Published online 17 September 2020

Nucleic Acids Research, 2020, Vol. 48, No. 18 ***e108***
doi: 10.1093/nar/gkaa748

A super-sensitive auxin-inducible degron system with an engineered auxin-TIR1 pair

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Received May 31, 2020; Revised August 19, 2020; Editorial Decision August 25, 2020; Accepted August 28, 2020

ABSTRACT

The auxin-inducible degron (AID) system enables rapid depletion of target proteins within the cell by applying the natural auxin IAA. The AID system is useful for investigating the physiological functions of essential proteins; however, this system generally requires high dose of auxin to achieve effective depletion in vertebrate cells. Here, we describe a super-sensitive AID system that incorporates the synthetic auxin derivative 5-Ad-IAA and its high-affinity-binding partner OsTIR1^{F74A}. The super-sensitive AID system enabled more than a 1000-fold reduction of the AID inducer concentrations in chicken DT40 cells. To apply this system to various mammalian cell lines including cancer cells containing multiple sets of chromosomes, we utilized a single-step method where CRISPR/Cas9-based gene knockout is combined with insertion of a pAID plasmid. The single-step method coupled with the super-sensitive AID system enables us to easily and rapidly generate AID-based conditional knockout cells in a wide range of vertebrate cell lines. Our improved method that incorporates the super-sensitive AID system and the single-step method provides a powerful tool for elucidating the roles of essential genes.

INTRODUCTION

Gene knockout is a common method for examining the functions of gene products; however, for essential genes, it is difficult to generate knockout cell lines, as knockouts can lead to cell death. To avoid the lethality, conditional knockout must be achieved. Transcription of a target gene can be conditionally turned off under the control of a conditional promoter such as a tetracycline responsive promoter (1). However, it usually takes more than two days to deplete a preexisting target protein within cells after turning off transcription. To rapidly and conditionally deplete preexisting target proteins, we previously developed the auxin-inducible degron (AID) system that allows target proteins to be directly degraded within the cells (2). Since then, the AID system has been widely used for conditionally knocking out essential target proteins in yeasts and various vertebrate cell lines (2–6).

The plant hormone auxin (indole-3-acetic acid, IAA) promotes the degradation of Aux/IAA transcriptional repressors through the ubiquitin proteasome pathway in plants (7–10). This auxin-dependent degradation is utilized by the AID system for rapid degradation of target proteins in yeasts and various vertebrate cell lines. In the AID system, an auxin receptor F-box protein (TRANSPORT INHIBITOR RESPONSE1, TIR1) is exogenously expressed to form a chimeric E3 ubiquitin ligase complex (SCF^{TIR1}) in non-plant cells. In the presence of auxin, an AID-tagged target protein binds to SCF^{TIR1} and is then degraded through the ubiquitin proteasome pathway (2). In the AID system, the *Arabidopsis thaliana* IAA17 protein (AtIAA17) is used as an AID-tag and the natural auxin IAA is used as an AID inducer.

Generating AID-based knockout cell lines requires two steps that include 1) the establishment of a TIR1- expressing cell line and 2) replacement of the endogenous gene with the gene encoding the AID-tagged target protein. In the second step, the DNA sequence of the AID-tag must be inserted at the amino or carboxyl terminus of the protein coding region

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of the endogenous gene through either homologous recombination or Cas9-mediated homology-directed repair (Figure 1A) (11). However, it is difficult to add the AID-tag to all of the endogenous target alleles in cancer cell lines (such as HeLa cells) that possess multiple sets of chromosomes (12,13). This presents a problem for utilizing the AID system.

Other systems that use chemical compounds other than IAA have also been developed for regulating the stabilities of target proteins. For example, target proteins fused with a destabilized form of FKBP (DD_{FKBP}) are stable in the presence of the compound Shield-1 and are degraded after removal of Shield-1 (14–17). Another example is the Halo-tagHyT system, where Halo-tagged target proteins are destabilized in the presence of HyT13 (18,19). The working concentrations of Shield-1 and HyT13 are low (0.1–1 and 0.5–10 μM, respectively). Compared to these systems, the conventional AID requires high IAA concentrations (100–500 μM) (20) that may cause cytotoxicity in some cell lines. In fact, we found that 500 μM IAA concentrations caused growth delays in some human cell lines (HeLa, U2OS, and RPE1) and resulted in increased apoptosis and necrosis in U2OS cells (Supplementary Figure S1).

In this study, to overcome these two problems in the conventional AID system, we introduce an improved method to generate AID-based conditional knockout cell lines. In our method, to reduce the AID inducer concentration, we utilized an engineered pair consisting of a synthetic auxin and a modified TIR1 that was developed using the bump-and-hole strategy (21). We succeeded in establishing a super-sensitive AID system that incorporates a high-affinity-binding pair that includes an auxin derivative (5-Ad-IAA) and a modified TIR1 from *Oryza sativa* ($OsTIR1^{F74A}$). We also succeeded in the generation of AID-based conditional knockout cell lines from cancer cells by applying a single-step method (Figure 1B and C). Finally, we provide a system that is more efficient system than the conventional AID system by combining the super-sensitive AID system with the single-step method.

MATERIALS AND METHODS

Yeast two-hybrid assay

Yeast assays were performed according to the previous report (21). Briefly, EGY48 strain was transformed with pSH18–34 (LexA-operon:LacZ reporter), pGLex313-based plasmid (LexA-DNA-binding-domain-fused TIR1 series), and pJG4–5-based plasmid (B42-transcriptional-activator-fused AtIAA17 protein). Transformed strains were grown at 30°C on agar plates containing minimal SD base (Clontech, cat. 630411) and –His/–Trp/–Ura dropout supplement (Clontech, cat. 630424). Colonies were grown in SD/–His/–Trp/–Ura medium for one night at 30°C, and then medium was replaced with liquid medium containing minimal SD/Gal/Raf base (Clontech, cat. 630420), –His/–Trp/–Ura dropout supplement, 50 mM Na-phosphate buffer (pH 7.0), 80 mg/ml X-gal (Wako) and compounds. After 3-day incubation at 30 or 37°C, the cultures were transferred to white 96-well plates and observed.

Cell culture

HeLa, HeLa-S3, HT1080, U2OS, RPE1 cells are cultured in DMEM (Nakalai tesque) supplemented with 10% FBS, 100 U/ml of penicillin/streptomycin (Nakalai tesque) at 37°C in 5% CO_2. DT40 cells are cultured in DMEM (Nakalai tesque) supplemented with 10% FBS, 1% chicken serum (Gibco), 100U/ml of penicillin/streptomycin (Nakalai tesque) and 50 μM of 2-mercaptethanol (Sigma) at 38.5°C in 5% CO_2. Feeder Free Mouse ES cells (E14tg2a) are cultured on Gelatinized dish in DMEM (Nakalai tesque) supplemented with 10% FBS, 5% Knockout Serum Replacement, 2 mM of L-glutamine (Nakalai tesque), 100 U/ml of penicillin/streptomycin, 1% of non-essential amino acid (Nakalai tesque) and LIF protein at 37°C in 5% CO_2.

Auxin derivative stock solutions

Auxin derivatives (IAA, cvxIAA, 5-Ph-IAA and 5-Ad-IAA) were diluted into dimethyl sulfoxide (DMSO) at 500 mM or 5 mM concentration and stored at −30 °C. cvxIAA and 5-Ph-IAA were chemically synthesized (21). 5-Ad-IAA is commercially available from Tokyo Chemical Industry (Product Number A3390, https://www.tcichemicals.com/AT/en/search/?text=5-adamantyl-IAA).

Plasmid constructions

We used pX330-U6-Chimeric_BB-CBh-hSpCas9 (pX330) (Addgene #42230) (22) for the construction of CRISPR/Cas9 vectors following the protocol (23). Specific primer pairs for CRISPR/Cas9 vectors are shown in Supplementary Table S1. To construction of pAID plasmids, pAID1.1N-T2A-BSR (Addgene ID: #105985) and pAID1.1C-T2A-BSR (Addgene ID: #105986) (24) were modified. Codon optimized OsTIR1 DNA was obtained from Masato Kanemaki. pmScarlet-c1 plasmid was obtained from Addgene (Addgene ID: #85044) (25). pAID plasmids for DT40 cells contained OsTIR1 and $OsTIR1^{F74A}$ under the control of the Cytomegalovirus (CMV) promoter. cDNA of the target protein was inserted into the EcoRV site using InFusion technology (Takara). pAID plasmids for human or mouse cells contained OsTIR1 and $OsTIR1^{F74A}$ under control of the eukaryotic translation elongation factor 1 Alpha 1 (EF1a) promoter. cDNA of the target protein was inserted into the EcoRV site using the InFusion technology, as was the case for the pAID plasmids in DT40 cells. Specific oligo pair (Supplementary Table S1) was used for amplification of cDNA of the target gene by PCR. PCR products were inserted in the EcoRV site of pAID plasmid by In-Fusion cloning system (Takara).

We deposited following plasmids to Addgene: pAID1.2-CMV-NmScarlet-mAID (Addgene ID: #140618), pAID1.2-EF1a-NmScarlet-mAID (Addgene ID: #140617), pAIDFA-CMV-NmScarlet-mAID (Addgene ID: #140616), pAIDFA-EF1a-NmScarlet-mAID (Addgene ID: #140615), pAID-CMV linearizing in pX330 (Addgene ID: #140609), and pAID-EF1a linearizing in pX330 (Addgene ID: #140610).

