An improved auxin-inducible degron system for fission yeast

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Fusion of a conditional degron to a target protein is a powerful approach to control the level and function of a protein of interest. Auxin-inducible degron (AID) system, which utilizes the plant auxin signaling components to control protein degradation in non-plant species, is the most widely used small-molecular-controlled degradation method in yeasts and animals. We have improved the AID system for the fission yeast *Schizosaccharomyces pombe* by altering its three key components: the AID degron, the auxin receptor F-box protein, and the small-molecule inducer (Fig. 1). This document describes the design and the components of our system.

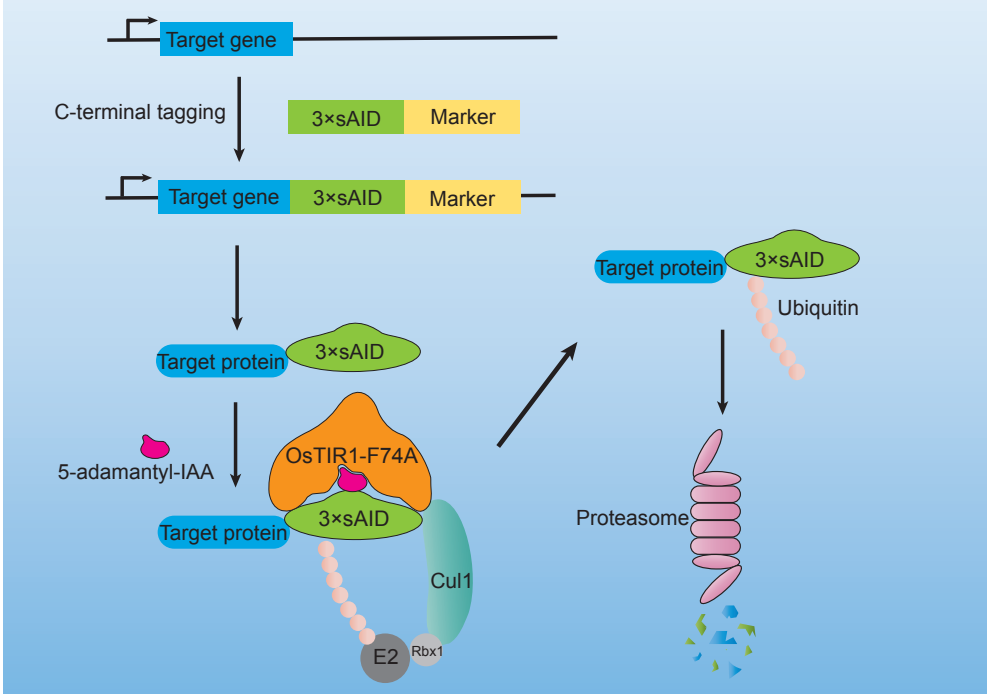


Figure 1. Schematic of our improved AID system for *S. pombe*.

1. The improvements on the AID degron.

In the previously published AID system for *S. pombe*, full-length *Arabidopsis thaliana* IAA17 (AtIAA17) protein is added at the C-terminus of a target protein as a degron (Kanke et al. 2011). Reducing the size of the degron may lower the possibility of interfering the functions of the target protein. Based on sequence alignment and previously published reports on the structure-sequence relationship of Aux/IAA proteins, we chose a 36-amino-acid sequence from the AtIAA17 protein (amino acids 71-106 of AtIAA17) as a minimized degron. We named this 36-

amino-acid sequence the short AID (sAID) (Fig. 2). It is shorter than previously reported minimal degrons including AID*, AID[47], and "mini-AID" (Kubota et al. 2013; Morawska and Ulrich 2013; Brosh et al. 2016; Natsume et al. 2016; Yesbolatova et al. 2019). Based on a previous report showing that three copies of "mini-AID" worked better for some proteins than a single copy of "mini-AID" (Kubota et al. 2013), we decided to use three tandem copies of the sAID (Fig. 2).

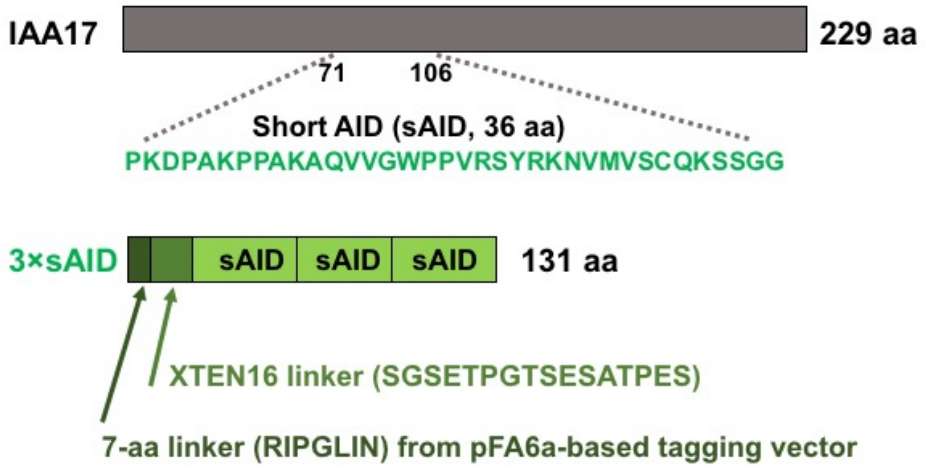
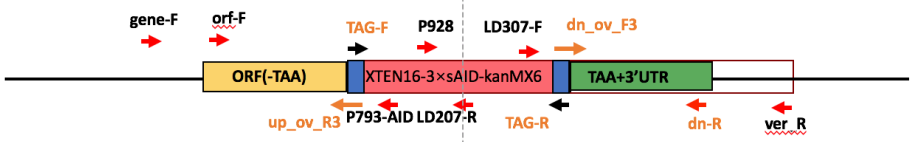


