

An auxin-based degron system for the rapid depletion of proteins in nonplant cells

Kohei Nishimura¹, Tatsuo Fukagawa², Haruhiko Takisawa¹, Tatsuo Kakimoto¹ & Masato Kanemaki¹

Plants have evolved a unique system in which the plant hormone auxin directly induces rapid degradation of the AUX/IAA family of transcription repressors by a specific form of the SCF E3 ubiquitin ligase. Other eukaryotes lack the auxin response but share the SCF degradation pathway, allowing us to transplant the auxin-inducible degron (AID) system into nonplant cells and use a small molecule to conditionally control protein stability. The AID system allowed rapid and reversible degradation of target proteins in response to auxin and enabled us to generate efficient conditional mutants of essential proteins in yeast as well as cell lines derived from chicken, mouse, hamster, monkey and human cells, thus offering a powerful tool to control protein expression and study protein function.

Studies of protein function *in vivo* are greatly helped by systems that allow conditional inactivation or depletion of the protein of interest. Ideally, inactivation of the target protein would be rapid and efficient so that the immediate consequences can be assessed before the phenotype is complicated by the accumulation of secondary effects. Reversibility of inactivation would be an additional advantage. In practice, these features are hard to achieve in most eukaryotic species, unless a small-molecule inhibitor has already been developed for the target protein.

In many species including mammalian cells, protein expression can be controlled at the DNA or mRNA levels^{1–3}. In these systems, however, protein depletion is indirect and thus depends on the stability of the target protein itself. In many cases this is a major limitation to the analysis of protein function. In addition, gene deletion is not reversible, and RNAi systems can suffer from off-target effects and leakiness of silencing.

Alternatively, the coding sequence of the gene can be altered so that the protein is modulated directly. By fusing the target protein with the binding domain of steroid hormones or rapamycin, systems to modulate protein localization or function have been developed^{4,5}. These systems have been used to generate cell lines and transgenic organisms⁶ but only work for certain classes of proteins, such as those involved in macromolecular interactions or proteins localized in the nucleus.

Other systems aim to deplete a target protein directly by exploiting specific protein degradation pathways^{7,8}. Some of these have been achieved by modulating ubiquitin ligases such as SCF (Skp1, Cullin and F-box) complexes^{9–11}. An advantage of these systems is that endogenous proteins can be rapidly controlled as long as a specific domain or small molecule that interacts with the target protein is known^{12,13}. As a more general approach to protein knockdown, a domain to induce degradation (known as a ‘degron’) can be fused to the protein of interest. The temperature-sensitive degron system, using a degradation pathway based on the N-end rule^{14,15}, has been used to characterize many essential proteins in budding yeast^{16,17}. This approach is, however, of limited use in mammalian cells that are less resistant to changes in temperature, although a recent study described its application to chicken DT40 cells¹⁸. Another method involves fusion with the destabilizing domain derived from FKBP12 (ddFKBP)¹⁹. This method has been used to control protein expression in mammalian cells, their parasites and transgenic organisms^{20–22}. We compare current degradation-based methods in **Supplementary Table 1**.

Here we describe an alternative system for rapid protein depletion, based on transplanting the auxin-dependent degradation pathway from plants into other eukaryotic species, which we call the auxin-inducible degron (AID) system. This system allowed us to induce rapid depletion of a protein of interest (within 30 min) in the presence of auxin, and the reaction was reversible and tunable. This system worked in budding yeast as well as in cell lines derived from chicken, mouse, hamster, monkey and human, implying that it could be applicable to most eukaryotes, except plant species.

RESULTS

Principles of the auxin-inducible degron system

Auxin represents a family of plant hormones that control gene expression during many aspects of growth and development²³. Auxin family hormones, such as indole-3-acetic acid (IAA; a natural auxin) and 1-naphthaleneacetic acid (NAA; a synthetic auxin), bind to the F-box transport inhibitor response 1 (TIR1) protein^{24,25} and promote the interaction of the E3 ubiquitin ligase SCF-TIR1 (a form of SCF complex containing TIR1)

¹Department of Biological Sciences, Graduate School of Science, Osaka University, Osaka, Japan. ²Department of Molecular Genetics, National Institute of Genetics and The Graduate University of Advanced Studies, Shizuoka, Japan. Correspondence should be addressed to M.K. (mkanemaki@bio.sci.osaka-u.ac.jp).