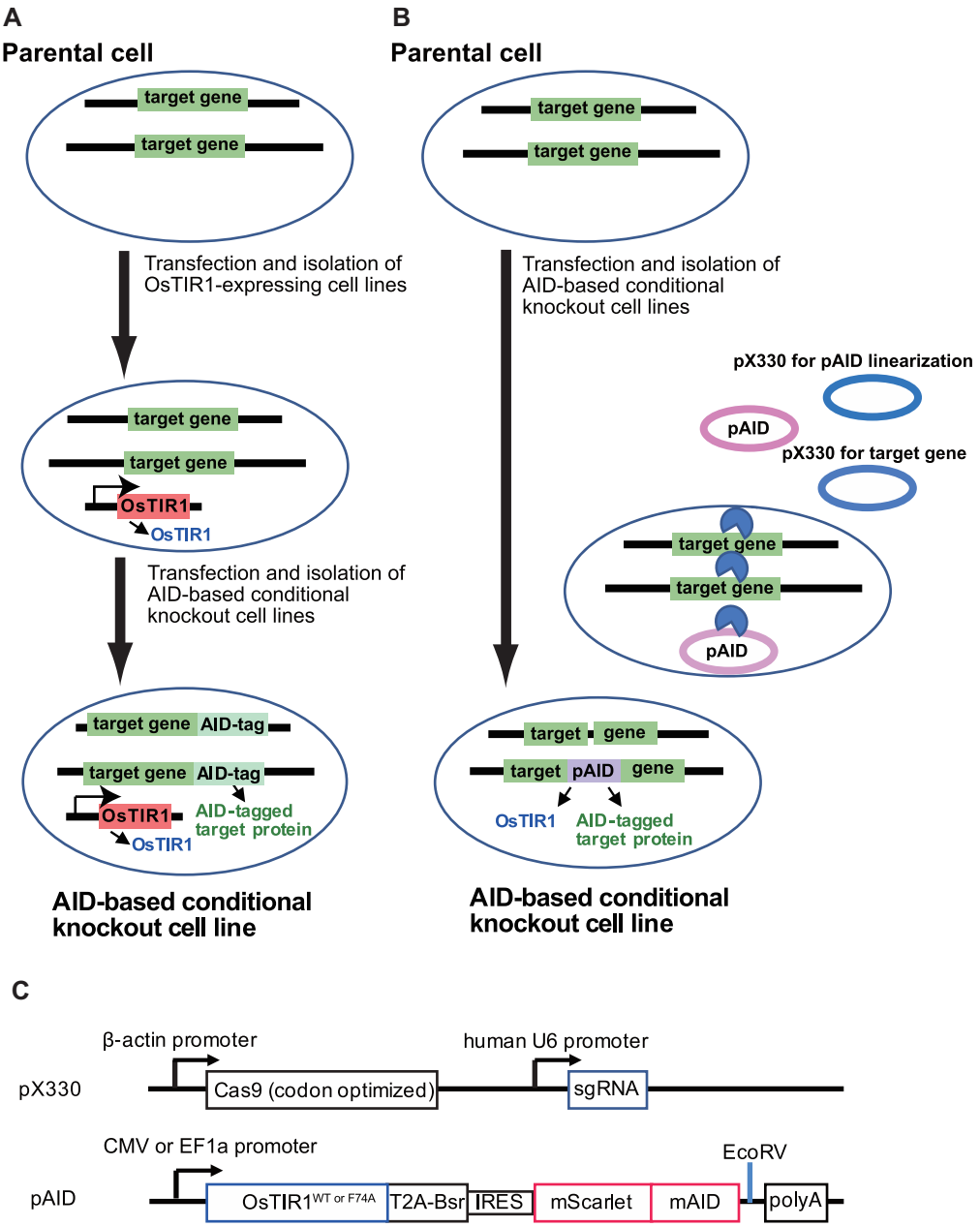

Figure 1. Comparison of the conventional and single-step methods for generating an AID-based conditional knockout cell lines. (**A**) The conventional method is composed of two steps that include establishing an OsTIR1-expressing cell line and replacing the endogenous protein with the AID-tagged protein. (**B**) A single-step method. CRISPR/Cas9-based gene targeting is coupled with pAID-plasmid integration to express both OsTIR1 and an AID-tagged target protein. Parental cells are transfected simultaneously with three different plasmids that include (i) the pAID plasmid encoding OsTIR1, an AID-tagged target protein, and a protein that confers resistance to the drug blasticidin, (ii) the pX330 Crispr/Cas9 plasmid for disrupting a target gene, and (iii) the pX330 CRISPR/Cas9 plasmid for linearizing the pAID plasmid. After transfection, the Cas9 protein induces DNA double-strand breaks in the target locus and pAID. Target genes are disrupted by pAID-plasmid integration and/or internal deletion/insertion. (**C**) Plasmid maps for pX330 and pAID that are used for the single-step method in (B).

Transfection and cloning colonies for generation of AID-based conditional knockout cell lines

pAID plasmid (1 μg), pX330 for target gene (2 μg), and pAID-EF1a-linearlizing in pX330 (1μg) were diluted in 50 μl of 20 mM Glutamic acid solution (pH4.0). 18 μg of Polyethylenimine (PolySciences) was diluted in 50 μl of 20 mM glutamic acid solution (pH4.0). DNA solution was mixed with polyethylenimine solution and vortexed for 5 s. Mixture solutions were incubated at room temperature for 15 min. Cells (1×10^6) (HeLa, U2OS, HT1080, and E14tg2a) were diluted with 300 μl of serum-free DMEM. Cells were mixed with DNA-polyethylenimine solution and vortexed for 5sec. After 15-min incubation at room temperature, cells (diluted 1, 1/5, 1/25 or 1/125) were plated to 10-cm plates and cultured at 37°C. Selection started by adding 10 μg /ml of blastsidin at 24 h after the transfection. Selec-

tion media were changed every 3 days. Surviving colonies were isolated at 1 to 2 weeks after selection.

pAID plasmid (6 μg), of pX330 for target gene (12 μg), and pAID-CMV-linearlizing in pX330 (6μg) were pelleted by ethanol precipitation. Pelleted DNA was resuspended with 125 μl of R buffer of Neon transfection system (Thermo Fisher). DT40 cells (2×10^5) were diluted in DNA solution and Neon transfection was performed at indicated condition (1400 V, 5 ms, 6 times). 2 days after transfection, cells were plated to 96-well plates and selection started by adding 30 μg/ml of blastsidin. At 1 week after selection, surviving colonies were picked up.

pAID plasmid (6 μg), of pX330 for target gene (12 μg), and pAID-EF1a-linearlizing in pX330 (6μg) were pelleted by ethanol precipitation. Pelleted DNA was resuspended with 125μl of R buffer of Neon transfection system (Thermo Fisher). RPE1 cells (2×10^5) were diluted in DNA solution and Neon transfection was performed at indicated condition (1400 V, 30 ms, 1 time). At 2 days after transfection, cells (diluted 1, 1/5, 1/25 or 1/125) were plated to 10-cm plates. Selection started by adding 10 μg/ml of blastsidin at 24 h after the transfection. Selection media were changed every 3 days. Surviving colonies were isolated at 1 to 2 weeks after selection.

Luciferase assay

DT40 cells (0.5×10^4 cells) expressing the AID-tagged luciferase (mScarlet-mAID-luciferase) and each OsTIR1 ($OsTIR1^{WT,}$ $OsTIR1^{F74A}$, or $OsTIR1^{F74G}$) were cultured with various concentrations of the AID inducers IAA and/or 5-Ad-IAA. Luciferase activity was measured with Steady-Glo Luciferase Assay System (Promega) according to a manufacture protocol.

Fluorescence microscopy

Cells were fixed with 3% paraformaldehyde. After DAPI staining, cells were washed with PBS twice and mounted with Vectashield mounting reagent (Vector Laboratories). Fluorescence images were captured by sCMOS camera (Zyla 4.2; Andor) mounted on ECLIPSE Ti microscope (Nikon) with an objective lens (Plan Apo lambda 100×/1.45 NA; Nikon) and CSU-W1 confocal scanner unit (Yokogawa) controlled by NIS elements (Nikon). Z stacked images were obtained.

Cell growth assay and cell viability assay

DT40 cells (1×10^5 cells/ml) were cultured with or without an auxin derivative. Cell concentrations are calculated by Countess II (Thermo Fisher) at indicated time point after adding auxin derivatives. DT40 cells (0.5×10^5 cells/ml) were cultured with a various concentration of auxin derivatives. Cell viability was measured by beta-Glo assay (Promega) at 48 h after adding each auxin derivative.

Colony formation assay

Mammalian cells (500–1000 cells) were plated to 6-well plates with or without an auxin derivative at indicated concentrations and incubated at standard cultured conditions for 1–2 weeks. Cells were fixed with cold 100% methanol for 2 min and air-dried. Cells were stained with Crystal Violette and washed with water.

Immunoblotting analysis

Protein samples were denatured by boiling in SDS sample buffer containing 10% β-mercaptoethanol and then subjected to SDS-PAGE by using 5–15% and 5–20% gradient gels. Proteins were transferred onto PVDF membranes and the blots were blocked with 1% BSA at room temperature for 0.5–1 h and incubated with each specific antibody [rabbit anti-chicken CENP-A, rabbit anti-chicken CENP-H, rabbit anti-chicken CENP-T, sheep anti-AID antibody, rat anti-RFP antibody (5F8, Chromotek), mouse anti-human CENP-A, mouse anti-human CENP-H (sc-365222, SANTA CRUZ), guinea pig anti-human CENP-C, and mouse anti-mouse CENP-H (sc-136403, SANTA CRUZ) antibodies] at 4°C for overnight or at room temperature for 1 h. After washing three times with TBST, the membrane blots were incubated with each secondary antibody conjugated with HRP (goat anti-rabbit IgG, goat anti-mouse IgG, goat anti-sheep IgG, goat anti-rat IgG, and goat anti-guinea pig IgG antibodies). Detections were performed with ECL Prime (GE Healthcare) and all images were acquired with a ChemiDoc MP Imaging system (Bio-rad). Signal intensities on the immunoblots were determined using ImageJ.