Figure 2. Design of the improved AID degron.

For C-terminal 3×sAID tagging, we constructed a pFA6a-based tagging plasmid, which has the same 7-aa linker (RIPGLIN) as other pFA6a-based tagging plasmids (Fig. 2). To reduce the chance that tagging may affect function, we added another 16-aa linker sequence (XTEN16 linker, SGSETPGTSESATPES) (Fig. 2), which was initially developed by David Liu's lab for linking Cas9 and FokI (Guilinger et al. 2014) and was adopted by Jonathan Weissman's lab as a linker between the AID and proteins being tagged by the AID (Costa et al. 2018). We obtained the DNA encoding XTEN16 and 3×sAID by gene synthesis. When designing the DNA sequence, we chose codons optimized for expression in *S. pombe*. To reduce the chance of recombination between the three copies of sAID coding sequences, codons were chosen so that pairwise nucleotide identities between the three copies are lower than 80%. The DNA was cloned into a kanMX-marked pFA6a plasmid backbone. The resulting C-terminal tagging plasmid is pDB4581 (see the SnapGene file provided with this file).

Tagging can be performed by transforming cells with an overlap PCR product containing an upstream homology arm (up), a 2085-bp tagging cassette (TAG), and a downstream homology arm (down) (Fig. 3).



Fragment	primer	Template	Fragment length	Note
TAG	P121 (TAG-F); P122 (TAG-R)	pDB4581	2085 bp	up_ov_R3 (remove the stop codon) and dn_ov_F3 (start from the stop codon) primers are designed by LJ according to overlap PCR; Primers for PCR verification: LD307+ver-R; gene-F + P793 (AID), gene-F + ver-R (>3kb),but for WT (600 bp) ; P928+LD207 (368 bp) AID tagged protein can be detected by western-blot (anti-AID)
up	orf-F; up_ov_R3	Genomic DNA	~300 bp	
down	dn_ov_F3; dn_R	Genomic DNA	~300 bp	
Overlap fragment	orf_F; dn_R	up, TAG, down	~3000 bp	

Figure 3. C-terminal tagging with 3×sAID.

Primers used in this step:

P121 (TAG-F): CGGATCCCCGGGTTAATTAA

P122 (TAG-R): GAATTCGAGCTCGTTTAAAC

P793 (AID-R): TCCACCGGAAGACTTTTGGCAAG

P928 (AID-F3): GGCCACCTGTCCGTTCTTACCG

LD307: CATCTGCCCAGATGCGAAGTTAAG

LD207: GGTATTCTGGGCCTCCATGTCGC

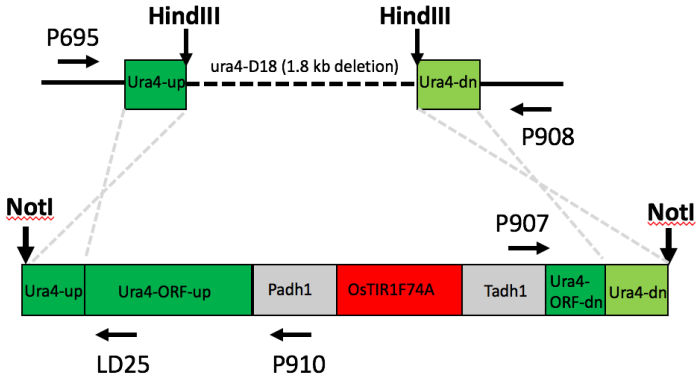
2. The improvements on the F-box protein and the small molecule inducer.

For the auxin receptor F-box protein used in the AID system, Kanke et al. developed a strategy of fusing Skp1 and nuclear localization signal (NLS) to auxin receptor F-box protein for degrading nuclear proteins in *S. pombe* (Kanke et al. 2011). This strategy has two possible shortcomings: first, targeting the F-box protein to the nucleus may reduce the efficiency of degrading cytoplasmic proteins; second, the strongest promoters (e.g. Pnmt1 and Padh1) cannot be used because of a growth inhibitory effect, possibly due to the Skp1 fusion. To avoid these shortcomings, we decided to not fuse the F-box protein with NLS or Skp1. We obtained the DNA encoding *S. pombe* codon-optimized auxin receptor F-box protein *Oryza sativa* TIR1 (OsTIR1) by

gene synthesis. The codon-optimized OsTIR1 (without Skp1 or NLS fusion) expressed from the strong P_{adh1} promoter shows no obvious toxicity in the absence of auxin and the AID degron.

