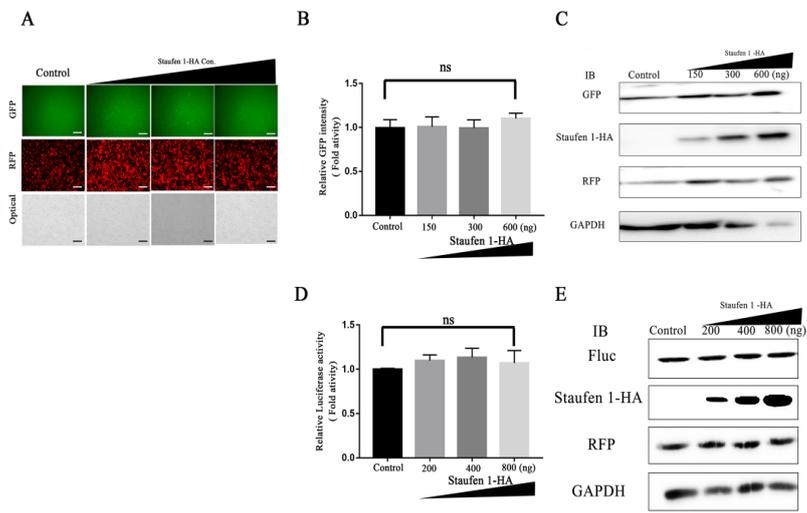


Fig. 3.

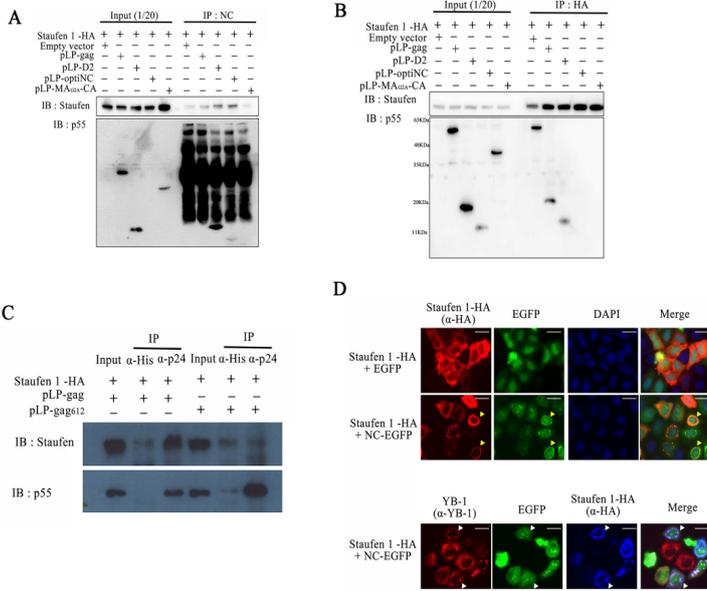


Fig. 4.