SCREENING SUMO E3 LIGASE USING QFRET ASSAY FOR INFLUENZA VIRUS

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ABSTRACT

Influenza is one of the most prevalent viruses that has plagued millions worldwide. Every year health organizations encourage the public to get their flu vaccines to combat the flu season. Although the flu vaccines and medicines are widely available, it is estimated that flu viruses still cause 300,000-500,000 deaths every year. The two most common influenza virus strains are influenza A and B viruses, or IAV and IBV, respectively. It has been discovered that drug resistance develops very soon after a new drug is launched. It is highly demanded that anti-flu virus drugs with novel mechanisms be developed. Our lab has discovered that SUMOylation, a post-translational modification, is essential to the viral IAV and IBV life cycle. In this study, we have screened all the E3 ligases in the human genome to discover the SUMO E3 ligase responsible for the essential SUMOylation of IAV M1 protein using our Quantitative Fluorescence Energy Transfer(qFRET). We first determined the FRET spectrum of all E3 ligases with M1 protein and then quantified the FRET signals to provide a first-line examination of interactions. We then determined the E3-M1 interaction affinities, KD, to ensure the real interactions. We found the E3 ligase PIAS1 has the highest affinity to M1 among other E3s. By understanding the interaction affinity between IAV M1 protein with SUMOylation E3 ligase, we hope to block the interaction between the PIAS1-M1 for novel anti-flu medicine development.

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1. Introduction

Influenza virus has posed a significant challenge to public health for decades. The highly contagious respiratory virus is categorized into four distinct subtypes - A, B, C, and D - and is responsible for many various widespread epidemics and pandemics. The two most notable ones being the Spanish Flu of 1918 and the pandemic of H1N1 in 2009 with Influenza A virus (IAV) being known to trigger most of the outbreaks, due to its ability to mutate quickly. The National Foundation of Infectious Diseases has estimated that the flu infection has financially affected both employers and businesses around $11.2 billion annually through easily calculated funds and indirect costs[1]. The World Health Organization (WHO) has estimated that there has been 1 billion infections, 3-5 million severe cases, and 300,000-500,000 deaths annually[2].

Although there has been significant strides made in the development of seasonal flu vaccines, the efficacy remains suboptimal, especially against the IAV variant. The persistent threat of the disease has consistently posed a threat against public health, as the occurrence of pandemic strains cycles every 10-50 years from varying existing types. The influenza virus continues to evolve, developing a drug resistance to the already existing therapeutics, necessitating the need for further research[3].

Understanding the molecular mechanisms underlying influenza infection requires elucidating human-virus interactions represented as protein-protein interactions, which are fundamental to numerous biochemical and physiological processes. However, studying these interactions faces significant challenges, as over 80% of proteins exist in complex formations rather than in isolation, and it is very difficult to express viral proteins in other systems, such as bacterial cells or mammalian cells, necessitating sophisticated technologies for analysis. Förster Resonance Energy Transfer (FRET) is a phenomenon in which two fluorophores with overlapped excitation

and emission spectrums can transfer energy through dipole-dipole interaction when they are close enough. As depicted in Figure 1, when proteins are in close proximity of 1-10 nm, FRET occurs between the two fluorophores, and the FRET spectrum can be elucidated[5,14]. FRET is a distance-dependent photophysical process, in which positioning of the vectors highly affects the FRET efficiency meaning that the two vectors in parallel with one another is most favorable. Meaning that the FRET efficiency is zero if the two vectors are perpendicular to one another even if the two fluorophores are within the optimal FRET distance of 1-10 nm. It was found that if the two proteins are less than 1 nm apart, then the collision between the donor and acceptor will exist, whereas if it is greater than 10 nm the emission by the donor will be more dominant[16].

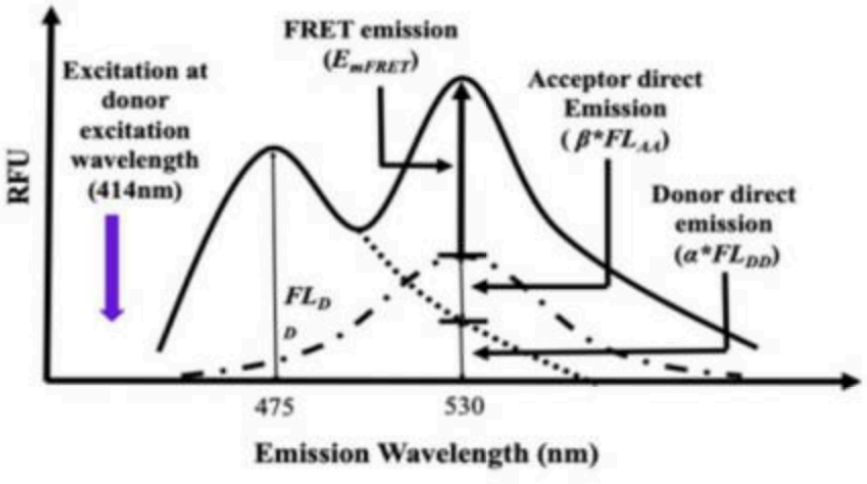

Figure 1: Fluorescent emission spectra explaining the energy transfer phenomenon between FRET donor and acceptor when the two fluorophores with overlapped excitation and emission spectrums are close to each other[12].

