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Quantitative Förster resonance energy transfer analysis for kinetic determinations of SUMO-specific protease

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ABSTRACT

Förster resonance energy transfer (FRET) technology has been widely used in biological and biomedical research, and it is a very powerful tool for elucidating protein interactions in either dynamic or steady state. SUMOylation (the process of SUMO [small ubiquitin-like modifier] conjugation to substrates) is an important posttranslational protein modification with critical roles in multiple biological processes. Conjugating SUMO to substrates requires an enzymatic cascade. Sentrin/SUMO-specific proteases (SENPs) act as an endopeptidase to process the pre-SUMO or as an isopeptidase to deconjugate SUMO from its substrate. To fully understand the roles of SENPs in the SUMOylation cycle, it is critical to understand their kinetics. Here, we report a novel development of a quantitative FRET-based protease assay for SENP1 kinetic parameter determination. The assay is based on the quantitative analysis of the FRET signal from the total fluorescent signal at acceptor emission wavelength, which consists of three components: donor (CyPet–SUMO1) emission, acceptor (YPet) emission, and FRET signal during the digestion process. Subsequently, we developed novel theoretical and experimental procedures to determine the kinetic parameters, k_{cat}, K_M, and catalytic efficiency (k_{cat}/K_M) of catalytic domain SENP1 toward pre-SUMO1. Importantly, the general principles of this quantitative FRET-based protease kinetic determination can be applied to other proteases.

Reversible posttranslational modifications of proteins with ubiquitin or ubiquitin-like proteins (Ubls)[1] are one of general mechanisms to regulate protein activity and have diverse roles in many important biological events. SUMO (small ubiquitin-like modifier) covalently modifies proteins with important roles in diverse cellular processes, including regulation of cell cycle, cell survival and death, DNA damage response, and stress response [1–5]. Like ubiquitination, SUMOylation (the process of SUMO conjugation to substrates) occurs through a multienzyme-catalyzed cascade, involving E1, E2, and E3 ligases, after the SUMO is processed into mature form from its precursor, pre-SUMO, by its Sentrin/SUMO-specific proteases (SENPs). Conjugated SUMO is then removed from the conjugated substrate by SENPs to refresh the SUMOylation cycle [1,3,6,7]. Seven SENPs have been identified in the human genome: SENP1–3 and 5–8 (SENP8 is not a SUMO protease but functions on another small Ubl known as Nedd8).

Enzyme kinetic parameters, such as k_{cat}, K_M, and their ratio, are most general terms used to compare the efficiencies of enzymes or the enzyme specificities of different substrates for a particular enzyme. The catalytic efficiency or specificity of an enzyme is best characterized by the ratio of the kinetic constants, k_{cat}/K_M. Several methods are commonly used to determine k_{cat}/K_M, such as the enzymatic digestion in solution, followed by the polyacrylamide gel-based Western blot method, radioactive-labeled substrate, dialysis of digested substrate, fluorescent compound-labeled peptide substrate, and fluorescent protein-labeled substrate.

The kinetic parameters of SUMO–SENP pairs have been determined in several studies [8–11]. The pre-SUMOs digested by SENP2 in solution followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis were used to determine the kinetic parameters of SENP2 for different pre-SUMO1, -2, and -3, resulting in k_{cat}/K_M values of 2.6×104 to $3.8 \times 105\ M^{-1}\ s^{-1}$ [8]. An organic fluorophore, ACC (7-amino-4-carbamoylmethylcoumarin), was used to label tetrapeptide substrate, and the fluorescence signal of ACC as a leaving group was monitored after cleavage by SENPs. The range of the k_{cat}/K_M values from this assay was 34–203 $M^{-1}\ s^{-1}$, several orders of magnitude lower

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[1] *Abbreviations used:* Ubl, ubiquitin-like protein; SUMO, small ubiquitin-like modifier; SENP, sentrin/SUMO-specific protease; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ACC, 7-amino-4-carbamoylmethylcoumarin; AMC, 7-amino-4-methylcoumarin; FRET, Förster resonance energy transfer; DUB, deubiquitinating enzyme; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; DTT, dithiothreitol; HPLC, high-performance liquid chromatography.

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than the natural substrates [7,9,12]. To determine the kinetic parameters of different SENP paralogs, another organic fluorophore, AMC (7-amino-4-methylcoumarin), was tagged onto mature SUMOs. The k_{cat}/K_M from this assay is $2.4 \times 106\ M^{-1}\ s^{-1}$. Because there is no specific sequence of either SUMO tail or SUMO-specific substrate after the AMC, this system cannot clearly differentiate the iso- and endopeptidase activities of SENPs [13]. Recently, Förster resonance energy transfer (FRET)-based protease assays were used to study the deubiquitinating enzymes (DUBs) or SENPs. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were used as the FRET pair to study endopeptidase activities of SENP1 and SENP2 toward pre-SUMO1, -2, and -3 [10,11,14,15]. One of these studies revealed the k_{cat}/K_M of SENP1 toward SUMO1 as $3.8 \times 107\ M^{-1}\ s^{-1}$. However, in this study the ratio of acceptor emission versus donor emission was used as the quantitative parameter for FRET signal monitor for protease activity determination. The ratio of acceptor emission versus donor emission in FRET assay is not an accurate measurement for FRET analysis because there are signal cross-contaminations at both acceptor and donor emission wavelengths by acceptor and donor self-fluorescence; therefore, this analysis is not an accurate FRET signal analysis [16,17]. The resulting FRET ratiometric signal analysis does not directly correlate with the amount of digested substrate; therefore, the kinetic parameters determined are not accurate. In addition, the low FRET efficiencies of these fluorescent proteins and the complexity of fluorescence emissions of the donor and acceptor limit assay reliability and sensitivity. Recently, emerging studies have focused on efforts to develop quantitative FRET techniques with differentiations of donor, acceptor, and net FRET signal contributions in order to estimate the free and bound complex of protein interaction for steady-state parameter determination [18,19]. In these approaches, the fluorescence contributions of free donor, free acceptor, and net FRET were obtained through either calibration [18] or direct estimation by direct coefficiency measurements [19].

