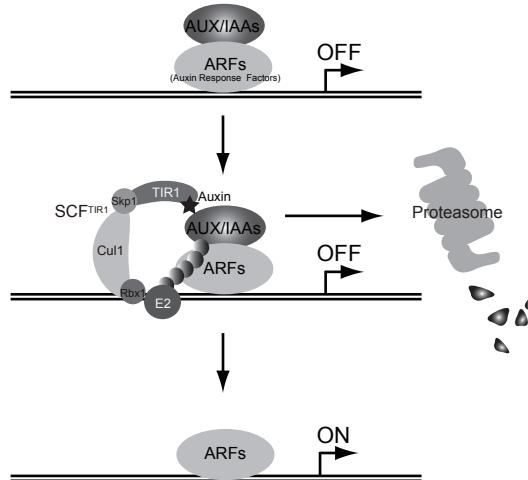


An auxin-based degron system for the rapid depletion of proteins in nonplant cells

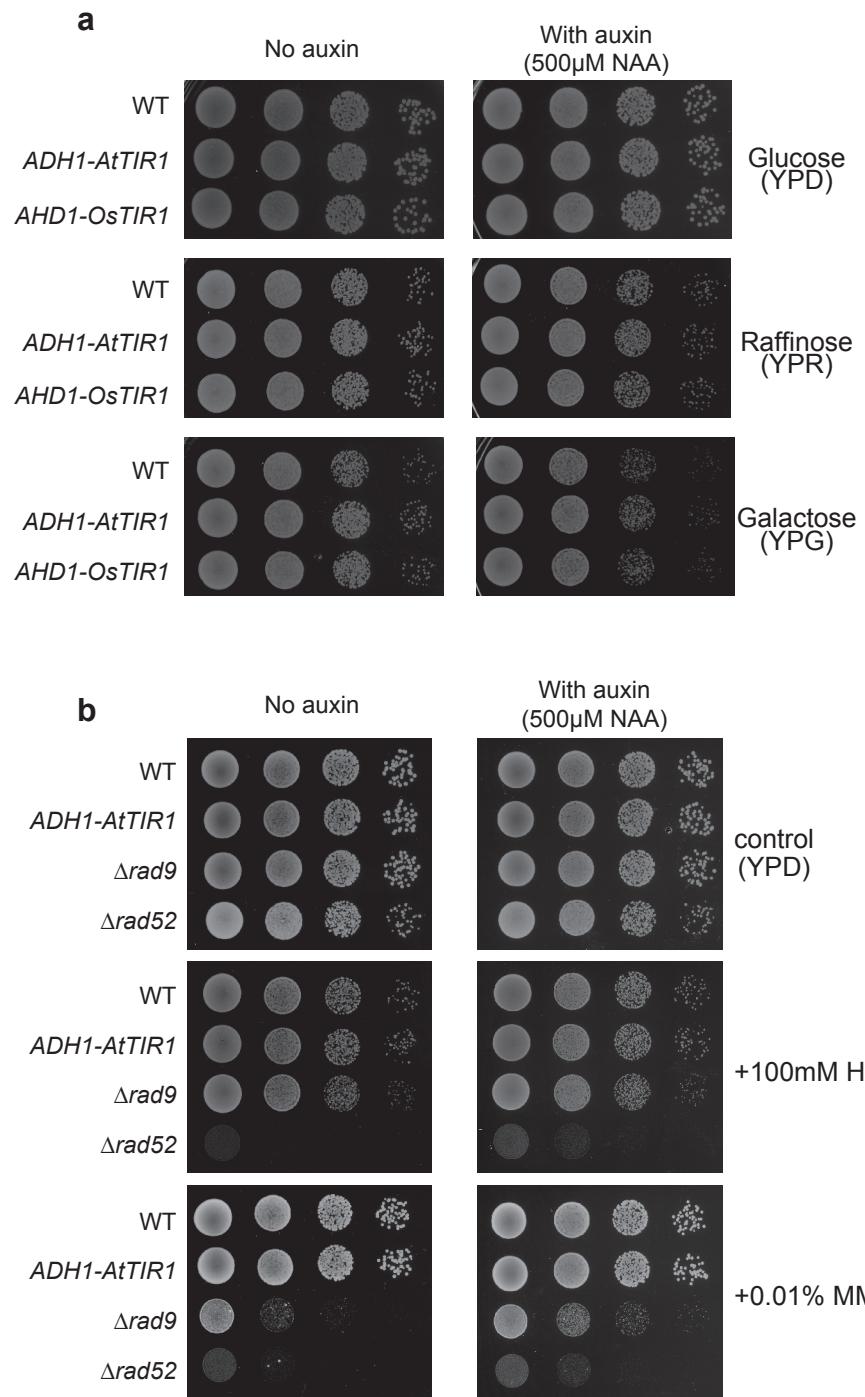
Kohei Nishimura, Tatsuo Fukagawa, Haruhiko Takisawa, Tatsuo Kakimoto & Masato Kanemaki

Supplementary figures and text:

