Dissecting Human and Influenza Virus Interaction with qFRET Technology

Chuchu Liu, *Department of Biology*
Runrui Dang, *Ph.D. Student, Department of Bioengineering*
Jiayu Liao, *Ph.D., Department of Bioengineering*

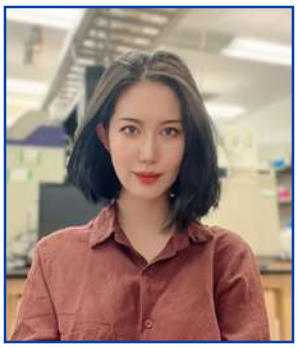
ABSTRACT

Influenza viruses cause seasonal epidemics and occasional pandemics around the world. During each flu season, IAV and IBV viruses are circulated widely in the community, with IAV being the dominant circulating virus and IBV accounting for 25% of all flu cases on average. Due to the significant threat posed by the flu virus, international organization, including the World Health Organization (WHO), have risen to prominence in limiting its global effect. Despite the vaccinations and anti-flu medications that have been developed to combat influenza, drug resistance development highlights the necessity of further studies for influenzas virus pathogenesis and new therapeutic development. Förster resonance energy transfer (FRET) is a technique for detecting protein interactions *in vitro* and *in vivo* that is widely employed in biological and biomedical research. Here we report that the IBV M1 protein has a high affinity with human SUMOylation enzymes, the conjugating enzyme UBC9 and the ligase PIAS1, and conform M1 can be SUMOylated determined with a quantitative FRET (qFRET) assay developed in our lab. Understanding the viral infection process and developing new treatment methods requires identifying and deciphering the host route of viral infection. It is critical to comprehend the viral infection process and develop new therapeutics. Blocking the host human SUMOylation pathway is particularly effective for IBV reduction. Our research provides a direct interaction of human proteins with influenza B protein, providing new insights into human-virus interactions for future therapeutics development. .

KEYWORDS: influenza virus, protein interaction affinity, SUMOylation, quantitative FRET assay

FACULTY MENTOR - Dr. Jiayu Liao

Dr. Jiayu Liao is an assistant professor in Bioengineering at UCR. Dr. Liao received his Ph.D. at UCLA in 1999 and was a founding faculty member of the Bioengineering University's department of California, Riverside. Dr. Liao's research at UCR has focused on the host-virus interaction and the creation of unique highly sensitive and quantitative FRET technology platforms for fundamental and biochemical engineering in SUMOylation and other protein-protein interactions. Dr. Liao was a senior research fellow at the Novartis Research Foundation's Genomic Institute and the Scripps Research Facility before coming to UCR.



Chuchu Liu

Chuchu Liu is a fourth-year Biology student in the Department of Natural and Agricultural Sciences. She is a volunteer in Dr. Liao's Lab and does research on high-throughput screening. She has previously worked in a Molecular Imaging Laboratory at the West China Second University Hospital of Sichuan University (WCSUH-SCU). Her work focused on targeting heart inflammatory molecules caused by diabetes with nanoprobe technology, MR imaging of myocardial infarction tract by neutrophilic granulocyte, and T-cell tracking based on magnetic hyperthermia. Chuchu's interests include science promotion, STEM education, and research. She hopes to get her Ph.D. in Bioengineering in the future.

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INTRODUCTION

One of the two types of viruses causing the COVID-19 pandemic in the 21st century is influenzas, which are classified as influenza A, B, C, and D (IAV, IBV, ICV and IDV)[1]. Despite the development of vaccines and antiviral medications, the number of deaths caused by influenza remains high each year, notably high specifically during the 2009 H1N1 pandemic. In the recent flu season of 2017-2018, it is estimated that flu caused approximately 51,000 deaths and 710,000 hospitalizations, making it one of the most life-threatening infectious disease[2]. The origins of IBV were initially discovered in 1940, and later, the second lineage was found in 1983, the Yamagata- and Victoria-strains[3]. During each influenza season, these two IBV subtypes are co-circulated, contributing considerably to the influenza illness burden over the years. Although IBV's ability to cause serious disease and mortality in particular groups has increased, the science and medical communities have underestimated IBV's contributions to the disease burden, despite the fact that it is considered to produce less severe symptoms than other IAV strains. IBV, for example, has been found to cause serious sickness in children and has a substantially higher fatality rate than IAV[4]. Several studies, however, have found IBV significantly improved their ability to cause severe disease and mortality in specific groups, such as infants and young children, and that they contribute considerably to yearly sickness, accounting for 37% of the overall economic losses of influenza[5]. A recent study indicated that, during 2004 to 2013, IBV-related morbidity was substantially greater than that caused by IAV in infants[6]. For HIV patients, IBV was likewise linked to a greater rate of hospitalization than IAV. Also, during the 2010-2011 influenza season, IBV accounted for just 26% of prevalent influenza strains, yet they were responsible for 38% of pediatric mortality. These findings disprove the theory that IBV produces fewer and milder symptoms than IAV.

