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Corresponding Author: Jeong Keun Ahn

Authors: Ji Ae Kim[1,#], Mi Sun Choi[1,2,#], Jung Sun Min[1], Inho Kang[1], Jeongho Oh[1], Jin Chul Kim[1,3], Jeong Keun Ahn[1,*]

Institution: [1]Department of Microbiology & Molecular Biology, Chungnam National University,
[2]Department of predictive toxicology, Korea Institute of Toxicology (KIT),
[3]Division of Biological Sciences, University of California,

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Author's name: Ji Ae Kim[a]†, Mi Sun Choi[a, b]†, Jung Sun Min[a], Inho Kang[a], Jeongho Oh[a], Jin Chul Kim[a, c] and Jeong Keun Ahn[a]*

Affiliation:

[a] Department of Microbiology & Molecular Biology, College of Biological Science and Biotechnology, Chungnam National University, Daejeon, 34134, KOREA

[b] Current address: Department of predictive toxicology, Korea Institute of Toxicology (KIT), 141 Gajeong-ro, Yuseong, Daejeon, 34114, KOREA

[c] Current address: Division of Biological Sciences, University of California, San Diego, La Jolla, CA, USA

†Both authors contributed equally to this work.

Running Title: Inhibition of NF-κB by HSV-1 ICP27

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Corresponding Author's Information:

To whom correspondence should be addressed.

Tel.: 82-42-821-6418; Fax: 82-42-822-7367; Email: jkahn@cnu.ac.kr

ABSTRACT

Herpes simplex virus type 1 ICP27 is a multifunctional protein which is responsible for viral replication, late gene expression, and reactivation from latency. It has been reported that ICP27 interacts with various cellular proteins including Daxx. However, the role of interaction between ICP27 and Daxx is largely unknown. Since Daxx has been reported to repress NF-κB activity, there is a possibility that ICP27 may influence the inhibitory effect of Daxx on NF-κB activity. In this study, we tested whether ICP27 affects NF-κB activity through its interaction with Daxx. Interestingly, ICP27 enhanced Daxx-mediated repression of NF-κB activity. In addition, we found that sumoylation of Daxx regulates its interaction with p65. It turned out that ICP27 binds to Daxx, inhibits Daxx sumoylation, and enhances p65 deacetylation induced by Daxx. Therefore ICP27 represses NF-κB activity by elevating the inhibitory effect of Daxx on NF-κB activity through desumoylation of Daxx.

Introduction

Herpes simplex virus type 1 (HSV-1) is a neurotropic DNA virus that establishes latency within sensory neurons (1). During viral infection, the viral genes are expressed in sequential cascade consisting of three major classes: immediate early (IE), early (E), and late (L) genes. Transcription of the immediate early genes (ICP0, ICP4, ICP22, ICP27, and ICP47) is initiated by the cooperation between host transcription factor Oct-1 and VP16 protein which is packed in the tegument of HSV-1 particle (2). These immediate early proteins are important in subsequent viral early protein expression by serving as regulatory proteins that initiate the transcription of early genes (3).

Among the immediate early proteins of HSV-1, ICP27 is a multifunctional regulatory protein required for virus replication and the switch from early to late virus gene expression (4). ICP27 also activates viral DNA replication and stimulates the transcription of late genes (5). In addition to gene expression regulation, ICP27 controls cell cycle and apoptosis through modulating signaling pathways such as NF-κB and MAPK pathways (6, 7). The activity of NF-κB was strongly augmented in macrophages infected with the ICP27-deletion mutant HSV-1, suggesting that ICP27 represses NF-κB (8).

Transcription factor NF-κB acts as a key regulator of cellular processes such as cell proliferation, survival, apoptosis, inflammation, and immune responses (9). The most abundant and ubiquitously expressed NF-κB is a heterodimer consisting of two subunits p50 and p65, while p65 is the main transcriptional activator (10). In most unstimulated cells, NF-κB proteins remain inactive through direct interaction with inhibitors of NF-κB (IκB) (11). While inducible degradation of IκB proteins by various stimuli including proinflammatory cytokines, T- and B-cell receptor signals, and viral and bacterial products is a central mechanism regulating NF-κB transcriptional activity, recent studies indicate that

posttranslational modification of NF-κB subunits, especially p65, is important to control NF-κB activity (12). It has been reported that acetylation and phosphorylation of p65 play significant role in the regulation of NF-κB activity (13).

NF-κB p65 subunit is reversibly acetylated at a number of sites and this modification has different effects on its transcriptional activity (14). Acetylation of p65 also modulates its DNA binding, subcellular localization, and assembly with IκBs. The transcriptional coactivators p300/CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF) acetylate p65, while histone deacetylase 3 (HDAC3) deacetylates p65 (15). Especially, p300/CBP mediated acetylation of p65 is inhibited by Daxx (16).