Quantitative FRET (qFRET) technology emerges as a promising tool, as it is quicker, cheaper, and often less destructive to proteins than alternative assays, and it can be implemented as a high-throughput assay format. Specifically, qFRET can be used to assess protein interaction affinity (K_D), allowing further study into mitigating these interactions. K_D is the equilibrium constant used to evaluate the strength of the binding affinity between two proteins. A high K_D

value can tell researchers that the protein does not exhibit a high binding affinity for the other, while a low K_D value will indicate a high affinity. This technology is developed explicitly towards post-translational modifications, such as SUMOylation, a pathway critical for the IAV M1 life cycle, as it utilizes the pathway for replication[4]. Using qFRET, we characterized the interaction affinities of E3 ligase molecules within the SUMOylation pathway with the influenza IAV virus M1 protein.

SUMOylation is a process that is directed by an enzyme cascade consisting the E1-activating enzyme, E2-conjugating enzyme and E3 ligase in which SUMO molecules are covalently conjugated to the lysine residue of the target substrate. A quick overview of the SUMOylation pathway is that the SUMO precursor is cleaved at its carboxyl-terminal tails by the protease SENP to expose the gly-gly motif leading to a matured SUMO molecule. The C terminus of the matured SUMO molecule will bind to the E1 activating enzyme heterodimer AOS1-Uba2 through a thioester bond in an ATP dependent reaction. The SUMO molecule is transferred from Uba2 to the E2- conjugating enzyme Ubc9 to form a thioester linkage between the Cys residue of Ubc9 to the carboxyl group of the SUMO molecule. The Ubc9 protein will then attach the C-terminal gly residue of the SUMO molecule to the lysine residue of the target substrate. The SUMO molecule will bind to the lysine side chain by removing one hydrogen to form an isopeptide bond. The role of the E3 ligase in the SUMOylation pathway is to recruit and accelerate the transfer of SUMO molecules onto the target substrate, as well as to help with the binding specificity and enhance the SUMO transfer[18]. It has been observed that there are 9 binding lysine sites in the IAV M1 sequence that can be SUMOylated, however it is still unknown which E3 ligase it is able to bind with. SUMOylation can only occur on lysine residues.

This research holds the potential to identify novel targets for antiviral drug development and deepen our understanding of the host-virus interactions, ultimately aiding in developing more effective strategies for combating influenza infections without drug resistance.

2. Material and Methods

2.1. Molecular Cloning of Constructs

pET28b plasmids encoding the fluorescent fusion protein, CyPet-TRAF6, PIAS3, PIAS4, RHES, hSTUB1, hRNF4, hMApl, parkin, PIAS1, hCBX4, hHDAC4, hHDAC4, hTRIM28, Dcst, Fbxw7, hCRBN, SOCS1, YPet-hSCNA, and YPet-M1, were cloned into *E.Coli* using varying strains of Electrocomp *E.Coli* cells via electroporation. Followed by a 1-hour recovery, Luria-Bertani (LB) agar plates with 50 µg/mL kanamycin were used to plate the transformed *E.Coli* cells. The following strains were used to amplify all the transformed plasmid DNA constructs followed by an extensive screening protocol to determine the highest strain value used for protein expression: BL21-CodonPlus (DE3)-RIL, ArticExpress(DE3)RP, BL21-CodonPlus, OverExpress™ C43(DE3), BL21(DE3), Shuffle®T7, BL21(DE3) pLysS, Rosetta (DE3)pLysS and OverExpress™ C41(DE3).

2.2. Protein Expression and Characterization of IAV M1 and E3 Ligases

The previously identified highest expressing strain was inoculated into a starting culture at 1:8 v/v of LB broth with 50 µg/mL Kanamycin, for resistance selectivity. Grown at a smaller culture overnight at 37 ℃ and placed in a shaker at 250 RPM overnight, the culture was then transferred to 1 L of 2XYT media supplemented with 50 µg/mL Kanamycin and placed into a shaker at 250 RPM at 37 ℃ until a proper optical density (O.D.) of 0.4-0.6 was reached at 600 nm absorbance. At the desired OD, protein expression was induced with 1M IPTG, a final concentration of 0.375 mM, and left to shake overnight at 16 ℃ and 200 RPM.