Protein interactions are important in all physiological processes and pathogenicity in all organisms, ranging from transcription, signal transduction, and the cell cycle to cancer and neurodegenerative illnesses[7]. A number of approaches to determine protein interaction affinities have been developed[8]. Surface plasmon resonance (SPR), nuclear magnetic resonance (NMR), calorimetry (e.g., ITC-isothermal titration calorimetry and DSC-differential scanning calorimetry), radio-labeled binding tests, ultracentrifugation, and fluorescence polarization (FP) are a few examples of these methods. In fundamental science and translational study, these technologies have substantially enhanced the understanding of protein interactions and dynamics. Protein associations and affinity variations play a key role in normal living and illness. Expensive assay tools and lengthy procedures, on the other hand, cannot always yield trustworthy and consistent findings. One of the issues of current methods for protein interaction affinity determination is that they all require large amounts of purified proteins. Large-scale proteome systems' affinity and dynamics for complicated proteins, on the other hand, are mostly unexplored. As a result, protein-protein interaction experiments are currently quite scarce.

Quantitative tricubic analysis or ratiometric fluorescence signals were previously employed in quantitative Förster resonance energy transfer (qFRET) imaging and biochemical approaches to produce Förster resonance energy transfer (FRET) signals; theoretically, these methods could be used to measure protein interaction affinities (K_D)[9]. The quantitative tricubic technique, on the other hand, necessitates the determination of both the fluorophore's molar extinction coefficient and FRET effectiveness. It is also challenging to transform the approach into a methodological framework since it demands an estimate of the FRET efficiency and several instrument-related factors during the measurement. Without omitting the direct emission of the donor and acceptor signals, FRET analysis methods employ point-to-

point subtraction to assess the FRET signal as well as the ratio of fluorescence emitted at the acceptor and donor wavelengths. When using titration ratio FRET tests to measure protein interaction affinity, the K_D values are often greater than when using SPR or ITC[10].

Because of the availability of numerous fluorescence parameter estimations and the difficulties of determining absolute FRET signal, current approaches for K_D measurement in mixtures lack precision and robustness. We recently developed a cross-wavelength correlation coefficiency method to dissect the absolute FRET signal from the direct emissions of free donor and acceptor and determine the protein-protein interaction affinity K_D, which is very consistent with the values determined with SPR or ITC, but with a significant reduction of time and process[11-13]. FRET-based K_D determination is very sensitive and reliable technique. K_D determination approach based on the qFRET has a number of benefits over other approaches. First, protein interaction measurement is carried out in solution, mimicking the physiological milieu of live cells. Other approaches, such as SPR or ITC, on the other hand, need coupling proteins on solid chip surface, all of which may disrupt protein conformation. The qFRET assays is also extremely sensitive. Because the quantity of fluorescent proteins in FRET tests may range from nM to mM, depending on the detector used, a high concentration of protein is not necessary to determine the K_D. Third, FRET-based K_D tests are non-hazardous to the environment, and protein labeling techniques are ubiquitous. Here, we report that we used the qFRET method to determine the interaction affinity of human SUMOylation E2 conjugating enzyme, Ubc9, and E3 ligase, PIAS1, with the IBV M1 protein.

MATERIAL AND METHODS

1.1 Molecular cloning of DNA constructs

The pET28b (+) constructs for CyPet-SUMO1, UBA2, AOS1, and UBC9 were cloned as described in earlier work [1]. The Ypet-M1 was created by amplifying the open reading frame of YPet using primers and a Linker sequence and ligating it into the pET28b (+) vector (Millipore Corporation, Billerica, MA). TOP10 DH5a *E. coli* bacteria were used to amplify all plasmid DNA constructs.