Daxx was initially identified as a protein that specifically binds to the death domain of Fas receptor and enhances Fas-mediated apoptosis via JNK activation (17). In addition to apoptosis, Daxx is known to repress several transcription factors, including ETS, Pax3, p53, Smad4, and NF-κB (18). Also, Daxx interacts with ICP4 and ICP27 to lead to nuclear domain 10 (ND10) deposition of HSV-1 viral genome (19).

We previously demonstrated that HSV-1 infection represses NF-κB activity by ICP27 which suppresses NF-κB activity through stabilizing IκBα, while Daxx inhibits NF-κB acetylation and represses NF-κB transcriptional activity (16, 20). These findings have led to the hypothesis that ICP27 may repress NF-κB through interaction with Daxx. In this study, we report that ICP27 inhibits p65 acetylation and NF-κB transcriptional activity by repressing Daxx sumoylation which increases the interaction between p65 and Daxx.

RESULTS

ICP27 represses NF-κB activity by enhancing p65 deacetylation through its direct interaction with Daxx

Previously it has been reported that ICP27, ICP4, and Daxx possibly form a complex at ND10 in HSV-1 infected cells (19). We tested the physical interaction between ICP27 and Daxx by pull-down assay (Fig. 1A). It turned out that ICP27 directly binds to Daxx. Since ICP27 inhibits NF-κB activity through regulating IκBα (16) and Daxx suppresses acetylation of p65 (20), we assumed that ICP27 might be also involved in the regulation of NF-κB through Daxx. Therefore, we examined whether ICP27 regulates NF-κB activity affected by Daxx. As expected, Daxx represses NF-κB activity and ICP27 further suppresses NF-κB activity repressed by Daxx (Fig. 1B).

Since Daxx has been previously reported to inhibit p65 acetylation (16), we examined whether ICP27 affects p65 acetylation through interaction with Daxx or not. To check the effect of ICP27 on p65 acetylation regulated by Dax, 293T cells were cotransfected with expression plasmids for p65, CBP, Daxx, and ICP27, and then treated with TSA, which is a HDACs inhibitor, to diminish the artifacts by HDACs. Daxx–mediated inhibition of p65 acetylation is not affected by TSA (16), while HDACs are able to deacetylate p65 (14). As expected, the level of p65 acetylation is decreased by Daxx and further reduced dramatically by ICP27 in a dose dependent manner (Fig. 1C). To exclude the possibility that ICP27 directly inhibits p65 acetylation, we analyzed the physical interaction between ICP27 and p65 by pull-down assay and identified that ICP27 does not directly bind to p65 (data not shown). Because ICP27 does not physically interact with p65, we examined whether ICP27 affects the binding between Daxx and p65 to regulate p65 acetylation. As seen in Figure 1D, ICP27 augments the interaction between Daxx and p65. Taken together, these data suggest that

ICP27 affects p65 acetylation through enhancing the interaction between Daxx and p65.

ICP27 changes cellular localization of Daxx

To further understand how ICP27 enhances the interaction between Daxx and p65, we next examined whether ICP27 affects the subcellular localization of Daxx. Because Daxx mainly exists in the nucleus and p65 is predominantly localized in the cytoplasm at normal state, while ICP27 is shuttling between the nucleus and the cytoplasm, we speculated that ICP27 might change the cellular localization of Daxx. We performed subcellular fractionation assay to analyze the intracellular distribution of Daxx in the presence of ICP27. Interestingly, ICP27 elevates the translocation of Daxx from the nucleus to the cytoplasm (Fig. 2A). Immunofluorescence analysis also demonstrated that Daxx is relocated to the cytosol in the presence of ICP27 (Fig. 2B). These data suggest that ICP27 increases co-localization of Daxx and p65 in the cytoplasm.

ICP27 inhibits Daxx sumoylation

Recently it has been shown that Daxx sumoylation is important for Daxx localization. Sumoylation-defective Daxx mutant localizes in the cytoplasm, whereas wild type Daxx localizes in the nucleus (21). Because ICP27 changes subcellular localization of Daxx, we tested whether Daxx sumoylation is regulated by ICP27. Interestingly, Daxx sumoylation is inhibited by ICP27 (Fig. 3A) and Daxx sumoylation induced by SUMO E2 conjugation enzyme Ubc9 is also inhibited by ICP27 (Fig. 3B). To further substantiate the effect of Daxx sumoylation on the interaction between Daxx and p65, we performed pull-down assay using lysis buffer containing NEM which is commonly used as a desumoylation inhibitor. As shown in Figure 3C, NEM abolishes the physical binding of Daxx to p65 suggesting that the elevated sumoylation of Daxx interfers the interaction between Daxx and p65. Moreover, the

inhibition of Daxx sumoylation by ICP27 increases the interaction between Daxx and p65 (Fig. 3D). These results suggest that the sumoylation level of Daxx is critical for the interaction between Daxx and p65.