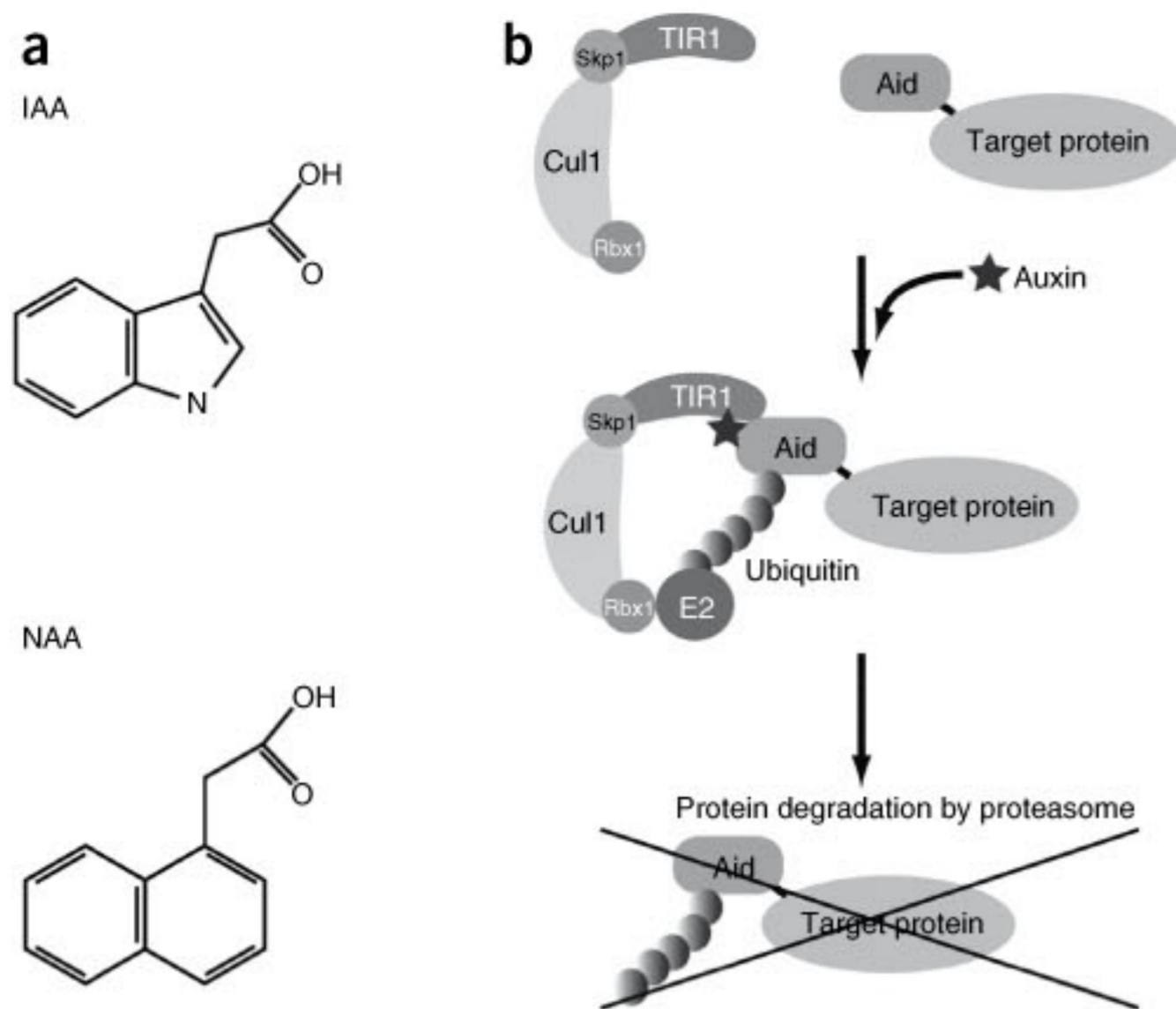


Figure 1 | Schematic illustration of the AID system. **(a)** Structure of indole-3-acetic acid (IAA) and 1-naphthaleneacetic acid (NAA). **(b)** Auxin binding to TIR1 promotes the interaction between TIR1 and the aid degron (aid) of the target protein. SCF-TIR1 acts as an E3 ubiquitin ligase to recruit an E2 ligase resulting in polyubiquitylation of the aid degron. Finally, the target is degraded by the proteasome.

and the auxin or IAA (AUX/IAA) transcription repressors^{26–28} (Fig. 1a and Supplementary Fig. 1a). SCF-TIR1 recruits an E2 ubiquitin conjugating enzyme that then polyubiquitylates AUX/IAAs resulting in rapid degradation of the latter by the proteasome²⁹. All eukaryotes have multiple forms of SCF in which an F-box protein determines substrate specificity, but orthologs of TIR1 and AUX/IAAs are only found in plant species. As a degron to induce degradation of a target protein, we chose IAA17 (also known as AXR3) of *Arabidopsis thaliana* owing to its short half-life (8–10 min) in seedlings³⁰. Our idea was to recreate the SCF-TIR1 complex in the cells of interest so that a target protein fused

to the IAA17 degron (hereafter called ‘aid’ degron) would be degraded in an auxin-dependent manner (Fig. 1b).

GFP fused to the aid degron is degraded in budding yeast

The F-box family of proteins bind via their F-box domain to the evolutionarily conserved Skp1 protein³¹. Because of the high conservation of Skp1 among all eukaryotes, especially at the C-terminus region to which the F-box domain binds (Supplementary Fig. 1b), we expected that SCF-TIR1 might be able to form using endogenous Skp1 even when TIR1 is ectopically expressed in cells of other eukaryotic species. We expressed the *Arabidopsis thaliana* TIR1 gene (*AtTIR1*) under the control of the galactose-inducible *GAL* promoter in the budding yeast *Saccharomyces cerevisiae*. We found that *AtTIR1* immunoprecipitated specifically with the budding yeast Cul1 homolog Cdc53, suggesting that the SCF-TIR1 E3 ubiquitin ligase could form in budding yeast (Fig. 2a). We confirmed that expression of *AtTIR1* and/or addition of 500 μ M NAA affects neither cell growth in medium containing various sugars nor sensitivity to DNA-damaging reagents (Supplementary Fig. 2).

Next we tested whether a protein fused to the aid degron would be depleted in an auxin- and TIR1-dependent manner. We fused the aid degron to the N terminus of GFP followed by an SV40 nuclear localization signal (aid-GFP-NLS) and expressed the fusion protein in cells with or without *GAL-AtTIR1* (Supplementary Fig. 3a). Notably, aid-GFP-NLS was undetectable within 30 min after addition of auxin to cells expressing *AtTIR1* (Fig. 2b,c). An alternative construct with the degron at the C terminus of GFP, GFP-aid-NLS, was also depleted in an *AtTIR1*- and auxin-dependent manner (Supplementary Fig. 3b,c), indicating that the degron works at either end of GFP. We estimated the amount of residual GFP-aid-NLS in cells after induced degradation to be less than 3% of that in cells before induction (Supplementary Fig. 3d). Furthermore, GFP-aid expressed in the cytoplasm could also be depleted (Supplementary Fig. 4). We also found that the AID system can be used to reversibly control expression of nuclear GFP reporter (Fig. 2d).