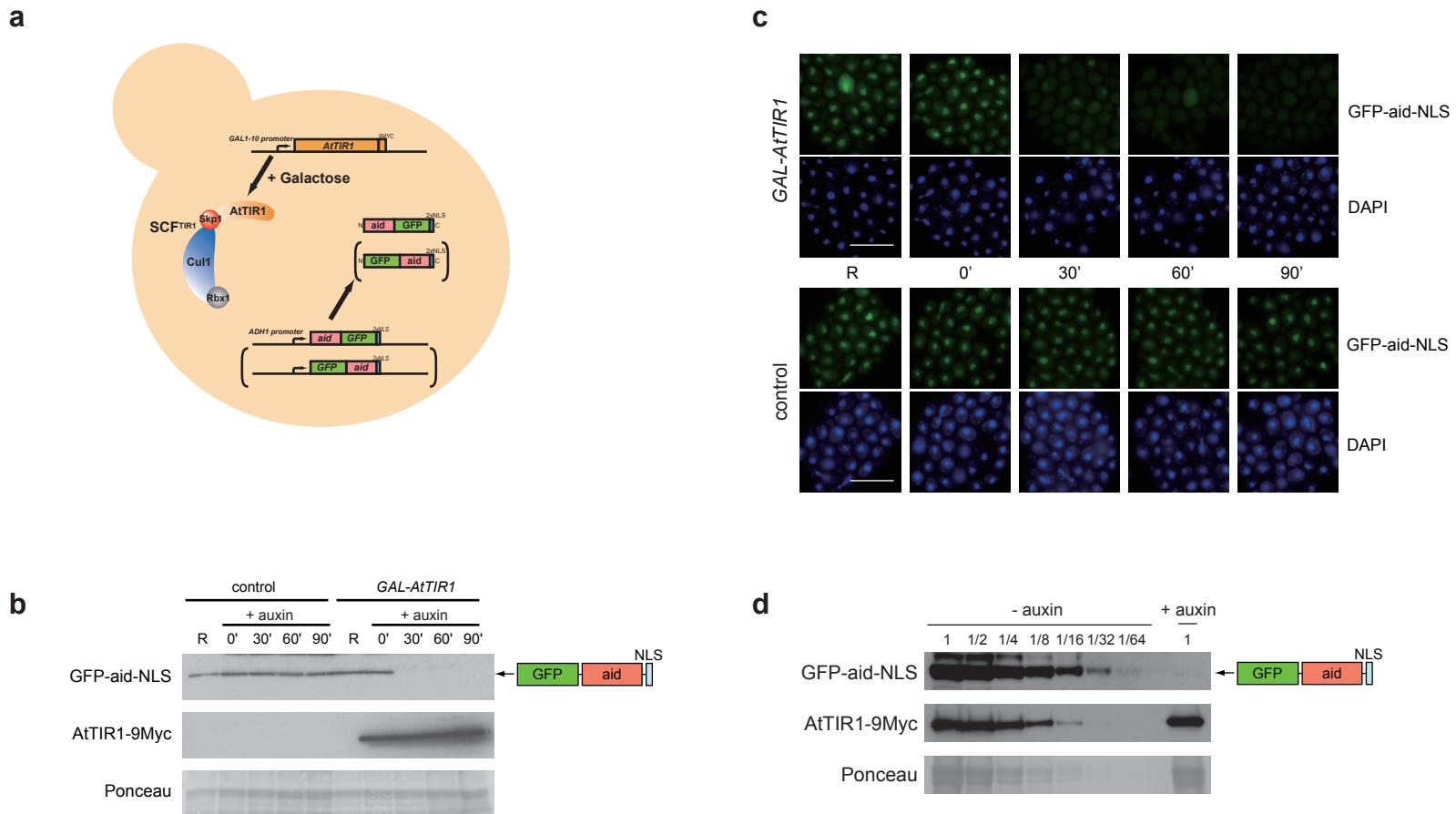
Supplementary Figure 1	The auxin response in plants.
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Supplementary Figure 3	The aid degron works at the carboxy terminus of GFP.
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Supplementary Table 1	Comparison of degradation based methods to control protein expression.
Supplementary Table 2	Yeast strains used in this study.
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a**b**

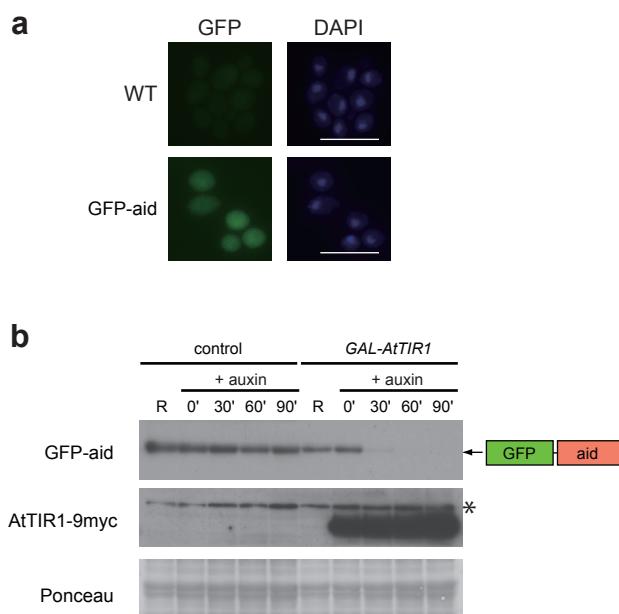
Supplementary Figure 1: The auxin response in plants. (a), The AUX/IAA transcription repressors bind to ARFs (auxin response factors) to inhibit the auxin responsive genes¹. Auxin induces poly-ubiquitylation of AUX/IAAs by activating the SCFTIR1 E3 ubiquitin ligase, resulting rapid degradation of AUX/IAAs by the proteasome. The auxin responsive genes therefore would be activated by ARFs in the presence of auxin. (b), Skp1 homologous in *A.thaliana* (GenelID 843928), *S.cerevisiae* (GenelID 851928), *S.pombe* (GenelID 2540917), mouse (GenelID 21402) and human (GenelID 6500) were aligned using the ClustalW software. Black and grey boxes indicate identical and similar amino acids, respectively. Underline shows the F-box binding region² (56 % identical and 71 % similar from *Arabidopsis* to human including yeast species).



Supplementary Figure 2: Neither expression of TIR1 nor addition of auxin affect cell growth of budding yeast. (a), Addition of auxin, expression of TIR1 or both wouldn't affect cell growth of budding yeast in a medium containing glucose, raffinose or galactose. WT cells and the cells constitutively expressing AtTIR1 or OsTIR1 were grown on the indicated agar medium with or without 500 μ M NAA. Cells were grown at 24 °C for two days. (b), Addition of auxin, expression of TIR1 or both wouldn't affect sensitivity to DNA damaging reagents. WT cells or cells expressing AtTIR1 were grown on a YPD plate containing 100 mM hydroxyurea (HU) or 0.001 % MMS. Cells were grown at 24 °C for two days. While $\Delta rad9$ and $\Delta rad52$ are sensitive to MMS and to the both reagents, respectively, expression of AtTIR1 didn't affect to cell growth in the presence or absence of 500 μ M