1.2 Protein expression and purification

The pET28b (+) constructs encoding CyPet-SUMO1, AOS1, UBA2, UBC9, YPet-Linker3-M1, CyPet-UBC9, and CyPet-PIAS1 were transformed into BL21 DE3 *E. coli* cells. LB plates with 50 g/mL kanamycin were used to plate the transformed *E. coli*. A single colony was inoculated into 5 mL LB media with a starting culture of 50 µg/mL kanamycin. Each starting culture was transferred to 1 L 2XYT media with 50 µg/mL kanamycin and cultured for 3 hours at 37 °C with 180 RPM in shaker. 0.35 mM IPTG was used to induce recombinant protein expression overnight at 20 °C with 180 RPM in shaker. The next day, the bacterial cells were collected at 4 °C for 5 minutes at 8000 xg in centrifuge. The bacterial pellet was resuspended in 30 mL binding buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl, and 5 mM imidazole). An ultrasonic liquid processor was used to lyse the cell suspension (Misonix, Farmingdale, NY). After centrifugation at 4°C for 30 minutes at 35,000 xg, the supernatant was transferred to Column with Ni^{2+}-NTA agarose beads

(QIAGEN, Valencia, CA). Two-column volumes of Wash Buffer 1 (20 mM Tris-HCl pH 7.4, 0.5% (v/v) TritonX-100, and 1.5 M NaCl), two-column volumes of Wash Buffer 2 (20 mM Tris-HCl pH 7.4, 0.5 percent TritonX-100, and 1.5 M NaCl), and one column volume of Wash Buffer 3 (20 mM Tris-HCl pH 7.4, 500 mM NaCl, and 10 mM imidazole) was used to purify before adding 700µl elution buffer to elute the protein (20 mM Tris-HCl pH 7.4, 20 mM NaCl, and 400 mM imidazole). The dialysis buffer was used to dialyze recombinant proteins overnight at 4°C (20 mM

Tris- HCl pH 7.4, 50 mM NaCl, and 1 mM DTT). Protein concentrations were assessed using the Bradford assay with known quantities of bovine serum albumin (Thermo-Fisher Scientific Inc., Rockford, IL) as standards, and protein purity was evaluated using SDS-PAGE following Coomassie G-250 staining (Bio-Rad, Hayward, CA). Fluorescence intensities recorded on a FlexStationII384 were used to calculate fluorescent-fusion protein concentrations (CyPet-SUMO1 and Ypet-Linker3-M1) (Molecular Devices, Sunnyvale, CA).

1.3 qFRET dissociation constant (K_D)

The dissociation constant K_D was determined by making the receptor concentration at a content number, 0.20μM ([R]Total, the concentration of CyPet binding protein UBC9 or PIAS1) and increasing the ligand concentration ([L]Total, the concentration of YPet binding protein M1) from 0 to 4μM. In a total volume of 60 μl, CyPet and YPet binding proteins were combined and interacted with each other. Each condition (0.2 μM CyPet binding protein UBC9 or PIAS1 interact with 0, 0.05, 0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0μM YPet binding protein M1, respectively) was repeated three times. The samples were incubated for 15 minutes at 37°C. The samples were then transferred to a Greiner 384-well plate (Sigma-Aldrich). $FlexstationII_{384}$ was used to determine the fluorescence emissions.

Emission intensities were recorded at three wavelengths following excitation at 414 nm and 530 nm after excitation at 475 nm. The relationship between K_D and E_{mFRET} is shown in the **Equation 1**.[3]

$$E_{mFRET} = E_{mFRET} * \frac{[L]_{Total}-[R]_{Total}-K_D+\sqrt{([R]_{Total}+K_D-[L]_{Total})^2+4*K_D*[L]_{Total}}}{[R]_{Total}+K_D-[L]_{Total}+\sqrt{([R]_{Total}-[L]_{Total}+K_D)^2+4*K_D*[L]_{Total}}} \quad Equation1$$

1.4 qFRET in vitro SUMOylation assay

In a total volume of 60 μL, all components of the SUMOylation experiment (0.5 μM CyPet-SUMO1, 0.1 μM E1, 0.2 μM E2, 0.25 μM E3 PIAS1, and 2 μM YPet-Linker3-M1) were mixed in SUMOylation buffer (50 mM Tris-HCl pH 7.4, 1 mM DTT, and 4 mM $MgCl_2$). The sample reaction mixture was incubated in an Eppendorf tube at 37 °C after adding 6 μL of ATP (20mM stock). All sample mixes were then placed in a Greiner 384-well plate (Sigma-Aldrich). $FlexstationII_{384}$ was used to monitor fluorescence emissions (Molecular Devices, Sunnyvale, CA). Following excitation at 414 nm, emission intensities were measured at 475 and 530 nm, including 530 nm after excitation at 475 nm [3].