In HSV-1 infection, ICP27 represses p65 acetylation by inhibiting Daxx sumoylation

Next, we tested whether ICP27 influences p65 acetylation during the early period of HSV-1 infection. 293T cells were infected with HSV-1 wild type virus (wt) and ICP27 deletion mutant virus (Δ27) at MOI 5. In HSV-1 wt infected cells, p65 acetylation is repressed as compared with mock infected cells, whereas in HSV-1 Δ27 infected cells, p65 acetylation is elevated as compared with HSV-1 wt infected cells (Fig. 4A). Then, we examined whether HSV-1 infection affects Daxx sumoylation. At 6 h post infection, 293T cells were harvested with lysis buffer containing NEM to inhibit desumoylation of proteins. In HSV-1 Δ27 infected cells, Daxx sumoylation is more increased as compared with HSV-1 wt infected cells (Fig. 4B and C) suggesting that ICP27 represses Daxx sumoylation when HSV-1 virus is infected. Our studies revealed that ICP27 represses p65 acetylation through inhibiting Daxx sumoylation at early stage of HSV-1 infection.

DISCUSSION

NF-κB plays a crucial role in regulating cellular processes such as apoptosis, proliferation, inflammation, and immune responses (22, 23). Viruses are pathogens challenging the host immune system and have evolved different strategies to regulate NF-κB signaling (24). Some viruses modulate NF-κB in a bidirectional manner for immune surveillance, optimization of viral infection, and replication (25). Especially, in HSV-1 infection, diverse viral proteins maintain a delicate balance between activation and inhibition of NF-κB.

In general, HSV-1 activates NF-κB in a biphasic way. The first wave of NF-κB activation is mediated by glycoprotein D and viral tegument protein UL37 which binds to herpesvirus entry mediator (HVEM) (26) and TRAF6 respectively (27). At second wave, IE viral proteins ICP4 and ICP27 activate NF-κB in IKK-dependent manner (28). However, recent studies showed that HSV-1 IE proteins have a repressible role in NF-κB signaling. ICP0 inhibits TLR2-dependent activation of NF-κB signaling by degradation of myeloid differentiation primary response (MYD88) (29). Also, at early viral infection suppresses NF-κB activity while HSV-1 Δ27 virus does not suppress it. However, at late HSV-1 infection, HSV-1 wt and HSV-1 Δ27 promoted NF-κB activities (20). These results suggest that ICP27 plays a bifunctional role in NF-κB regulation. Thus, more precise study on the molecular mechanism of NF-κB modulation by ICP27 is required.

In this study, we provide experimental evidence of novel mechanism for ICP27 to inhibit NF-κB activity through regulating Daxx sumoylation suggesting that ICP27 physically interacts with Daxx to inhibit Daxx sumoylation possibly by steric interference. Sumoylation is an important post-translational modification that regulates protein activity, stability, and subcellular localization. Also, Daxx itself is sumoylated by SUMO-1 and Ubc9 E2-conjugating enzyme (30). Several recent publications demonstrate that Daxx sumoylation is

associated with its subcellular localization. Ubc9 dominant-negative mutant induces accumulation of Daxx to cytoplasm (31). Sumoylation-defective Daxx KA mutant showed that physiological role of Daxx sumoylation is regulating its nuclear anchoring (21). Our data also imply that Daxx sumoylation is crucial for its subcellular localization. ICP27 changes cellular localization of Daxx from the nucleus to the cytoplasm through repression of Daxx sumoylation and subsequently ICP27 increases the interaction between p65 and Daxx. In addition, Daxx plays a role as a regulatory protein in viral gene expressions and intrinsic immunity against viral infection. Many viruses modulate Daxx for viral replications and immune escapes in host cells (32).