Here, we report a novel development of a highly sensitive and quantitative FRET-based protease assay for the determination of the kinetic parameters of pre-SUMO1 maturation by SENP1. An engineered FRET pair, CyPet and YPet (derived from CFP and YFP), with significantly improved FRET efficiency and fluorescence quantum yield, were used to generate the CyPet–(pre-SUMO1)–YPet substrate [20]. In contrast to traditional ratiometric analysis of FRET signal, we differentiated and quantified absolute fluorescence signals contributed by the donor and acceptor and FRET at the acceptor and emission wavelengths, respectively. The absolute fluorescent signals enabled us to convert them into protein concentrations by preestablished fluorescent protein standard curves. The K_M, k_{cat}, and k_{cat}/K_M of SENP1 toward pre-SUMO1 were determined by this approach, and the value of k_{cat}/K_M, $(2.49 \pm 0.37) \times 107\ M^{-1}\ s^{-1}$, is in agreement with general enzymatic kinetic parameters. Therefore, this methodology is valid and can be used as a general approach to characterize other proteases as well.

Materials and methods

Plasmid constructs

The open reading frames of the genes were amplified by PCR, and the PCR products were cloned into PCRII–TOPO vector (Invitrogen). After confirming the constructs by sequencing, the complementary DNAs (cDNAs) encoding CyPet–(pre-SUMO1)–YPet, CyPet–SUMO1, YPet, and the catalytic domains of SENP1 and SENP7 were cloned into the pET28(b) vector (Novagen) with an N-terminal polyhistidine tag.

Protein expression and purification

Escherichia coli cells of strain BL21(DE3) were transformed with pET28 vectors encoding CyPet–(pre-SUMO1)–YPet, CyPet–SUMO1, YPet, and the catalytic domains of SENP1 and SENP7. The transformed bacteria were grown in 2 × YT medium to an optical density at 600 nm of 0.4–0.5 by induction with 100 μM isopropyl β-D-thiogalactoside (IPTG) for 16 h at 25 °C. The polyhistidine-tagged recombinant proteins were purified from bacterial lysates with nickel agarose affinity chromatography (Qiagen) and eluted in 20 mM Tris–HCl (pH 7.4), 50 mM NaCl, and 2 mM dithiothreitol (DTT). Recombinant proteins CyPet–SUMO1 and YPet were further purified by gel filtration high-performance liquid chromatography (HPLC) with a Superdex75 10/300 GL column on an HPLC purification system (GE Healthcare, ÄKTA purifier). Protein purity was examined by SDS–PAGE, and concentrations of the purified proteins were determined by Bradford assay (Thermo Scientific).

Protease kinetic assay and Western blot

FRET-based SUMO processing assays were conducted by measuring the emission intensity of CyPet at 475 nm and of YPet at 530 nm with an excitation wavelength of 414 nm in a fluorescence multiwell plate reader (Molecular Devices, FlexStation II 384).

CyPet–(pre-SUMO1)–YPet was incubated with the recombinant catalytic domain of SENP1 at 37 °C in buffer containing 25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20, and 2 mM DTT to a total volume of 30 μl and added to each well of a 384-well plate (Greiner, glass bottom). The final concentration of CyPet–(pre-SUMO1)–YPet was fixed at 300 nM, and the final concentration of SENP1 was varied as 12, 2.5, 1.2, 0.3, 0.15, and 0.12 nM. Reactions were stopped at 4 min and analyzed by fluorimeter and the Western blot.

For initial velocity determination of CyPet–(pre-SUMO1)–YPet digested by SENP1, substrate with different concentrations was digested with 0.8 nM SENP1. Reactions were tested within the original 5 min. A one-phase association model was used to fit the exponential increased reaction velocity. Five samples were repeated in each concentration.

Specificity test

CyPet–(pre-SUMO1)–YPet was incubated at 37 °C in buffer containing 25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20, and 2 mM DTT and injected into a 384-well plate (Greiner, glass bottom) with the recombinant catalytic domain of either SENP1 or SENP7. The final concentration of CyPet–(pre-SUMO1)–YPet was fixed at 300 nM, and the final concentration of SENP1 and SENP7 was either 300 or 3 nM. Reactions were stopped at 1 h and were analyzed by fluorimeter and Western blot.

Standard curve for digested substrate

The recombinant protein CyPet–(pre-SUMO1)–YPet was incubated at 37 °C in buffer containing 25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20, and 2 mM DTT to a total volume of 100 μl and added to each well of a 96-well plate (Greiner, glass bottom). The emission signals at 475 nm were collected after excitation at 414 nm, and the amount of protein was varied from 0.5 to 7 μg.

For the standard curve, the recombinant proteins CyPet–SUMO1 and YPet were incubated at 37 °C in buffer containing 25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20, and 2 mM DTT to a total volume of 100 μl with a 1:1 M ratio and added to each well of a 96-well plate (Greiner, glass bottom). The emission signals at

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475 nm were collected after excitation at 414 nm, and the amount of CyPet–SUMO1 was varied from 1 to 9 μg.

Spectrum analysis (quantitative FRET analysis)

M is the total amount of CyPet–SUMO1–YPet (μg) in 80 μl, and x is the amount of digested CyPet–SUMO1–YPet in 80 μl.

Undigested CyPet–SUMO1–YPet:

$$\frac{M-x}{M}\times I_{da}=\frac{M-x}{M}\times\left(FL_{530/414}-\alpha I_d-\beta I_a\right),$$

where I_d is CyPet fluorescence at 475 nm under excitation of 414 nm, I_{da} is FRET-induced YPet emission at 530 nm under excitation of 414 nm, and I_a is direct YPet emission at 530 nm under excitation of 475 nm. YPet contribution $\beta I_a = y = kM$.

CyPet contribution $\alpha I_d = \alpha(I_{d1} + I_{d2})$

I_{d1} : undigested CyPet—SUMO1—YPet $I_{d1} = y = k(M - x)$

$$I_{d2}\text{ : digested CyPet—SUMO1 }I_{d2}=Z=j\times\frac{40.76}{68.16}x,$$

where I_{d1} (= y in standard curve) is emission of CyPet–(pre-SUMO1)–YPet at 475 nm under excitation of 414 nm, k is slope of the standard curve for I_{d1} to amount of CyPet–(pre-SUMO1)–YPet (μg), I_{d2} (= z in standard curve) is emission of CyPet–SUMO1 at 475 nm under excitation of 414 nm, and j is slope of the standard curve for I_{d2} to amount of CyPet–SUMO1 (μg).