1.5 Em_{FRET} Analysis

The CyPet and YPet were fused to the SUMO1 and M1, respectively. Excitation and emission peak wavelengths for CyPet and YPet are 414 nm / 475 nm and 475 nm /530 nm, respectively. When the FRET pair (CyPet and YPet) is close together (between 1 and 10 nm), the donor's excitation at 414 nm causes an energy transfer from the donor to the acceptor, resulting in the donor's quenching and the acceptor's excitation. FRET can occur when YPet-M1 is SUMOylated with a CyPet-SUMO1, culminating in a 530 nm emission with a 414 nm excitation. Anything that hinders SUMOylation (such as the lack of ATP or the addition of STE-025), on the other hand, has no effect on the emission at 530 nm.

The development of the SUMO1-M1 complex was tracked using actual FRET emission (E_{mFRET}). E_{mFRET} was defined as illustrated in **Equation 2** [4]. To calculate the true FRET emission, direct emissions at 530 nm from free CyPet-SUMO1 and YPet-M1 must be calculated and subtracted from the overall emission intensity at 530 nm. The E_{mFRET} was calculated using a previously published

spectrum analysis to account for the components to the exhaust emissions at 530 nm. Real FRET emission (E_{mFRET}), CyPet direct emission, and YPet direct emission are three fractions of total fluorescent emissions at 530 nm given a 414 nm excitation (E_{mTotal}).

With a ratio coefficient of $\alpha = 0.368$, the direct fluorescence contribution of the CyPet at 530 nm is proportionate to its peak emission at 475 nm (FL_{DD}) when stimulated at 414 nm. With a ratio value of $\beta = 0.029$, YPet's direct emission at 530 nm is proportionate to its emission at 530 nm given a 475 nm excitation (FL_{AA}). For each sample, the fluorescence signal was measured from 400 to 600 nm.

$$Em_{FRET} = Em_{Total} - \alpha * FL_{DD} - \beta * FL_{AA} \quad Equation2$$

RESULTS

This research demonstrates that the human SUMOylation pathway is required for the IBV life cycle[14]. The SUMOylation pathway, a pathway required for the IBV life cycle, can impede IBV viral replication. SUMO is a member of the superfamily of ubiquitin-like polypeptides that uses a multistep enzymatic cascade to modify protein function and stability. The whole SUMOylation process involves a SUMO-activating enzyme E1 (Aos1/Uba2), a SUMO-conjugating enzyme E2 (Ubc9), and SUMO ligases E3 (PIAS1 family, RanBP2/Nup358, Pc2)[15]. It is generally assumed that E3 is not needed *in vitro* SUMOylation and plays an essential role *in vivo* SUMOylation for efficiency and specificity[16]. Thus, understanding the affinities of SUMOylation E2 and E3 for the IBV proteins is critical for further study of IBV M1 functionality during the infection process.

A straightforward way of tracing the reaction and collecting the results is to use the FRET signal to monitor the SUMOylation process. In the first study, the FRET signal was greatly significant when the CyPet-SUMO1 was

