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Development of destabilized mCherry fluorescent proteins for applications in the model yeast *Saccharomyces cerevisiae*

Yu Chyuan Heng [a,b,c], Jee Loon Foo [a,b,c,*]

[a] NUS Synthetic Biology for Clinical and Technological Innovation (SynCTI), National University of Singapore, Singapore, Singapore
[b] Synthetic Biology Translational Research Programme, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore
[c] Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

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ABSTRACT

Fluorescent proteins are widely used molecular reporters in studying gene expression and subcellular protein localization. To enable the monitoring of transient cellular events in the model yeast *Saccharomyces cerevisiae*, destabilized green and cyan fluorescent proteins have been constructed. However, their co-utilization is limited by an overlap in their excitation and emission spectra. Although red fluorescent protein is compatible with both green and cyan fluorescent proteins with respect to spectra resolution, a destabilized red fluorescent protein is yet to be constructed for applications in *S. cerevisiae*. To realize this, we adopted a degron-fusion strategy to prompt destabilization of red fluorescent protein. Specifically, we fused two degrons derived from Cln2, a G_1-specific cyclin that mediates cell cycle transition, to the N- or C-terminus of mCherry to generate four destabilized fluorescent proteins that are soluble and functional in *S. cerevisiae*. Importantly, the four mCherry fluorescent proteins are highly differential with regards to fluorescence half-life and intensity, which provides a greater choice of tools available for the study of dynamic gene expression and transient cellular processes in the model yeast.

1. Introduction

Fluorescent proteins are fluorophores that spontaneously emit light at a higher wavelength upon absorbing energy at a lower wavelength. Such proteins do not require any exogenous substrate to function and are readily expressed in a wide range of organisms without significant interference to their growth. Due to these properties, fluorescent proteins are now one of the most widely used molecular reporters, especially in gene expression, subcellular protein localization studies and biosensing applications.[1] Although their high stability benefits the detection of switched-on genes, it also delays the conveying of information related to gene switch-off. Therefore, stable fluorescent proteins are unsuitable for monitoring transient cellular events that involve dynamic gene expression.[2,3]

To enable transient gene expression studies, various destabilized fluorescent proteins have been developed, usually through fusion to degradation domains that confer rapid protein turnover. In *Saccharomyces cerevisiae*, destabilized green fluorescent protein (GFP) and cyan fluorescent protein (CFP) with fluorescence half-lives ranging from 5 to 74 min have been constructed.[2-4] Although the availability of different spectral classes of destabilized fluorescent proteins permits the parallel monitoring of the transient expression of more than one gene, the simultaneous use of the currently available destabilized GFP and CFP is unsuitable due to a large overlap in the excitation and emission spectra. Accordingly, red fluorescent protein (RFP) is a better candidate for pairing with both GFP or CFP given the greater spectra resolution.[5] However, there is no destabilized RFP reported for applications in *S. cerevisiae*. Therefore, the development of a destabilized RFP for *S. cerevisiae* is set to be a valuable tool for studying transient events in the model yeast.

To this end, we aimed to develop a destabilized RFP by accelerating the protein's decay using a degron derived from a protein native to *S. cerevisiae*. One such degron candidate is the one from Cln2, a G_1-specific cyclin that mediates the G_1/S phase transition of the cell cycle through periodic binding and activation of the cyclin-dependent kinase Cdc28. As cell cycle progression requires the rapid decay of Cln2 upon exiting G_1 to release Cdc28, Cln2 is very unstable, with a protein half-life as short as 8 min.[6,7] Its instability is modulated by the C-terminus degron

Abbreviations: 5-FOA, 5-fluoroorotic acid; CFP, cyan fluorescent protein; GFP, green fluorescent protein; MCS, multiple cloning site; RFP, red fluorescent protein.

* Corresponding author. NUS Synthetic Biology for Clinical and Technological Innovation, 28 Medical Drive, 117456, Singapore.
E-mail address: jeeloon.foo@nus.edu.sg (J.L. Foo).