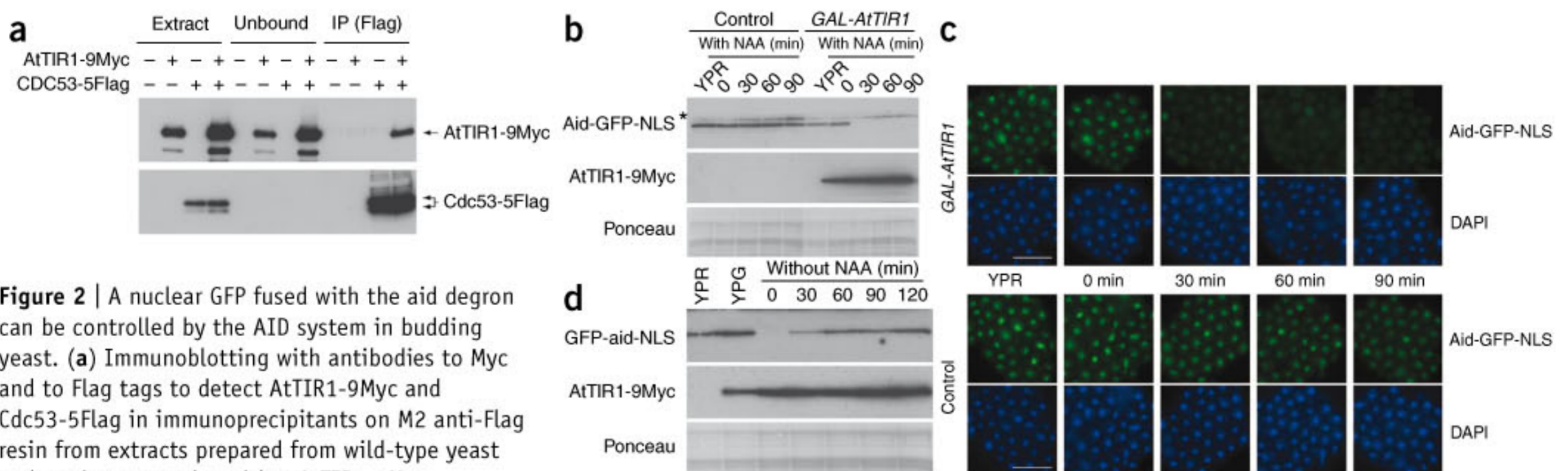


Figure 2 | A nuclear GFP fused with the aid degron can be controlled by the AID system in budding yeast. **(a)** Immunoblotting with antibodies to Myc and to Flag tags to detect *AtTIR1*-9Myc and *Cdc53*-5Flag in immunoprecipitants on M2 anti-Flag resin from extracts prepared from wild-type yeast and strains expressing either *AtTIR1*-9Myc, *Cdc53*-5Flag or both. **(b)** Aid-GFP-NLS and *AtTIR1*-9Myc detected by immunoblotting using antibodies to GFP and to Myc, respectively. Yeast strains expressing aid-GFP-NLS in control and *GAL-AtTIR1* backgrounds were initially grown in YP medium supplemented with raffinose (YPR), and the cells were transferred to YP medium with galactose (YPG) for 1 h before addition of 500 μ M NAA. Asterisk shows a background protein. Ponceau staining shows the loading control (bottom). **(c)** Fluorescence images of yeast expressing aid-GFP-NLS in control and *GAL-AtTIR1* backgrounds treated as described in **b**, fixed in 4% paraformaldehyde for 10 min and stained with 2 μ g ml⁻¹ DAPI nuclear stain. Scale bars, 10 μ m. **(d)** GFP-aid-NLS and *AtTIR1*-9Myc detected by immunoblotting using antibodies to GFP and to Myc, respectively. Cells expressing GFP-aid-NLS and *AtTIR1* were initially grown in YPR medium. After growing cells in YPG medium for 1 h to induce *AtTIR1*, 500 μ M NAA was added to deplete GFP-aid-NLS for another 1 h (0 min). Cells were washed and released in YPG medium without NAA and cells were collected at indicated time points for immunoblotting.

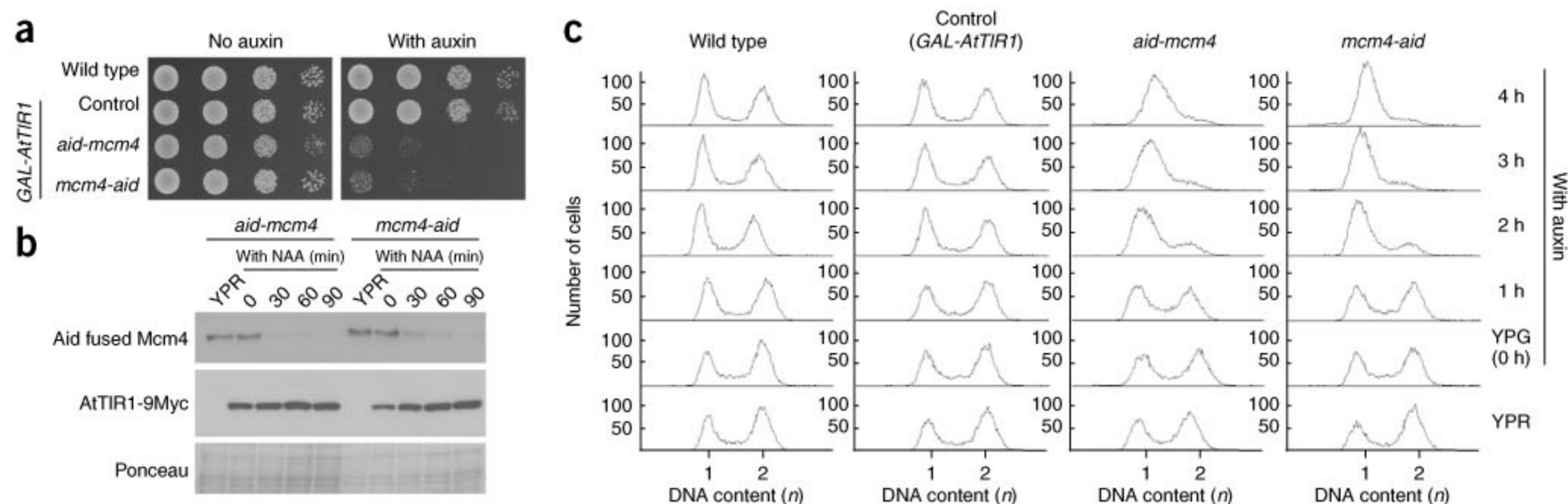


Figure 3 | Cell cycle-defective phenotype after Mcm4 depletion using the AID system. **(a)** Serial dilutions of wild type and control, *aid-mcm4* or *mcm4-aid* in the *GAL-AtTIR1* background on a YPG plate containing CuSO_4 with or without 500 μM NAA. Plates were incubated at 24 °C for 2 d. **(b)** Immunoblotting to detect aid-fused Mcm4 and AtTIR1-9Myc with antibodies to aid and to Myc, respectively. Yeast strains used in **a** were initially grown in YPRCu and then were transferred to YPGCu for 1 h. Cells were collected for analysis at indicated time points after addition of 500 μM NAA. Ponceau staining shows the loading control (bottom). **(c)** Flow cytometry analysis of *aid-mcm4* and *mcm4-aid* used in **b**, collected at indicated time points after addition of 500 μM NAA.

The AID system allows essential yeast protein inactivation

We next tested whether the AID system could be used to construct tight conditional mutants for essential proteins in budding yeast. Previous work showed that the temperature-sensitive degron could be used to generate very tight alleles of the MCM2-7 (minichromosome maintenance) proteins, a hexameric DNA helicase essential for both initiation and elongation steps of DNA replication¹⁵. We modified the endogenous *MCM4* gene to introduce the aid degron either at the N or C terminus of the encoded protein, in cells harboring *GAL-AtTIR1* (*aid-mcm4* or *mcm4-aid*, respectively). Both mutants did not form colonies at all in the presence of auxin showing that they had a tight growth defect even though the wild-type strain and a control strain expressing AtTIR1 grew well on the same plate (**Fig. 3a**). We found that depletion of aid-Mcm4 and Mcm4-aid proteins occurred within about 30 min at 24 °C (**Fig. 3b**), and both strains arrested with one copy of the chromosome within a single generation time (**Fig. 3c**), indicating that DNA synthesis was efficiently blocked.