Remaining FRET:

$$\begin{aligned}FL'_{530/414}&=\text{Remaining FRET}_{530/414}+\text{CyPet}_{530/414}+\text{YPet}_{530/414}\\&=\frac{M-x}{M}\times\left(FL_{530/414}-\alpha I_d-\beta I_a\right)+I_a\\&\quad+k(M-x)+j\times\frac{40.76}{68.16}x.\end{aligned}$$

Spectrum analysis (ratiometric FRET analysis)

M is the total amount of CyPet–(pre-SUMO1)–YPet (μg) in 80 μl, and x is the amount of digested CyPet–(pre-SUMO1)–YPet in 80 μl.

The ratio of fluorescent emission at 530–475 nm under excitation at 414 nm (r) was obtained from CyPet–(pre-SUMO1)–YPet and CyPet–SUMO1 with YPet (1:1 M ratio). In that way, the $FL_{530/475}$ ratio is directly proportional to the SUMO substrate concentration:

$$\frac{M-x}{M}=\frac{FL'_{530/414}/FL'_{475/414}}{r_{(\text{CyPet}-\text{preSUM01}-\text{YPet})}-r_{(\text{CyPet}-\text{SUM01}+\text{YPet})}},$$

where $FL'_{530/414}$ and $FL'_{475/414}$ are detected fluorescent emission at 530 and 475 nm under excitation of 414 nm at different time points, r(CyPet–pre-SUMO1–YPet) is the ratio of recombinant protein CyPet–(pre-SUMO1)–YPet's emission at 530 nm to emission at 475 nm under excitation of 414 nm, r(CyPet–SUMO1 + YPet) is the ratio of recombinant protein CyPet–SUMO1 and YPet's emission (1:1 M ratio) at 530 nm to emission at 475 nm under excitation of 414 nm.

k_{cat} and K_M determination by FRET:

CyPet–(pre-SUMO1)–YPet was incubated with recombinant catalytic domain of SENP1 at 37 °C in buffer containing 25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20, and 2 mM DTT to a total volume of 80 μl and added to each well of a 384-well plate (Greiner, glass bottom). The final concentration of SENP1 was fixed at 0.8 nM, and the final concentration of CyPet–(pre-SUMO1)–YPet was varied as 115.3, 241.2, 406.9, 594.2, and 725.3 nM and as 1.4713, 1.8991, and 2.2998 μM. Runs were conducted by measuring the emission intensity of YPet at 530 nm and of CyPet at 475 nm with an excitation wavelength of 414 nm in a fluorescence multiwell plate reader (Molecular Devices, FlexStation II 384) at the original 5 min with 10-s intervals. Data were analyzed by the developed method and plotted in GraphPad Prism V software fitting the Michaelis–Menten equation.

Results

Designing a highly sensitive FRET-based assay for SENP1 endopeptidase activity

The general strategy for the FRET-based protease assay was based on fluorescent protein-tagged substrate (Fig. 1). The SENP1 substrate, pre-SUMO1, was flanked by a FRET pair, CyPet and YPet. This pair has more than 20-folds of improved energy transfer efficiency achieved by engineering CFP and YFP, respectively, to yield a high dynamic range and sensitivity for the FRET assay [20]. When the fusion protein CyPet–(pre-SUMO1)–YPet is mixed with SENP1, it is cleaved by the protease, resulting in two products: the CyPet–SUMO1 and the SUMO tail with YPet. Therefore, the FRET will be disrupted, resulting in an increase of CyPet's emission and a dramatic decrease of YPet's emission at the CyPet excitation wavelength. The decreased fluorescent emission of acceptor YPet after the cleavage can be used to characterize kinetic properties of SENP1 in real time.

The fusion substrate, CyPet–(pre-SUMO1)–YPet, and catalytic domain of SENP1 were cloned into the bacterial protein expression vector, pET28(b). The recombinant proteins were expressed and purified on an affinity column of Ni beads (Fig. 1B). To test the sensitivity and dynamics of this FRET assay, we incubated the CyPet–(pre-SUMO1)–YPet with the catalytic domain of SENP1 (1:1 M ratio). A significant signal change was observed from the CyPet–(pre-SUMO1)–YPet substrate after digestion (Fig. 1C, left). After incubation with SENP1, the emission ratio (E_{530}/E_{475} when excited at 441 nm) of the CyPet–YPet pair shows more than six-folds of signal changes (4.23–0.63). In contrast, the control fusion protein, CFP–(pre-SUMO1)–YFP, shows only more than twofolds of signal changes (1.10–0.49) (data not shown). Because the two constructs used in this study, CFP–(pre-SUMO1)–YFP and CyPet–(pre-SUMO1)–YPet, have the same length and similar structures, this result suggests that the CyPet–YPet pair has a higher energy transfer efficiency and provides substantially more sensitivity than the CFP–YFP pair.

We hypothesized that this improved sensitivity would allow us to be able to determine reliable kinetic parameters of SENP1. To explore this possibility, the recombinant protein SENP1 was mixed with different molar ratios of CyPet–(pre-SUMO1)–YPet, and SUMO processing was examined after 4 min. The processed SUMO substrates correlated very well with the ratios of protease (see Supplement Fig. 1A, left, in supplementary material), and the results were confirmed by Western blots (Supplement Fig. 1A, right). These results suggest that the highly sensitive FRET-based protease assay can be used to monitor the pre-SUMOs' processing in real time as well as possibly to measure the initial rates if we can convert FRET signal to digested substrate concentration.

SENPs belong to the family of cysteine proteases. The SENP protease family contains seven members in the human genome with different specificities for SUMO substrates [7]. Previous studies suggested that SENP1 catalytic residue mutant (C603) increases the level of SUMO1's conjugation [21]. SENP6 and SENP7 have no endopeptidase activity on pre-SUMOs [9,12]. The digestion specificities of pre-SUMO1 by SENP1 and SENP7 were tested in this FRET-based protease assay. Pre-SUMO1 was specifically processed