After the induced culture was left to shake for 12-15 hours, the bacterial cells were collected by centrifugation at 4℃, 8000 xg for 5 minutes. The collected bacterial pellet was resuspended in centrifuge bottles physically, with the addition of 30 mL of Binding Buffer (20 mM Tris HCl, pH 7.4, 0.5 mM NaCl, 5 mM Imidazole). The resuspended bacterial pellet undergoes sonication at ultrasonic frequencies to lyse the cells at alternating on and off phases of pulses for 7 minutes. Subsequently, the sonicated cells underwent 2 cycles of centrifugation at 4℃, 35,000 xg for 30 minutes, after which the supernatant was transferred into columns containing Ni^{2+}-NTA agarose beads while ensuring that pellet fibers were not included to prevent clogging. The attached protein and beads will undergo two-column volumes of Wash Buffer 1 (20mM Tris HCl, pH 7.4, 300mM NaCl), two-column volumes of Wash Buffer 2 (20mM Tris HCl, pH 7.4, 1.5 M NaCl, 0.5% Triton-100), one-column volume of Wash Buffer 3 (20 mM Tris HCl, pH 7.4, 0.5M NaCl, 10mM Imidazole), and one-column volume of Interaction Buffer (150mM NaCl, 25mM Tris HCl pH 8, 5% glycerol) to strip unwanted bounded components to reduce non-specific binding.

After washing, proteins were eluted with 300 µL to 1mL 450 mM Elution Buffer (1 M Imidazole, Milliq Water) depending on the expected yield; 300 µL of Elution Buffer was allowed to flow through before collection. Dialysis Buffer (150 mM NaCl, 25 mM Tris HCl pH 8, 5% glycerol, DTT to a final concentration of 1 mM) was prepared in a glass beaker with a dialysis membrane bag prepared for each protein. Eluted proteins were pipetted into the dialysis bags and left to dialyze overnight at 4℃ to remove excess salts.

Protein concentration was determined using the FlexStationI1384 to measure fluorescence intensities at Excitation 414 nm / Emission 475 nm (CyPet range) and Excitation 475 nm / Emission 530 nm (YPet Range). Purified protein underwent a 1:6 dilution before being pipetted into Greiner 384-well plates. The acquired fluorescence readings were calculated based on CyPet

and YPet fluorescence standards to determine the concentration of the purified fluorescent-tagged proteins. Protein size was determined and confirmed via SDS gel electrophoresis; gel samples were prepared by taking 5 µg of the protein sample, 15 µL of SDS, and 15 µL of MilliQ H_2O. Samples were heated at 100℃ for 5 minutes before loading into the polyacrylamide gel (Acrylamide, 10% APS, Temed, 1.5M Tris HCl pH 8.8, 1.5M Tris HCl pH 7.4, 10% SDS) with 3 µL of the DNA ladder. Electrophoresis was conducted at 100V for 3 hours; the gels were stained overnight with a Staining Buffer (Coomassie Blue R350, Methanol, Acetic Acid), then with a Destaining Buffer (Methanol, Acetic Acid) to better visualize the gel.

2.3. Em_{FRET} Assay

A 1µM:1µM CyPet and YPet fused with E3 Ligase and M1, respectively, assay was performed to generate preliminary interaction data. Varying excitation and emission peak wavelengths at 414 nm /475 nm and 475 nm/ 530 nm, were used for CyPet and YPet, respectively. When the fluorescent pair (CyPet and YPet) are placed in close contact at 2-10 nm with favorable orientations, then the excitation of the donor will excite the energy transfer from the acceptor. The coupling between the two fluorophores occurs due to the excitation of the donor, CyPet, which induces an energy transfer to the emission of the acceptor, YPet. As a result, it quenches the donor while exciting the acceptor. Preparing a 1 µM sample of CyPet and YPet with Interaction Buffer (150 mM NaCl, 25 mM Tris HCl pH 8, 5% glycerol) and 1 M DTT. Control samples were prepared with CyPet alone for the alpha value (α) and with YPet alone for the beta values (β); the ratio coefficient, α, is calculated to account for the emission peak of CyPet at 475 nm, as the ratio coefficient, β, is calculated to account for the emission peak of YPet at 530 nm. The prepared samples were pipetted in triplicate into 384 well plates.

Em_{FRET} was determined by utilizing **Equation 1** to calculate true FRET emission.