RESULTS

$OsTIR1^{F74A}$ interacts with AtIAA17 at extremely low concentrations of the auxin derivative 5-Ad-IAA

In the AID system, the auxin receptor TIR1 interacts with an AID-tagged protein to promote its degradation in the presence of IAA. Recently, we identified a highly active engineered auxin-TIR1 pair that is composed of the synthetic auxin derivative 5-Adamantyl-IAA (5-Ad-IAA, also known as pico-cvxIAA) and the modified *A. thaliana* TIR1 ($AtTIR1^{F79A}$) that possesses a single amino acid substitution at Phe (F)-79 in the auxin binding pocket (26). This raised the possibility of developing a more efficient AID system that uses 5-Ad-IAA and $AtTIR1^{F79A}$ compared to the conventional AID system that uses IAA and *O. sativa* TIR1 (OsTIR1) (Figure 2A).

To examine AID-inducer-dependent interactions between TIR1 proteins and AtIAA17 (AID-tag), we performed a yeast two-hybrid assay (Figure 2B) by using AtIAA17 and AtTIR1 ($AtTIR1^{WT}$ or $AtTIR1^{F79A}$). We also used *O. sativa* TIR1 ($OsTIR1^{WT}$ or $OsTIR1^{F74A}$ that has a corresponding amino acid modification to $AtTIR1^{F79A}$) (Figure 2C). Both $AtTIR1^{F79A}$ and $OsTIR1^{F74A}$ interacted with AtIAA17 in the presence of either IAA or 5-Ad-IAA at 30 °C (Figure 2C, left). Surprisingly, the effective concentrations of 5-Ad-IAA were >100 000 times lower than that of IAA. Similar high-affinity interactions were detected at 37 °C (Figure 2C, right), which is a suitable temperature for vertebrate cells. These results suggested that both $AtTIR1^{F79A}$ and $OsTIR1^{F74A}$ were useful for the improved AID system with 5-Ad-IAA in vertebrate cells.

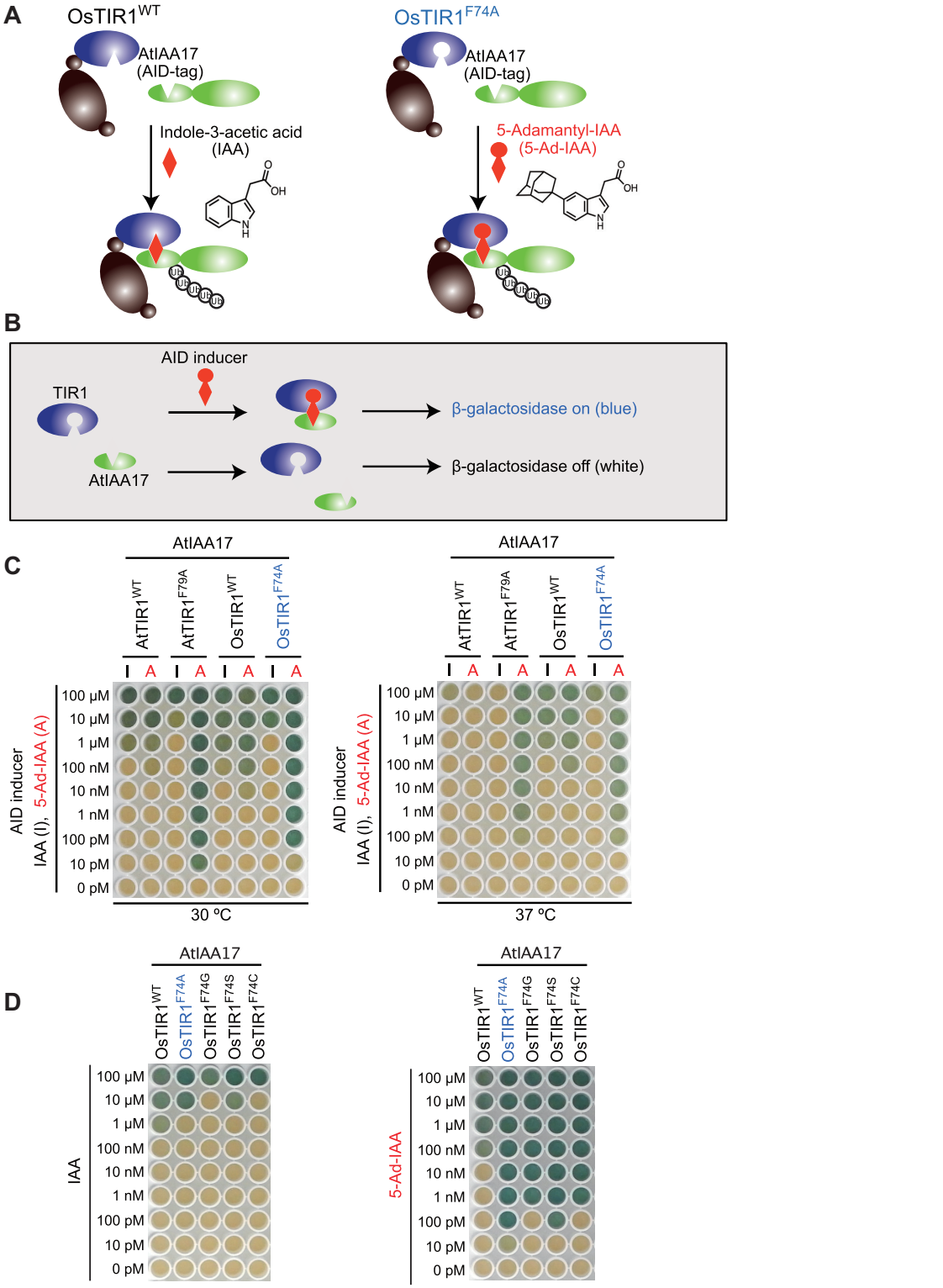

Figure 2. AID inducer-mediated interactions of AtIAA17 (AID-tag) with modified TIR1 proteins. (**A**) Schematic illustrations of the conventional AID system incorporating IAA and OsTIR1^{WT} (left) and the super-sensitive AID system with the synthetic auxin derivative (5-Ad-IAA) and OsTIR1^{F74A} (right). OsTIR1 (blue), a functional subunit of the E3 ubiquitin ligase SCF complex, efficiently interacts with an AID-tagged target protein (green) through an AID inducer (IAA or 5-Ad-IAA) (red) to ubiquitinate the tareget protein for rapid proteasome-dependent degradation. (**B**) Schematic diagram of the yeast two-hybrid assay to evaluate AID-inducer-dependent interactions between TIR1 and AtIAA17 (AID-tag). (**C**) A yeast two-hybrid assay showing interactions of AtIAA17 with each of four TIR1s (AtTIR1^{WT}, AtTIR1 ^{F79A}, OsTIR1^{WT} and OsTIR1^{F74A}) in the presence of various concentrations of AID inducers (IAA or 5-Ad-IAA) at 30°C (left) or 37°C (right). (**D**) A yeast two-hybrid assay showing interactions of AtIAA17 with each of five OsTIR1s (OsTIR1^{WT}, OsTIR1^{F74A}, OsTIR1^{F74G}, OsTIR1^{F74S} and OsTIR1^{F74C}) in the presence of various concentrations of AID inducers (IAA or 5-Ad-IAA) at 30°C.

To evaluate the suitability of AtTIR1^{F79A} and OsTIR1^{F74A} for our AID system in vertebrate cells, we generated AID-based conditional knockout chicken DT40 cell lines through the use of the single step method (Figure 1B and 1C; see details in later sections). By selecting an essential centromere protein (CENP-H) as a target protein for degradation, we generated CENP-H conditional knockout cell lines expressing either AtTIR1^{F79A} (AtTIR1^{F79A}-AID cell line) or OsTIR1^{F74A} (OsTIR1^{F74A}-AID cell line) (Supplementary Figure S2A). AID-tagged CENP-H was more efficiently degraded in the OsTIR1^{F74A}-AID cell line than it was in the AtTIR1^{F79A}-AID cell line (Supplementary Figure S2B). Consistent with this result, severe growth defects were observed in the OsTIR1^{F74A}-AID cell line, and not in the AtTIR1^{F79A}-AID cell line (Supplementary Figure S2C). Importantly, extremely low concentrations of 5-Ad-IAA (5–50 nM) were sufficient to promote the function of the AID system with OsTIR1^{F74A}, and these concentrations were much lower than the AID inducer concentrations used in the conventional AID system (100–500 μM IAA) (20). These results indicate that OsTIR1^{F74A} is more suitable than AtTIR1^{F79A} for the sensitive AID system with 5-Ad-IAA in vertebrate cells.