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(Cln2^{CT}; Fig. 1A), which directs Cln2 degradation through the ubiquitin-proteasome machinery.[6,8-10] Cln2^{CT} degron fusion has been previously shown to destabilize other proteins, including a C-terminus Cln2^{CT}-tagged GFP that has a fluorescence half-life of 34 min - a significant reduction from 7 h 23 min.[3,6,7] Structurally, Cln2^{CT} degron contains a PEST motif rich in proline, glutamic acid, serine, and threonine[11] and a D domain with four Cdc28 consensus phosphorylation sites[6] (Fig. 1A). The protein destabilization effect of the individual PEST or D domain was reportedly marginal.[6] In contrast, the PD domain (Cln2^{PD}) encompassing both the PEST and the D domains was shown to accelerate protein decay, albeit at a lower rate compared to the full-length Cln2^{CT} degron. Thus, Cln2^{PD} was established to be a functional minimized degron that can sufficiently initiate rapid protein degradation in *S. cerevisiae*.

In this study, we developed destabilized RFPs for applications in *S. cerevisiae* by adopting a degron-fusion strategy. Among the different RFP variants available, we chose monomeric mCherry for its faster maturation time of 15 min.[12] By fusing Cln2^{CT} and Cln2^{PD} degrons to either the N- or C-terminus, we generated a family of four destabilized mCherry fluorescent proteins varied in fluorescence half-life and intensity. Our work therefore marks the successful generation of destabilized mCherry fluorescent proteins for different applications in the model yeast *S. cerevisiae*.

2. Materials and methods

2.1. Strain and culture conditions

S. cerevisiae haploid BY4741 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). YPD medium (1.0% yeast extract, 2.0% peptone and 2.0% glucose) and synthetic defined medium (6.7 g/L yeast nitrogen base, 2.0% glucose, 1.92 g/L yeast synthetic drop-out medium supplements without uracil and 85.6 mg/L uracil) were used for the non-selective cultivation of yeast strains. Synthetic minimal medium lacking uracil consisting of 6.7 g/L yeast nitrogen base, 2.0% glucose and 1.92 g/L yeast synthetic drop-out medium supplements without uracil was used for the selection of uracil prototrophs. To select for *URA3* marker loss, 5-fluoroorotic acid (5-FOA) pre-dissolved in dimethyl sulfoxide at a stock concentration of 100 mg/mL was added to synthetic defined medium at a final concentration of 1.0 mg/mL. Solid culture media were similarly prepared with the addition of 2.5% agar. All *S. cerevisiae* strains were grown at 30 °C.

2.2. Plasmid and strain construction

A yeast codon-optimized *mCHERRY* gene was synthesized by Integrated DNA Technologies (Singapore) and amplified by PCR using primer pairs end-tagged with *BamHI* or *EcoRI* restriction sites. DNA fragments containing the 3′-terminus 531 nucleotides (*CLN2^{CT}*) and 186 nucleotides (*CLN2^{PD}*) of the *CLN2* gene were similarly amplified from yeast genomic DNA and fused to either the 3′- or 5′-terminus of *mCHERRY* gene through splicing-by-overlap PCR. The resulting DNA fusion constructs (Table S1) were digested with BamHI and EcoRI prior to insertion into the multiple cloning site (MCS) of the pMFA vector predigested with the same restriction enzymes, creating *MFa1* locus-specific integration plasmids pMFA1-5 (Table 1). The pMFA vector was generated by substituting a 2.4 kbp fragment containing the *2μ* origin, *f1* origin and *GAL1* promoter in pYES2/CT vector (Thermo Fisher Scientific, Waltham, MA, USA) with a 1.5 kbp fragment containing 619 bp upstream and 700 bp downstream flanking sequences of the *MFa1* locus gapped by an MCS (Fig. S1).

