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Fluorescent resonance energy transfer -based biosensor for detecting conformational changes of Pin1

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ABSTRACT

Pin1, a peptidyl prolyl cis/trans isomerase (PPIase), regulates the activity and stability of various phosphorylated proteins. Pin1 consists of a PPIase domain and WW domain, both of which are required for the function of Pin1. However, how the behavior of these domains changes upon binding to phosphorylated proteins has not been analyzed. We created a Fluorescent Resonance Energy Transfer (FRET)-based biosensor "CPinY", which is composed of Pin1 flanked by CFP and YFP, and analyzed the interaction between Pin1 and c-Myc. Our results indicated that the dual phosphorylation of c-Myc at Thr58 and Ser62 is essential for tight interaction with Pin1. Additionally, this interaction caused a significant conformational change in Pin1. Our CPinY biosensor also detected a novel type of inhibitor of Pin1 function. We believe that his biosensor will be a novel drug screening technology targeting Pin1.

1. Introduction

Pin1 is a peptidyl prolyl isomerase (PPIase) that specifically binds to phosphorylated proteins. Analysis of Pin1 knockout mice [1] has suggested that the connection between Pin1 and a variety of diseases is regulated by its phosphorylation status [2], such as cancer [3–8], Alzheimer's disease [9,10], chronic kidney disease [11], thrombocytopenia [12], heart disease [13], infertility [14], metabolic diseases [15–17], and so on. Although Pin1 is an attractive drug target to treat these diseases, no drugs targeting Pin1 are currently available.

Pin1 contains two domains connected by an unstructured linker: an N-terminal WW domain and a C-terminal catalytic PPIase domain [18]. Several models have been proposed to explain the molecular mechanism of action of these domains [19]. The "binding-first model" postulates that the WW domain binds the target molecule prior to isomerization by the PPIase domain. The "catalysis-first model" postulates that the PPIase domain isomerizes the target molecule before the WW domain caps the pSer-Pro site. The "allosteric model" postulates that binding to WW domain allosterically induces PPIase activity [20–23]. Although each model suggests that both domains play an important role in Pin1 function, the molecular movement of Pin1 after it binds each substrate is still unclear.

Jacob et al. reported that the RNA polymerase C-terminal domain (CTD) and Cdc25 bind Pin1 and reduce the flexibility of its domains (depicted in supplementary Figure 1) [24]. Their results suggested that the conformational changes of Pin1 are induced by binding to phosphorylated proteins. We hypothesize that this binding and subsequent change in movement may be detectable by FLET technology, because it can quantitatively measure the structural changes of proteins.

Abbreviation: FRET, Fluorescent Resonance Energy Transfer; PPIase, peptidyl prolyl isomerase; CPinY, FRET-based biosensor composed of Pin1 sandwiched by CFP and YFP (Venus); CTD, RNA polymerase C-terminal domain; pMyc, phosphorylated c-Myc.

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2. Materials and methods

2.1. Gene construction

A polymerase chain reaction (PCR) was performed using KOD-Plus Neo polymerase (Toyobo, Japan). All of the oligonucleotide primers used in this study are listed in supplementary Table 1. The DNA fragment assembly was performed using the In-Fusion HD Cloning Kit (Takara Bio, Japan). The cDNA of seCFP [25], YFP (Venus) [26], circular permutation variants [27], and the pCold I vector (Takara Bio) were amplified by PCR. These amplified genes were then assembled to obtain pCold_CPinY for expansion in *Escherichia coli*. CPinY was expressed as N-terminal $(His)_6$-tagged constructs. The insertion of a flexible linker sequence (GGSGGGGSGG) between the fluorescent proteins and Pin1 and the construction of the CPinY_R68-69A mutant was done using PCR-based mutagenesis. Quikchange (Stratagene) was used to construct the CPinY_W34A mutant.

2.2. Purification of proteins

$(His)_6$-tagged proteins, CPinY, and the CPinY variants were expanded and purified from *E. coli* JM109 using Ni-NTA superflow. Column chromatography using a HiLoad 16/600 Superdex (200 pg) was then used according to manufacturer's instructions. Appropriate fractions were dialyzed using 10 mM HEPES (pH 7.5). The homogeneity of purified proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined using A_{515} and a molar extinction coefficient of 84,000 $M^{-1}cm^{-1}$ for YFP [28].