Previously we reported that Daxx inhibits p65 acetylation (16). NF-κB transcriptional activity is regulated by diverse posttranslational modification including phosphorylation, methylation, ubiquitination, and acetylation. Seven acetylated lysines have been identified within p65, especially acetylation of K221 and K310 is important for NF-κB transcriptional activity. Acetylation of K221 enhances NF-κB DNA binding affinity and reduces interaction with IκBα. Acetylation of K310 is required for full NF-κB activity, but does not affect either NF-κB DNA binding affinity or its assembly with IκBα (33). Daxx binds to the N-terminal domain of p65 including major acetylation sites and it seems that Daxx interferes p65 acetylation by steric hindrance (16). In this report, we demonstrated that ICP27 inhibits p65 acetylation through increasing the interaction between Daxx and p65. Owing to this increased interaction induced by ICP27 through Daxx desumoylation, ICP27 represses NF-κB transcriptional activity. This is consistent with our previous report that NF-κB transcriptional activity was more repressed at early phase of viral infection in HSV-1 wt infected cells compared with in HSV-1 Δ27 infected cells (20). Because activation of NF-κB is an essential step of immune response activation, many viruses disrupt NF-κB pathway. Therefore, here we suggest a possible mechanism that HSV-1 is able to escape immune surveillance in

infected cells through modulating NF-κB activity by regulation of Daxx sumoylation.

MATERIALS AND METHODS

Cells and Reagents

HEK 293T, Vero, 3-3 (HSV-1 ICP27 expressing Vero), and SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. 3–3 cells were kind gifts from Dr. P. Schaffer (Harvard University, USA). Cells were transfected using PEI (Sigma-Aldrich) according to the manufacturer's instruction. Trichostatin A (TSA) and N-ethylmaleimide (NEM) were purchased from Sigma-Aldrich.

Viruses and virus infection

HSV-1 wild type strain KOS and ICP27 deletion mutant virus (34) were propagated and titrated on Vero cells and 3–3 cells respectively. For virus infection, cell monolayers were infected with either KOS or mutant virus at a multiplicity of infection (MOI) of 5 plaque forming units (PFU)/cell.

Plasmids

ICP27, Daxx, p65, and CBP expression plasmids have been described previously (20). pBH-ICP27 was provided by Dr. Stephen A. Rice (35). Plasmids expressing EGFP-SUMO-1 and HA-UBC9 were provided by Dr. Hsiu-Ming Shih (36) and Dr. H. Ariga (37) respectively.

GST and Ni-NTA Pull-down assay

Transiently transfected 293T cells were lysed in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for 30 min at 4 °C. Cell lysates were incubated with glutathione-Sepharose 4B beads or Ni-NTA beads (Incospharm) overnight at 4 °C. The bead complexes were washed three times with modified RIPA buffer and bound proteins were analyzed by Western blotting.

Immunoprecipitation and Western Blot Analysis

Cell lysates were prepared in modified RIPA buffer and incubated with specific antibody overnight at 4 °C. The immunoprecipitated complexes were isolated using protein-A Sepharose, resolved by SDS-PAGE, and transferred to nitrocellulose membrane. Immunoblotting was performed with various antibodies. Anti-GST, anti-His, anti-HA, anti-GFP, anti-ICP27, rabbit anti-goat IgG-HRP, and goat anti-mouse IgG-HRP antibodies were purchased from Santa Cruz. Anti-Flag antibody was purchased from Sigma-Aldrich.

Luciferase Assay

All luciferase assays were performed at 24 h after transfection using a Dual Luciferase Assay kit (Promega) according to the manufacturer's instruction. 293T cells were transiently transfected with luciferase reporter construct NF-kB-Luc and pTK Renilla luciferase plasmid (Promega) using PEI according to the manufacturer's instruction.

Subcellular Fractionation Assay

Briefly, cells were suspended in fractionation buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Cell

lysate was passed through a 25 Ga needle 10 times using a 1 ml syringe and stored on ice for 20 min. The lysates were centrifuged at 720 × *g* for 5 min at 4 °C and supernatants were collected as cytosolic fractions. The nuclear pellets were resuspended in nuclear buffer (RIPA buffer with 10% glycerol and 0.1% SDS) and sonicated in three 10 sec bursts at 40% intensity with 30 sec break on ice.

Immunofluorescence assay

Transfected cells on sterile glass coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with PBS containing 0.2% Triton X-100 for 5 min. Cells were immunostained with primary antibody (anti-Daxx, Santa Cruz) for overnight at 4 °C, and then treated with fluorescent secondary antibody (donkey anti-rabbit Alexa Fluor 488, Invitrogen) for 1 h at room temperature. Fluorescence microscopy was performed using a Leica TCS SL system.

Statistical Analysis

Statistical comparisons of results were evaluated by one-way analysis of variance (ANOVA) followed by Student's *t* test for unpaired values. A *P* value of less than 0.001 was considered significant.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig. 1 ICP27 represses NF-κB activity by enhancing p65 deacetylation through its direct interaction with Daxx.

(A) 293T cells were transfected with plasmids expressing Flag-ICP27 and GST-Daxx. GST-Daxx (3 μg each) was pulled-down with glutathione-Sepharose 4B and protein complex was analyzed by immunoblotting using anti-Flag antibody.