Equation 1:

$$EmFRET = EmTotal - ((\alpha * FLdonor) + (\beta * FLacceptor))$$

Spectrum reading is generated for qualitative interaction data collection. The following parameters are set for CyPet and YPet with 414 nm excitation, 455 nm cutoff and 475 nm excitation, 515 nm cutoff, respectively. For both samples, it was measured from 400 to 600 nm. Spectrum readings are to be performed separately for CyPet and YPet intensities.

2.4. K_D Determination

The dissociation constant (K_D) was determined by keeping the donor protein concentration at a constant of 0.1 μM and titrating the acceptor protein concentration from 0 μM to 25 μM. The fluorescent fusion protein pairs were combined into a total volume of 60 μL with Interaction Buffer (150mM NaCl, 25mM Tris HCl pH 8, 5% glycerol) and 1M DTT. Each titration was repeated in triplicates, again, to account for errors introduced due to pipetting variation. The prepared samples were incubated for 15 minutes in a 55℃ water bath before being transferred to a Greiner 384-well plate. FlexStationII384 was used to measure fluorescence intensities at 414 nm /475 nm, 475 nm /530 nm, and 414 nm/ 530 nm. Selecting the "Endpoint" settings, set the correct fluorescence intensities, and select the wells to be analyzed, PMT constant gain at "Low" and to allow for mixing.

Three wavelengths were recorded and the relationship between K_D and Em_{FRET} was determined by **Equation 2**.

Equation 2:

$$EmFRET * \left(\frac{[Acceptor]total - [Donor]total - KD + \sqrt{([Donor]total + KD - [Acceptor]total)^2 + 4 * KD * [Acceptor]\,total}}{[Donor]total + KD - [Acceptor]total + \sqrt{([Donor]total - [Acceptor]total + Kd)^2 + 4 * KD * [Acceptor]total}}\right)$$

Prism5 (GraphPad Software) was used to fit the Em_{FRET} values into **Equation 2** to determine the

K_D value. A non-linear regression fit was set with the donor concentration set at 0.1 uM and the initial K_D and $Em_{FRETmax}$ conditions set to zero.

3. Results

The potential all human SUMOylation E3 ligases and as control, Ubiquitin E3 ligase, in human genome, TRAF6, PIAS3, PIAS4, RHES, hSTUB1, hRNF4, hMApl, parkin, PIAS1, hCBX4, hHDAC4, hHDAC4, hTRIM28, Dcst, Fbxw7, hCRBN, SOCS1, hSCNA, and IAV M1 genes were synthesized and cloned into pET28b vector with CyPet and YPet tag, respectively. Following an extensive screening process, strains exhibiting an expression ratio above 3(induced vs. uninduced) were chosen to express proteins. Among these, hTRIM28 and PIAS1 were chosen for K_D determination because their FRET spectrum and EmFRET signals were positive for potential interaction. The proteins underwent purification through Ni-His affinity columns, and the eluted samples were collected for qFRET determination. An SDS gel electrophoresis was performed to confirm the fluorescent full-length proteins were maintained.

The FRET spectral analyses were conducted prior to determining the dissociation constant (K_D) for the interaction between the E3 ligase and the IAV M1 protein. The interaction spectra were examined at concentrations of 0.1 μM, 0.5 μM, and 1.0 μM for each protein, aiming to discern the specificity of the binding between the two proteins. In the initial investigation, CyPet-tagged E3 ligase at a concentration of 0.1 μM was engaged with YPet-tagged IAV M1 protein (Figure 2a-f). Subsequently, a parallel study was conducted wherein both CyPet-tagged E3 ligase and YPet-tagged IAV M1 protein were present at concentrations of 0.5 μM (Figure 3a-f). Finally, a third examination involved both entities at concentrations of 1.0 μM (Figure 4a-f). The graphical representations derived from these experiments offer qualitative insights into the interaction

dynamics before the quantitative determination of K_D. An increase of each of the substrate loadings shows a clearer emission peak at the YPet fluorescent emission at 475 nm indicating an energy transfer between the two protein pairs.