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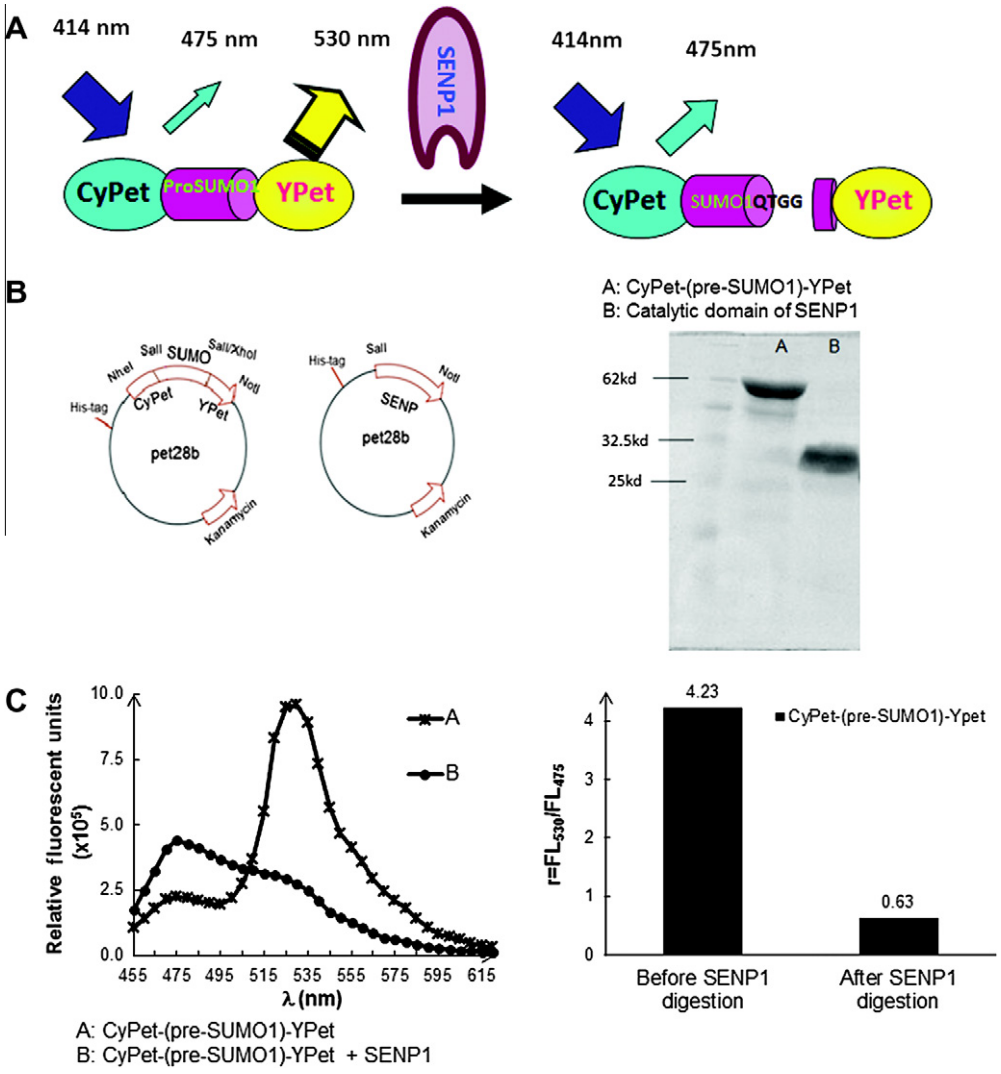

Fig.1. Design of FRET-based protease assay and demonstration of CyPet–YPet advantages. (A) Schematic graph of FRET-based assay for SENP cleavage. (B) Cloning diagrams of CyPet–(pre-SUMO1)–YPet and SENP1 catalytic domain in protein expression vector pET28(b) (left) and coomassie staining of purified CyPet–(pre-SUMO1)–YPet and SENP1 catalytic domain in polyacrylamide gel (right). (C) Emission spectrum of CyPet–(pre-SUMO1)–YPet (300 nM) with incubation of catalytic domain of SENP1 (300 nM) for 1 h under excitation at 414 nm (left) and ratiometric measurement ($r = FL_{530}/FL_{475}$) of CyPet–YPet pair under excitation at 414 nm (right).

by the SENP1 in either a 1:1 or 1:100 ratio of enzyme/substrate mixture after a 1-h incubation, whereas the SENP7 showed almost no processing activity toward pre-SUMO1 (Supplement Fig. 1B, left). These results were confirmed by Western blotting (Supplement Fig. 1B, right), indicating that this assay can be applied to study the substrate specificities of different SENP family members.

Fluorescence emission spectrum analysis and standard curves for donor and acceptor direct emissions and FRET signals

To determine the parameters of SENP1 kinetics by FRET assay, two issues must be addressed: how to determine the absolute FRET signal that is corresponding to digested substrate concentration and how to convert the absolute FRET signal into a protein concentration. For the first issue, we must distinguish the fluorescence signal of FRET from the direct fluorescence signals of donor and acceptor at the emission wavelength (Fig. 2A). The absolute FRET signal will determine the amount of undigested substrate, excluding interference of donor and acceptor direct emissions from both digested and undigested substrates. For the second issue, standard curves are needed to convert FRET signal to concentrations of corresponding proteins.

Before digestion, under excitation at 414 nm, the total fluorescence emission of CyPet–(pre-SUMO1)–YPet at 530 nm = the FRET emission + YPet direct emission + CyPet direct emission or (Fig. 2A):

$$FL_{530/414} = FL_{\mathrm{FRET}} + FL_{\mathrm{CyPet(cont)}} + FL_{\mathrm{YPet(cont)}}, \quad (1)$$

where $FL_{530/414}$ is total fluorescence emission at 530 nm when excited at 414 nm, FL_{FRET} is absolute FRET signal, $FL_{\mathrm{CyPet(cont)}}$ is CyPet direct emission when excited at 414 nm, and $FL_{\mathrm{YPet(cont)}}$ is YPet direct emission when excited at 414 nm.

The direct emission of CyPet at 530 nm was proportional to its emission at 475 nm when excited at 414 nm ($FL_{\mathrm{CyPet(475/414)}}$) with a constant ratio of α. The direct emission of YPet at 530 nm is proportional to its emission at 530 nm when excited at 475 nm ($FL_{\mathrm{YPet(530/475)}}$) with a constant ratio of β:

$$FL_{530/414} = FL_{\mathrm{FRET}} + \alpha FL_{\mathrm{CyPet(475/414)}} + \beta FL_{\mathrm{YPet(530/475)}}, \quad (2)$$

where $FL_{\mathrm{CyPet(475/414)}}$ is CyPet emission at 475 nm when excited at 414 nm and $FL_{\mathrm{YPet(530/475)}}$ is YPet emission when excited at 475 nm. From the spectrum analysis of CyPet alone and YPet alone at excitation of 414 and 475 nm, respectively, we determined that α was 0.378 and β was 0.026 (data not shown).