2.3. Pin1 PPIase assay

The PPIase assay was performed as previously described [29]. The total sample volume was 1500 μL; sample buffer was 35 mm HEPES (pH 7.5). Pin1 stock solutions of AEPF-pNA (30 mg/mL in trifluoroethanol containing 470 mm LiCl) and proteases (100 mg/mL for chymotrypsin in 35 mm Hepes (pH 7.8)) were also prepared as described [29]. The increase of pNA liberated by proteolysis was monitored at 5 °C. Absorbance was detected by the signal difference at 390 nm to calculate the first order rate constants, and was measured using an Agilent 8453 UV–vis spectrophotometer (Santa Clara, CA).

2.4. Characterization of the CPinY system

The fluorescence emitted by CPinY was measured using an FP-8200 spectrofluorometer (Jasco) at 25 °C in 50 mM HEPES (pH 7.5). To obtain the fluorescence spectra, CFP was excited with

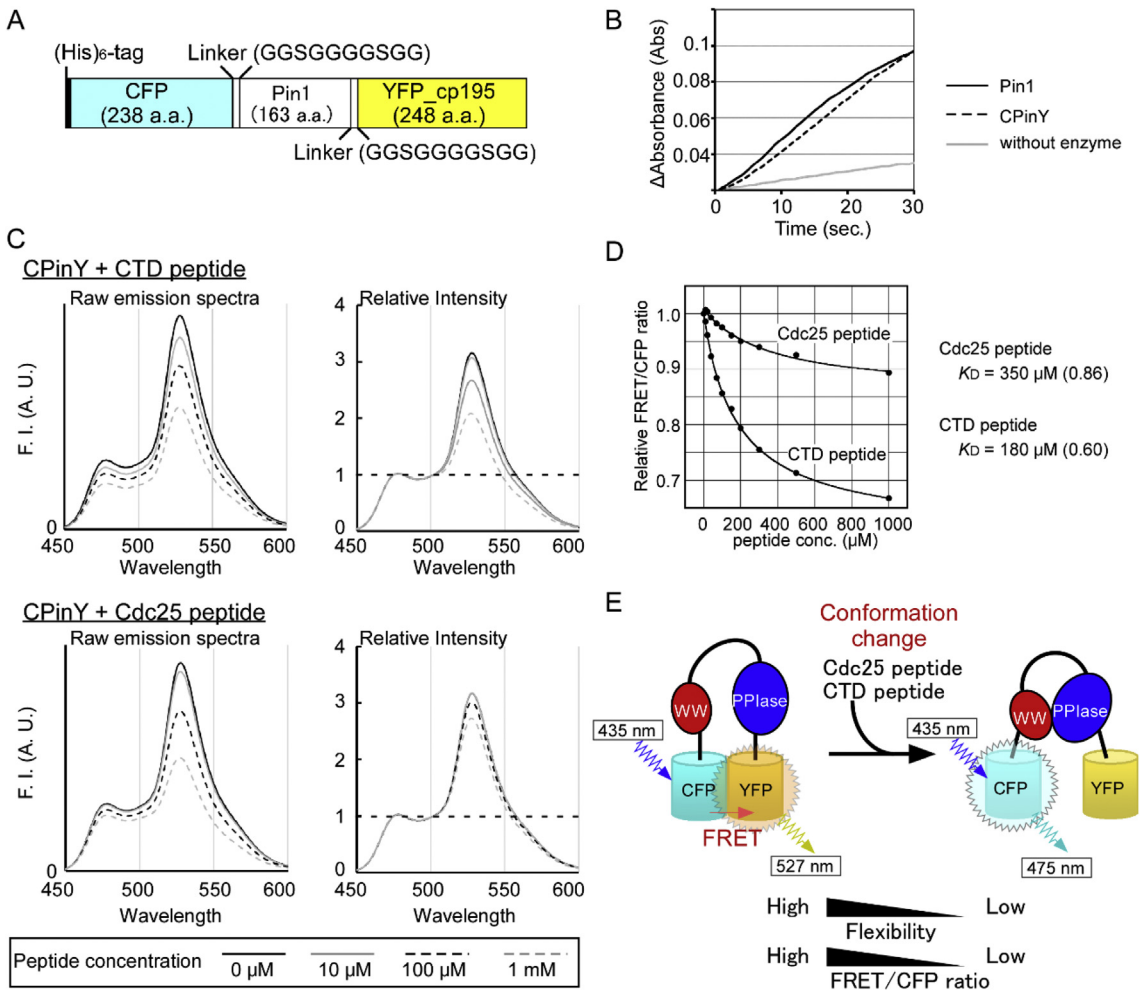

Fig. 1. Construction and evaluation of a biosensor CPinY. A) Construction of CPinY. CPinY was composed of Pin1 sandwiched by CFP and YFP_cp195. **B**) PPIase activity of Pin1 and CPinY. CPinY showed similar level of PPIase activity as that of the wild type control, indicating that the addition of CFP and YFP did not influence the PPIase activity of Pin1. **C**) (left) Emission spectrum of CPinY in the presence of (top) CTD peptide and (bottom) Cdc25 peptides. (right) The spectrum was scaled by the intensity at 435 nm. **D)** Titrations of relative FRET/CFP ratios in the presence of phosphorylated peptide. **E)** Model of the conformational changes of CPinY upon addition of phosphorylated peptides.

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435 ± 10 nm light and the emission from 450 to 600 nm was scanned [30].

Titration analyses compared the FRET/CFP ratios between different concentrations of tested peptides. Analysis plots were fitted with the following equations:

$$\textit{Relative FRET Ratio} = R_{sat} + (1 - R_{sat}) \times \frac{K_D^n}{K_D^n + [\text{peptide}]^n}$$

where n is the Hill coefficient, K_D is an apparent dissociation constant, R_{sat} is an estimated value of FRET/CFP ratio upon peptide saturation, and square brackets indicate the peptide concentration. The relative FRET ratio is equal to the calculated FRET/CFP ratio divided by the FRET/CFP ratio of a peptide-free condition.

3. Results

3.1. Construction of the biosensor: CPinY

We constructed our biosensor from a circularly permutated Venus having the 195th amino acid as its N-terminus (CFP-Pin1-YFP_cp195) [27]. This construct contained a flexible linker sequence (GGSGGGGSGG) between Pin1 and FPs (Fig. 1A). Our biosensor (herein "CPinY") maintained PPIase activity comparable to that of wild type Pin1 (Fig. 1B). The emission spectrum was recorded following the addition of the Pin1-interacting peptides,