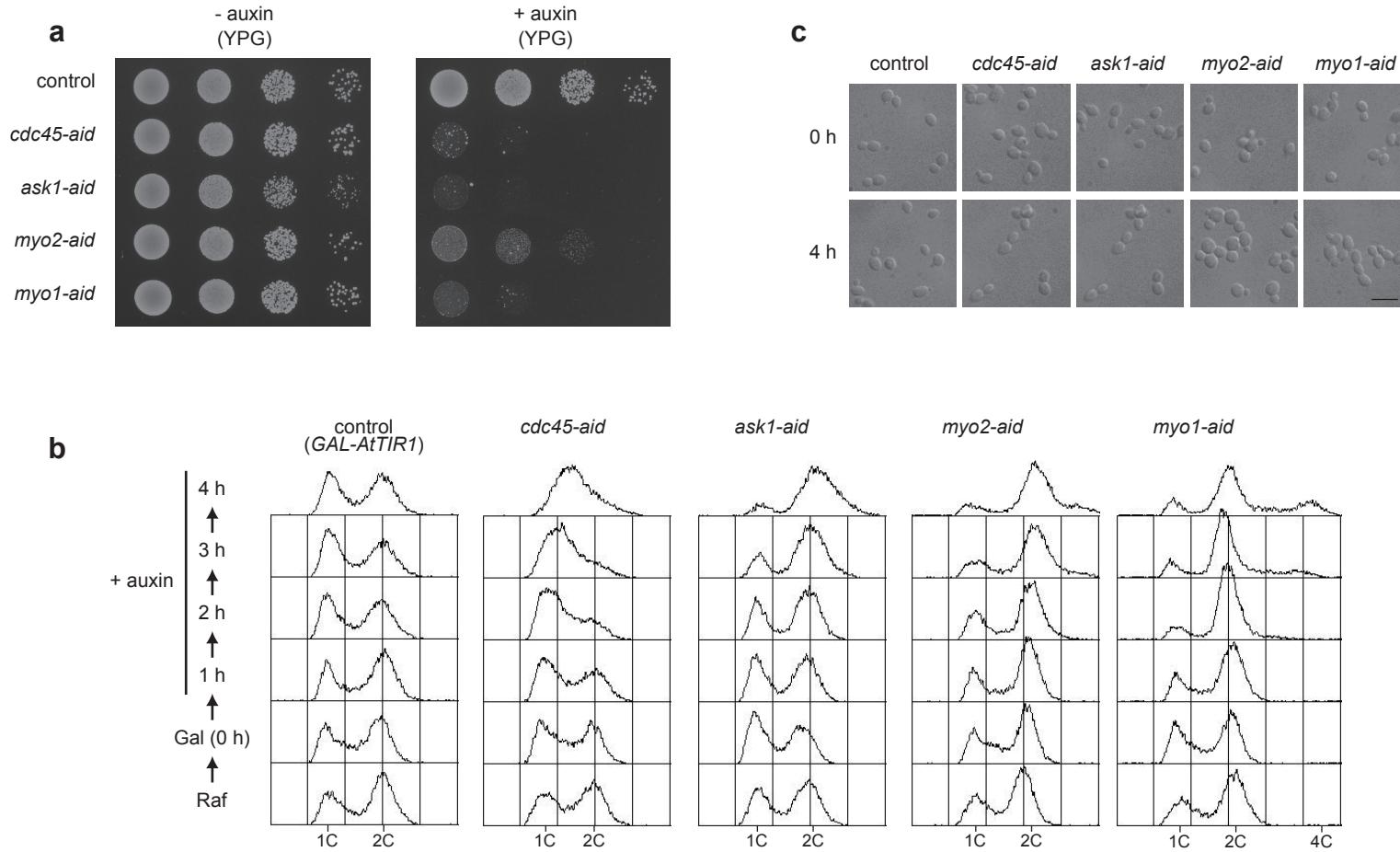


Supplementary Figure 3: The aid degron works at the carboxy terminus of GFP. (a), Schematic illustration of yeast cell to test GFP depletion by the AID system. One copy of *AtTIR1* under control of the galactose-inducible *GAL1-10* promoter was integrated at the *URA3* locus. One copy of either *aid-GFP-NLS* or *GFP-aid-NLS* under control of the constitutive *ADH1* promoter was integrated at the *TRP1* locus. (b), GFP fused with the aid degron at its carboxy terminus is depleted by AID. Cells expressing GFP-aid-NLS were treated and processed in the same way as in Figure 2b. (c), Cells used in b were analysed in the same way as in Figure 2c. The scale bars correspond to 10 μ m. (d), Quantification of residual GFP-aid-NLS in yeast cells. Serially diluted yeast extracts before addition of NAA and undiluted extracts after a 500 μ M NAA treatment for 90 minutes were separated by SDS-PAGE and immunoblotted using anti-GFP and anti-Myc antibodies.

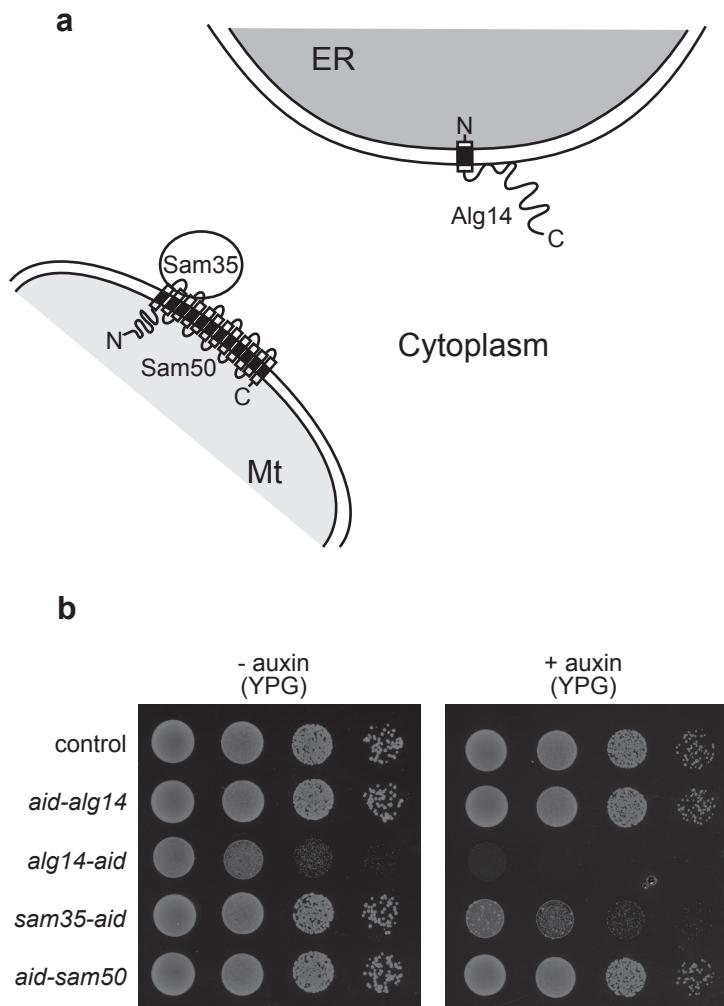


Supplementary Figure 4: AID works in the budding yeast cytoplasm.