Integrant strains BY41m1-5 (Table 1) expressing a single chromosomal copy of *mCHERRY* and the four fusion proteins were constructed by substituting the *MFa1* gene in the *MFa1* locus using the "pop in/pop out" gene replacement strategy as described previously.[13] For this, the integration plasmids pMFA1-5 (with *URA3* selection marker) were first linearized at the upstream *MFa1* flanking sequence by digestion with BglII restriction enzyme before transformation into BY4741 competent cells using the standard lithium acetate/single-stranded carrier DNA/-polyethylene glycol method.[14] Positive integrant colonies were selected

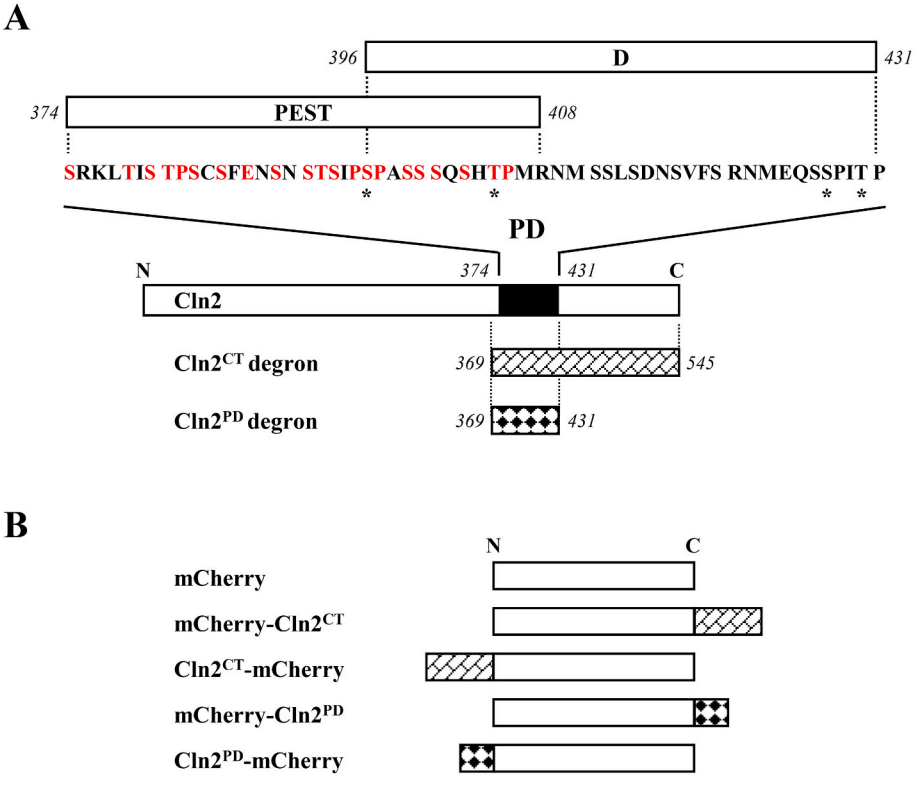

Fig. 1. A degron-fusion strategy to construct destabilized mCherry fluorescent proteins. **(A)** The C-terminus of cyclin Cln2. The PEST domain encompasses a 35-amino-acid sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (highlighted in red). The D domain overlaps partly with the PEST domain and includes a 36-amino-acid sequence containing four Cdc28 consensus phosphorylation sites (marked with asterisk). Cln2^{CT} degron includes the whole C-terminus of Cln2, while Cln2^{PD} degron comprises only the region containing the PEST and the D domains. **(B)** Four mCherry fusion protein were constructed by tagging Cln2^{CT} or Cln2^{PD} degrons to either the N- or C-terminus of mCherry fluorescent protein.

Table 1
Lists of plasmids and strains used in this study.