After the digestion by SENP1, CyPet–(pre-SUMO1)–YPet was cleaved to release CyPet–SUMO1 and the SUMO1 tail with tagged

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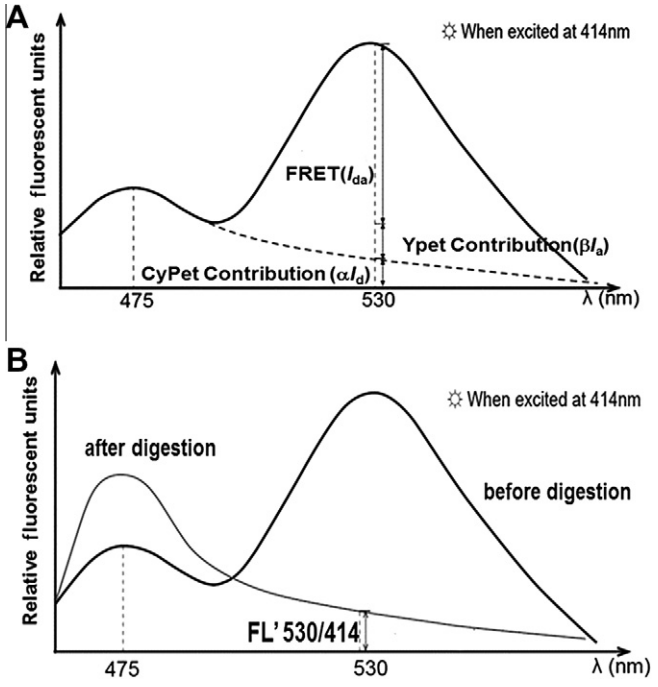

Fig.2. Fluorescence signal analysis of the contributions by acceptor, donor, and FRET signal. (A) Dissection of emission spectra from engineered protein CyPet–(pre-SUMO1)–YPet under excitation at 414 nm. I_d is CyPet fluorescence at 475 nm under excitation of 414 nm, I_{da} is FRET-induced YPet emission at 530 nm under excitation of 414 nm, and I_a is direct YPet emission at 530 nm under excitation of 475 nm. $FL_{530/414} = I_{da} + \alpha I_d + \beta I_a$, where $\alpha = 0.332$ and $\beta = 0.026$. (B) Diagram of fluorescence emission spectrum of substrate CyPet–(pre-SUMO1)–YPet before and after digestion by SENP1 under excitation at 414 nm.

YPet. The fluorescent signal at 530 nm was decreased and fluorescent signal at 475 nm was increased when excited at 414 nm (Fig. 2B). Therefore, the remaining total fluorescent emission at 530 nm = remaining FRET emission + CyPet direct emission from digested CyPet–SUMO1 + CyPet direct emission from remaining CyPet–(pre-SUMO1)–YPet + YPet direct emission or:

$$FL'_{530/414} = FL'_{\mathrm{FRET}} + FL'_{\mathrm{CyPet(530/414)\text{-}cs}} + FL'_{\mathrm{CyPet(530/414)\text{-}csy}} + FL_{\mathrm{YPet(cont)}} \quad (3)$$

or

$$FL'_{530/414} = FL'_{\mathrm{FRET}} + \alpha\left[FL'_{\mathrm{CyPet(475/414)\text{-}cs}} + FL'_{\mathrm{CyPet(475/414)\text{-}csy}}\right] + \beta\left(FL_{\mathrm{YPet(530/475)}}\right), \quad (4)$$

where $FL'_{530/414}$ is total fluorescence emission at 530 nm after digestion, FL'_{FRET} is remaining FRET signal, $FL'_{\mathrm{CyPet(530/414)\text{-}cs}}$ is direct emission of free CyPet–SUMO1, $FL'_{\mathrm{CyPet(530/414)\text{-}csy}}$ is direct emission of remaining fusion protein CyPet–(pre-SUMO1)–YPet, $FL'_{\mathrm{CyPet(475/414)\text{-}cs}}$ is emission of free CyPet–SUMO1 at 475 nm when excited at 475 nm, and $FL'_{\mathrm{CyPet(475/414)\text{-}csy}}$ is emission of CyPet in remaining fusion protein CyPet–(pre-SUMO1)–YPet.

Standard curves were created by plotting the fluorescent emission signals against amount of protein. For undigested CyPet–(pre-SUMO1)–YPet, the fluorescence emissions of various concentrations at 475 nm under excitation at 414 nm were determined and the fluorescent signal was plotted with protein amounts (Fig. 3A). For the digested CyPet–SUMO1, different amounts of CyPet–SUMO1 were mixed with YPet with a molar ratio of 1:1 and the emissions at 475 nm with excitation at 414 nm were determined, and the fluorescent signals were plotted against the protein amounts. Slopes of $k = 18877$ and $j = 50876$, respectively, describe the linear relationship between the detected fluorescent signals and the protein amounts in these two standard curves.

After treatment with SENP1, the remaining FRET emission (FL'_{FRET}) is:

$$FL'_{\mathrm{FRET}} = \frac{M-x}{M}FL_{\mathrm{FRET}} = \frac{M-x}{M\left(FL_{\frac{530}{414}} - \alpha FL_{\mathrm{CyPet}\left(\frac{475}{414}\right)} - \beta FL_{\mathrm{YPet}\left(\frac{530}{475}\right)}\right)}, \quad (5)$$

where M is used to present the total amount of CyPet–(pre-SUMO1)–YPet (μg) in 100 μl and x is the amount of digested CyPet–(pre-SUMO1)–YPet (μg) in 100 μl.

The YPet direct emission did not change whether the fusion protein was digested or not; therefore, the fractions $\beta FL_{\mathrm{YPet(530/475)}}$ remained the same.

The CyPet direct emission was divided into two parts: one from the digested CyPet–SUMO1 and one from the undigested CyPet–(pre-SUMO1)–YPet. After treatment with SENP1, the CyPet direct emission from undigested CyPet–(pre-SUMO1)–YPet ($FL'_{\mathrm{CyPet(475/414)\text{-}csy}}$) was:

$$\alpha FL'_{\mathrm{CyPet}\left(\frac{475}{414}\right)\text{-}\mathrm{csy}} = \alpha k(M-x) \quad (6)$$

and the CyPet direct emission from digested CyPet–SUMO1 ($FL'_{\mathrm{CyPet(475/414)\text{-}cs}}$) was:

$$\alpha FL'_{\mathrm{CyPet}\left(\frac{475}{414}\right)\text{-}\mathrm{cs}} = \alpha j\frac{38}{65}x \quad (7)$$

Here, 38/65 gives the molecular mass ratio of CyPet–SUMO1 to CyPet–(pre-SUMO1)–YPet.