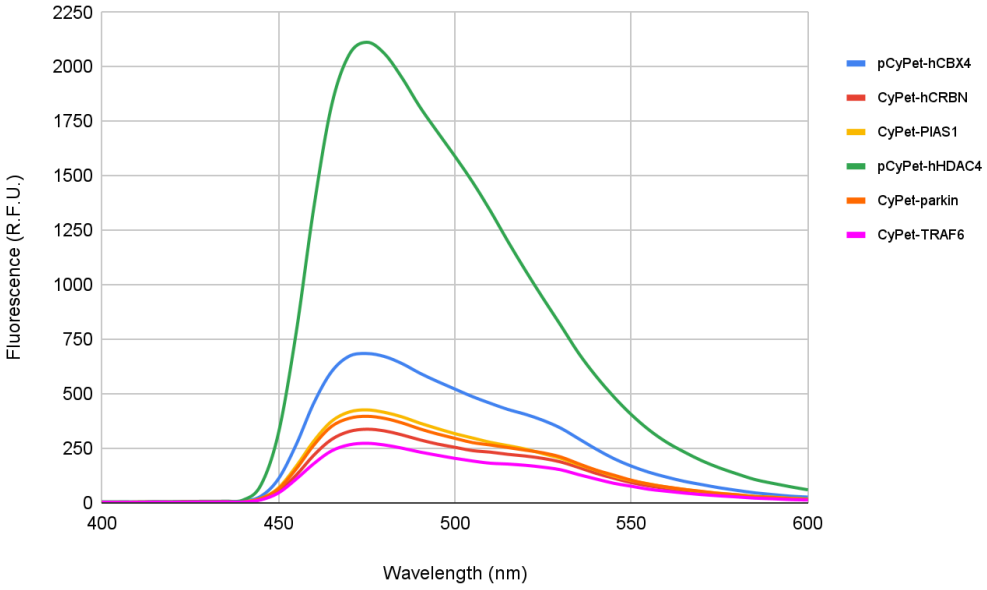

Figure 2: 0.1 uM CyPet : 0.1 uM YPet IAV M1 interaction spectrum graph determined from Em_{FRET} assay. CyPet-hCBX4 (blue), CyPet-hCRBN (red), CyPet-PIAS1 (yellow), CyPet-hHDAC4 (green), CyPet-parkin (orange), CyPet-TRAF6 (pink).

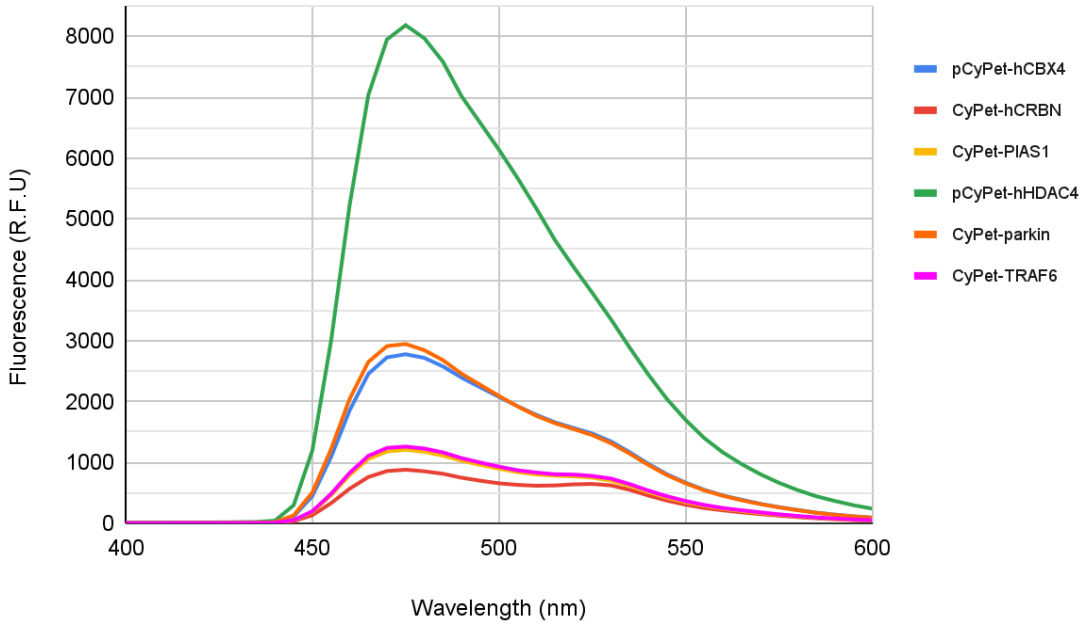

Figure 2: 0.5 uM CyPet : 0.5 uM YPet IAV M1 interaction spectrum graph determined from Em_{FRET} assay. CyPet-hCBX4 (blue), CyPet-hCRBN (red), CyPet-PIAS1 (yellow), CyPet-hHDAC4 (green), CyPet-parkin (orange), CyPet-TRAF6 (pink).

A select set of E3 ligases were chosen for assessment at lower concentrations of 0.1 µM and 0.5 µM, in addition to the standard 1.0 µM, to discern potential concentration-dependent effects on their interactions. However, all E3 ligases were evaluated at the 1.0 µM concentration alongside the determination of Förster resonance energy transfer (FRET) efficiency (Em_{FRET}) to investigate their binding characteristics comprehensively.