Plasmid	Genotypes	Source
pYES2/CT	P_{GAL1}-*MCS*-T_{CYC1}; pUC *ori*; *AmpR*; *URA3*; *2μ ori*; *f1 ori*	Thermo FS
pMFA	Modified from pYES2/CT by replacing region from *2μ ori* to P_{GAL1} with 619 bp upstream and 700 bp downstream flanking sequences of the *MFa1* locus, gapped by an MCS; $MFa1_{up}$-*MCS*-$MFa1_{down}$	This study
pMFA1	pMFA with *mCHERRY* inserted into the MCS; $MFa1_{up}$-*mCHERRY*-$MFa1_{down}$	This study
pMFA2	pMFA with *mCHERRY-CLN2*^{CT} inserted into the MCS; $MFa1_{up}$-*mCHERRY-CLN2*^{CT}-$MFa1_{down}$	This study
pMFA3	pMFA with *CLN2*^{CT}*-mCHERRY* inserted into the MCS; $MFa1_{up}$-*CLN2*^{CT}*-mCHERRY*-$MFa1_{down}$	This study
pMFA4	pMFA with *mCHERRY-CLN2*^{PD} inserted into the MCS; $MFa1_{up}$-*mCHERRY-CLN2*^{PD}-$MFa1_{down}$	This study
pMFA5	pMFA with *CLN2*^{PD}*-mCHERRY* inserted into the MCS; $MFa1_{up}$-*CLN2*^{PD}*-mCHERRY*-$MFa1_{down}$	This study
Strain	**Genotypes**	**Source**
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ATCC
BY41m1	BY4741 *mfa1Δ::mCHERRY*	This study
BY41m2	BY4741 *mfa1Δ::mCHERRY-CLN2*^{CT}	This study
BY41m3	BY4741 *mfa1Δ::CLN2*^{CT}*-mCHERRY*	This study
BY41m4	BY4741 *mfa1Δ::mCHERRY-CLN2*^{PD}	This study
BY41m5	BY4741 *mfa1Δ::CLN2*^{PD}*-mCHERRY*	This study

on synthetic minimal medium as uracil prototrophs. Marker rescue was performed by growing the positive integrant colonies overnight in YPD medium prior to 5-FOA selection. Colony PCR was conducted to verify gene insertion and *URA3* marker loss in the *MFa1* locus.

2.3. Flow cytometry

To characterize the steady-state fluorescence intensity of mCherry and the four fusion proteins, overnight seed cultures of integrant strains were inoculated to an initial OD_{600} of 0.2 in synthetic defined medium. Triplicate cultures (100 μL) of each strain were cultivated on a 96-well microplate at 30 °C, 999 rpm for 24 h before measuring cellular fluorescence. For the cycloheximide chase assay to examine fluorescence half-life, overnight seed cultures of integrant strains were inoculated to an initial OD_{600} of 0.5 in synthetic defined medium. Triplicate cultures (5 mL) of each strain were cultivated at 30 °C, 225 rpm on a 6-well microplate. Cell cultures were treated with 50 μg/mL cycloheximide after 2 h. Cellular fluorescence was measured every 30 min thereafter for a duration of 7 h. A BD Accuri™ C6 flow cytometer equipped with BD CSampler™ (BD Biosciences, Franklin Lakes, NJ, USA) was used to measure mean fluorescence of 100,000 cells per sample at a flow rate of 35 μL/min (core size 16 μm). Red fluorescence was detected by the FL4 (675/25 nm) channel. All data acquired was analyzed using the FlowJo V10 software (BD Biosciences, Franklin Lakes, NJ, USA).

2.4. Western blotting

Crude lysates of integrant strains were prepared according to the manufacturer's instructions using Y-PER reagent (Thermo Fisher Scientific, Waltham, MA, USA). The soluble protein fractions were collected as the supernatants of the crude lysates (total protein fractions) after centrifugation at 13,500 rpm for 10 min. Protein samples were denatured by resuspension in Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA) pre-added with 355 mM 2-mercaptoethanol and heated at 95 °C for 10 min. The denatured samples (25 μg) were fractionated on 12.5% SDS-PAGE gels before being transferred to 0.45 μm nitrocellulose membranes. The membranes were probed with mouse monoclonal anti-mCherry antibody (1C51; 1:2000 dilution; Novus Biological, Saint Charles, MO, USA) and horse anti-mouse IgG, horseradish peroxidase-linked antibody (1:2000 dilution; Cell Signalling Technology, Danvers, MA, USA). Protein bands were detected by ultraviolet exposure after the addition of 200 μL SignalFire™ ECL reagent (Cell Signalling Technology, Danvers, MA, USA).