(B) 293T cells were transfected with NF-κB-luciferase reporter plasmid (0.4 μg) and expression plasmids for p65 (0.2 μg), Daxx (0.2 μg), and ICP27 plasmid (0.2 μg). At 24 h after transfection, NF-κB activities were determined via a Dual-luciferase reporter assay system. Data represent three independent experiments performed in duplicate. Statistical significance was evaluated using Student's *t* test (***, $p<0.001$).

(C) 293T cells were transfected with combinations of plasmids expressing GST-p65, Flag-Daxx, HA-CBP, and ICP27 (1.5 μg each). At 24 h after transfection, cells were treated with 2 μm TSA for 30 min. Cell extracts were pulled down with glutathione-Sepharose 4B and analyzed by SDS-PAGE. The level of p65 acetylation was detected by immunoblotting using anti-acetylated lysine antibody.

(D) 293T cells were transfected with plasmids expressing Flag-Daxx, His-p65 and ICP27 (2 μg each). At 24 h after transfection, cell extracts were pulled down with Ni-NTA Chelating Agarose CL-6B. Protein complex was separated by SDS PAGE and analyzed by Western blotting with specific antibodies.

Fig. 2 ICP27 changes subcellular distribution of Daxx.

(A) 293T cells were transfected with plasmid for GST- ICP27 (6 μg). At 24 h after transfection, cells were fractionated into nuclear and cytoplasmic fraction. Fractionated

proteins were immunoblotted with anti-GST and anti-Daxx antibodies. Tubulin was used as a cytosolic marker.

(B) SH-SY5Y cells were transfected with plasmid for GST-ICP27 (3 μg). At 24 h after transfection, subcellular distribution of Daxx (green) was determined by immunofluorescence microscopy using specific antibody against Daxx. Scale bar = 5 μm.

Fig. 3 ICP27 increases the interaction between Daxx and p65 by regulating Daxx sumoylation.

(A) 293T cells were transfected with plasmids expressing GST-Daxx, GFP-SUMO-1, and Flag-ICP27 (2 μg each). At 24 h after transfection, cell extracts were prepared with lysis buffer containing 20 mM NEM and pulled down with glutathione-Sepharose 4B. Sumoylated Daxx was detected by immunoblotting using anti-GFP and anti-GST antibodies.

(B) 293T cells were transfected with plasmids expressing GST-Daxx, GFP-SUMO-1, HA-Ubc9, and Flag-ICP27 (1.5 μg each). At 24 h after transfection, cell extracts containing 20mM NEM were pulled down with glutathione-Sepharose 4B and analyzed by Western blotting.

(C) 293T cells were transfected with plasmids expressing Flag-Daxx and His-p65 (3 μg each). At 24 h after transfection, cell extracts containing NEM were pulled down with Ni-NTA Chelating Agarose CL-6B and analyzed by immunoblotting using anti-Flag antibody.

(D) 293T cells were transfected with plasmids expressing Flag-Daxx, GST-p65, GFP-SUMO, and ICP27 (1.5 μg each). At 24 h after transfection, cell extracts were pulled down and analyzed by Western blotting with specific antibodies.

Fig. 4 ICP27 represses p65 acetylation through interaction with Daxx at early stage of HSV-1 infection.

(A) 293T cells were infected with wild type HSV-1 and HSV-1 Δ27 virus at MOI of 5. At 6 h post infection, cells were treated with 1 μm TSA. At 6 h post infection, cells were harvested in modified RIPA buffer containing 1 μm TSA and precipitated with anti-p65 antibody. The immunocomplex was analyzed by Western blotting with the indicated antibodies.

(B) 293T cells were infected with wild type HSV-1 and HSV-1 Δ27 virus at MOI of 5. At 6 h post infection, cells were harvested in modified RIPA buffer with 20 mM NEM and analyzed by Western blotting with the indicated antibodies.

(C) 293T cells were infected with wild type HSV-1 and HSV-1 Δ27 virus at MOI of 5. At 6 h post infection, cells were harvested in modified RIPA buffer with 20 mM NEM and precipitated with anti-Daxx antibody. The immunocomplex was analyzed by Western blotting with the indicated antibodies.