Take all of the fractions together, the detected fluorescent signals at 530 nm under excitation of 414 nm when CyPet–(pre-SUMO1)–YPet was treated with SENP1:

$$FL'_{\frac{530}{414}} = \frac{M-x}{M}\left(FL_{\frac{530}{414}} - \alpha FL_{\mathrm{CyPet}\left(\frac{475}{414}\right)} - \beta FL_{\mathrm{YPet}\left(\frac{530}{475}\right)}\right) + \alpha\left[k(M-x) + \frac{38}{65}jx\right] + \beta FL_{\mathrm{YPet}\left(\frac{530}{475}\right)}. \quad (8)$$

During the experiments, we first determined the CyPet and YPet direct emissions and total emissions at 530 nm. The emission of the recombinant protein CyPet–(pre-SUMO1)–YPet was measured at 475 nm when excited at 414 nm to determine the CyPet direct emission $\left(\alpha FL_{\mathrm{CyPet}\left(\frac{475}{414}\right)}\right)$, the emission was measured at 530 nm when excited at 475 nm to determine the YPet direct emission ($\beta FL_{\downarrow}\mathrm{YPet}(530/475)$), and the emission was measured at 530 nm when excited at 414 nm to determine the total emission $\left(FL_{\frac{530}{414}}\right)$. After treatment of SENP1, the total emission ($FL_{\downarrow}(530/414)^{\uparrow}$) was obtained at 530 nm when excited at 414 nm (Eq. (8)). We then obtain the digested concentrations of recombinant protein CyPet–(pre-SUMO1)–YPet (x) from the parameters determined above.

To compare with the other most used ratiometric analysis method of FRET signal, we also determined the ratios of FL_{530}/FL_{475} during the digestion process [11].

Determining the initial velocity of CyPet–(pre-SUMO1)–YPet cleavage by SENP1

The pre-SUMOs' maturation by SENP1 can be determined by monitoring the changes of fluorescence signal at 530 nm during the digestion. Changes of absolute FRET signal and other fluorescence components can be analyzed according to Eq. (8) with calculations from standard curves (Fig. 3). Different amounts of the fluorescent substrate, ranging from 0.115 to 2.300 μM, were cincubated with 0.8 nM catalytic domain of SENP1. The remaining fluorescence intensity $\left(FL'_{\frac{530}{414}}\right)$ was monitored and then digested substrate, x, was calculated according to Eq. (8). The digested

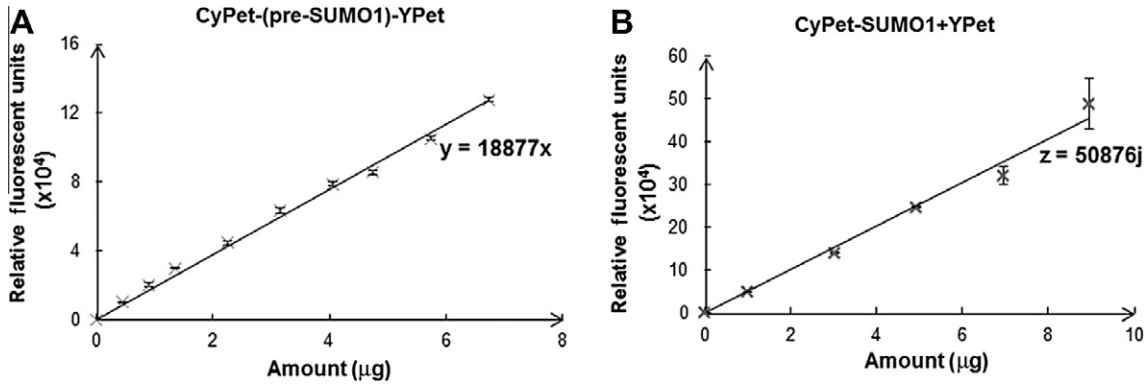

Fig.3. Standard curves of fluorescent signal related to protein amount. (A) Emission of CyPet–(pre-SUMO1)–YPet at 475 nm under excitation at 414 nm. (B) Emission of CyPet–SUMO1 + YPet (1:1 M ratio) under excitation at 414 nm (the *x* axis is the protein amount of CyPet–SUMO1).

substrate concentration showed very good dose-dependent digestion with the amount of substrate (Fig. 4). This dose-dependent cleavage of fluorescent substrate suggests that the catalytic domain of SENP1 shows excellent activities even at a 1:5000 ratio of enzyme/substrate.

To determine the reaction velocity of SENP1, the reaction rate (v) is correlated with the change in the amount of substrate (S):

$$V = -\frac{d[S]}{dt} = \frac{d[P]}{dt}. \tag{9}$$

As the digested substrate (or product) concentration increases exponentially from 0, when $t = 0$, to $[S]0$ at infinite time,

$$[P] = [S]_0(1 - e^{-kt}). \tag{10}$$

Accordingly, the original velocity (V_0) of CyPet–(pre-SUMO1)–YPet's maturation by SENP1 is:

$$V_0 = \frac{d[P]}{dt}_{t=0} = k[S]_0. \tag{11}$$

The original velocities were calculated by Eq. (11) under different substrate concentrations (Table 1). The initial reaction velocity displayed a good substrate dose-dependent relationship.