3. Results and discussion

3.1. A degron-fusion strategy for mCherry destabilization

The cyclin Cln2 habours a 177 amino-acid C-terminus degron, $Cln2^{CT}$, that promotes rapid protein turnover in *S. cerevisiae*[3] (Fig. 1A). Within $Cln2^{CT}$ lies a 62 amino-acid PD domain, $Cln2^{PD}$, that also functions as a degron, although with a weaker effect than $Cln2^{CT}$.[6,7] To construct destabilized mCherry fluorescent proteins, we fused $Cln2^{CT}$ and $Cln2^{PD}$ degrons to either the N- or C-terminus of mCherry to create four fusion proteins, namely $Cln2^{CT}$-mCherry, mCherry-$Cln2^{CT}$, $Cln2^{PD}$-mCherry and mCherry-$Cln2^{PD}$ (Fig. 1B and Table S1). Integrant strains BY41m1-5 were constructed for single chromosomal expression of mCherry and the four fusion proteins under the medium strength constitutive *MFa1* promoter in MATa mating type haploid BY4741.

3.2. Characterization of fluorescence intensity and protein abundance

The steady-state fluorescence intensity of mCherry and the four fusion proteins were assessed quantitatively by flow cytometry. As shown in Fig. 2A, all four mCherry fusion proteins gave fluorescence signals that were at least three-fold weaker than the stable counterpart. The highest signal was found with $Cln2^{PD}$-mCherry (3.2-fold lower), followed by mCherry-$Cln2^{PD}$ (14.2-fold lower), $Cln2^{CT}$-mCherry (24.5-fold lower) and mCherry-$Cln2^{CT}$ (84.6-fold lower). To examine the correlation between the reduced fluorescence signal and protein abundance, western blotting was conducted using a monoclonal anti-mCherry antibody. As shown in Fig. 2B, mCherry, mCherry-$Cln2^{CT}$, $Cln2^{CT}$-mCherry, mCherry-$Cln2^{PD}$, and $Cln2^{PD}$-mCherry were all soluble and observed at their predicted molecular weights (26.7, 45.7, 45.7, 33.5 and 33.5 kDa, respectively). Consistent with fluorescence intensity, the protein bands of mCherry (in both the total and soluble protein fractions of the crude lysate) were also the most intense, followed by $Cln2^{PD}$-mCherry, mCherry-$Cln2^{PD}$, $Cln2^{CT}$-mCherry and finally mCherry-$Cln2^{CT}$. As the steady-state protein abundance reflect the equilibrium between protein synthesis and degradation, the lower protein level of the four fusion proteins imply that they are all relatively unstable compared to mCherry.

3.3. Characterization of fluorescence half-life

Cycloheximide is a eukaryote protein synthesis inhibitor that interferes with the ribosomal translocation step during translation elongation in the cytoplasm. Adding cycloheximide inhibits protein synthesis leading to a decrease in intracellular protein abundance over time due to degradation. The rate by which protein abundance decreases correlates to protein stability and the half-life. As fluorescence intensity is correlated with the abundance of fluorescent protein inside the cells, changes in fluorescence intensity upon cycloheximide treatment could be used to deduce the fluorescence half-life of the fluorescent protein. With this rationale, we treated integrant strains BY41m1-5 with 50 μg/mL of cycloheximide and tracked changes in the fluorescence intensity over a 7-h chase period to characterize the fluorescence half-lives of mCherry and the four fusion proteins.

Fig. 3A shows changes in the fluorescence intensity of integrant strains BY41m1-5 during the 7-h chase period. All strains including the one expressing stable mCherry exhibited a rise in average fluorescence in the first 30–90 min. This was contributed by signals emerged from fluorescent proteins synthesized prior to cycloheximide treatment