We also applied the AID system to other cell cycle regulators that act in the nucleus or the cytoplasm. We made auxin-sensitive mutants of *CDC45*, *ASK1*, *MYO2* and *MYO1*, which showed a tight cell-cycle defect (**Supplementary Fig. 5**). Furthermore, we generated conditional mutants of membrane proteins localized at the endoplasmic reticulum or the mitochondria as long as the degron moiety was exposed to the cytoplasm (**Supplementary Fig. 6**). Thus the AID system supports the generation of yeast strains allowing the rapid and efficient depletion of essential proteins present either in the nucleus or the cytoplasm. We noticed that proteins expressed at relatively low amounts are less efficiently depleted by the AID system, and IAA works slightly better than NAA for some proteins (**Supplementary Fig. 7**).

Improvement of the AID system to confer thermostability

To apply the AID system to animal cells, we had to develop the approach to work well at higher temperatures, such as 37 °C. As initially designed, our approach did not work at higher temperatures (**Supplementary Fig. 8a**), suggesting that some component of the AID system does not work efficiently. Consistent with this

view, it had been previously reported that the auxin-dependent interaction between AtTIR1 and GST-IAA7, another of the AUX/IAA proteins, is reduced at 37 °C compared to that at 25 °C in a pull-down assay²⁶.

To overcome the problem, we sought to find a thermostable TIR1 and hypothesized that plant species which grow in a warmer environment might have such a TIR1 protein. We cloned putative *TIR1* orthologs from rice *Oryza sativa* (*OsTIR1*) and cotton *Gossypium hirsutum* (*GhTIR1*) (**Supplementary Fig. 8b**). GhTIR1 was very closely related to AtTIR1 (**Supplementary Fig. 8c**; 80% identical), whereas OsTIR1 was more divergent (61% identical). Although we could use both GhTIR1 and OsTIR1 to construct auxin-sensitive *mcm4-aid* mutants at 24 °C as we did with AtTIR1, only OsTIR1-based mutant was functional at a wide range of temperatures (**Supplementary Fig. 8a**). When we added lower concentrations of NAA than the 500 μM NAA used above, OsTIR1 worked better than AtTIR1 even at 24 °C (**Supplementary Fig. 9a,b**). Thus OsTIR1 and *Arabidopsis* IAA17 were an optimal AID system for use in yeast.

Depletion of a GFP reporter protein in mammalian cells

To test the modified AID system in mammalian cells, we constructed a plasmid to express OsTIR1 and GFP-aid-NLS simultaneously (**Supplementary Fig. 10a**). We exposed HeLa cells transfected with this vector to either 500 μM IAA or NAA for 5 h in the presence or absence of a proteasome inhibitor, MG132. GFP-aid-NLS was undetectable after treatment with either IAA or NAA, and the depletion was suppressed by MG132 (**Fig. 4a**). We also found that OsTIR1 lacking the F-box domain does not deplete GFP-aid-NLS (**Supplementary Fig. 10b**), and Cdc34, the E2 ubiquitin ligase for SCFs, was required to deplete GFP-aid-NLS in yeast (**Supplementary Fig. 9c**). These findings indicate that GFP-aid-NLS was degraded through the SCF-proteasome pathway. We also transiently transfected the GFP-aid-NLS construct into COS1 (monkey-derived), CHO-K1 (hamster-derived) and NIH3T3 (mouse-derived) cell lines. In all cases, expression of GFP-aid-NLS was greatly reduced after treating cells with the auxins (**Fig. 4b**).