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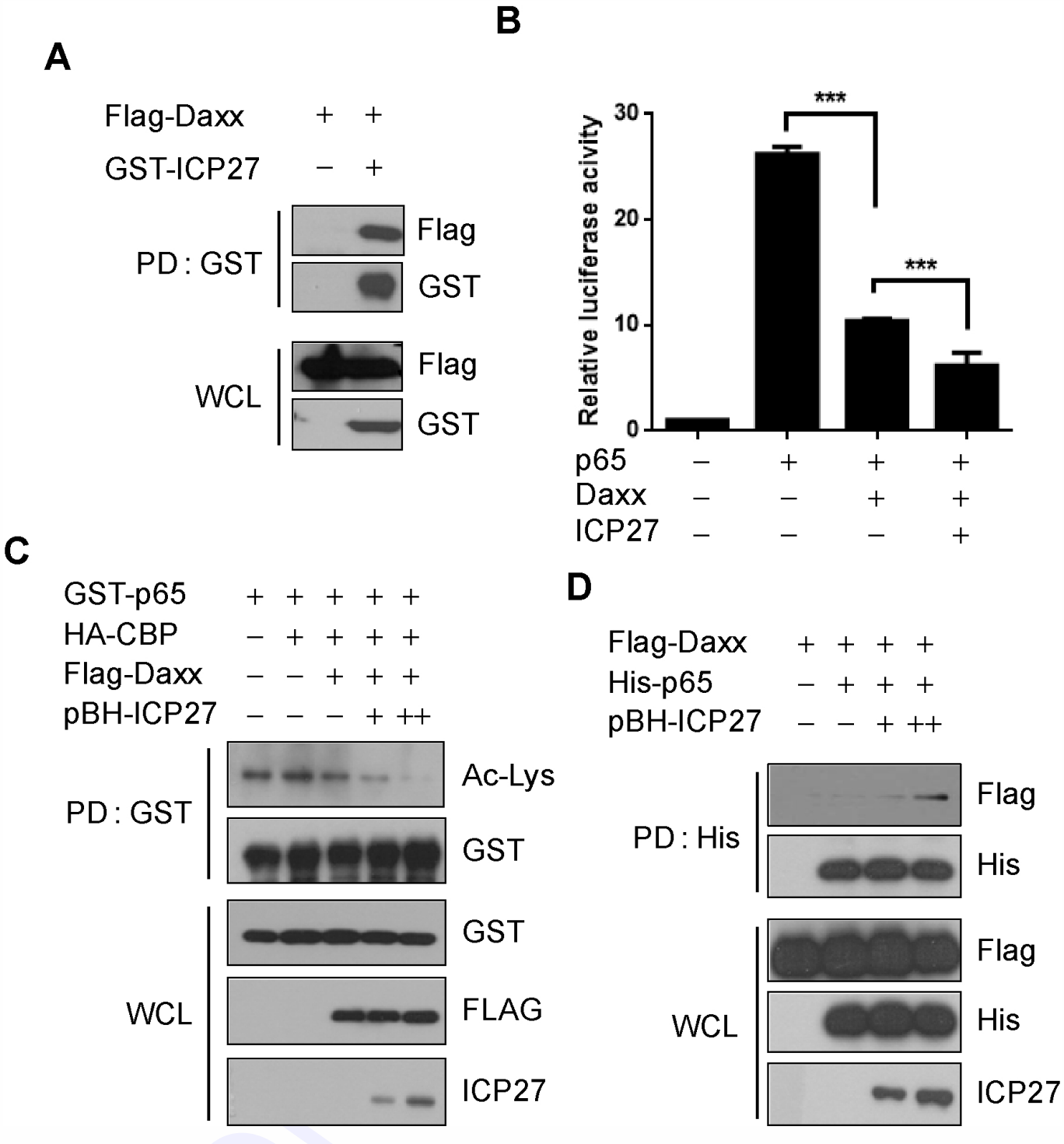
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ICP27