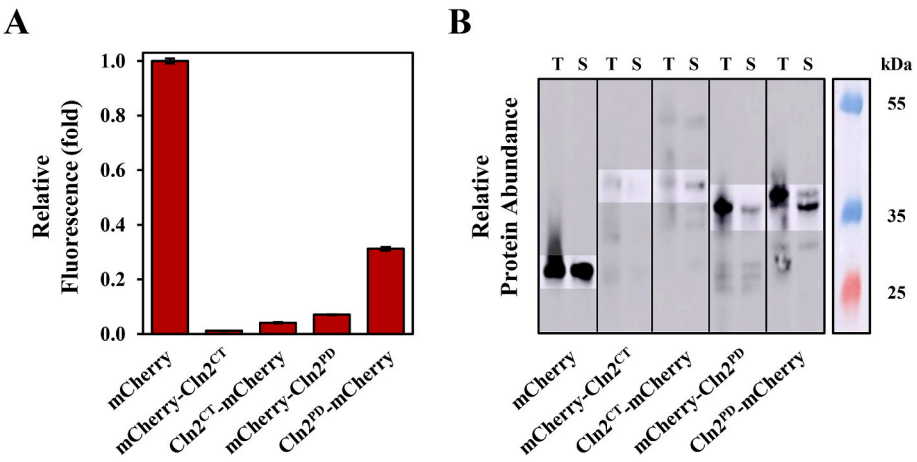

Fig. 2. (A) Characterization of the relative steady-state fluorescence intensity of mCherry and the four fusion proteins. The values presented are the average fold ± standard deviation of three independent replicates. **(B)** Characterization of the relative steady-state protein abundance and solubility of mCherry and the four fusion proteins. Approximately 25 μg of the total protein fraction (T) and the soluble protein fraction (S) of the crude lysate of each integrant strain were fractionated by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane, and finally immunostained with a monoclonal anti-mCherry antibody. Predicted molecular weights: mCherry, 26.7 kDa; mCherry-Cln2^{CT} and Cln2^{CT}-mCherry, 45.7 kDa; mCherry-Cln2^{PD} and Cln2^{PD}-mCherry, 33.5 kDa.

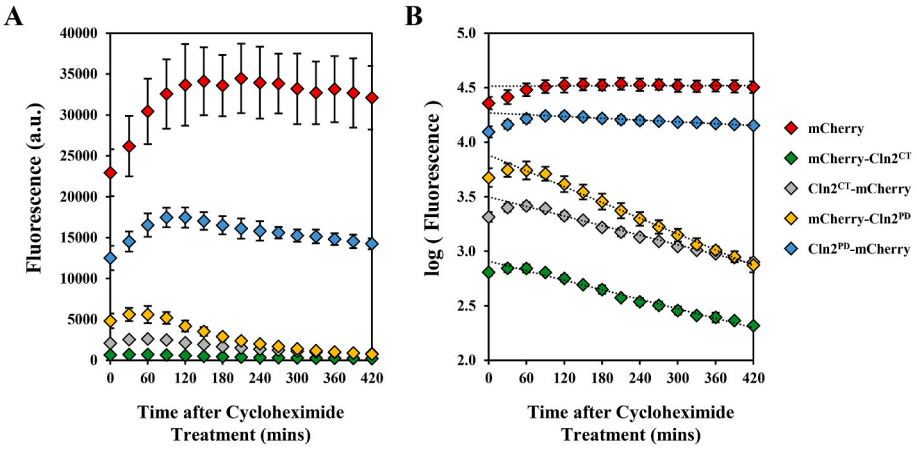

Fig. 3. Characterization of the fluorescence half-lives of mCherry and the four fusion proteins. **(A)** Changes in the average fluorescence of integrant strains BY41m1-5 expressing a single chromosomal copy of mCherry and the four fusion proteins over a course of 7 h after 50 μg/mL cycloheximide treatment. **(B)** The average fluorescence was plotted as a logarithm against time for calculation of fluorescence half-life from the slope of the linear plot. Values presented in panels A and B are the average ± standard deviation of six independent replicates.