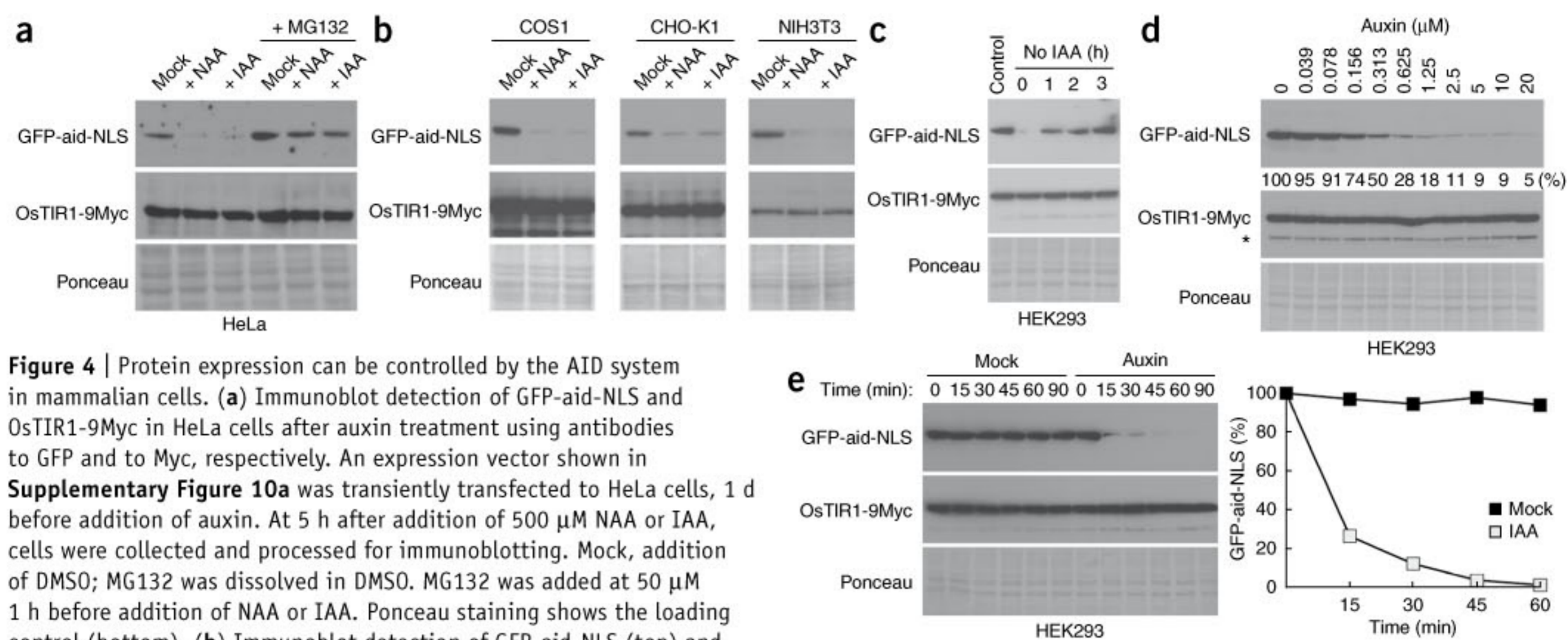


Figure 4 | Protein expression can be controlled by the AID system in mammalian cells. **(a)** Immunoblot detection of GFP-aid-NLS and OsTIR1-9Myc in HeLa cells after auxin treatment using antibodies to GFP and to Myc, respectively. An expression vector shown in **Supplementary Figure 10a** was transiently transfected to HeLa cells, 1 d before addition of auxin. At 5 h after addition of 500 μM NAA or IAA, cells were collected and processed for immunoblotting. Mock, addition of DMSO; MG132 was dissolved in DMSO. MG132 was added at 50 μM 1 h before addition of NAA or IAA. Ponceau staining shows the loading control (bottom). **(b)** Immunoblot detection of GFP-aid-NLS (top) and OsTIR1 (middle) in COS1, CHO-K1 and NIH3T3 cells. These cell lines were treated as in **a**. **(c)** Immunoblots to detect expression of GFP-aid-NLS. A stable HEK293 cell line expressing both OsTIR1-9Myc and GFP-aid-NLS were treated with 500 μM IAA for 2 h before growth in medium without IAA for indicated amounts of time. **(d)** Immunoblot detection of GFP-aid-NLS in HEK293 cells used in **c** treated with indicated IAA concentrations for 3 h. Expression of GFP-aid was quantified and indicated taking the 0 μM IAA sample as 100%; percentages are indicated below the top gel. Asterisk indicates a background protein. **(e)** Immunoblot detection of GFP-aid-NLS and OsTIR1-9Myc in the HEK293 stable cell line used in **c** was mock-treated or treated with 500 μM IAA. Cells were collected at indicated time points for immunoblotting (left). Expression of GFP-aid-NLS was quantified and plotted with the 0 min time point sample as 100% (right).

We then tested whether depletion was reversible in human-derived HEK293 cell line. We stably expressed OsTIR1 and GFP-aid-NLS. Cell growth was equivalent to that of the parental cells, indicating that expression of OsTIR1 was not harmful (data not shown). In addition, proteins of major SCF targets were unaffected both in HEK293 and the stable cells expressing OsTIR1 and GFP-aid-NLS in the presence or absence of auxin (**Supplementary Fig. 10c**). After degradation of GFP-aid-NLS, we transferred the cells to medium lacking auxin. As expected, GFP-aid-NLS was re-expressed within 1 h (**Fig. 4c**), showing that expression of a target protein can be controlled reversibly. To test whether expression could be modulated by altering auxin concentration in the culture medium, we grew the same strain in medium containing 0–20 μM IAA. The amount of GFP-aid-NLS was inversely correlated with the auxin concentration (**Fig. 4d**), and at 20 μM IAA was only 5% of the amount of GFP-aid-NLS in cells grown without auxin. Finally, we monitored the depletion kinetics of GFP-aid-NLS in the presence of auxin. The majority of GFP-aid-NLS was depleted within 15 min of auxin addition (**Fig. 4e**), indicating that the half-life of GFP-aid-NLS in the presence of the auxin was about 10 min. Residual GFP-aid-NLS in cells after depletion was estimated to be about 3% of the amount in untreated cells (**Supplementary Fig. 10d**).

Rapid depletion of CENP-H in chicken DT40 cells using AID

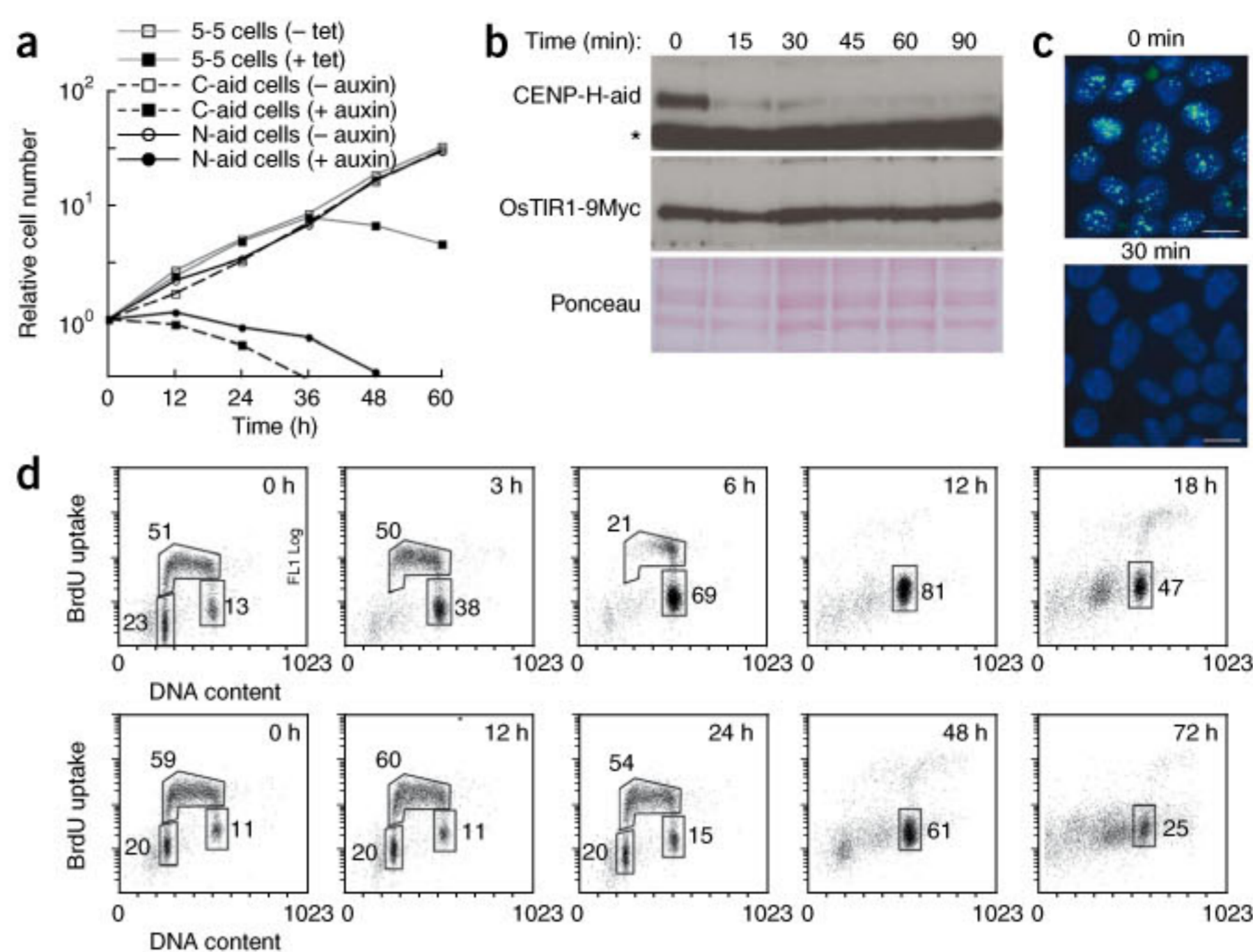
To test whether the AID system is suited to the rapid and conditional depletion of endogenous proteins in animal cells, we took advantage of chicken DT40 cells in which endogenous genes can be modified efficiently by homologous recombination. We found that addition of 500 μM IAA did not affect growth of wild-type DT40 cells or a stable DT40 cell line expressing OsTIR1 (**Supplementary Fig. 11a,b**). Furthermore, in a global gene

expression profile analysis, we found that addition of 500 μM IAA caused very few effects in DT40 cells and the DT40 cells expressing OsTIR1 (**Supplementary Fig. 12**).