In addition to AtTIR1^{F79A}, both AtTIR1 ^{F79G} and AtTIR1 ^{F79S} are known to provide high affinity interactions with the AtIAA3 (an AtIAA17 homologue) fragment in the presence of auxin derivatives (21,26). We therefore performed a yeast two-hybrid assay to test OsTIR1s possessing amino acid substitutions (F74G, F74S, and F74C). These OsTIR1s interacted with AtIAA17 at 10 to 100 μM IAA and at 10–1000 pM with 5-Ad-IAA (Figure 2D). Among the OsTIR1s, OsTIR1^{F74A} interacted with AtIAA17 at the lowest concentration of 5-Ad-IAA. This result is consistent with a previous finding that AtTIR1^{F79A} exhibited a higher binding affinity to auxin derivatives than did AtTIR1^{F79G} and AtTIR1^{F79S} (21,26). Based on this, OsTIR1^{F74A} and 5-Ad-IAA would provide the best combination for the sensitive AID system (Figure 2A, right).

Evaluation of the combination of OsTIR1^{F74A} and 5-Ad-IAA for target degradation in AID system

To evaluate the improved AID system using OsTIR1^{F74A} and 5-Ad-IAA in chicken DT40 cells, we further employed a luciferase reporter assay for its highly sensitive and quantitative detection of target protein degradation by measuring the luciferase activity. We generated chicken DT40 cell lines that expressed OsTIR1 (OsTIR1^{WT}, OsTIR1^{F74A}, or OsTIR1^{F74G}) and luciferase that was tagged with a minimal AID-tag derived from AtIAA17 (27,28) (Figure 3A). The AID-tagged luciferase was degraded in the OsTIR1^{F74A}-cell lines at 50 nM 5-Ad-IAA concentrations (Figure 3B). In contrast, much higher concentrations of AID inducers were required to degrade the luciferase protein in the OsTIR1^{WT}- and OsTIR1^{F74G}-cell lines (Figure 3B). The luciferase activities in cells expressing OsTIR1^{F74A} were rapidly lost in the presence of 5-Ad-IAA (5 μM), and this result was similar to the result from cells expressing OsTIR1^{WT} when treated with IAA (500 μM) (Figure 3C). These results indicate that extremely low concentrations of 5-Ad-IAA can facilitate the degradation of target proteins in DT40 cells expressing OsTIR1^{F74A}. Therefore, we concluded that the combination of OsTIR1^{F74A} and 5-Ad-IAA allows for the most efficient function of the improved AID system that is highly sensitive in DT40 cells.

The ability of OsTIR1^{F74A} to tightly bind to 5-Ad-IAA results in the efficient depletion of target proteins in AID-based conditional knockout DT40 cell lines

Using the highly sensitive AID system, we can generate AID-based conditional knockout DT40 cell lines with loss of interesting essential genes by combining our system with the single-step method we previously developed (24) (Figure 1B and 1C). The single-step method simultaneously accomplishes three processes that include i) target gene disruption, ii) TIR1 protein expression, and iii) AID-tagged target protein expression (Figure 1B). In the single-step method, we combined CRISPR/Cas9-based gene knockout (22,30) with integration of an AID plasmid (pAID) to express both OsTIR1 and an AID-tagged target protein. Expression of the AID-tagged target protein compensates for disruption of the target gene expression that is caused by either insertion of the pAID plasmid or an internal deletion/addition. It was previously shown that the efficiency of direct insertion of pAID plasmid in to the chicken *CENP-H* locus was ∼75% and that the rate of internal insertion/deletion in the target locus was ∼25% (24).

To determine which modification of OsTIR1 is optimal for use in AID-based conditional knockout DT40 cell lines, we generated four CENP-H conditional knockout cell lines expressing either OsTIR1^{F74A}, OsTIR1^{F74G}, OsTIR1^{F74S} or OsTIR1^{F74C} and the mAID-tagged CENP-H (mScarlet-mAID-CENP-H) as the target protein (Figure 4A). Immunoblot analyses revealed that in each AID-based conditional knockout cell line, the AID-tagged CENP-H protein was degraded by 5-Ad-IAA in a dose-dependent manner (Figure 4B). Degradation of the AID-tagged CENP-H was more efficient in the OsTIR1^{F74A}- and OsTIR1^{F74S}-AID cell lines than in the other AID cell lines (Figure 4B). CENP-H is essential for cell growth (29) and based on this, we evaluated the growth curves and survival rates of the AID-based conditional knockout cell lines. We examined growth defects in four AID cell lines after treating these cells with 5-Ad-IAA (Figure 4C). Consistent with the levels of protein degradation, the OsTIR1^{F74A}-AID cell line exhibited the most severe growth defects, even at 0.5 μM 5-Ad-IAA (Figure 4C). These results demonstrate that the degradation profile of AID-tagged CENP-H is positively correlated with the growth defect phenotype.

We also generated AID-based conditional knockout DT40 cell lines using either OsTIR1^{F74G} or OsTIR1^{F74A}, and each of these cell lines expressed full-length-AtIAA17-tagged CENP-H (CENP-H-IAA17). Consistent with the luciferase assay shown in Figure 3, immunoblot analyses revealed that CENP-H-IAA17 was degraded at concentrations as low as 5 nM 5-Ad-IAA in the OsTIR1^{F74A}-AID cell line but not in the OsTIR1^{F74G}-AID cell line (Supplementary Figure S3A). Survival rates indicated that the OsTIR1^{F74A}-AID cells were 100-fold more sensitive to 5-Ad-IAA than were the OsTIR1^{F74G}-AID cells (Supplementary Figure S3B and S3C). The CENP-H-IAA17 degrada-

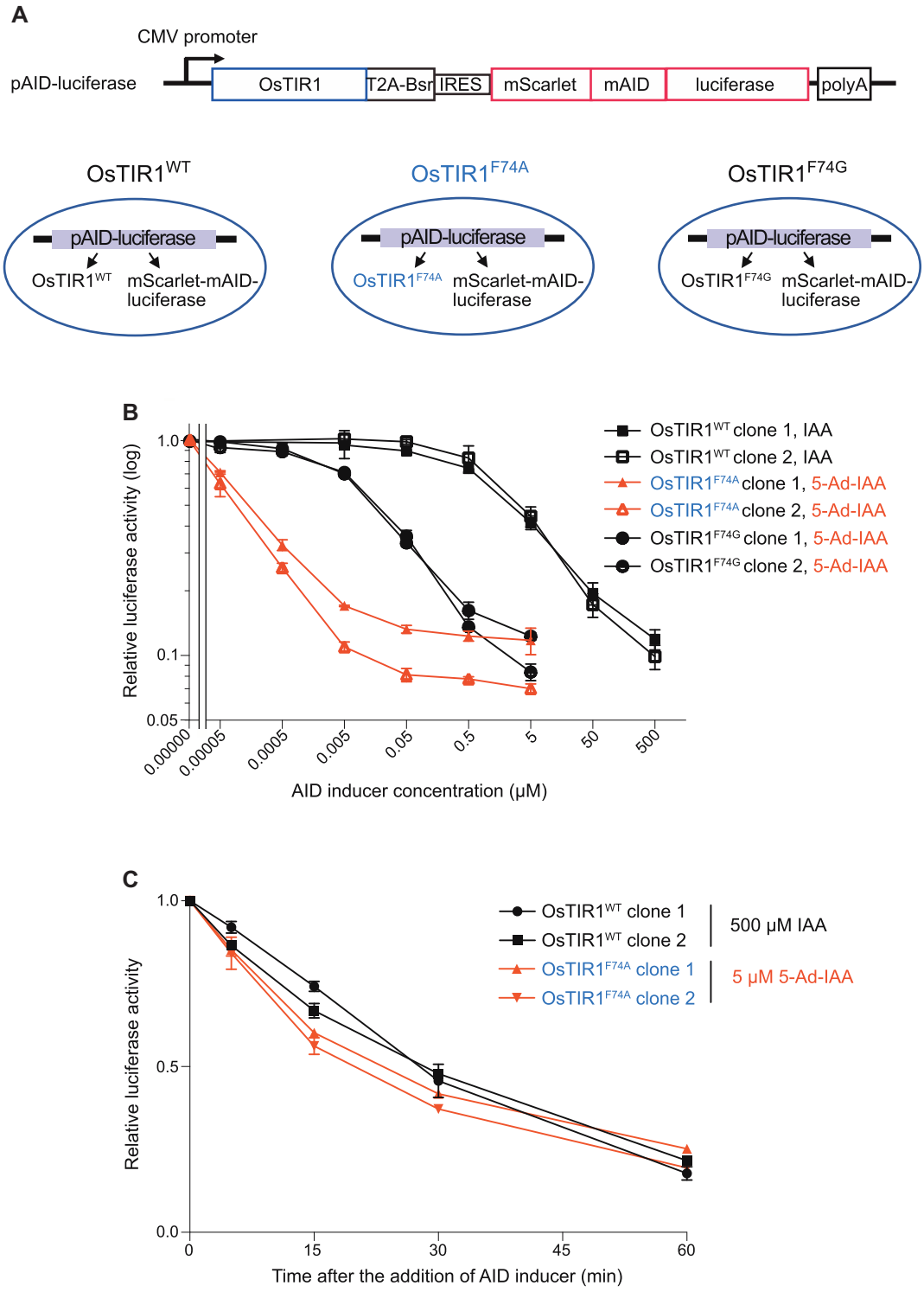

Figure 3. Efficient degradation of AID-tagged luciferase in DT40 cells expressing OsTIR1^{F74A} in the presence of 5-Ad-IAA. (**A**) Schematic illustrations of a plasmid map of pAID-luciferase and AID cell lines expressing OsTIR1^{WT}, OsTIR1^{F74A} or OsTIR1^{F74G} to evaluate the degradation of AID-tagged luciferase. (**B**) AID-inducer-dependent reduction of luciferase activities in various AID cell lines shown in (A). 5-Ad-IAA was used in cell lines expressing OsTIR1^{F74A} or OsTIR1^{F74G} and IAA was used in cells expressing OsTIR1^{WT} as AID-inducers. Luciferase activities were measured at 3 h after adding various concentrations of AID inducers. Relative luciferase activities were shown with a log scale. Two independent clones were examined. Error bars indicate the standard deviation of three independent experiments. (**C**) Time-dependent reduction of luciferase activities in each AID-cell line. 5-Ad-IAA (5 μM) was used in cells expressing OsTIR1^{F74A} and IAA (500 μM) was used in cells expressing OsTIR1^{WT}. Two independent clones were examined. Error bars indicate the standard deviation of three independent experiments.