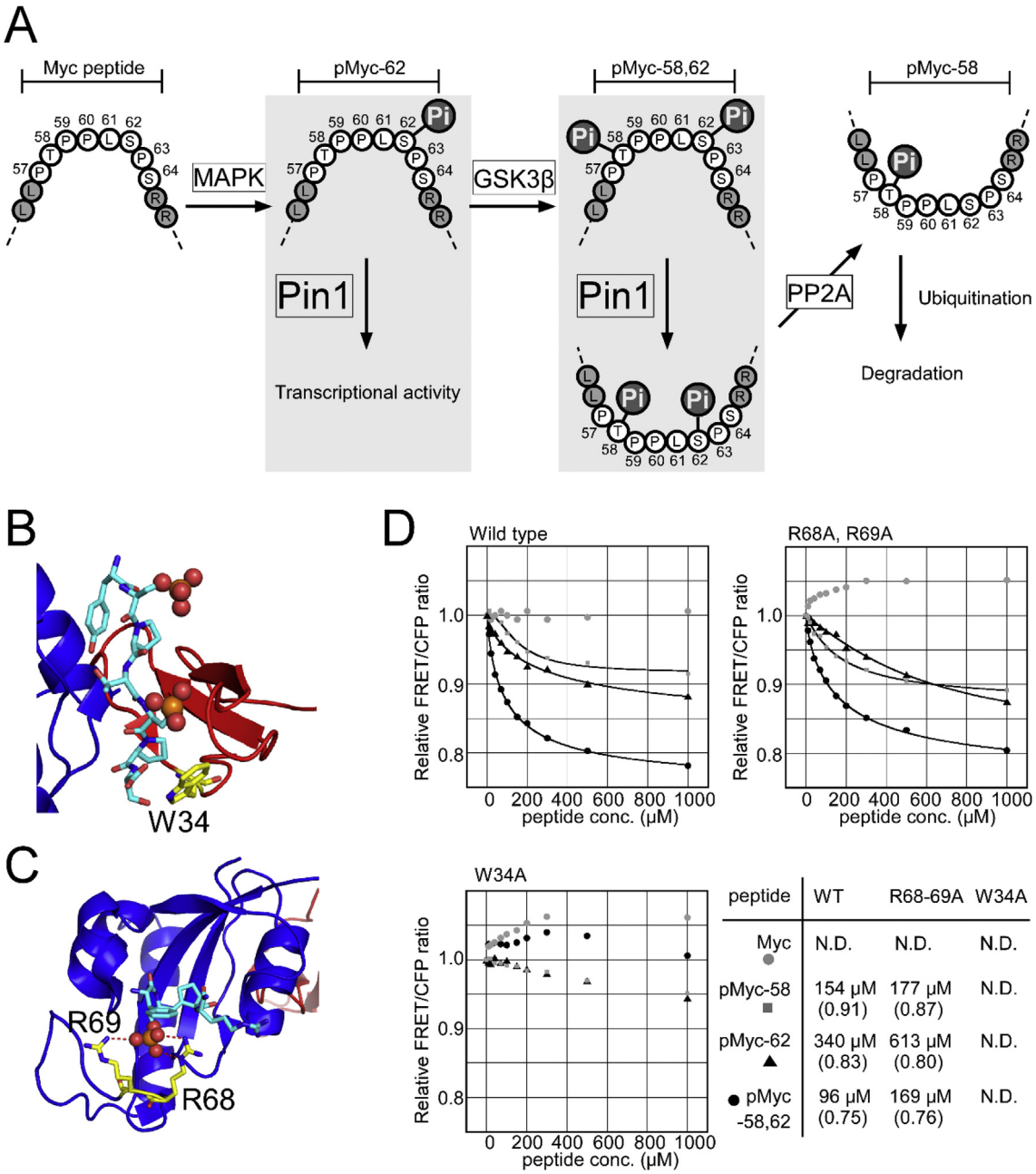

Fig. 2. Analysis of interaction between Pin1 and c-Myc using CPinY. A) Physiological interaction between Pin1 and c-Myc. **B, C)** Phosphorylated peptide binding to WW domain (B) and PPIase domain (C). W34 interacted with the proline residue whereas R68, R69 residues interacted with the phosphorylated group of peptides. **D)** Titrations of CPinY and its mutants with peptides derived from c-Myc.

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Cdc25 and CTD. (Fig. 1C, left). The sum of the emission level between 450 and 600 nm was decreased following peptide addition. Obviously, decrease of FRET should result in increase of cyan fluorescence. The change in FP positions induced by Pin1 conformational change was evaluated by the FRET/CFP ratio. This ratio is widely used to evaluate FRET-based readings, and is calculated as the emission values at 527 nm divided by the values at 435 nm [30] (Fig. 1C, right). The FRET/CFP ratio decreased as the peptide concentration increased and reached saturation (Fig. 1D). The apparent K_D values of Cdc25 and CTD were 350 μM and 180 μM, respectively. The saturated FRET/CFP ratios (denoted as R_{sat}) for Cdc25 and CTD were 0.86 and 0.60, respectively. A model of how these ligands bind Pin1 is shown in Fig. 1E. CPinY was further characterized by titrating peptides that mimicked Cdc25 and CTD, and the resulting apparent K_D values were similarly compared to NMR analysis [24].