The CENP-H protein is a constitutive component of the kinetochore and is required for faithful chromosome segregation^{32,33}. We have previously shown that in a conditional DT40 cell line called 5-5, expression of mRNA encoding CENP-H (controlled by the tetracycline-responsive *TRE* promoter) arrests the cell cycle at prometaphase in the presence of tetracycline before subsequently dying³³. We stably introduced an expression vector containing *OsTIR1* and *CENP-H* tagged with sequence encoding the aid degron at either N or C terminus into DT40 5-5 cells (**Supplementary Fig. 11c**). Growth of the resultant strains (hereafter referred to as N-aid and C-aid, respectively) plus tetracycline resembled the parental DT40 5-5 cells without tetracycline, showing that both versions of CENP-H tagged with the aid degron are functional (**Supplementary Fig. 11d**).

To investigate the growth defect after CENP-H depletion, we added auxin (500 μM IAA) to N-aid and C-aid cells that had been grown in the presence of tetracycline. Remarkably, both N-aid and C-aid cells ceased to grow immediately and subsequently started to die (**Fig. 5a**). In contrast, the parental DT40 5-5 cells arrested growth at 36 h (up to three cell cycles) after tetracycline addition³³. Immunoblotting showed that CENP-H-aid was depleted within 15 min (**Fig. 5b**). Similarly, localization of CENP-H-aid at the centromere disappeared within 30 min (**Fig. 5c**). However, we detected CENP-H up to 24 h after we added tetracycline to deplete *CENP-H* mRNA in the DT40 5-5 cells. To compare cell cycle defects after depletion of *CENP-H* mRNA or direct depletion of CENP-H protein using the AID system, we treated the C-aid line (grown in the presence of tetracycline) with 500 μM IAA and treated the DT40 5-5 strain with tetracycline. We pulse-labeled the cells with 5-bromodeoxyuridine (BrdU) and processed them

Figure 5 | Cell cycle phenotype of chicken DT40 cells after depletion of *CENP-H* mRNA or *CENP-H* protein. **(a)** Growth of conditional strains of *CENP-H* (DT40 5-5, N-aid and C-aid cells) after addition of IAA. N-aid and C-aid strains had been maintained in the presence of $2 \mu\text{g ml}^{-1}$ tetracycline to shut off transcription from the *CENP-H* transgene under control of the tetracycline-responsive *TRE* promoter. Tetracycline (tet) was added at $2 \mu\text{g ml}^{-1}$ to exponentially growing DT40 5-5 cells and $500 \mu\text{M}$ IAA was added to N-aid and C-aid cells. Living cells were counted after Trypan blue staining. **(b)** Immunoblot detection of CENP-H-aid and OsTIR1-9Myc using antibodies to aid and to Myc, respectively, in C-aid cells at indicated times after $500 \mu\text{M}$ IAA addition. Asterisk indicates a background protein. **(c)** Fluorescence image of fixed C-aid cells expressing CENP-H-aid before and after addition of $500 \mu\text{M}$ IAA. Cells were stained with antibody to CENP-H (green) and with DAPI (blue). Scale bars, $10 \mu\text{m}$. **(d)** Cell cycle distribution following the protein or mRNA depletion of *CENP-H* analyzed by flow cytometry. C-aid cells had been maintained in the presence of $2 \mu\text{g ml}^{-1}$ tetracycline before addition of auxin. *CENP-H* depletion was induced at time 0 by adding $500 \mu\text{M}$ IAA to C-aid cells (top) or $2 \mu\text{g ml}^{-1}$ tetracycline to DT40 5-5 cells (bottom). Cells were stained with FITC-anti-BrdU (y axis, log scale) to detect BrdU incorporation and with propidium iodide to detect total DNA (x axis, linear scale). The lower left, the upper and the lower right boxes represent G1, S and G2-M phase cells, respectively. The numbers given in the boxes indicates the percentage of gated events.



for flow cytometry. Even 3 h after addition of auxin, 38% of the C-aid cells accumulated at G2-M phase, 81% of them arrested at G2-M phase by 12 h, and the cells started dying by 18 h (Fig. 5d). In contrast, the cell cycle seemed not to be affected up to 24 h after addition of tetracycline to DT40 5-5 cells (Fig. 5d). These cells started to accumulate at G2-M phase at 48 h and to die at 72 h³³. These results demonstrate that direct and rapid depletion of *CENP-H* using the AID system produces a clearer and stronger phenotype than can be achieved by depletion of the mRNA.

DISCUSSION

Previous studies have illustrated the importance of rapid depletion of a protein for interpretation of the subsequent phenotypes. Transcriptional repression of the yeast *MCM4* gene leads to slow depletion of the protein and an accumulation of cells in G2-M phase, probably due to subtle defects in chromosome replication¹⁶. In contrast, rapid depletion of *Mcm4* by the temperature-sensitive degron¹⁶ or by the AID system (this study) caused a profound and obvious defect in chromosome replication, which reveals the protein's function more easily. Phenotypic differences between mRNA depletion and protein depletion were also evident in the case of the *CENP-H* protein in DT40 cells. In the case of proteins of unknown function, the advantages of rapid and efficient depletion are likely to aid greatly the analysis of protein function.

Ideally, a small molecule used to control protein expression should be biologically silent. It is generally believed that auxin is only active in plants and appears to be relatively silent in nonplant eukaryotes. As the reporter GFP-aid-NLS protein was depleted to 5% of its starting level in the presence of $20 \mu\text{M}$ IAA in HEK293 cells, we suggest that 20 – $500 \mu\text{M}$ IAA (or NAA) should be enough to induce maximum depletion for most animal cells. However, it has been reported that IAA can be oxidized by peroxidases and the products can be toxic to

cells³⁴. In addition, cells with very high peroxidase activity such as neutrophils have been reported to be sensitive to 1 mM IAA³⁵. It is therefore important to check the sensitivity to auxins when the AID system is applied to a new cell line or to other organisms.