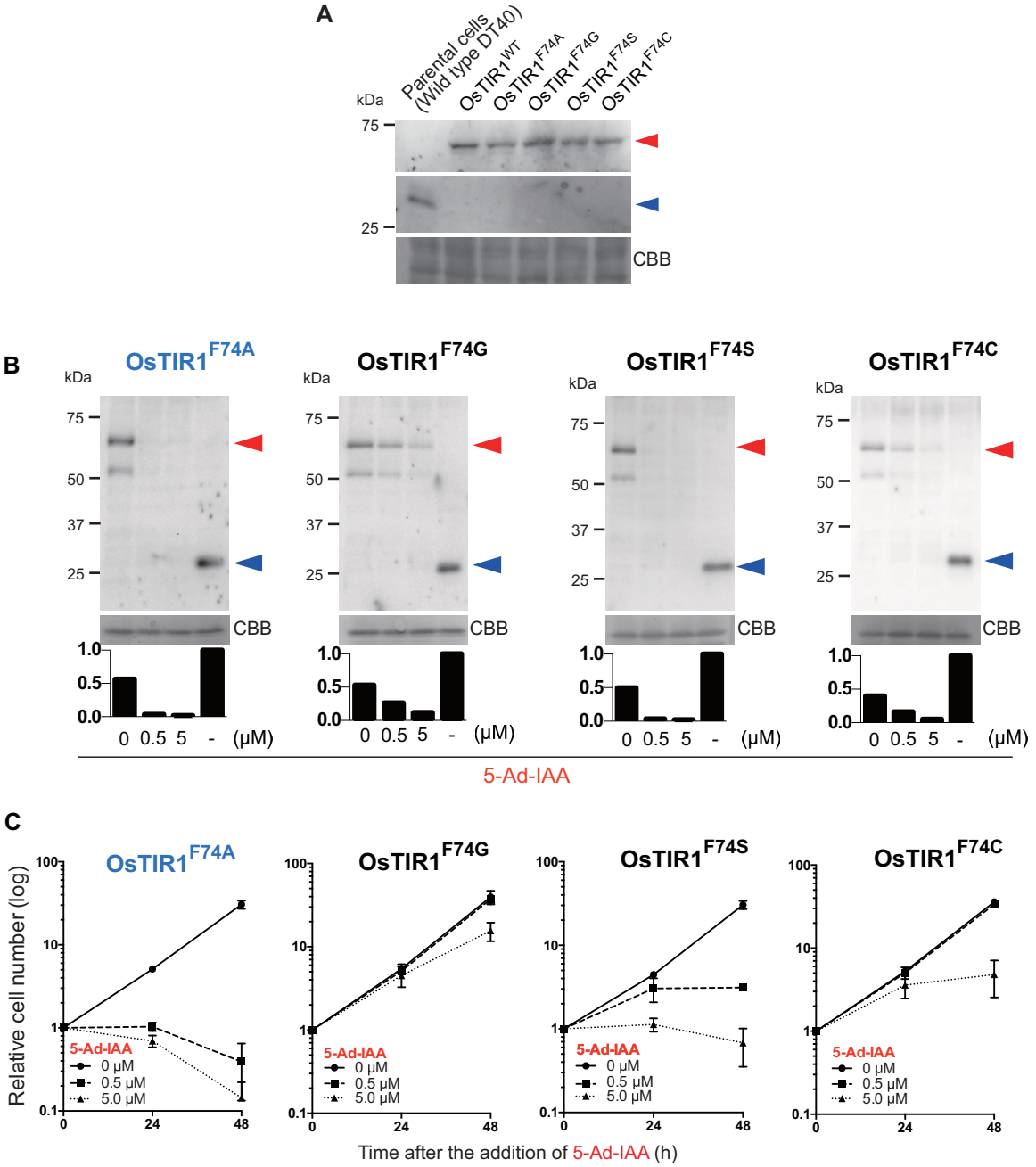

Figure 4. OsTIR1^{F74A} induces efficient degradation of an essential CENP-H protein in AID-based conditional knockout DT40 cell lines and this results in severe cell growth defects. (**A**) Immunoblots for endogenous CENP-H in parental DT40 cells (blue arrowhead) and AID-tagged CENP-H in five AID-based conditional knockout DT40 cell lines (red arrowhead) in the absence of the AID inducer. AID cell lines lacked the endogenous CENP-H and expressed both AID-tagged CENP-H and each of the OsTIR1s (OsTIR1^{WT}, OsTIR1^{F74A}, OsTIR1^{F74G}, OsTIR1^{F74S} and OsTIR1^{F74C}). (**B**) Immunoblots for evaluating the degradation of AID-tagged CENP-H (red arrowheads) in four AID-based conditional knockout DT40 cell lines at 3 h after the addition of various concentrations of 5-Ad-IAA. Endogenous CENP-H levels in parental DT40 cells are also shown (blue arrowheads). Relative signal intensities of CENP-H on the immunoblots are graphically shown. (**C**) Growth curves of four AID-based conditional knockout cell lines after the addition of various concentrations of 5-Ad-IAA. Error bars indicate the standard deviation of three independent experiments.

tion was correlated with the survival rates of the AID conditional knockout DT40 cells. Taken together, these results indicate that OsTIR1^{F74A} functions most efficiently in 5-Ad-IAA-dependent degradation of a target protein.

We also evaluated two other auxin derivatives, 5-(3-MeOPh)-IAA (cvxIAA) and 5-Phenyl-IAA (5-Ph-IAA), as AID inducers (Figure 5A). These auxin derivatives and 5-Ad-IAA were applied to an OsTIR1^{F74A}-AID conditional knockout DT40 cell line expressing mScarlet-mAID-CENP-H. Compared to cvxIAA and 5-Ph-IAA, 5-Ad-IAA was more effective for the degradation of AID-tagged CENP-H (Figure 5B) and for inhibiting the growth of the OsTIR1^{F74A}-AID cell line (Figure 5C). The effect of these auxin derivatives was examined regarding viabilities of OsTIR1^{F74G}-AID cells. Although 5-Ad-IAA was also the most effective AID inducer in the OsTIR1^{F74G}-AID cell line (Supplementary Figure S4A, lower), OsTIR1^{F74G}-AID cells were 100-fold less sensitive to 5-Ad-IAA than were OsTIR1^{F74A}-AID cells (Supplementary Figure S4). The effective concentration of 5-Ad-IAA in the OsTIR1^{F74A}-AID cell line was 1000-fold lower than that of IAA in the OsTIR1^{WT}-AID cell line using the conventional AID system (Figure 5D). Hence, the combination of OsTIR1^{F74A} and 5-Ad-IAA was the most efficient to promote the function of the super-sensitive AID system, where 0.5 μM 5-Ad-IAA was sufficient for CENP-H depletion in the AID-based conditional knockout chicken DT40 cells.

Application of the super-sensitive AID system to other proteins in chicken DT40 cells

To demonstrate the versatility of the super-sensitive AID system incorporating OsTIR1^{F74A} and 5-Ad-IAA, we examined two additional target proteins that included the centromeric proteins CENP-A and CENP-T that are essential for cell growth. Using the single-step method, we generated AID-based conditional knockout DT40 cell lines expressing OsTIR1^{F74A} and each of the fluorescent target proteins fused with a mAID-tag (mScarlet-mAID-CENP-A and mScarlet-mAID-CENP-T). Immunoblot analyses revealed that all of the target proteins (CENP-A, CENP-T, and CENP-H) were undetectable within 2 h after the addition of 5.0 μM 5-Ad-IAA; however, they could still be detected after the addition of 5.0 μM IAA (Figure 6A and Supplementary Figure S5A). The degradation of these proteins was also confirmed by fluorescence microscopy. mScarlet fluorescence signals from the target proteins became undetectable within the nuclei at 3 h following the addition of 5.0 μM 5-Ad-IAA, while these signals remained detectable at 3 h after the addition of 5.0 μM IAA (Figure 6B).

To evaluate the phenotypes of the AID-based conditional knockout DT40 cells, we monitored the survival rates. The survival rates of all the AID cell lines decreased in a dose-dependent manner in response to increasing concentration of the AID inducer (5-Ad-IAA). The concentrations of 5-Ad-IAA that were sufficient to induce complete cell death were 0.05 μM for CENP-A, 0.5 μM for CENP-H and 0.5 μM for CENP-T (Figure 6C). To compare the super-sensitive and conventional AID systems, we generated OsTIR1^{WT}-AID-based conditional knockout cell lines expressing CENP-H, CENP-A or CENP-T by using the single-step method. In these cell lines, the concentrations of IAA required for complete cell death were 50 μM for CENP-A, 500 μM for CENP-H, and 500 μM for CENP-T, respectively (Figure 6C). These results indicate a sufficient concentration of an AID inducer in the super-sensitive AID system is 1000-fold lower than that of the conventional AID system in chicken DT40 cells. We also compared the degradation profiles of AID-tagged CENP proteins in the super-sensitive and conventional AID systems. In the super-sensitive AID system with OsTIR1^{F74A}, AID-tagged CENP proteins were degraded at as low as 0.05 μM 5-Ad-IAA concentrations (Figure 6D). In contrast, AID-tagged CENP protein levels at 5 μM IAA were still comparable to those in the absence of IAA in the conventional AID system (Figure 6D). Taken together, these results indicate that the AID system with OsTIR1^{F74A} and 5-Ad-IAA is a super-sensitive system that is applicable for use on various target proteins in chicken DT40 cells.