(a), Cells expressing GFP-aid without NLS were fixed in 4 % PFA for 10 minutes and stained with DAPI before microscopic observation. The scale bars correspond to 10 μ m. (b), Cells expressing GFP-aid without NLS in appropriate control and the *GAL-AtTIR1* strain were grown and processed as in Figure 2b. Asterisk shows a background protein detected by anti-Myc antibody.

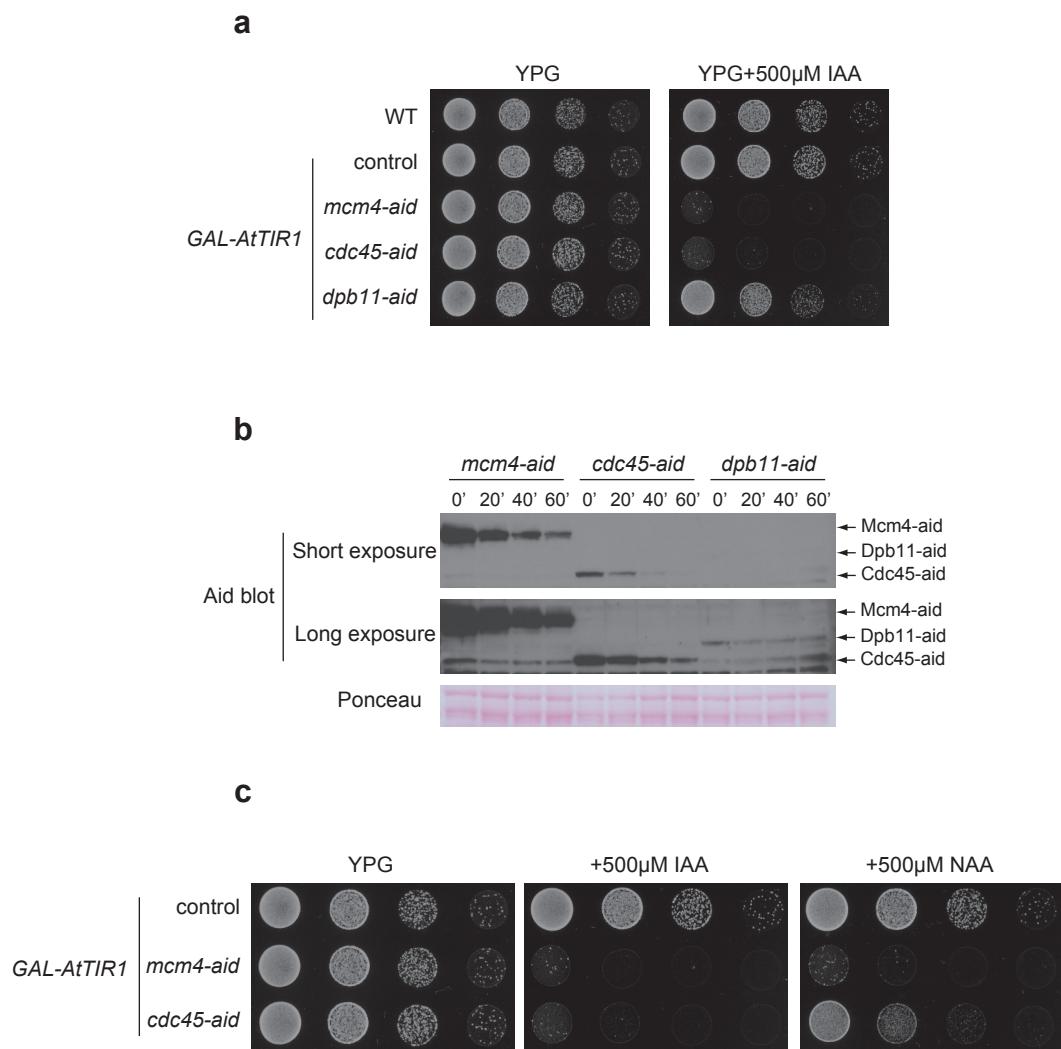


Supplementary Figure 5: AID mutants of the nuclear or cytoplasmic cell-cycle regulators show a tight cell cycle defect. (a), Cdc45 is a DNA replication protein that is thought to activate the Mcm2-7 helicase as a part of the CMG complex³. The Ask1 protein is essential for chromosome segregation and is a component of the DASH complex at the kinetochores⁴. Myo2 and Myo1 that are respectively the type V and type II myosin homologues in budding yeast, and that are both localized in the cytoplasm. Myo2 is required for localized cell surface growth (such as budding) by transporting cargos on actin cables⁵. Myo1 forms an essential part of the actomyosin ring structure at the bud-neck and is required for cytokinesis⁶. The aid degron was placed at their carboxy terminus in the *GAL-AtTIR1* background. Resultant strains were spotted on a YPG plate containing 500 μ M IAA and were grown for two days at 24 °C. (b), Cells were grown and treated in the same way as in Figure 3c. Depletion of *Cdc45-aid* caused a rapid and sustained block to chromosome replication. Both the *ask-aid* and *myo1-aid* mutant accumulated with a 2C DNA content, indicative of a failure in mitosis. At late time points some *myo1-aid* cells accumulated with a 4C DNA content, as previously reported for a *myo1-td* strain⁷. (c), Cells at 0 h and 4 h used in b were observed under a DIC microscope. The scale bar shows 10 μ m.