One limitation of the AID system is that it cannot be used to control endogenous proteins without manipulation of the gene of interest. Given the ease of homologous recombination, however, the application of AID is simple and straightforward in budding yeast. By transforming yeast cells that express TIR1, it should be possible to make a new AID mutant within one week. A great advantage of the AID system is that the culture can be maintained at a constant temperature. This should facilitate the combined use of temperature-sensitive degron and the AID system to target different proteins. In addition, the AID system should be particularly useful for studying processes like meiosis that do not take place at $37 \text{ }^\circ\text{C}$ or avoid problems in other processes that might arise from stress responses caused by a temperature shift.

In animal cells, it is more challenging to use the AID system because homologous recombination is generally much less efficient. We suggest three examples for future use of the AID system in studies of animal cells. (i) We suggest using cell lines in which genes can be targeted by homologous recombination (for example, mouse embryonic stem cells, human HCT116 cells and chicken DT40 cells). Using these cell lines, it should be possible to knock out or modify endogenous gene of interest to construct AID mutants. (ii) Expression of the endogenous gene could be repressed by an RNA interference-based method and replaced with exogenous expression of OsTIR1 and an RNA interference-resistant version of the target gene fused to the aid degron. In this way, rapid depletion of the target protein should be achieved after adding an auxin and the resultant phenotype could be observed in many types of cells. (iii) Using cell lines that lack or do not express a functional gene for a protein that is not essential for

cell viability. Those cells could be rescued by introducing *OsTIR1* and a gene expressing an aid degraon-fused protein, allowing the subsequent analysis of rapid depletion. The AID system should allow us to apply more sophisticated genetic studies to a wider range of eukaryotic cells in the future. To our knowledge, this system is the first practical use of a plant hormone for the study of protein function in nonplant eukaryotes.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemethods/>.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We thank K. Labib (Cancer Research UK) for critical reading of the manuscript, for support and for providing Myc and HA antibodies, yeast strains and tagging plasmids for budding yeast; S. Mimura (Nagoya University) for providing the *cdc34-2* yeast strain; H. Kanazawa (Osaka University) for providing a *GFP* plasmid; T. Hori for help with flow cytometry analysis; and H. Araki, H. Masukata and K. Sugawara for discussion. M.K. thanks Y. Tak for her support. This work was funded by a Challenging Exploratory Research and a Grant-in-Aid for Young Scientists (A) from the Ministry of Education, Science, Sports and Culture of Japan and by a Research Promotion Grant from the Uehara Memorial Foundation to M.K. K.N. is funded by a Japan Society for the Promotion of Science fellowship (DC2).

AUTHOR CONTRIBUTIONS

K.N. and M.K. designed and performed all experiments. T.F. supervised experiments using DT40. T.K. created the basic concept of AID to control protein levels in nonplant cells. M.K. and H.T. supervised and led this project. K.N. and M.K. wrote the manuscript. K.N., T.F., H.T., T.K. and M.K. discussed and checked the manuscript.

Published online at <http://www.nature.com/naturemethods/>.

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>.

- Sauer, B. & Henderson, N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc. Natl. Acad. Sci. USA* **85**, 5166–5170 (1988).
- Gossen, M. & Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89**, 5547–5551 (1992).
- Elbashir, S.M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
- Picard, D., Salser, S.J. & Yamamoto, K.R. A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. *Cell* **54**, 1073–1080 (1988).
- Haruki, H., Nishikawa, J. & Laemmli, U.K. The anchor-away technique: rapid, conditional establishment of yeast mutant phenotypes. *Mol. Cell* **31**, 925–932 (2008).
- Picard, D. Posttranslational regulation of proteins by fusions to steroid-binding domains. *Methods Enzymol.* **327**, 385–401 (2000).
- Zhou, P. Targeted protein degradation. *Curr. Opin. Chem. Biol.* **9**, 51–55 (2005).
- Banaszynski, L.A. & Wandless, T.J. Conditional control of protein function. *Chem. Biol.* **13**, 11–21 (2006).
- Gosink, M.M. & Vierstra, R.D. Redirecting the specificity of ubiquitination by modifying ubiquitin-conjugating enzymes. *Proc. Natl. Acad. Sci. USA* **92**, 9117–9121 (1995).
- Zhou, P., Bogacki, R., McReynolds, L. & Howley, P.M. Harnessing the ubiquitination machinery to target the degradation of specific cellular proteins. *Mol. Cell* **6**, 751–756 (2000).
- Sakamoto, K.M. *et al.* Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. *Proc. Natl. Acad. Sci. USA* **98**, 8554–8559 (2001).
- Schneekloth, J.S. Jr. *et al.* Chemical genetic control of protein levels: selective *in vivo* targeted degradation. *J. Am. Chem. Soc.* **126**, 3748–3754 (2004).
- Zhang, J., Zheng, N. & Zhou, P. Exploring the functional complexity of cellular proteins by protein knockout. *Proc. Natl. Acad. Sci. USA* **100**, 14127–14132 (2003).
- Dohmen, R.J., Wu, P. & Varshavsky, A. Heat-inducible degraon: a method for constructing temperature-sensitive mutants. *Science* **263**, 1273–1276 (1994).
- Labib, K., Tercero, J.A. & Diffley, J.F.X. Uninterrupted MCM2–7 function required for DNA replication fork progression. *Science* **288**, 1643–1647 (2000).
- Kanemaki, M., Sanchez-Diaz, A., Gambus, A. & Labib, K. Functional proteomic identification of DNA replication proteins by induced proteolysis *in vivo*. *Nature* **423**, 720–724 (2003).
- Sanchez-Diaz, A. *et al.* Inn1 couples contraction of the actomyosin ring to membrane ingression during cytokinesis in budding yeast. *Nat. Cell Biol.* **10**, 395–406 (2008).
- Su, X., Bernal, J.A. & Venkitaraman, A.R. Cell-cycle coordination between DNA replication and recombination revealed by a vertebrate N-end rule degraon-Rad51. *Nat. Struct. Mol. Biol.* **15**, 1049–1058 (2008).
- Banaszynski, L.A., Chen, L.C., Maynard-Smith, L.A., Ooi, A.G. & Wandless, T.J. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* **126**, 995–1004 (2006).
- Banaszynski, L.A., Sellmyer, M.A., Contag, C.H., Wandless, T.J. & Thorne, S.H. Chemical control of protein stability and function in living mice. *Nat. Med.* **14**, 1123–1127 (2008).
- Herm-Gotz, A. *et al.* Rapid control of protein level in the apicomplexan *Toxoplasma gondii*. *Nat. Methods* **4**, 1003–1005 (2007).
- Armstrong, C.M. & Goldberg, D.E. An FKBP destabilization domain modulates protein levels in *Plasmodium falciparum*. *Nat. Methods* **4**, 1007–1009 (2007).
- Teale, W.D., Paponov, I.A. & Palme, K. Auxin in action: signalling, transport and the control of plant growth and development. *Nat. Rev. Mol. Cell Biol.* **7**, 847–859 (2006).
- Ruegger, M. *et al.* The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast *grr1p*. *Genes Dev.* **12**, 198–207 (1998).
- Gray, W.M. *et al.* Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes Dev.* **13**, 1678–1691 (1999).
- Dharmasiri, N., Dharmasiri, S. & Estelle, M. The F-box protein TIR1 is an auxin receptor. *Nature* **435**, 441–445 (2005).
- Kepinski, S. & Leyser, O. The *Arabidopsis* F-box protein TIR1 is an auxin receptor. *Nature* **435**, 446–451 (2005).
- Tan, X. *et al.* Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**, 640–645 (2007).
- Abel, S. & Theologis, A. Early genes and auxin action. *Plant Physiol.* **111**, 9–17 (1996).
- Dreher, K.A., Brown, J., Saw, R.E. & Callis, J. The *Arabidopsis* Aux/IAA protein family has diversified in degradation and auxin responsiveness. *Plant Cell* **18**, 699–714 (2006).
- Schulman, B.A. *et al.* Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. *Nature* **408**, 381–386 (2000).
- Sugata, N., Munekata, E. & Todokoro, K. Characterization of a novel kinetochore protein, CENP-H. *J. Biol. Chem.* **274**, 27343–27346 (1999).
- Fukagawa, T. *et al.* CENP-H, a constitutive centromere component, is required for centromere targeting of CENP-C in vertebrate cells. *EMBO J.* **20**, 4603–4617 (2001).
- Folkes, L.K., Dennis, M.F., Stratford, M.R., Candeias, L.P. & Wardman, P. Peroxidase-catalyzed effects of indole-3-acetic acid and analogues on lipid membranes, DNA, and mammalian cells *in vitro*. *Biochem. Pharmacol.* **57**, 375–382 (1999).
- de Melo, M.P., de Lima, T.M., Pithon-Curi, T.C. & Curi, R. The mechanism of indole acetic acid cytotoxicity. *Toxicol. Lett.* **148**, 103–111 (2004).