For the small molecule inducer of the AID system, we wanted to avoid the use of auxin because it is not an inert molecule for yeasts and other fungal species. Over 50 years ago, physiological effects of auxin on certain yeast species have been reported (Yanagishima and Masuda 1965; Kamisaka et al. 1967). It has been firmly established now that many fungal species can synthesize auxin and/or exhibit physiological responses to auxin (Fu et al. 2015; Chanclud and Morel 2016). In particular, it has been shown that *S. cerevisiae* can synthesize and secrete auxin (Rao et al. 2010). Auxin promotes the morphological transition of *S. cerevisiae* to a filamentous form, retards the growth of the most commonly used *S. cerevisiae* strain S288C, and inhibits the activity of TORC1 (Prusty et al. 2004; Liu et al. 2016; Snyder et al. 2019; Nicastro et al. 2021). High concentrations of auxin can inhibit the growth of *S. pombe* (Kanke et al. 2011). Thus, to avoid unintended consequences of using auxin, we adopted as the inducer an auxin analog that no longer binds to the wild-type auxin receptor F-box proteins and is unlikely to trigger in yeasts the auxin-induced physiological changes. This analog, 5-adamantyl-IAA, can mediate at picomolar concentrations the interaction between the F79A mutant form of AtTIR1 and IAA proteins (Uchida et al. 2018; Torii et al. 2018; Yamada et al. 2018). F74 in OsTIR1 is the equivalent of F79 in AtTIR1. We introduced the F74A mutation into the *S. pombe* codon-optimized OsTIR1.

For the expression of OsTIR1 and OsTIR1-F74A, respectively, we constructed plasmids pDB5050 and pDB4695, which are based on the integrating vector pDB4697 (SnapGene files of these three plasmids are provided together with this file). In pDB4697, a 2567-bp *ura4*-gene-containing sequence from the *S. pombe* genome (Chromosome III: 114,830-117,653) was placed between two NotI sites. This sequence corresponds to the 1.8-kb region deleted in *ura4-D18* and sequences flanking the 1.8-kb region (about 400 bp on each side). pDB5050 and pDB4695 were constructed by inserting the P_{adh1} promoter, OsTIR1 coding sequence, and a terminator sequence into an AvrII site about 70 bp downstream of the coding sequence of *ura4* in pDB4697. Transforming a *ura4-D18* strain with NotI-digested pDB4697, pDB5050, or pDB4695 converts the strain to *ura4*+. Integration at the *ura4* locus can be confirmed by PCR (Fig. 4).



Transform NotI digested pDB4695 [Padh1-OSTIR1-F74A (ura4+)] into a *ura4-D18* strain.
Verification: P695 (ura4-up-left)+LD25 (ura4-ORF-up);
P907 (adh1-ter-ver-F)+P908 (Ura4-dn-right-R);
P695 (ura4-up-left-F)+P910 (pro adh1-R);

Figure 4. Integrating the OsTIR1-expressing cassette at the *ura4* locus.

The primers used for PCR verification of the integration are as follows:

P695: GGCGTCTTCCTCTAGATGCTCGTC

LD25: CACAAATGCATACATATAGCCAGTG

P907: GTGTATACAAATTTTAAAGTGACTCTTAGG

P908: GCGTAGTGTAGTATTGCTGACAT

P910: CGATGGAGTGCGTTGAATGAGAG

3. 5-adamantyl-IAA

5-adamantyl-IAA is commercially available from Tokyo Chemical Industry Co., Ltd. (TCI).

Product Number: A3390

https://www.tcichemicals.com/OP/en/p/A3390

We dissolved 5-adamantyl-IAA in 1 M NaOH to make a 1 mM stock solution and stored at -20°C. The stock solution was diluted in water before an appropriate amount was added to the medium. The pH of the medium was not obviously affected by NaOH in the stock solution when the final dilution ratio was higher than 1000 folds (i.e. final concentration of 5-adamantyl-IAA < 1 μM in the medium).

4. Antibodies

3×sAID tagged proteins can be detected by immunoblotting using a monoclonal anti-mini-AID-tag antibody from MBL (Code No. M214-3):

https://www.mblintl.com/products/m214-3/

https://www.mblbio.com/bio/g/product/tag/pickup/mini-aid.html

https://ruo.mbl.co.jp/bio/e/dtl/A/?pcd=M214-3

OsTIR1 and OsTIR1-F74A can be detected by immunoblotting using an anti-OsTIR1 antibody (MBL, Code No. PD048, https://ruo.mbl.co.jp/bio/e/dtl/A/?pcd=PD048)

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