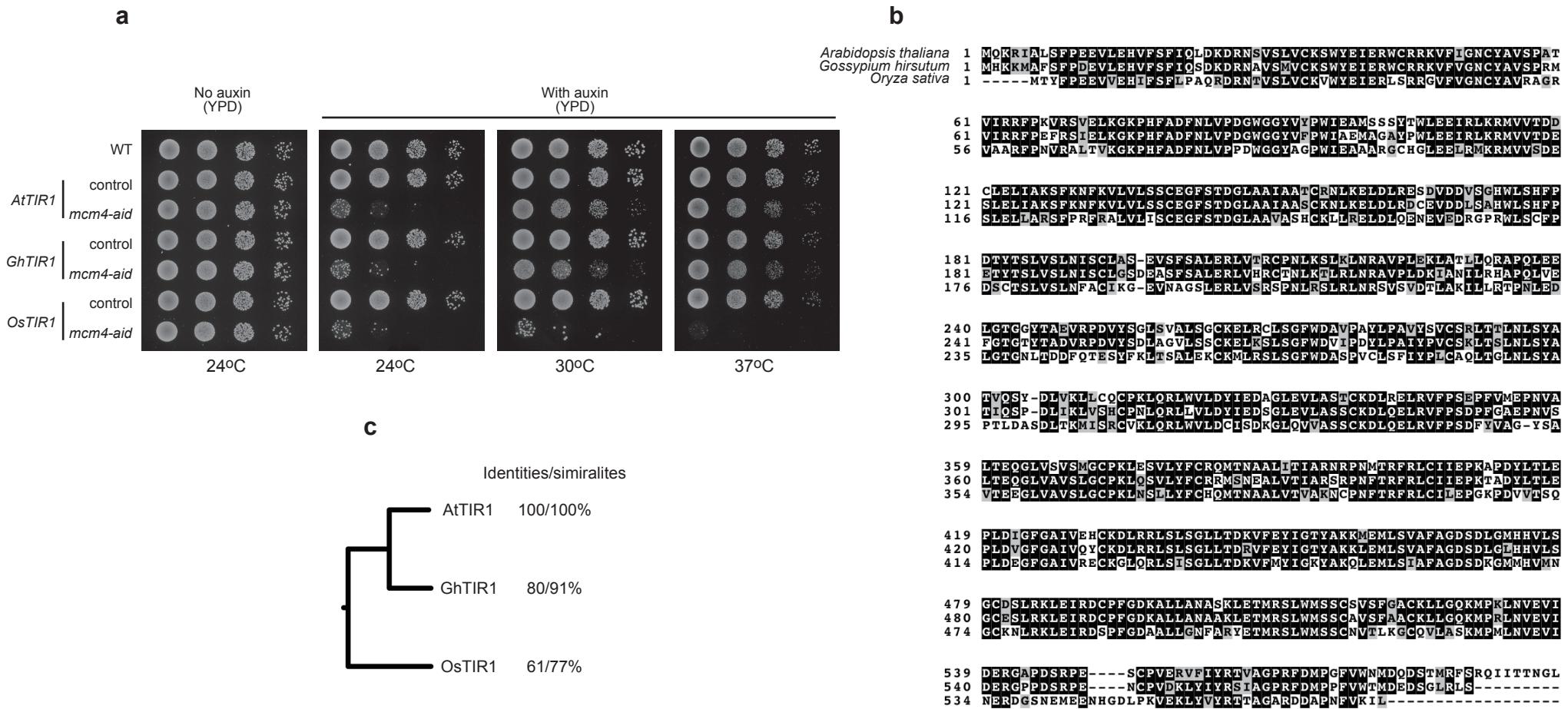
Supplementary Figure 6: AID mutants of proteins localized at the ER or the mitochondria.

(a), Schematic illustration showing subcellular localization of Alg14, Sam50 and Sam35 in yeast cell. Alg14, an essential protein involved in the lipid-linked oligosaccharide biogenesis, is localized at the endoplasmic reticulum (ER) exposing its amino and carboxy terminus to the ER lumen and the cytoplasm, respectively^{10, 11}. Sam50 and Sam35 (also known as Tob55 and Tob38, respectively) are essential proteins required for the biogenesis of beta-barrel membrane proteins of mitochondria (Mt). Sam50 is localized at the outer membrane of Mt exposing its amino terminus into the intermembrane space¹². Sam35 is localized on the surface of Mt binding to Sam50¹³⁻¹⁵. (b), Auxin sensitivity of AID mutants of Alg14, Sam50 and Sam35. Indicated strains in the *GAL-AtTIR1* background were spotted on a YPG plate with or without 500 μ M NAA and were grown for two days at 24 °C. The strains having the degron in the ER lumen or the intermembrane space of Mt didn't show sensitivity to auxin, suggesting that efficient degradation was not induced in ER and Mt. On the other hand, the strains containing *alg14-aid* or *sam35-aid*, in which the degron is exposed to the cytoplasm, showed a growth defect on the NAA plate.

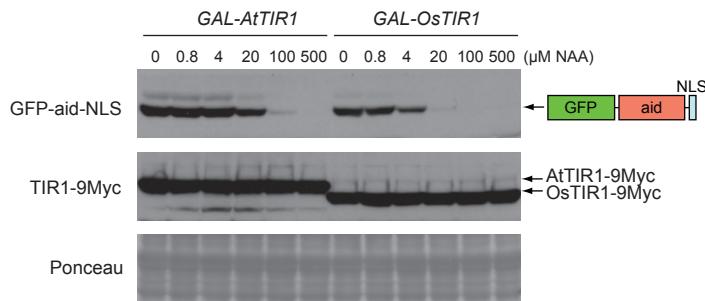
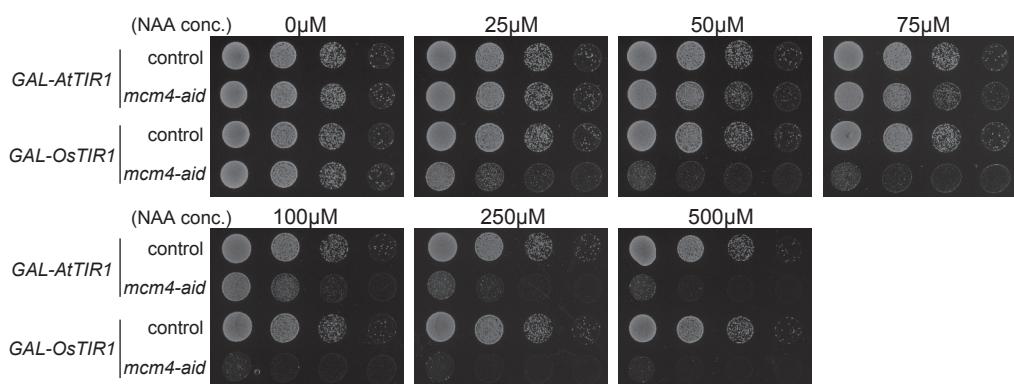
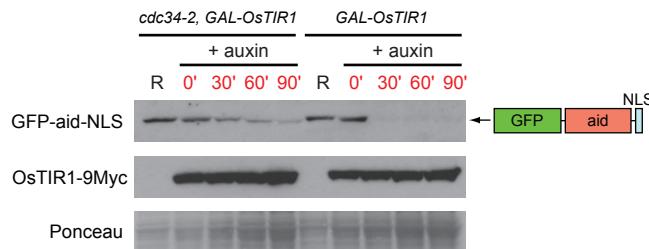