Michaelis–Menten analysis and k_{cat}/K_M determination

The catalytic specificity and efficiency of an enzyme for a specific substrate is best defined by the ratio of the kinetic constant, k_{cat}/K_M. This ratio is generally used to compare the efficiencies of different enzymes with one substrate or the use of different substrates by a particular enzyme. The K_M and V_{max} values can be obtained from the Michaelis–Menten equation by plotting the various velocities of SENP1 digestion versus the corresponding different concentrations of fluorescence substrate. We plotted the Michaelis–Menten graph for the data in Table 1 (Fig. 5). The V_{max} from the quantitative FRET analysis was determined to be 0.0058 ± 0.0002 μM s^{-1}, whereas the V_{max} from ratiometric analysis was determined to be 0.0045 ± 0.0002 μM s^{-1}. The k_{cat} was obtained from:

$$K_{cat} = \frac{V_{max}}{[E]}. \tag{12}$$

The k_{cat} from the quantitative FRET analysis was determined to be 7.27 ± 0.29 s^{-1} and the derived K_M was 0.29 ± 0.042 μM, whereas the k_{cat} from the ratiometric analysis was determined to be 5.57 ± 0.30 s^{-1} and the derived K_M was 0.067 ± 0.026 μM (Table 2). Then, the k_{cat}/K_M from the quantitative FRET analysis was $(2.49 \pm 0.37) \times 107$ M^{-1} s^{-1}, which is close to that in one of the previous SENP1 endopeptidase function studies, but the latter's K_M was nearly one log lower than the one we determined [11]. Then, the k_{cat}/K_M from the ratiometric analysis was $(8.31 \pm 3.27) \times 107$ M^{-1} s^{-1}. These results from the quantitative FRET analysis are similar to those obtained from the ratiometric method, but the rationale for the signal analysis is quite different. These results suggest that the quantitative FRET analysis is able to generate precise kinetic data.

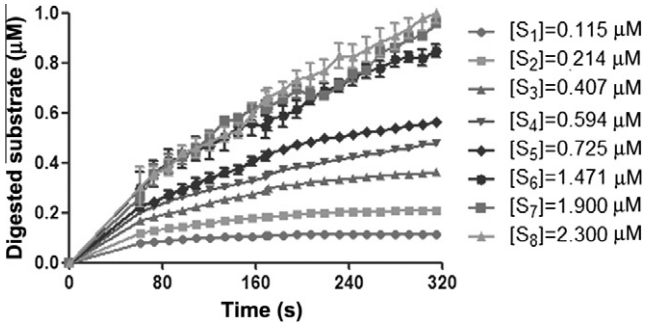

Fig.4. Quantitative analysis of CyPet–(pre-SUMO1)–YPet digested by different ratio of SENP1. Reactions were monitored within original 5 min.

Discussion

Here, we have reported the development of a highly sensitive and quantitative FRET-based protease assay for determination of the kinetic parameters of SENP1 in pre-SUMO1's maturation. We showed a robust approach for k_{cat}/K_M measurements in solution with a FRET-based protease assay in the steady state. In contrast to the previous ratiometric approach, we fundamentally improved the approach in both a new theory of FRET signal and kinetic analysis and an experimental procedure to derive kinetic parameters by deriving the quantitative contributions of absolute fluorescence signals from donor, acceptor, and real FRET at the acceptor's emission wavelength. This quantitative FRET analysis can differentiate the quantitative contributions of each component, whereas traditional ratiometric measurement of FRET cannot. The k_{cat}/K_M value from our quantitative FRET analysis study, $(2.49 \pm 0.37) \times 107$ M^{-1} s^{-1}, is more convergent and close to that from the previous ratiometric analysis (3.81×107 M^{-1} s^{-1}) using the CFP/YFP pair, but the individual measurements of K_M and k_{cat} are three to seven times larger than their apparent K_M and k_{cat}. The ratiometric method in our lab produced a slightly higher k_{cat}/K_M, $(8.31 \pm 3.27) \times 107$ M^{-1} s^{-1}, but with much higher variations.

The small numeric differences between these two approaches reflect a fundamental difference of the FRET data process. The discrep-

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Table 1
Initial velocity determined by quantitative FRET analysis and ratiometric methods.

[CyPet–SUMO1–YPet] (μM)	Quantitative FRET analysis		Ratiometric FRET analysis	
	V_0 ($\times 10^{-3}$ μM/s)	SD (V_0)	V_0 ($\times 10^{-3}$ μM/s)	SD (V_0)
0.115	1.96	0.03	2.66	0.04
0.214	2.54	0.03	3.36	0.04
0.407	3.20	0.06	3.84	0.07
0.594	3.58	0.09	3.93	0.07
0.725	4.12	0.11	4.82	0.10
1.471	5.15	0.38	4.58	0.17
1.900	5.18	0.31	4.09	0.24
2.300	5.00	0.41	3.70	0.56

Note: SD, standard deviation.

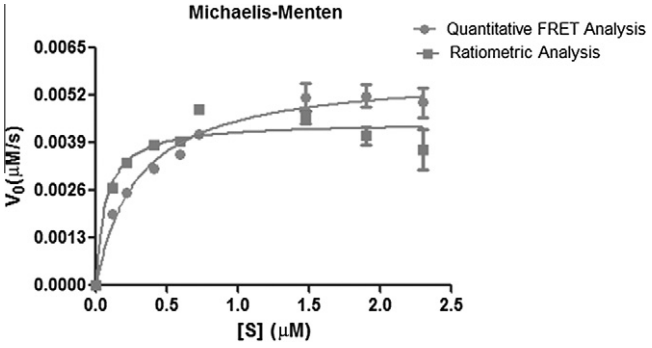

Fig.5. Michaelis–Menten graphical analysis of CyPet–(pre-SUMO1)–YPet's digestion by SENP1. Data were plotted and analyzed by GraphPad Prism 5 and nonliner regression.

Table 2
Kinetic parameters of SENP1 determined by quantitative FRET analysis and ratiometric FRET analysis.

Methods	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Quantitative FRET analysis	0.29 ± 0.042	7.27 ± 0.29	(2.49 ± 0.37) × 10^7
Ratiometric FRET analysis	0.067 ± 0.026	5.57 ± 0.30	(8.31 ± 3.27) × 10^7

ancy between these two approaches might be due to the inclusion of direct emission of donor and acceptor in the ratiometric analysis method. Based on our method, the donor's direct emission at the acceptor's emission peak wavelength (530 nm) is proportional to the donor's emission at its own emission peak wavelength (475 nm). Because the fluorescent emission of the donor is quantitatively related to the protein concentrations, and this relationship is different from the undigested CyPet–(pre-SUMO1)–YPet and digested CyPet–SUMO1, the donor's direct emission at 530 nm needs to be divided into two parts: the digested substrate and the remaining substrate, both of which are changed during the pre-SUMO's digestion process. The decreased fluorescent signal at 530 nm is correlated with the disrupted energy transfer from the donor to the acceptor and is also affected by the changes of the donor's direct emission as the two donor populations change during the digestion process. Traditional ratiometric measurements of FRET do not consider the direct emissions and simply convert all of the signal change to disrupted energy transfer, which may result in an overestimation of kinetic parameters from the Michaelis–Menten equation because of an overestimation of FRET emission signal (containing donor and acceptor direct emission) and an overestimation of FRET donor emission (increasing with digested substrate). The overestimations of FRET signal might not greatly affect the final k_{cat}/K_M kinetic ratio, but the effect is more obvious when studying the individual parameters, K_M and k_{cat}, which are important in determining the rate-limiting step and inhibitor potency of enzymes.