Fig. 1

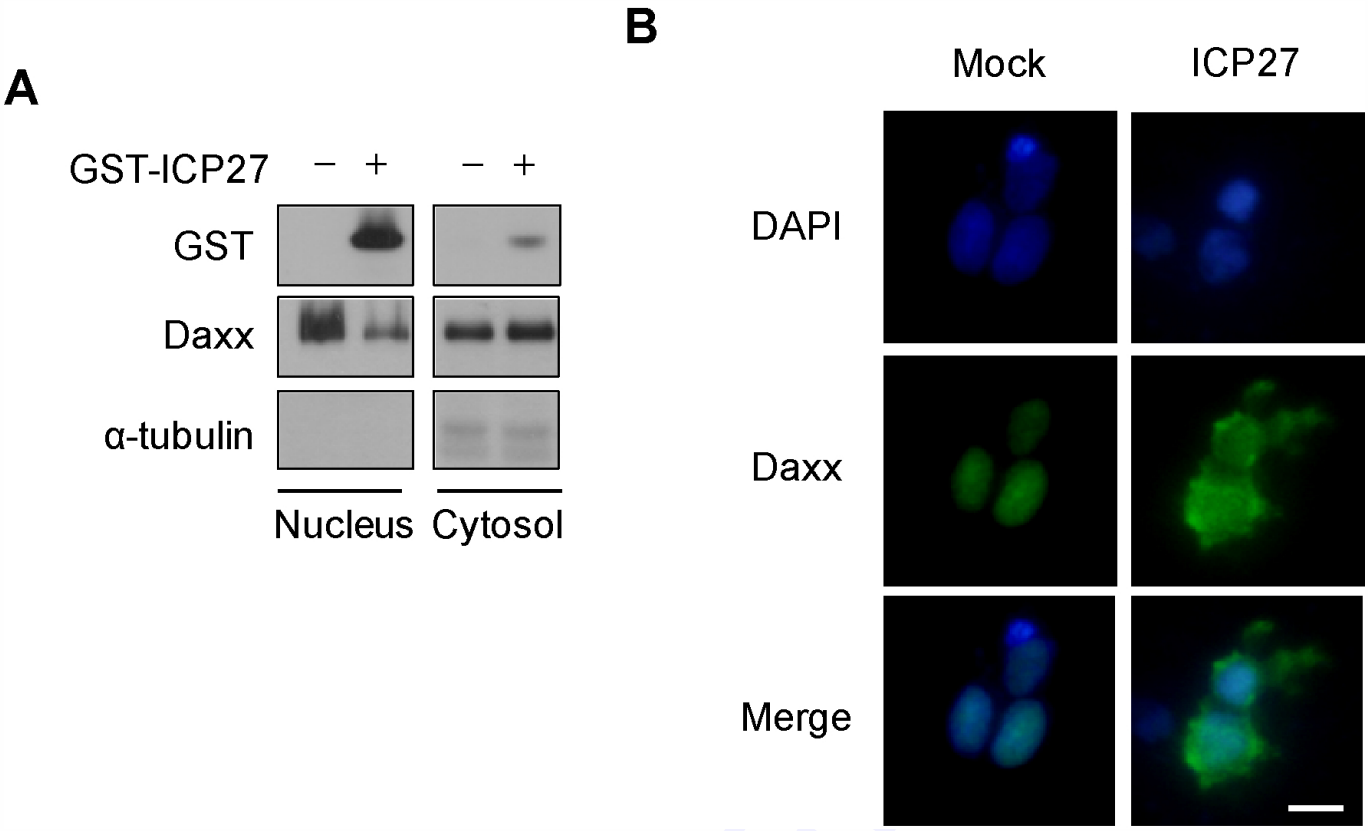
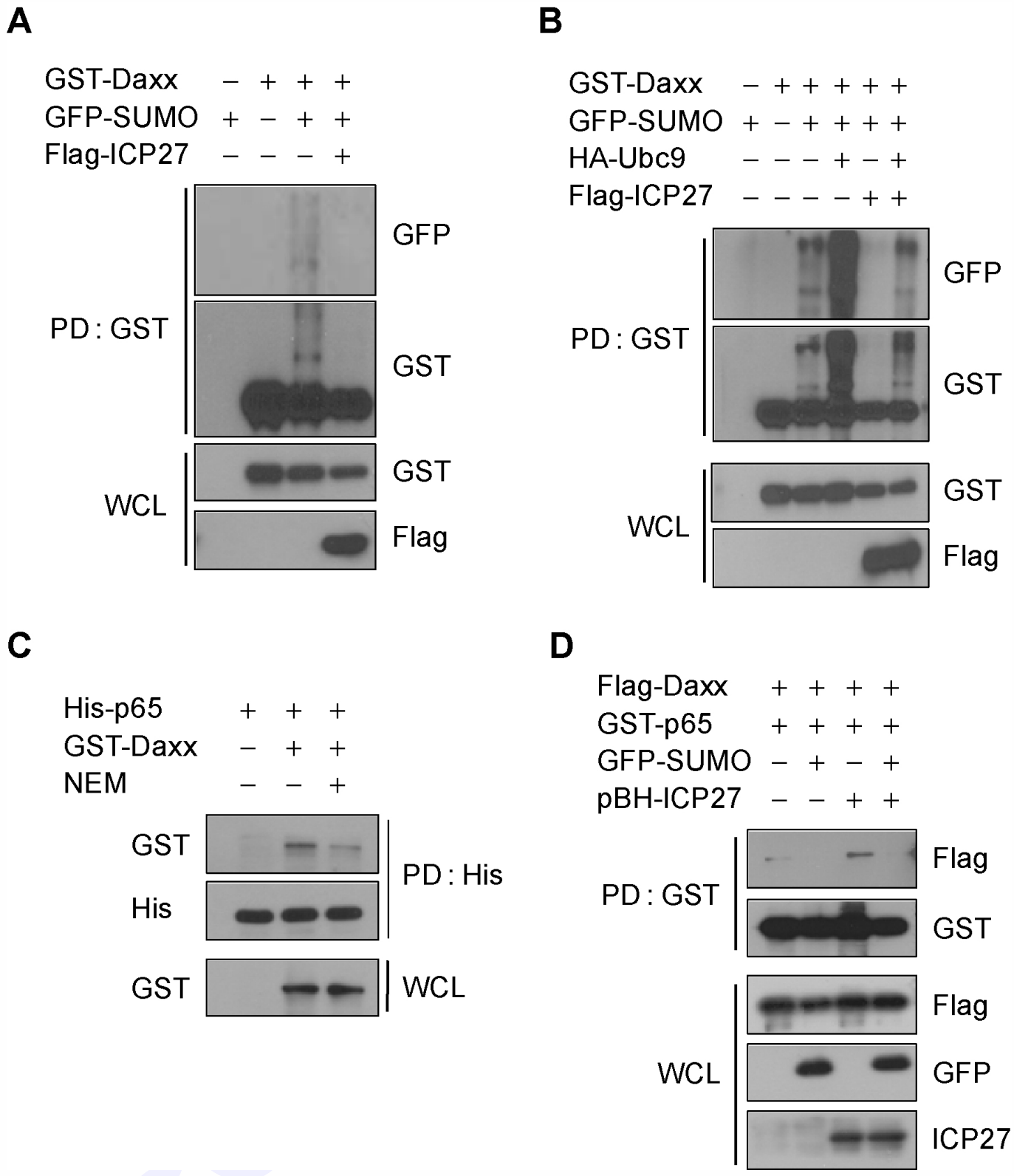