Supplementary Figure 7: Additional data of protein depletion by AID in budding yeast.

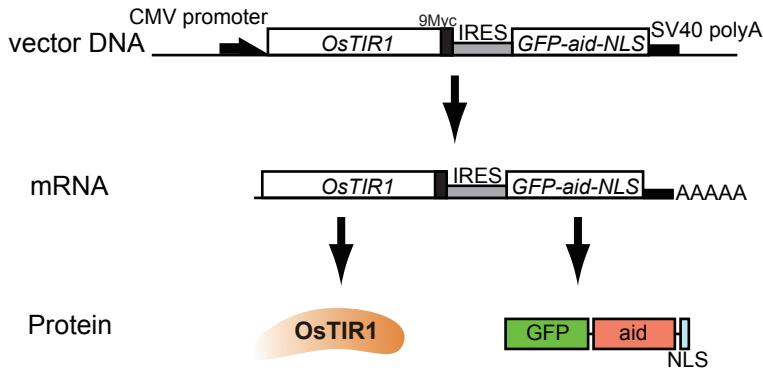
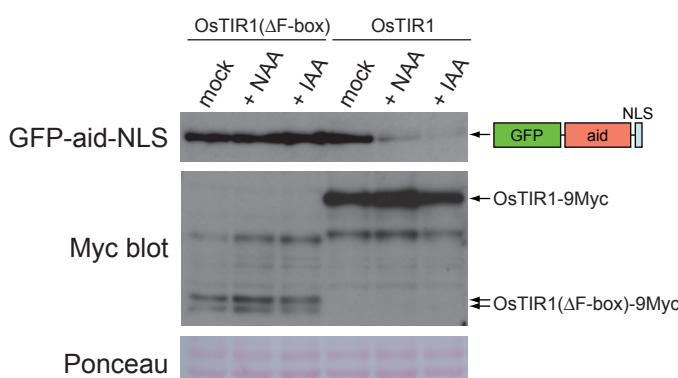
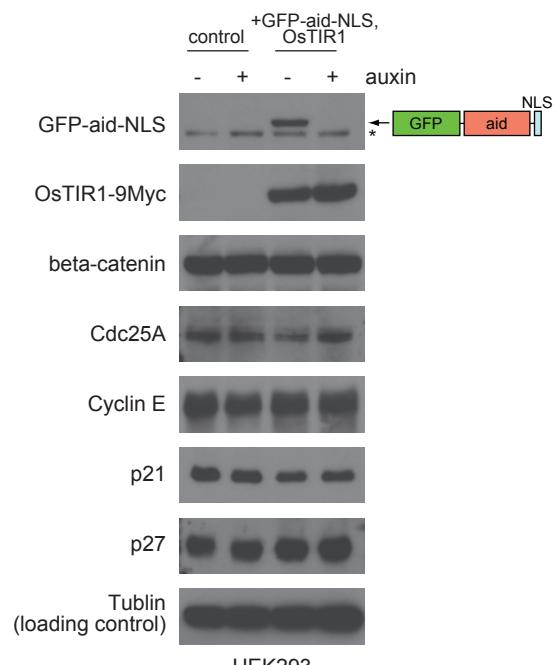
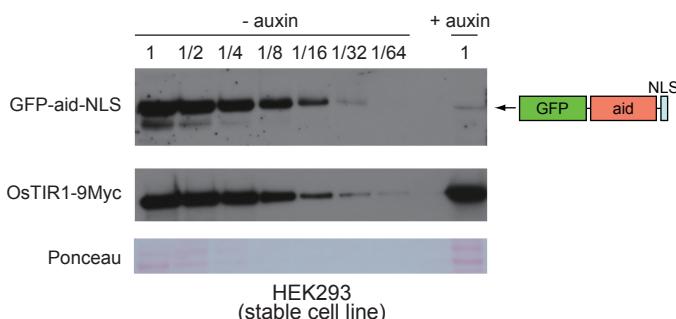
(a) Indicated strains in the GAL-*AtTIR1* background were grown on a YPG plate containing 500 μM IAA. While both *mcm4-aid* and *cdc45-aid* showed a tight growth defect, *dpb11-aid* was less sensitive to auxin. Dpb11, a BRCT-containing protein, is essential for the initiation of chromosome replication¹⁶. (b), Proteins expressed at a very low level are less efficient for degradation by AID. The strains used above were grown as in Figure 3b. Protein samples were prepared and the aid-fused proteins were detected by anti-aid antibody. Dpb11-aid, which only can be detected in the long exposure film, is expressed much less compared to Mcm4-aid and Cdc45-aid. Note that these proteins are expressed from their own promoters. In contrast to Mcm4-aid and Cdc45-aid, it seemed that Dpb11-aid were not efficiently depleted although some degradation of Dpb11-aid might have been induced. (c), IAA works better than NAA for some proteins. Indicated strains were grown on a YPG plate containing 500 μM NAA or IAA for two days at 24 °C. While *mcm4-aid* is highly sensitive to both NAA and IAA, *cdc45-aid* is more sensitive to IAA. A similar phenotype was observed with other essential proteins (data not shown).