Application of the super-sensitive AID system coupled with the single-step method to various mammalian cell lines

The super-sensitive AID system combined with the single-step method could also be applied to mammalian cells, and we tested this system on four human cultured cell lines (HeLa, HT1080, U2OS and RPE1) and a mouse embryonic stem (ES) cell line. We created a pAID plasmid possessing both a CENP-H cDNA and an OsTIR1^{F74A}-expression cassette, and we transfected the parental cells simultaneously with the pAID plasmid and the CRISPR/Cas9-based-CENP-H-targeting plasmid (Figure 1C), and then isolated positive single colonies (Figure 1B). Immunoblot analyses revealed that these isolated cell lines depleted endogenous CENP-H and expressed mAID-tagged CENP-H (Figure 7A, 0 h). Based on this method, we succeeded in establishing AID-based conditional knockout cell lines for mouse ES cells and various human cancer cells that possess multiple sets of chromosomes.

In each of the AID cell lines, mScarlet-mAID-CENP-H was degraded at 2–4 h following the addition of 5.0 μM 5-Ad-IAA, but not after the addition of 5.0 μM IAA (Figure 7A and Supplementary Figure S5B). The degradation was also confirmed by fluorescence microscopy. mScarlet fluorescence signals from the target protein (mScarlet-mAID-CENP-H) became undetectable in nuclei at 3 h after the addition of 5.0 μM 5-Ad-IAA; however, these signals remained detectable at 3 h after the addition of 5.0 μM IAA (Figure 7B). Consistent with the degradation profiles of CENP-H, these cell lines did not form any colonies in the presence of 5.0 μM 5-Ad-IAA; however, they did form colonies in the presence of 5.0 μM IAA (Figure 7C).

Next, we examined the effective concentration of the AID inducer 5-Ad-IAA using colony formation assays and immunoblots (Figure 8). The concentrations of 5-Ad-IAA that were required to prevent colony formation were 5 nM for HeLa, HT1080 and RPE1 cells, 50 nM for U2OS cells, and 500 nM for mouse ES cells (Figure 8A). Immunoblots revealed that the target protein CENP-H was degraded by 5-Ad-IAA in a dose-dependent manner in each cell line (Figure 8B), and this is consistent with the observed re-

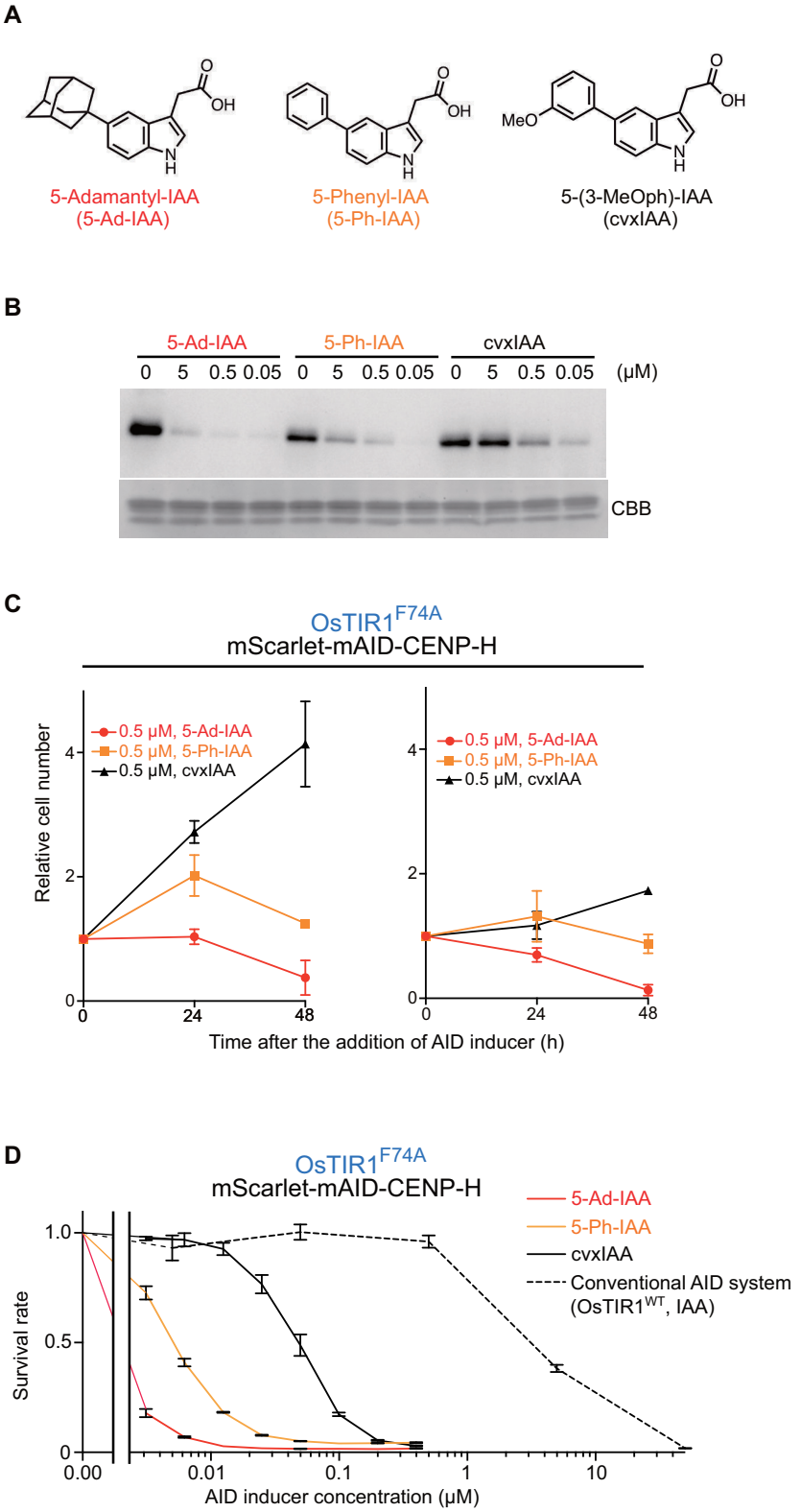

Figure 5. The auxin derivative 5-Ad-IAA induces efficient degradation of AID-tagged CENP-H in AID-based conditional knockout DT40 cell lines, and this results in severe cell growth defects. (**A**) Chemical structures of three auxin derivatives (5-Ad-IAA, 5-Ph-IAA, and cvxIAA). (**B**) Immunoblots for evaluating the degradation of AID-tagged CENP-H in an AID-based conditional knockout cell line expressing OsTIR1^{F74A} at 4 h after the addition of various concentrations of three AID inducers (5-Ad-IAA, 5-Ph-IAA, and cvxIAA). (**C**) Growth curves of the OsTIR1^{F74A}-AID cell line after the addition of 0.5 or 5.0 μM AID inducers (5-Ad-IAA, 5-Ph-IAA, and cvxIAA). Error bars indicate the standard deviation of three independent experiments. (**D**) Survival rates of the OsTIR1^{F74A}-AID cell line at 48 h after the addition of various concentrations of AID inducers (5-Ad-IAA, 5-Ph-IAA and cvxIAA). A broken line shows a control OsTIR1^{WT}-AID cell line (see C). Error bars indicate the standard deviation of three independent experiments.