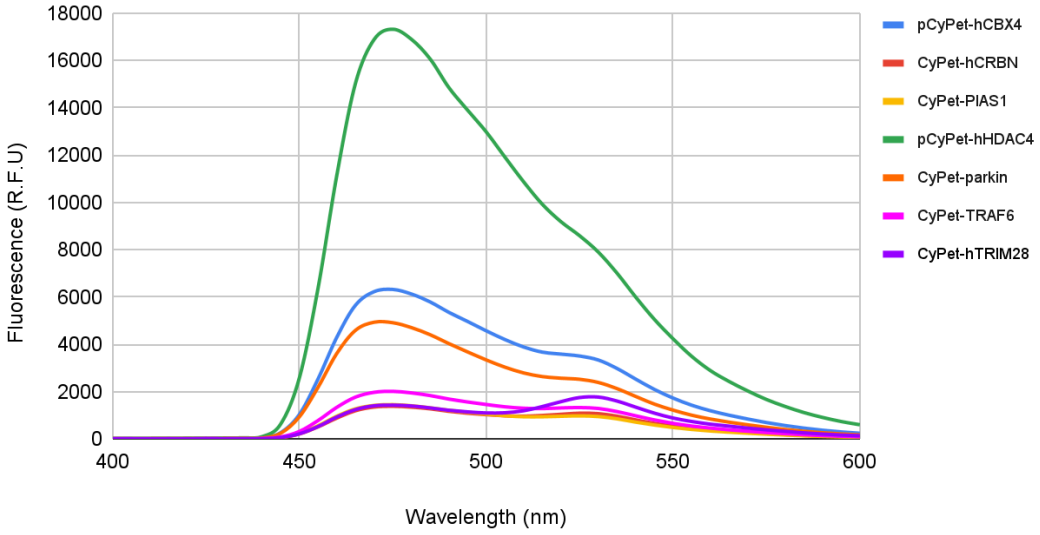

Figure 4: 1.0 uM CyPet : 1.0 uM YPet IAV M1 interaction spectrum graph determined from Em_{FRET} assay. CyPet-hCBX4 (blue), CyPet-hCRBN (red), CyPet-PIAS1 (yellow), CyPet-hHDAC4 (green), CyPet-parkin (orange), CyPet-TRAF6 (pink), CyPet-hTRIM28 (purple).

A quantitative absolute FRET signal value, Em_{FRET}, can provide additional information about the binding nature between the E3 ligase and IAV M1 protein. The Em_{FRET} was obtained to determine the sensitized FRET signal resulting from the binding of two proteins. The fluorescent pairs were excited at excitation wavelengths of 414 nm and 475 nm for CyPet and YPet, respectively. The α coefficient, necessary for determining Em_{FRET}, was derived from the donor fluorescent protein excited at 414 nm. Consequently, the β coefficient was derived from the acceptor fluorescent protein excited at 475 nm. The α and β coefficients were multiplied by the fluorescent emission of the donor and acceptor and subtracted by the total emission, as described in **Equation 1**. The acquired Em_{FRET} values were then subjected to comparative analysis, facilitating the assessment of the binding affinity between the E3 ligase and the IAV M1 protein. Consistent throughout **(Figure 2b, 3b & 4c)** there was a clear energy transfer between the pair with an evident peak at 414 nm and 475 nm, however the Em_{FRET} value is considerably low at 89.74 **(Figure 5)**. This comparative evaluation was depicted graphically in **Figure 5**, offering a

visual representation of the binding characteristics between the protein entities under investigation. The Em_{FRET} value for pCyPET-PIAS1 and pCyPet-M1 was 565.92, exhibiting a high binding affinity. In addition, the Em_{FRET} values for PIAS3, PIAS4 hSCNA, and TRM28 were very high too, indicating potential interactions. The generated Em_{FRET} values also provide a clue for further investigations.