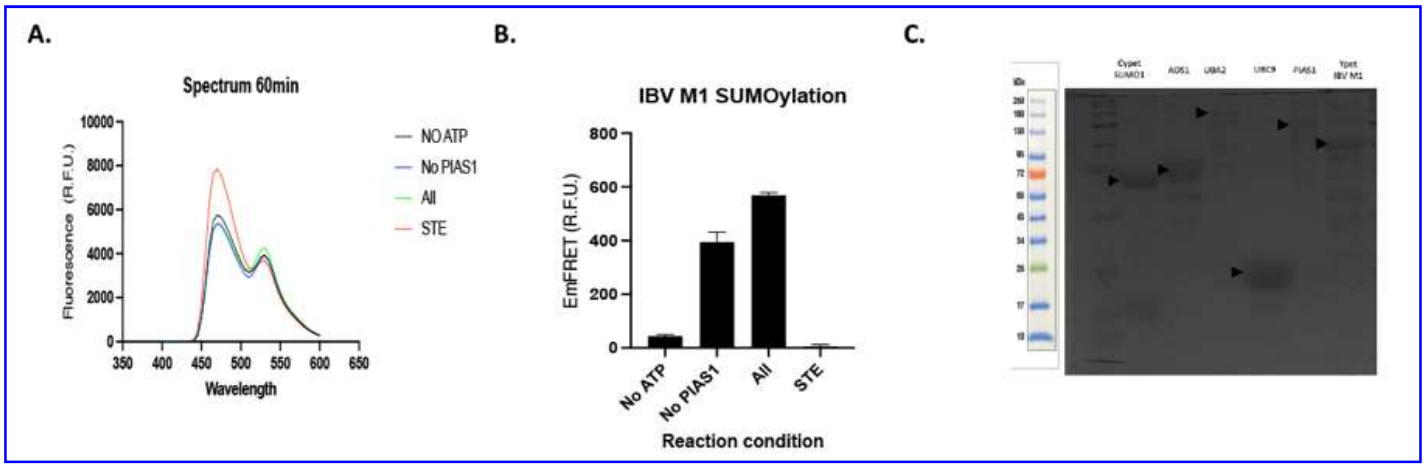

Figure 1. (A) Using the FRET assay, the FRET spectrum of the in vitro SUMOylation process of IBV M1 protein. CyPet-SUMO1, E1, E2, E3, YPet-M1, and ATP (ALL and green); CyPet-SUMO1, E1, E2, E3, YPet-M1, and ATP (no PIAS1 and blue); E1, E2, E3, YPet-M1, and no ATP (NO ATP and black); CyPet-SUMO1, E1, E2, E3, YPet-M1 (ALL plus STE and red). **(B)** IBV M1 SUMOylation quantitative FRET signal (EmFRET) from **(A)**. **(C)** An in vitro biochemical test of IBV M1 protein SUMOylation followed by a Western blot employing an anti-SUMO1 antibody. The SUMOylation processes were carried out in solution under different circumstances with and without the SUMOylation inhibitor, STE. Lane 1: CyPet-SUMO1, E1, E2, E3, YPet-M1, -ATP; Lane 2: CyPet-SUMO1, E1, E2, E3, YPet-M1, +ATP; Lane 3: CyPet-SUMO1, E1, E2, E3, YPet-M1, +ATP; Lane 4: CyPet-SUMO1, E1, E2, YPet-M1, +ATP+STE02

conjugated to the YPet-M1 due to the close closeness of the FRET donor and acceptor (**Figure 1A** ALL green). The SUMOylation inhibitor, STE025, blocks the SUMOylation process, as seen by the decrease of the FRET signal (**Figure 1A** STE and red). The SUMOylation E3 ligase PIAS1 was also included in this SUMOylation experiment. When protein levels are low, the SUMOylation E3 ligase plays a significant role in *in vivo* SUMOylation, although it is not required in the in *vitro* SUMOylation process. After adding PIAS1 to the reaction, we saw a significant increase in the FRET signal, which was consistent with previous findings (**Figure 1A** No PIAS1 in blue and ALL in green). We also examined the proteins expressed in bacterial cells, CyPet-SUMO1, ASO1, UBA2, PIAS1, and YPet-M1, in the SUMOylation reactions by SDS-PAGE gel followed with Coomassie-blue staining (**Figure 1C**). All the proteins are well expressed except the PIAS1, which expression level was low.

In this study, we used the qFRET to determine the affinities of Ubc9 and PIAS1 for the IBV M1 protein. The Ubc9 or PIAS1 was fused with FRET donor, CyPet, and IBV M1 was fused with FRET acceptor, YPet, respectively. Those three proteins were expressed in E. coli strain Bl21 (DE3). After proteins were purified through the Ni-His affinity column, the FRET donor, CyPet-Ubc9 or CyPet-PIAS1 was set in a constant concentration 0.2μM, and then the FRET acceptor, YPet-M1 was titrated in different concentrations from 0 to 4μM. The algorithm developed in our previous work can extract the absolute FRET signal, which corresponds to the interactions from the total fluorescence signal[11].

The **Figure 2** shows good sigmoidal curves of the absolute FRET signal (E_{mFRET}) of Ubc9 and PIAS1 with IBV M1. The K_D values calculated by our developed equation for Ubc9-M1 or PIAS1-M1 interactions were 0.20 μM and 0.22 μM, respectively, indicating a very high affinity of both SUMOylation enzymes E2 and E3 for the IBV M1. These K_D values show that the IBV M1 protein is a good substrate of SUMOylation enzymes.