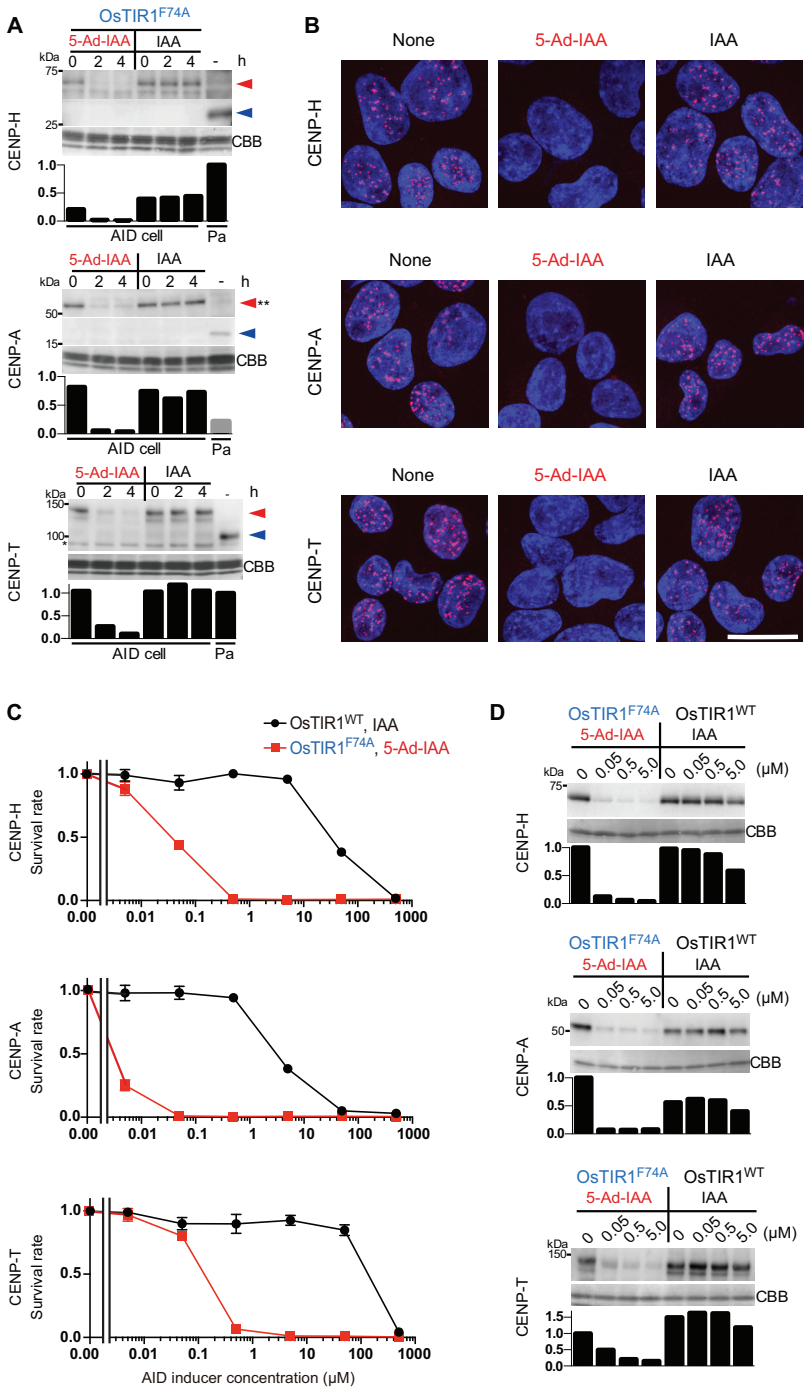

Figure 6. AID-based conditional knockout DT40 cell lines expressing OsTIR1^{F74A} exhibit 5-Ad-IAA-dependent degradation of target proteins in association with their biological phenotypes. (**A**) Immunoblots for evaluating the degradation of mScarlet-mAID-tagged CENP proteins (CENP-H, CENP-A and CENP-T; red arrowheads) in OsTIR1^{F74A}-AID cell lines at 0, 2 and 4 h after the addition of 5.0 μM AID inducers (5-Ad-IAA and IAA). Endogenous CENP proteins (blue arrowheads) in parental DT40 cells are also shown (Pa). Relative signal intensities of CENP proteins on the immunoblots are graphically shown. Note that AID-tagged CENP-A was detected with anti-mAID antibody (**), as it cannot be recognized by anti-chicken CENP-A antibody. This makes it impossible to compare their signal intensities. The uncropped gel images are shown in Supplementary Figure S5A. Black asterisks indicate non-specific signals. **(B)** Fluorescent images of mScarlet-mAID-tagged CENP proteins (CENP-H, CENP-A, and CENP-T) in OsTIR1^{F74A}-AID cells at 3 h after the addition of 5.0 μM AID inducers (5-Ad-IAA and IAA) or the solvent dimethyl sulfoxide (DMSO, none). Red fluorescence, mScarlet signals; and blue fluorescence, nuclei stained with DAPI. Scale bar, 20 μm. **(C)** Comparison between the super-sensitive AID system (OsTIR1^{F74A} and 5-Ad-IAA) and the conventional AID system (OsTIR1^{WT} and IAA). Survival rates of AID-based conditional knockout cell lines expressing AID-tagged CENP proteins (CENP-H, CENP-A, and CENP-T) were determined at 48 h after the addition of various concentrations of IAA or 5-Ad-IAA. Error bars indicate the standard deviation of three independent experiments. (**D**) Immunoblots for evaluating the degradation of AID-tagged CENP-H, -A, and -T proteins at 3 h after the addition of various concentrations of 5-Ad-IAA to AID cell lines expressing OsTIR1^{F74A} or IAA to AID cell lines expressing OsTIR1^{WT}. Relative signal intensities of CENP proteins on the immunoblots are graphically shown.

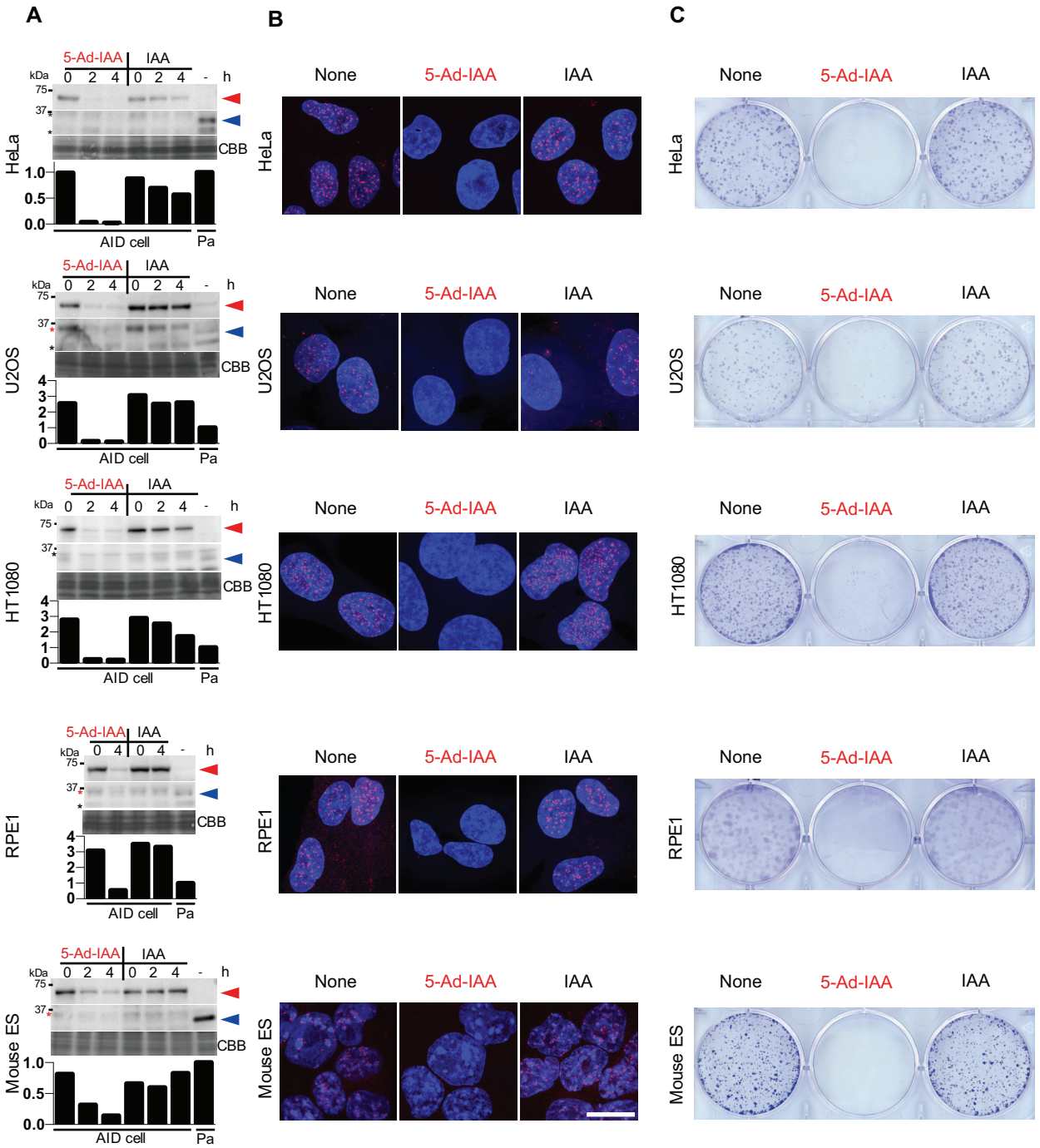

Figure 7. Mammalian AID-based CENP-H conditional knockout cell lines expressing OsTIR1^{F74A} showing 5-Ad-IAA-dependent degradation of target proteins in association with their biological phenotypes. (**A**) Immunoblots for evaluating the degradation of mScarlet-mAID-tagged CENP-H (red arrowheads) at 0, 2, and 4 h after the addition of 5.0 μM AID inducers (5-Ad-IAA or IAA) in five mammalian OsTIR1^{F74A}-AID cell lines (Hela, U2OS, HT1080, RPE1, and mouse ES cells). Endogenous CENP-H (blue arrowheads) levels in parental cells are also shown (Pa). Relative signal intensities of CENP-H on the immunoblots are graphically shown. The uncropped gel images are shown in Supplementary Figure S5B. Red asterisks indicate degradation products of AID-tagged CENP-H, and black asterisks indicate non-specific signals. (**B**) Fluorescent images of mScarlet-mAID-tagged CENP-H in OsTIR1^{F74A}-AID cells at 3 h after the addition of 5.0 μM AID inducers (5-Ad-IAA and IAA) or the solvent DMSO (None). Red fluorescence indicates mScarlet signals; and blue fluorescence indicates nuclei stained with DAPI. Scale bar, 20 μm. (**C**) Colony formation assay of the OsTIR1^{F74A}-AID cells that were cultured in the presence of 5.0 μM AID inducers (5-Ad-IAA and IAA) or DMSO (None). Colonies were stained with crystal violet.