ONLINE METHODS

cDNA and plasmid construction. *Arabidopsis* and rice cDNAs were obtained from the *Arabidopsis* Biological Resource Center and the Rice Genome Resource Center, respectively. *AtIAA17* (GeneID 839568), *AtTIR1* (GeneID 825473), *GhTIR1* (GenBank DQ659621.1) and *OsTIR1* (GeneID 4335696) cDNAs were cloned by standard methods. Details of the aid degron tagging vectors for budding yeast are available in **Supplementary Figure 13**. Expression vectors used for animal cells are based on pIRES2-AcGFP1 (Clontech). Genes encoding the target protein fused with the aid degron were cloned in the region between BstXI and NotI by swapping with genes encoding AcGFP. *OsTIR1-9Myc* was cloned between XhoI and SmaI in the multicloning site.

Yeast strains and cell growth. Yeast strains used in this study are listed in **Supplementary Table 2**. AID degron strains were made by a 'one-step PCR' approach using a yeast strain in which the only one copy of the *TIR1* gene was regulated by the galactose-inducible *GALI-10* promoter or the constitutive *ADHI* promoter. For each gene, a PCR-product was generated by using 70-mer oligos beginning with 50 nucleotides homology to each targeting region and ending with 20 nucleotides equivalent to sequences in the aid cassette (details in **Supplementary Fig. 13**). Cells were grown at 24 °C in YP medium (1% yeast extract (Difco) and 2% peptone (Oxoid)) supplemented with 2% raffinose (YPR medium), 2% galactose (YPG medium) or 2% glucose (YPD medium). In experiments using the *aid-mcm4* strain (**Fig. 3**), 0.1 mM CuSO₄ was added to culture medium to induce expression of *aid-mcm4* from the *CUP1* promoter.

Animal cell culture. Mammalian cells were obtained from the RIKEN BioResource Center. HeLa, COS1, NIH3T3 and HEK293 cells were cultured in DMEM (Sigma), supplemented with 10% FBS, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. CHO-K1 cells were maintained in the Ham's-F12 medium (Sigma) with the same supplements. Transfection was done using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. To establish a HEK293 stable cell line, transfected

cells were selected in the presence of 0.8 mg ml⁻¹ G418. DT40 strains used in this study are listed in **Supplementary Table 3**.

Detection of protein. Yeast cells extracts were prepared by the trichloroacetic acid (TCA) extraction method. Briefly, 1 × 10⁸ cells were lysed in 20% TCA using glass beads. The resultant pellet was extracted with the high-pH Laemmli buffer and denatured at 95 °C for 4 min. Cells were lysed in the Laemmli buffer before lysates were cleared through 0.45 µm Nylon filter (Spin-X centrifuge tube filter; Coster). After denaturation at 95 °C for 5 min, equal amounts of total protein were separated by SDS-PAGE. Antibodies used for immunoblotting are indicated in the figure legends. HA (monoclonal 12CA5) and Myc (monoclonal 9E10) antibodies were a gift from K. Labib. The aid polyclonal antibody was made in our lab.

FACS analysis. Yeast samples were fixed in 70% ethanol. After processing with 0.1 mg ml⁻¹ RNase A for 2 h at 37 °C and then 5 mg ml⁻¹ pepsin for 30 min at 37 °C, cells were stained with 2 µg ml⁻¹ propidium iodide. Stained cells were analyzed by using FACSscan (BD Biosciences). DT40 cells were pulse-labeled at the indicated time points with 20 µM BrdU for 20 min. After fixation in ice-cold 70% ethanol, cells were processed and analyzed as described previously³³.

Microscopy. Yeast cells were fixed in 4% paraformaldehyde with agitation for 10 min at room temperature (20 °C). Fixed cells were washed with PBS (pH 7.4) and suspended in Vectashield mounting medium (Vector Laboratories) containing 2 µg ml⁻¹ DAPI. Immunofluorescence staining of CENP-H in DT40 cells was performed as described previously³³.

Microarray analysis. DT40 cells and a stable DT40 cell line expressing *OsTIR1* were grown in the presence or absence of 500 µM IAA for 6 h. Total RNA was extracted using an RNeasy Midi kit (Qiagen) following the manufacturer's instructions. Gene expression profile analysis was conducted using a chicken single color microarray containing 43,083 probes (Agilent Technologies).