We will herein focus on two parameters to define the results of our experiments: i) the apparent K_D, which indicates the relative affinity between Pin1 and the peptide, and ii) the R_{sat}, which gives the structural information of Pin1 once saturated in peptides.

3.2. CPinY-based analysis of the interaction between Pin1 and c-Myc

Our novel biosensor was used to investigate the regulatory function of Pin1 on c-Myc. As shown in Fig. 2A, c-Myc is phosphorylated first at the Ser62 amino acid residue (herein pMyc-62) and then at the Thr58 residue (herein pMyc-58, 62). The mutants of Pin1 in WW domain (W34A) and PPIase domain (R68-69A) were used as the negative control (Fig. 2B and C). In the wild type CPinY, FRET/CFP ratios were reduced by titration on the increase of pMyc-58, pMyc-62, and pMyc-58,68, but not the non-phosphorylated c-Myc peptide (Fig. 3D).

The formation of pMyc-58,62 allows Pin1 to dephosphorylate Ser62 (herein pMyc-58) using Protein Phosphatase 2A (PP2A) [6]. First, the wild type version of CPinY was exposed to increasing levels of pMyc-58, pMyc-62, pMyc-58,62, and a non-phosphorylated c-Myc peptide. FRET/CFP ratios were reduced in the presence of only the phosphorylated proteins (Fig. 2D), indicating that conformational changes in Pin1 depend on the phosphorylation status of the c-Myc peptide. Next, a R68-69A mutant version of CPinY was also exposed to increasing c-Myc peptides. Similarly, the relative FRET/CFP ratios only decreased in the presence of the phosphorylated peptides pMyc-58, pMyc-62, and pMyc-58,62. However, the W34A mutant form of CPinY did not show changes in the relative FRET/CFP ratio following phosphorylated c-Myc addition. This indicates that this site is important for CPinY recognition of phosphorylated peptides.

From these experiments, the decrease of the relative FRET/CFP ratio by pMyc-58,62 was notably larger than that of pMyc-58 and pMyc-62. This implies that the binding of Pin1 and pMyc-58,62 is different from that of pMyc-58 or pMyc-62. These results thus suggest that the double phosphorylation of c-Myc at T58 and S62 is essential for the conformational change of Pin1, which is consistent with previous reports. These results strengthen the reliability of this biosensor.

3.3. Using CPinY for detecting Pin1 function

Next we sought to answer whether this conformational change inhibited the normal function of Pin1. We did this using the four previously described c-Myc peptides to assess the inhibition of Pin1 PPIase activity (Fig. 3A). The inhibition level was measured