(delay due to protein maturation). After 60 min, the average fluorescence of the strain expressing stable mCherry remained relatively high and stable, plateauing at >32,000 a. u. for the remaining 6 h. In contrast, a gradual decline in average fluorescence was observed for the strain expressing Cln2^{PD}-mCherry, reaching 14,251 a. u. in 7 h. On the other hand, the average fluorescence of strains expressing Cln2^{CT}-mCherry and mCherry-Cln2^{PD} substantially decreased over time, reaching <798 a. u. in 7 h. Similarly, a gradual decrease from an average fluorescence of 700 to 207 a. u. was observed for the strain expressing mCherry-Cln2^{CT}. The data obtained was plotted as a logarithm of fluorescence against time to calculate fluorescence half-life from the slope of the linear plot (Fig. 3B). Mean fluorescence half-life (Table 2) was calculated by averaging the fluorescence half-lives derived from the slopes of six independent replicates [log(2)/slope; $R^2 > 0.95$]. Among the four fusion proteins, mCherry-Cln2^{PD} was the most unstable, recording a mean fluorescence half-life of 124 min, followed by 205 and 207 min of Cln2^{CT}-mCherry and mCherry-Cln2^{CT}, respectively. Cln2^{PD}-mCherry was the most stable, reporting a mean fluorescence half-life of 1080 min.

Although it is still unclear, the longer fluorescence half-life of mCherry-Cln2^{CT} than the C-terminus Cln2^{CT}-tagged GFP[3] could plausibly be due to a higher protein stability of mCherry.[15]

Although the longer mean fluorescence half-life of Cln2^{PD}-mCherry relative to Cln2^{CT}-mCherry and mCherry-Cln2^{CT} corroborates with previous findings that Cln2^{CT} is a stronger degron than Cln2^{PD}, mCherry-Cln2^{PD} displays the shortest mean fluorescence half-life among the four fusion proteins (~40% shorter than Cln2^{CT}-mCherry and mCherry-Cln2^{CT}). This observation suggests that the destabilizing effect of the degrons might be protein dependent. Interestingly, fusion of the same degron to different protein termini also affects the degree of destabilization imposed. The difference is more pronounced for Cln2^{PD} which saw greater destabilization when fused to the C- rather than the N-terminus of a protein, as evidenced by the shorter mean fluorescence half-life of mCherry-Cln2^{PD} compared to Cln2^{PD}-mCherry (Table 2). Given that both Cln2^{CT} and Cln2^{PD} degrons are derived from the C-terminus of Cln2 protein, it is plausible that they might both function better at the C- than the N-terminus of a fusion protein. Nevertheless, such variations in

Table 2
Characteristics of mCherry and the four fusion proteins. aa, amino acids; MW, molecular weight; ND, not determined.

Protein	Length (aa)	Predicted MW (KDa)	Relative Fluorescence Intensity	Fluorescence Half-life (mins)	Relative Fluorescence Half-life
mCherry	236	26.7	1.00	ND	–
mCherry-Cln2^{CT}	413	45.7	0.01	207 ± 12	0.19
Cln2^{CT}-mCherry	413	45.7	0.04	205 ± 6	0.19
mCherry-Cln2^{PD}	299	33.5	0.07	124 ± 3	0.11
Cln2^{PD}-mCherry	299	33.5	0.31	1080 ± 206	1.00

destabilization effects are useful in permitting the development of four destabilized mCherry fluorescent proteins with dissimilar fluorescence intensity and half-life for various applications in *S. cerevisiae*. Among them, mCherry-Cln2^{PD} is suitable for all applications regardless of expression strength attributing to it shorter mean fluorescence half-life but moderate fluorescence intensity. Cln2^{CT}-mCherry and mCherry-Cln2^{CT}, on the other hand, are suitable for applications only when these destabilized fluorescent proteins are expressed from a strong promoter or at a high copy-number to compensate for their lower fluorescence intensity.

4. Conclusion

In summary, the fusion of Cln2^{CT} and Cln2^{PD} degrons to mCherry generated a family of four destabilized mCherry fluorescent proteins that are soluble and functional *in vivo* in *S. cerevisiae*. Among them, mCherry-Cln2^{PD} is the most unstable, followed by Cln2^{CT}-mCherry, mCherry-Cln2^{CT}, and finally Cln2^{PD}-mCherry. These newly developed destabilized fluorescent proteins are set to serve as valuable tools for the study of dynamic gene expression and transient cellular events in the model yeast. Importantly, their dissimilar fluorescence half-life and intensity also offers flexibility in tool selection based on the promoter strength and copy number.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotno.2022.12.001.

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