Fig. 2


A
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GFP-SUMO + – + +
Flag-ICP27 – – – +
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GFP
GST
WCL
GST
Flag
B
GST-Daxx – + + + + +
GFP-SUMO + – + + + +
HA-Ubc9 – – – + – +
Flag-ICP27 – – – – + +
PD : GST
GFP
GST
WCL
GST
Flag
C
His-p65 + + +
GST-Daxx – + +
NEM – – +
GST
His
PD : His
GST
WCL
D
Flag-Daxx + + + +
GST-p65 + + + +
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pBH-ICP27 – – + +
PD : GST
Flag
GST
WCL
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GFP
ICP27

Fig. 3

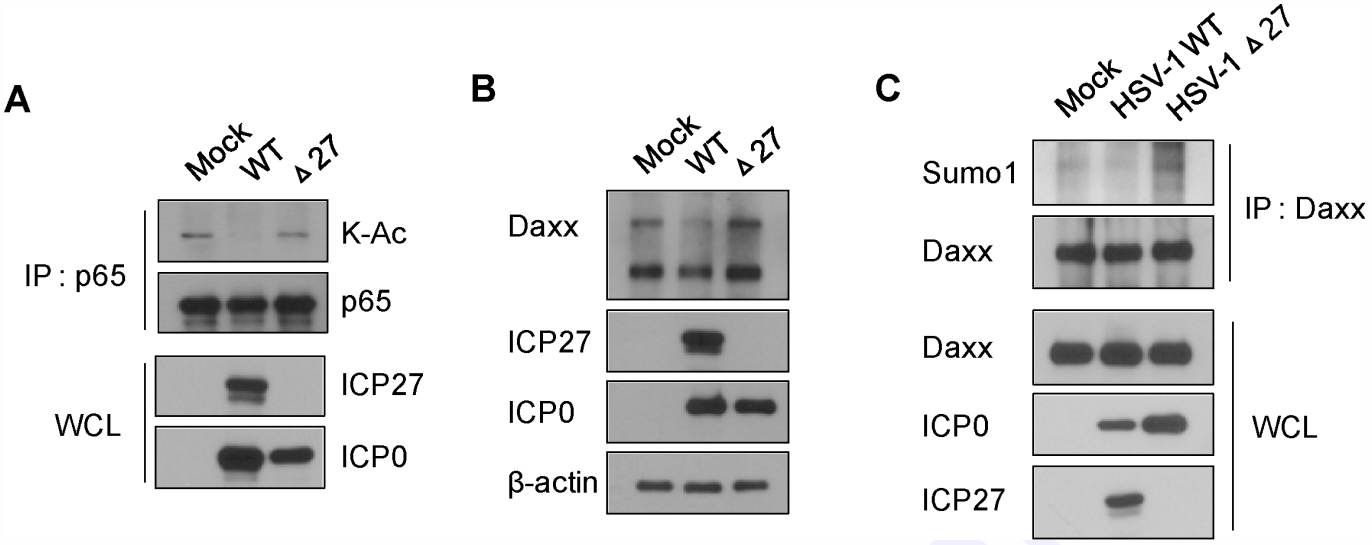

Fig. 4