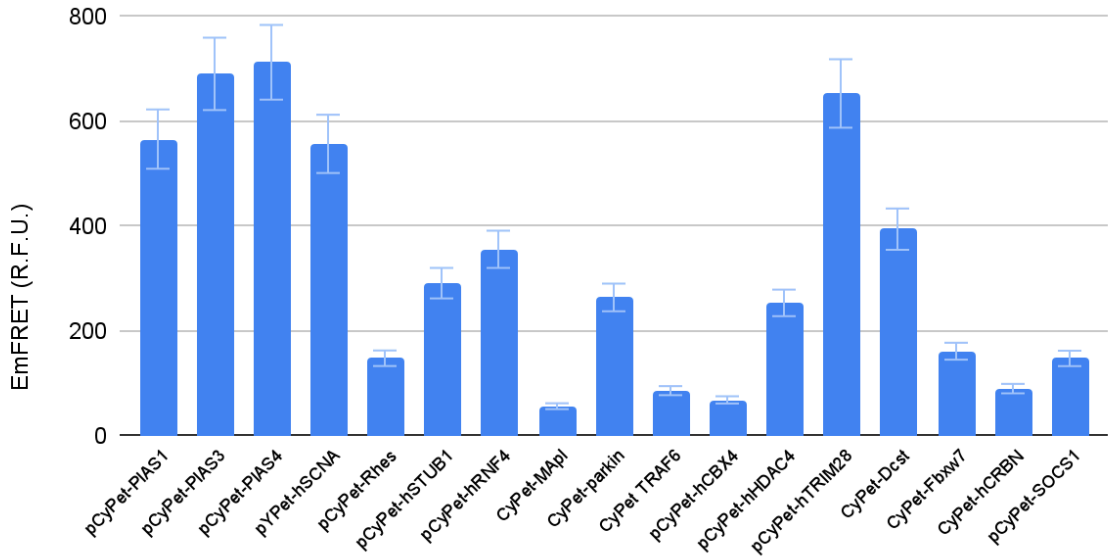

Figure 5: Results of varying 1uM E3 Ligase with 1uM YPet IAV M1 to check for interaction prior to K_D determination.

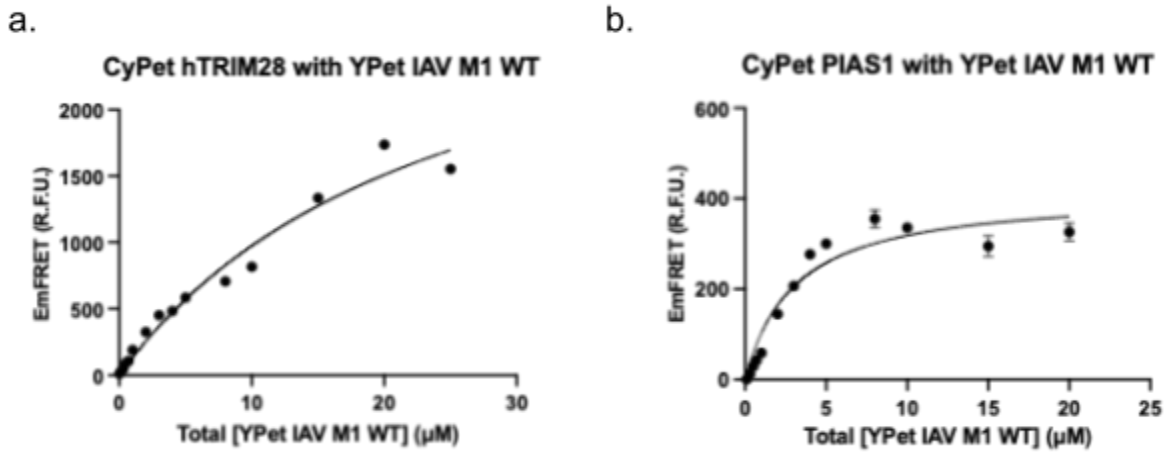

Figure 6: K_D determination results. (A) The interaction between IAV M1 and hTRIM28 was determined, with a K_D value of 24.2 μM. (B) The interaction between IAV M1 and PIAS1 was determined, with a K_D value of 2.9 μM.

We determined the K_D values between PIAS1 and TRIM28, and IAV M1 in order to determine its binding affinity for further characterizations. The binding affinity between the fused CyPet hTRIM28 and PIAS1 with YPet IAV M1 was determined by holding the FRET donor at a set concentration of 0.1 μM. The FRET acceptor was titrated in varying concentrations from 0 to 25 μM. The K_D value was determined by quantifying the absolute FRET signal between the interactions of the two fluorescent pairs[12]. The curves generated in **Figure 6** exhibit the binding affinity difference between hTRIM28 with IAV M1 and PIAS1 with IAV M1. The calculated K_D values for (**Figure 6a & b**), were 24.2 μM and 2.9 μM, respectively. The determined values indicate that the E3 ligase PIAS1 exhibits a notably higher affinity for IAV M1 protein, indicating a real SUMOylation E3 ligase for IAV M1, whereas the E3 ligase hTRIM28 is not. We will examine the E3 ligase activity of PIAS1 in the future study.