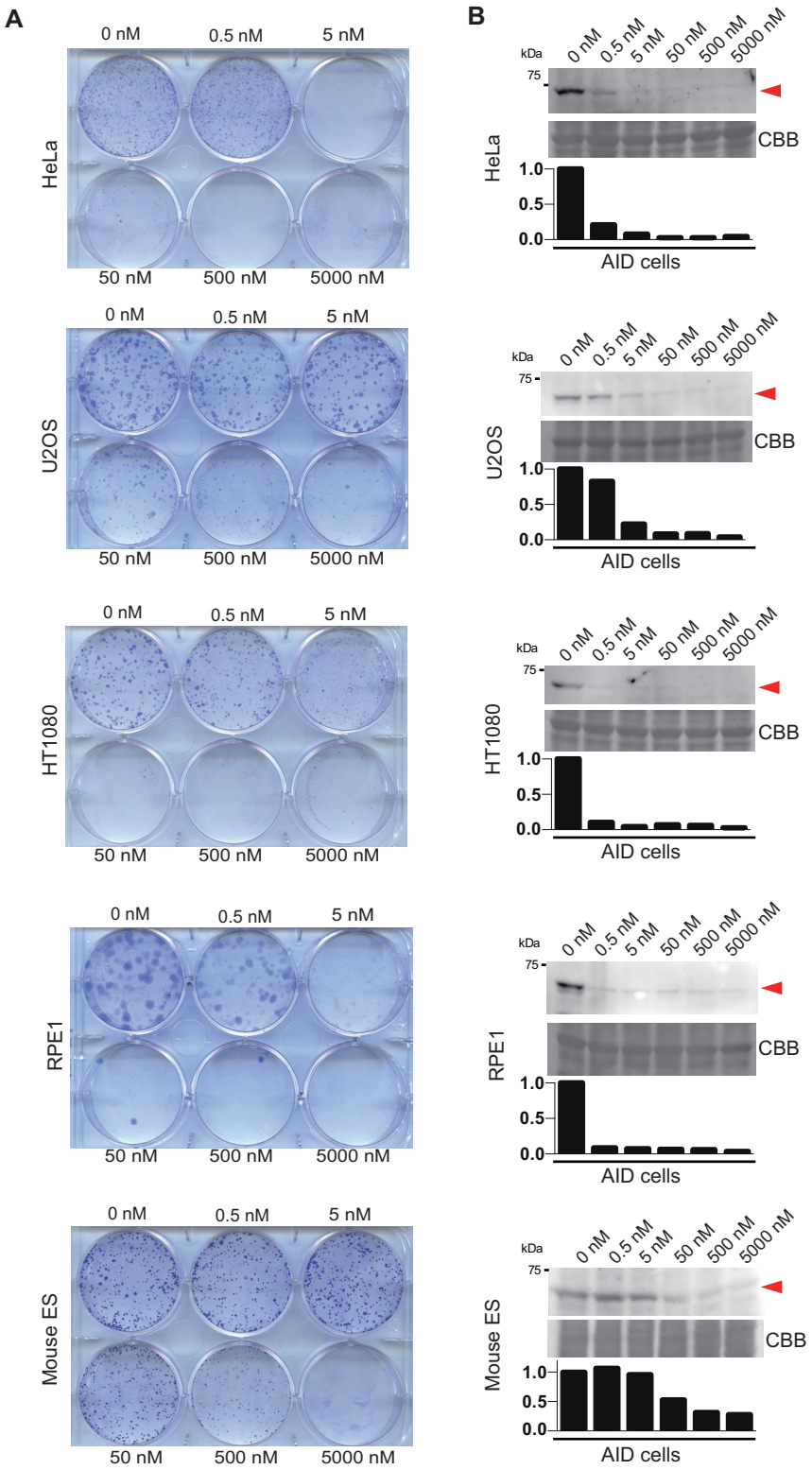

Figure 8. Mammalian AID-based CENP-H conditional knockout cell lines expressing OsTIR1^{F74A} exhibit efficient degradation of target proteins in low amounts of 5-Ad-IAA. (**A**) Colony formation assay of five mammalian AID-based CENP-H conditional knockout cell lines expressing OsTIR1^{F74A}-AID (Hela, U2OS, HT1080, RPE1, and mouse ES cells) that were cultured in the presence of various concentrations (0–5000 nM) of 5-Ad-IAA. Colonies were stained with crystal violet. (**B**) Immunoblots for evaluating the degradation of mScarlet-mAID-tagged CENP-H (red arrowheads) in five mammalian AID-based CENP-H conditional knockout cell lines expressing OsTIR1^{F74A} at 3 h after the addition of various concentrations of 5-Ad-IAA. Relative signal intensities of CENP-H on the immunoblots are graphically shown.

duction in cell viabilities (Figure 7A). All of the parental cells (HeLa, HT1080, U2OS, RPE1 and mouse ES) exibited no growth defects even at 5 μM of 5-Ad-IAA concentrations (Supplementary Figure S6), indicating that the working concentrations of 5-Ad-IAA are not cytotoxic.

We also used the single-step method to generate AID-based conditional knockout HeLa S3 cells that expressed each of CENP-A, CENP-H, and CENP-C as target proteins (Supplementary Figure S7). Immunoblot analyses and fluorescence microscopy revealed that all the target proteins became undetectable within 4 h after the addition of 5.0 μM 5-Ad-IAA, but not after the addition of 5.0 μM IAA (Supplementary Figure S7A and S7B). Consistent with their degradation profiles, no colonies were formed in any of the AID cell lines in the presence of 5-Ad-IAA (Supplementary Figure S7C), similar to observations in the other mammalian cells (Figure 7C). These results demonstrate that the super-sensitive AID system in combination with the single-step method can be applied to various target proteins in mammalian cell lines.

DISCUSSION

In this study, we established a super-sensitive AID system incorporation a combination of the synthetic auxin 5-Ad-IAA and the high-affinity-binding partner OsTIR1^{F74A}. Our improved method for generation of AID-based conditional knockout cell lines includes the super-sensitive AID system and the single-step method and possesses three advantages over the conventional method that include (i) extreme sensitivity to the AID inducer to enable a greater than 1000-fold reduction in the AID inducer concentrations, (ii) technical simplicity to enable a single-step generation of an AID-based conditional knockout cell line by simultaneously disrupting the target gene and expressing both the AID-tagged target protein and OsTIR1^{F74A}, and (iii) applicability to a wide range of vertebrate cell lines, including commonly-used cancer cells that have multiple sets of chromosomes.

A great advantage of the single-step method is its simplicity. All that is required is the construction of two plasmids, including pAID that possesses a cDNA of a target gene and the pX330 CRISPR/Cas9 plasmid for disruption of the target gene. In the single-step method, the resultant clones could exhibit clonal variations depending on the insertion site and copy number of the pAID plasmid. Therefore, in contrast to the easy selection of conditional knockout clones of essential genes according to their viability, it can be difficult to select conditional knockout clones of non-essential genes where their phenotypes are properly complemented.

Additionally, in this method, AID-tagged target proteins are expressed under the control of the internal ribosomal entry site (IRES), and this results in a variety of expression levels of target proteins within the clones. Thus, it is possible to use immunoblot analysis to select the clones with expression levels that are comparable to the expression levels of endogenous proteins. Conversely, higher expression levels might provide an advantage for the AID system as follows. The conventional AID system occasionally causes IAA-independent degradation of target proteins (basal degradation) due to their low expression levels (11,28,31–33). This makes it difficult to establish conditional knockout cell lines for essential genes. The single-step method overcomes this problem based on the ability of basal degradation (if there is any) to be compensated for by the high expression levels of target proteins.

Although we found that OsTIR1^{F74A} provides the best combination with 5-Ad-IAA in our super-sensitive AID system, another study (M. Kanemaki, personal communication) suggests that OsTIR1^{F74A} and OsTIR1^{F74G} exert similar effects on AID degradation. As the expression levels of target proteins varied in our system, it is possible that OsTIR1^{F74A} and OsTIR1^{F74G} may have similar effects in other systems. However, OsTIR1^{F74A} always exhibited better effects regarding protein degradation in the clones we used in the present study.

In cancer cell lines, genome rearrangements frequently occur and result in abnormal chromosome numbers (aneuploidy) (34). In the conventional AID system, AID-tags must be added to all of the target genes, and this is typically accomplished through homologous recombination (Figure 1A), a process that is often difficult in cancer cells. In contrast, the single-step method is able to disrupt multiple target genes through CRISPR/Cas9-based gene targeting (Figure 1B) and to generate AID-based conditional knockout cells lines in cancer cells.

Based on these various advantages, our improved method including the super-sensitive AID system and the single-step method will provide a powerful tool that can be used to elucidate the molecular mechanisms underlying a variety of biological processes.

DATA AVAILABILITY

All data supporting the findings in this study are available from the corresponding author upon reasonable request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful; to Tomomi Tsubouchi (NIBB) for providing mouse embryonic stem cell (E14tg2a strain); to Yasuhiro Kazuki (Tottori University) for providing HT1080 cell line; to Hiroshi Kimura (Tokyo Tech) for providing HeLa S3 cell lines; to Masato Kanemaki (NIG) for sharing unpublished manuscript and for providing a plasmid containing codon optimized OsTIR1 gene; to Yuko Fukagawa (Osaka University) and Reika Fukuoka (Osaka University) for their technical supports.

FUNDING

SPS KAKENHI [JP17K15041, JP19K06611, JP20K21423 to K.N., JP20H04883, JP20H05409 to N.U., JP17K07443 to K.T., JP19H04957, JP20H03208, JP20K21423 to T.K., JP17H06476 and JP19H00990 to K.U.T., JP17H06167, JP16H06279, JP15H05972 to T.F.] from the Japan Society for the Promotion of Science (JSPS); Hori Sciences

And Arts Foundation (to K.N.). Funding for open access charge: Japan Society for the Promotion of Science (JSPS) [JP17H06167 to T.F.].
Conflict of interest statement. None declared.

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