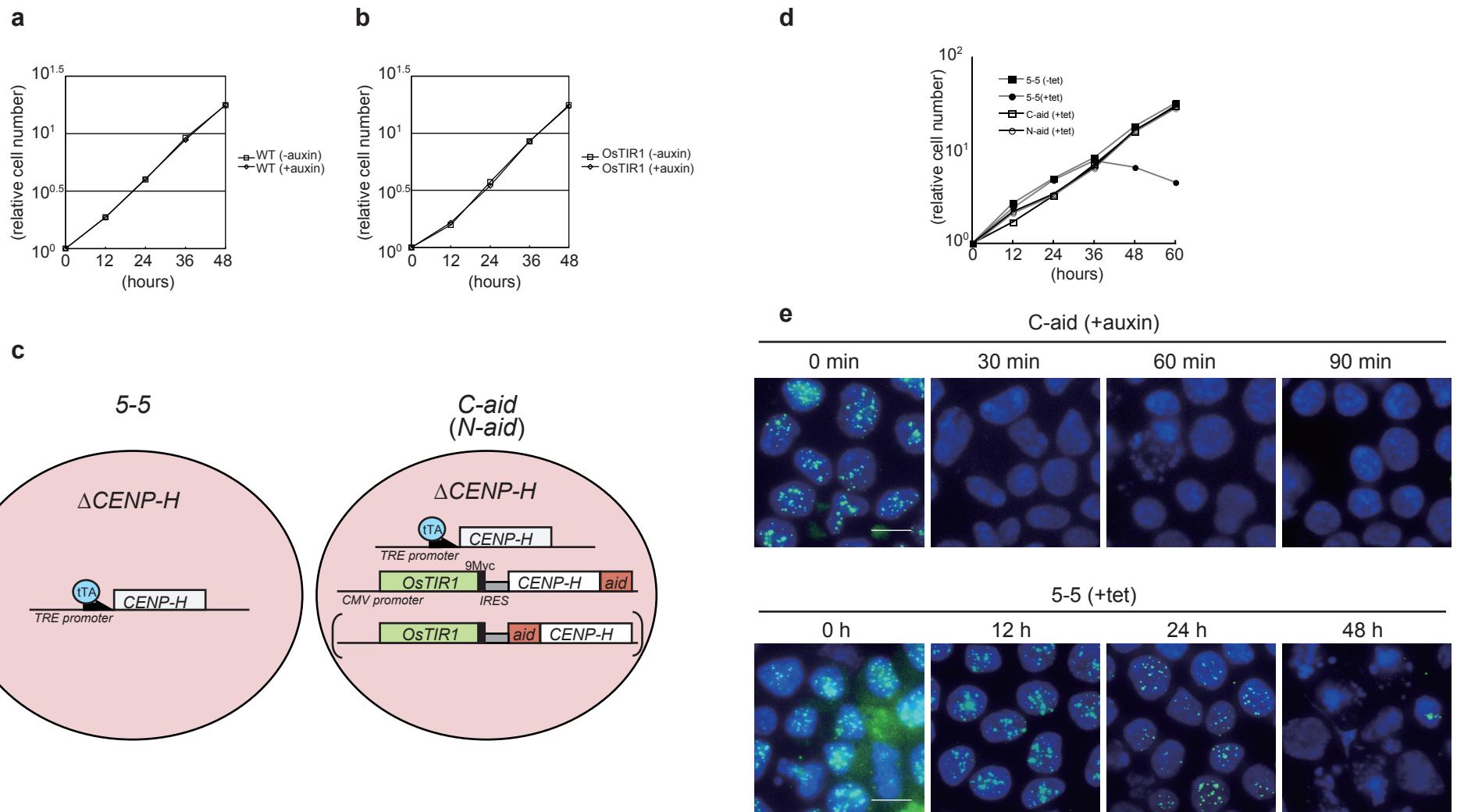
Supplementary Figure 8: OsTIR1 works even at high temperatures. (a) A thermo-stable AID system can be constructed using OsTIR1. Appropriate control and mcm4-aid cells expressing AtTIR1, GhTIR1 or OsTIR1 from the constitutive ADH1 promoter were spotted in serial dilution on a YPD plate with or without 500 µM NAA. Plates were incubated at indicated temperatures for two days. (b), Alignment of TIR1 orthologues. TIR1 from *A.thaliana* (GeneID 825473), *G.hirsutum* (GenBank DQ659621.1) and *O.sativa* (GeneID 4335696) were aligned using the ClustalW software. Black and grey boxes indicate identical and similar amino acids, respectively. (c), Phylogenetic tree of TIR1 homologues. TIR1 from *A.thaliana* (AtTIR1), *G.hirsutum* (GhTIR1) and *O.sativa* (OsTIR1) were analysed using the ClustalW software. Identities and similarities of amino acid sequences were indicated taking AtTIR1 as 100 %.