The catalytic efficiency of pre-SUMO1's maturation by SENP1 was considered as the highest among all of the SUMO–SENP digestions, both pre-SUMO processing and SUMO–substrate deconjugating [7]. The k_{cat}/K_M measurement in our study is approximately 107 M^{-1} s^{-1}, which is close to the diffusion limit (~108–109 M^{-1} s^{-1}), and the SENP2 digestion of pre-SUMO1 was much slower than the digestion of SENP1 (data not shown) and, thus, partially proves this point. Although we presented only two SENP paralogs here, comparing pre-SUMO1's maturation by SENP1 and SENP7 in our study demonstrated the specificity of SENPs to pre-SUMO's maturation. The preliminary results from SENP1 and SENP7 digestion indicate a potential application in studying various pre-SUMOs' maturation by different SENP paralogs and other substrate–protease digestion processes.

Fluorophores and fluorescent proteins have been widely used in various biological studies recently. The method we developed in this study is environmentally friendly and requires only molecular cloning and protein expression without radioactive labeling or expensive instruments. The fluorescent-tagged proteins are in the aqueous phase, which is mostly close to their natural environment in cells. Fluorescence intensity can be determined by general fluorescence spectroscopy or fluorescence plate readers, which are widely available. Compared with the traditional "gel-based" method, our FRET-based protease assay offers several advantages, including increased sensitivity, real-time measurement, and less time and labor needed. In addition, the highly sensitive FRET-based assay can be used in high-throughput biological assays such as protease inhibitor screenings. The kinetic study can also be used to characterize the properties of the inhibitors (e.g., K_i, IC50). Therefore, the highly sensitive quantitative FRET-based protease assays could be a powerful approach in developing genome-wide protease–substrate profiling and inhibitor screenings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.12.019.

References

[1] E.S. Johnson, Protein modification by SUMO, Annu. Rev. Biochem. 73 (2004) 355–382.
[2] G. Gill, SUMO and ubiquitin in the nucleus: different functions, similar mechanisms?, Genes Dev 18 (2004) 2046–2059.
[3] R.T. Hay, SUMO: a history of modification, Mol. Cell 18 (2005) 1–12.

[4] S. Müller, C. Hoege, G. Pyrowolakis, S. Jentsch, SUMO, ubiquitin's mysterious cousin, Nature 2 (2001) 202–210.
[5] M. Hochstrasser, SP–RING for SUMO: new functions bloom for a ubiquitin-like protein, Cell 107 (2001) 5–8.
[6] E.T.H. Yeh, SUMOylation and de-SUMOylation: wrestling with life's processes, J. Biol. Chem. 284 (2008) 8223–8227.
[7] M. Drag, G.S. Salvesen, DeSUMOylating enzymes–SENPs, IUBMB Life 60 (2008) 734–742.
[8] D. Reverter, C.D. Lima, Structural basis for SENP2 protease interactions with SUMO precursors and conjugated substrates, Nat. Struct. Mol. Biol. 13 (2006) 1060–1068.
[9] J. Mikolajczyk, M. Drag, M. Békés, J.T. Cao, Z. Ronai, G.S. Salvesen, Small ubiquitin-related modifier (SUMO)-specific proteases: profiling the specificities and activities of human SENPs, J. Biol. Chem. 282 (2007) 26217–26224.
[10] R. Horton, E.A. Strachan, K.W. Vogel, S.M. Riddle, A substrate for deubiquitinating enzymes based on time-resolved fluorescence resonance energy transfer between terbium and yellow fluorescent protein, Anal. Biochem. 360 (2007) 138–143.
[11] L. Shen, M.H. Tatham, C. Dong, A. Zagorska, J.H. Naismith, R.T. Hay, SUMO protease SENP1 induces isomerization of the scissile peptide bond, Nat. Struct. Mol. Biol. 13 (2006) 1069–1077.
[12] M. Drag, J. Mikolajczyk, I.M. Krishnakumar, Z. Huang, G.S. Salvesen, Activity profiling of human deSUMOylating enzymes (SENPs) with synthetic substrates suggests an unexpected specificity of two newly characterized members of the family, Biochem. J. 409 (2008) 461.
[13] N. Kolli, J. Mikolajczyk, M. Drag, D. Mukhopadhyay, N. Moffatt, M. Dasso, G. Salvesen, K.D. Wilkinson, Distribution and paralogue specificity of mammalian deSUMOylating enzymes, Biochem. J. 430 (2010) 335–344.
[14] I.H. Engels, C. Daguia, T. Huynh, H. Urbina, J. Buddenkotte, A. Schumacher, J.S. Caldwell, A. Brinker, A time-resolved fluorescence resonance energy transfer-based assay for DEN1 peptidase activity, Anal. Biochem. 390 (2009) 85–87.
[15] S.F. Martin, N. Hattersley, I.D. Samuel, R.T. Hay, M.H. Tatham, A fluorescence-resonance-energy-transfer-based protease activity assay and its use to monitor paralog-specific small ubiquitin-like modifier processing, Anal. Biochem. 363 (2007) 83–90.
[16] C. Berney, G. Danuser, FRET or no FRET: a quantitative comparison, Biophys. J. 84 (2003) 3992–4010.
[17] G.W. Gordon, G. Berry, X.H. Liang, B. Levine, B. Herman, Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy, Biophys. J. 74 (1998) 2702–2713.
[18] S.F. Martin, M.H. Tatham, R.T. Hay, I.D. Samuel, Quantitative analysis of multi-protein interactions using FRET: application to the SUMO pathway, Protein Sci. 17 (2008) 777–784.
[19] Y. Song, V. Madahar, J. Liao, Development of FRET assay into quantitative and high-throughput screening technology platforms for protein–protein interactions, Ann. Biomed. Eng. 39 (2011) 1224–1234.
[20] A.W. Nguyen, P.S. Daugherty, Evolutionary optimization of fluorescent proteins for intracellular FRET, Nat. Biotechnol. 23 (2005) 355–360.
[21] D. Bailey, Characterization of the localization and proteolytic activity of the SUMO-specific protease, SENP1, J. Biol. Chem. 279 (2004) 692–703.

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