4. Discussion

SUMOylation is an *in vivo* post-translational modification process that has the capability to regulate protein function and stability. The Influenza virus has been discovered to utilize the SUMOylation pathway for its replication once infecting the human cells. Similar to how the viral protein employs the post-translational pathway for replication, the host protein can offset the effects by modulating its immune response utilizing SUMOylation. By expressing the fluorescent labeled protein we were able to perform K_D determination to better understand the interaction. K_D is the equilibrium constant that is used to evaluate the binding affinity between two proteins and a higher K_D value would indicate that the two proteins do not exhibit a high binding affinity. Consistent through the spectral graph and Em_{FRET} determined data, hTRIM28 and PIAS1 had shown a clear energy transfer at 1.0 μM and a high Em_{FRET} value of 653 R.F.U.

and 565 R.F.U., respectively. The K_D values were determined by quantifying the absolute FRET signal between the interactions of the two proteins. hTRIM28, although exhibited a clear energy transfer and had a high Em_{FRET} value of 653 R.F.U. at 1 μM had a high K_D value of 24.2 μM indicating that the IAV M1 protein does not have a high binding affinity for it. Conversely, the PIAS1 protein stayed consistent with the preliminary data and with a high Em_{FRET} value of 565 R.F.U. had a K_D value of 2.9 μM meaning the IAV M1 protein exhibits a high affinity for it. The results presented indicate that the PIAS1 protein may be a real E3 ligase for the IAV M1 protein and, once validated, can be used to develop a chemical inhibitor to block the host-viral interactions.

The results presented showed that the three different methods of FRET: spectral, Em_{FRET} and K_D all exhibited varying degrees of affinity. The two methods of spectrum and Em_{FRET} suggested an interaction, but true interaction is determined by the evaluation of K_D. The reason supporting the variance is due to the presence of the non-specific binding between proteins, which can be observed when high concentrations can force two proteins to interact spatially, rather than by its true interaction affinity. Crowding is a term that refers to the effect of non-specific interactions when the solute is larger than that of the solvent[5]. This is evident in hTRIM28 protein with a size of 145 kDa, compared to the IAV M1 protein with a size of 53 kDa, which is more than double its size. Qualitatively assessing FRET interaction is not sufficient to accurately assess the binding affinity between the two proteins, especially for large-sized proteins at high concentrations. Hence, measuring the 1μM CyPet hTRIM28 and 1 μM YPet IAV M1 protein led to inaccurate preliminary results, due to the non-specific binding occurring. Quantitative Förster Resonance Energy Transfer (qFRET) solves this problem as the FRET values determined for the true target protein interactions were taken from subtracting the interference of nonspecific interactions and

the non-target protein interactions. Additionally, by keeping the CyPet-hTRIM28 at a constant concentration of 0.1 μM and titrating the YPet IAV M1 protein from 0-25 μM it will lead to a saturation point where binding can no longer occur even with the addition of more YPet IAV M1. Therefore, even if there is a strong FRET determined, more accurate results derived from qFRET can show that the interactions are weaker than they were anticipated.

The study performed is highly clinical and is applicable for drug development. The application is to better understand how the viral protein exploits the SUMOylation pathway to replicate itself to better engineer antiviral therapeutics without drug resistance[6]. The better understanding of E3 ligase within the SUMOylation pathway can lend itself to better understanding how the IAV M1 protein depends on the host factor for its viral assembly and replication[7,8]. Until the development of qFRET it was difficult to understand the mechanism of E3 ligases and its role in helping to facilitate the transfer of the SUMO molecule from the E2- conjugating enzyme Ubc9 to the target substrate. The results presented proved the importance of qFRET with the discrepancy between the interaction between CyPet hTRIM28 and YPet IAV M1 compared to its true biochemical binding affinity. However, the in vitro results are difficult to predict whether it will match in in vivo studies, however qFRET is a powerful tool that can be used to test the other E3 ligases in either studies[10,11].

The qFRET technology used in this study is carried out in a solution without a high purity requirement, and this condition can mimic physiological and pathological conditions. Therefore, the qFRET-based measurements are closer to physiological events in living cells. In addition, the qFRET assay is very sensitive, and the concentrations of fluorescence-tagged proteins required in the qFRET assay can be as low as nM; therefore, a minimal amount of proteins is needed for the interaction affinity determinations. Furthermore, the qFRET assay is environmentally

friendly and does not contain any radioisotopes or chemicals. Applications of the qFRET technology should provide high-quality protein interaction and catalytic affinities of systems, networks, and proteomes and provide comprehensive quantitative biological and biomedical maps without the need for laborious protein purification, especially for those difficult-to-be-expressed proteins, such as SUMOylation E3 ligase in this study. The genome-wide search for SUMOylation E3 ligase for influenza virus M1 protein is not only important for research but also for novel anti-virus therapeutics development in the future.

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