DISCUSSION

Viruses employ host factors to infect hosts and replicate their genomes[17,18]. Depending on the roles of host factors, these components can be important or essential for

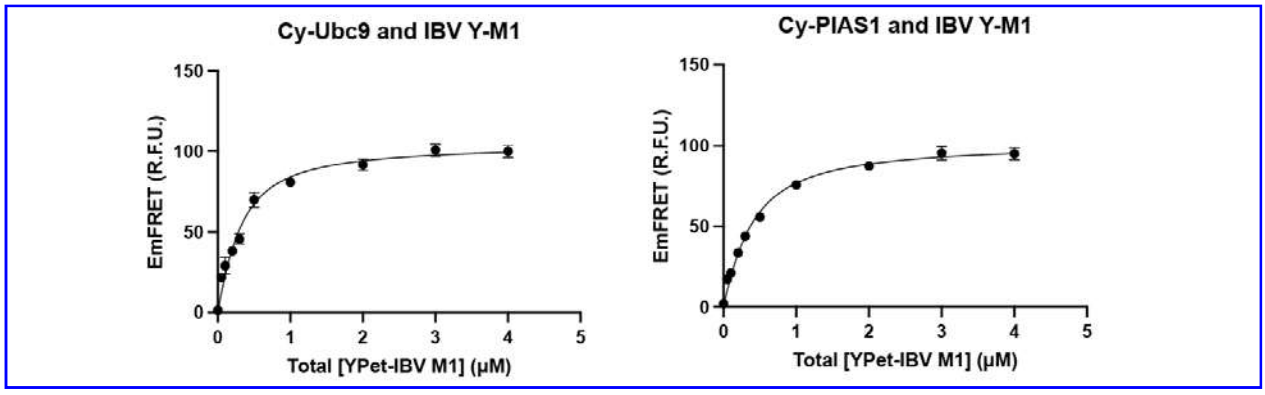

Figure 2. Ubc9, the SUMOylation E2 conjugation enzyme, and PIAS1, an E3 ligase, have a very high affinities for the IBV M1 protein, as demonstrated by a qFRET test. **(A)**. Using the qFRET assay in solution, the interaction affinity KD value of 0.2 M between Ubc9 and M1 was obtained. **(B)**. The qFRET assay was used to measure the contact affinity KD value of 0.22μM between PIAS1 and M1.

viruses. A novel antiviral method that targets host factors against infections has been proposed and developed in some efforts. SARS-CoV-2 patients are protected by antibodies that block the human receptor ACE2 and the viral S protein interaction. Here, we show that suppressing the human SUMOylation as a new approach for anti-IBV infection therapy, which is compatible with previous research and theory18. Identification and characterization of the essential host factors for viral replication offer considerable promise for revealing novel methods against IBV infection.

The IBV M1 protein is recognized by the SUMOylation E2 conjugation enzyme and E3 ligase at very high affinities, both around 0.2 μM in our qFRET assay, suggesting the potential SUMOylation of M1 under physiological conditions. Further research is required to explore the detailed functional requirement of SUMOylation for the M1 protein in viral life cycle. The IBV M1 protein is known to play essential roles in the formation of viral Ribonucleoprotein(vRNP) and the budding of the viral particle. The functional significance of M1 SUMOylation needs further investigation.

Clinical data shows that children infected with IBV are more susceptible and have higher hospitalization rates than those infected with IAV, reflecting different pathological processes and immune responses caused by IBV infection from IAV infection19. A study shows that IBV was revealed to be less sensitive to the Zanamivir and Oseltamivir than IAV in cellular assay[20]. Although there are still some differences between IBV and IAV needed to be explored, IBV has already been suggested to become a nonnegligible threaten for the anti-flu drug discovery. Therefore, novel therapies to treat influenza viruses need to be developed. Several studies have screened for host factors, factors important for IAV infection and replication. SUMOylation is a common factor identified among the genome-wide screens[21,22]. Since we confirm that the IBV M1 has a high affinity with SUMOylation E2 and E3, inhibiting SUMOylation can provide a valuable possibility for the development of a new class of inhibitors to treat influenza virus infections as well as other viruses that utilize SUMOylation. This approach illustrates the possibility of developing novel classes of anti-flu drugs.

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