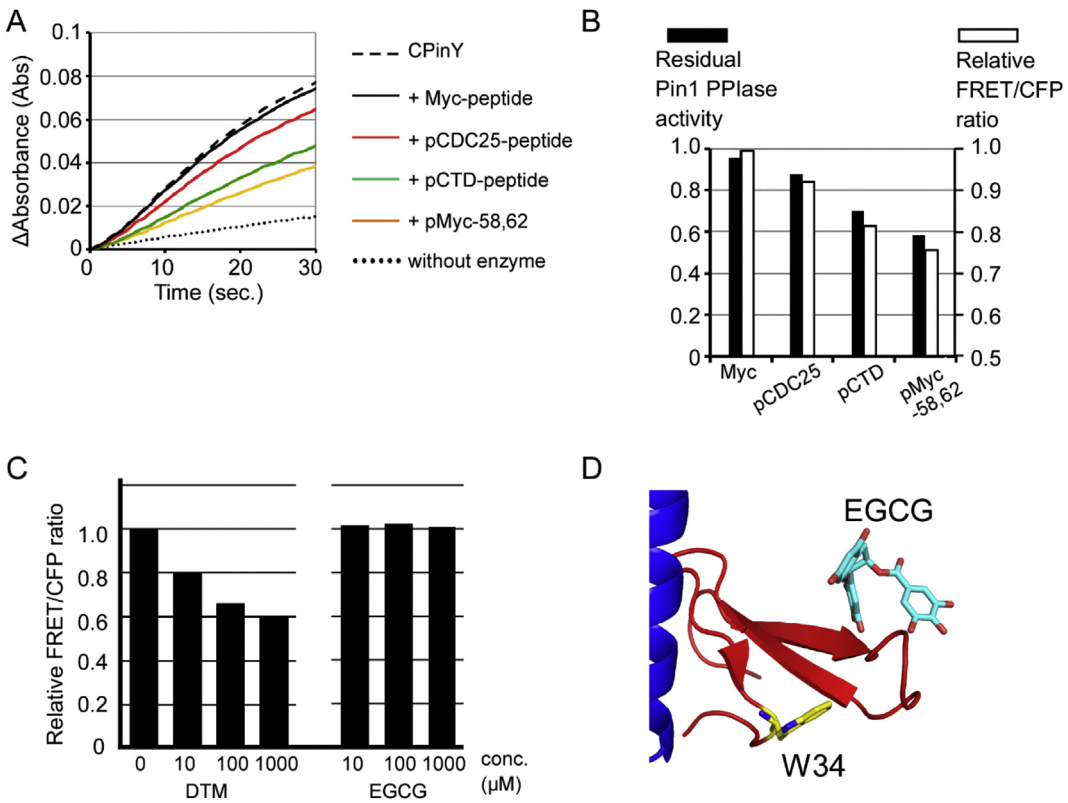

Fig. 3. Relationships between inhibition of Pin1 PPIase activity and CPinY FRET. A) Pin1 PPIase assay in the presence of phosphorylated peptides. **B)** Relative activity of Pin1 in the presence of phosphorylated peptide (black) and FRET/CFP ratio (white). **C)** Relative FRET/CFP ratio in the presence of known Pin1 inhibitors: DTM and EGCG. **D)** Binding site of EGCG in WW domain.

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relatively throu gh correlation with changes in the FRET/CFP ratio. As summarized in Fig. 3B, only the phosphorylated peptides appeared to significantly inhibit Pin1 PPIase activity.

Next, we sought to compare these peptide inhibitors with other known small molecule inhibitors Pin1 inhibitors, DTM [31] and EGCG [32,33]. Interestingly, only DTM caused a significant decrease in the FRET/CFP ratio (Fig. 3C). Therefore, EGCG might not cause a significant structural change of Pin1. This may be attributable to the fact that EGCG binds Pin1 at a site from the key W34 residue (Fig. 3D) [32]. This further supports the importance of the WW motif in leading to the conformational change in Pin1. Together, these results also showed that this novel biosensor is sufficiently sensitive to detect small molecule chemicals that inhibit the molecular motion of Pin 1.

4. Discussion

We estimated the structural changes that Pin1 could undergo with reference to a report by Jacob et al. (Fig. 1). They used NMR to show that phosphorylated peptides mimicking the known Pin1 target molecules Cdc25 and CTD significantly reduced the domain flexibility of Pin1. These results prompted us to investigate whether an intramolecular FRET-based biosensor could provide further structural information. As a result, the CPinY biosensor was engineered and tested using different screening assays in this study.

K_D values analyzed by this biosensor were similar to those analyzed by NMR [24]. Additionally, the R_{sat} decrease upon binding of these ligands indicates that CPinY becomes more flexible when interacting with other proteins.

At first, CPinY was used to analyze the interaction between Pin1 and c-Myc, which is fairly well-characterized [6]. CPinY had verified that the double phosphorylation at Thr58 and Ser62 was essential for the large conformational changes of Pin1, and that the WW domain of Pin1 interacted with the phosphorylated sites. These results are consistent with a model proposed previously based on the pull-down assay [6].

Then we applied CPinY to investigate the effect of the Pin1 molecular inhibitors DTM and EGCG on Pin1 structure. CPinY analysis revealed that DTM could induce molecular movement while EGCG could not. These results further strengthen CPinY as a novel method to screen for chemicals that can induce conformational changes in Pin1.

Author contributions

Conceptualization; TU, Resources; MH,TU,CU, Experiment; KH,EO, Supervision; MH,TU, Analysis; MH,TU, Writing; TU, MH, CU, Editing; HZ, Administration; TU, CU.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.09.123.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.09.123.

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