a**b****c**

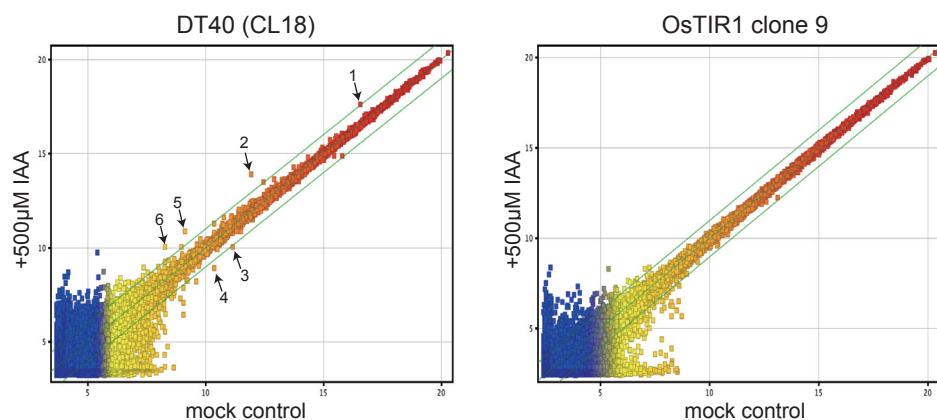
Supplementary figure 9: Protein depletion under different auxin concentrations. (a), Modulation of expression level by changing auxin concentration. GFP-aid-NLS in yeast of the *GAL-AtTIR1* or *GAL-OsTIR1* background was induced for degradation at the indicated NAA concentrations for 90 minutes at 24 °C. These results also show that OsTIR1 works better than AtTIR1 in budding yeast even at the lower temperature. (b), Auxin sensitivity of *mcm4-aid* strains can be modulated by changing auxin concentration. Yeast strains of *mcm4-aid* either in the *GAL-AtTIR1* or *GAL-OsTIR1* background were spotted on YPG plates containing the indicated concentration of NAA. After two days at 24 °C, intermediate growth defects can be observed at lower NAA concentrations. The *mcm4-aid*, *GAL-OsTIR1* strain showed more auxin-sensitivity than the *mcm4-aid* *GAL-AtTIR1* strain, suggesting that OsTIR1 works better than AtTIR1 for degradation of Mcm4-aid. (c), AID works thorough the SCF pathway. GFP-aid-NLS and *GAL-OsTIR1* were introduced into WT or cells harbouring the temperature-sensitive *cdc34-2* mutation. Cells were initially grown in YPR (R) at 24 °C and then released in YPG to induce expression of OsTIR1. Temperature was raised to 35 °C one hour before addition of 500 μM NAA. GFP-aid-NLS and OsTIR1-9Myc were detected using anti-GFP and anti-Myc antibodies, respectively. Depletion of GFP-aid-NLS was significantly suppressed in the *cdc34-2* strain showing that Cdc34 is required for AID to work.

a**b****c****d**

Supplementary figure 10: Additional data showing depletion of GFP-aid-NLS in mammalian cells. (a), An expression vector to test GFP degradation in mammalian cells. OsTIR1-9Myc and GFP-aid-NLS are expressed at the same time from transcripts driven by the CMV promoter. The encephalomyocarditis virus derived the internal ribosome entry sequence (IRES) allows expression of GFP-aid-NLS. (b), OsTIR1 lacking the F-box domain doesn't induce protein degradation. WT or a mutant lacking 1-260 aa of OsTIR1 were transiently transfected to COS1 cells. Cells were treated as in Figure 4b. (c), Neither addition of auxin nor activation of OsTIR1 affect expression of many SCF targets in mammalian cells. HEK293 and the stable strain expressing OsTIR1 were cultured in the presence or absence of 500 μM IAA for 3 hours. Cell extracts were prepared and separated for immunoblot. Anti-beta-catenin (12F7, MBL), anti-Cdc25A (DCS-121, MBL), anti-Cyclin E (HE12, MBL), anti-p21 (DCS-60, MBL) and anti-p27 (DCS-72, MBL) monoclonal antibodies were used for detection. Asterisk shows a background protein. (d), Quantification of residual GFP-aid-NLS in cells induced for degradation. The stable cell line expressing OsTIR1 and GFP-aid-NLS were treated as in the legend for Figure 4e. Serially diluted samples before addition of IAA and undiluted sample after 3 hours of a 500 μM IAA treatment were separated and blotted to detect GFP-aid-NLS. It was estimated that 1/32 (about 3 %) of GFP-aid-NLS was expressed in cells induced for degradation.



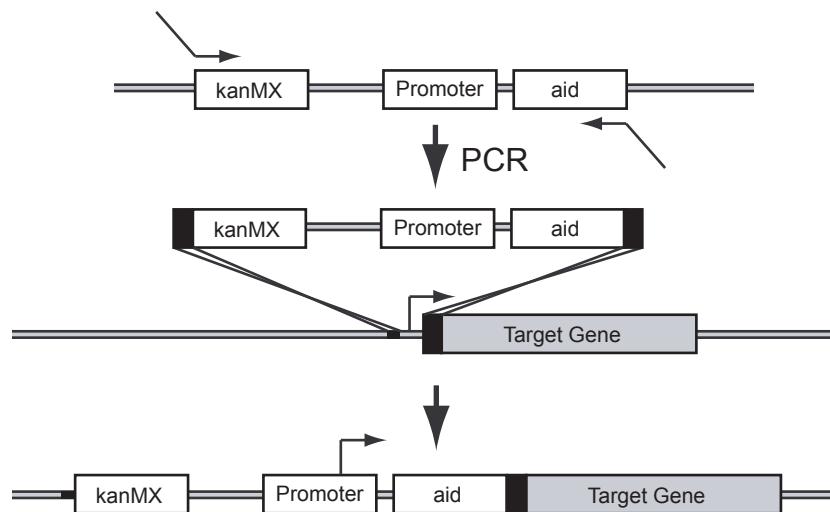
Supplementary Figure 11: Additional data for application of AID to DT40 cells. (a), Addition of auxin doesn't affect to growth of DT40. Wild type DT40 cells (CL18) were grown in the presence or absence of 500 μ M IAA. Living cells were counted after a Trypan blue staining at the indicated time points. (b), OsTIR1 activation doesn't affect to cell growth. Wild type DT40 was transformed with a construct that expresses OsTIR1-9Myc under the CMV promoter. A resultant clone (clone 9) was grown in the presence or absence of 500 μ M IAA. (c), Schematic illustration of DT40 cells in which expression of CENP-H is controlled by tetracycline (5-5) or auxin (C-aid and N-aid). C-aid and N-aid cells are maintained in the presence of 2 μ g/ml tetracycline through out growth. (d), The aid-fused CENP-H proteins are functional in DT40 cells. 5-5, C-aid and N-aid cells were grown and then 2 μ g/ml tetracycline was added at 0 time point. Cells were withdrawn at the indicated time and living cells were counted after a Trypan blue staining. (e), Immunofluorescence analysis of CENP-H after protein depletion by the AID system or mRNA depletion by the tetracyclin-repressive TRE promoter system. Auxin (500 μ M IAA) or 2 μ g/ml tetracycline was added to C-aid or 5-5 cells, respectively. Cells were fixed at the indicated time points and processed for microscopic analysis. Green dots and blue staining indicate CENP-H at the centromeres and chromosomal DNA, respectively. The scale bars correspond to 10 μ m.

a**b**

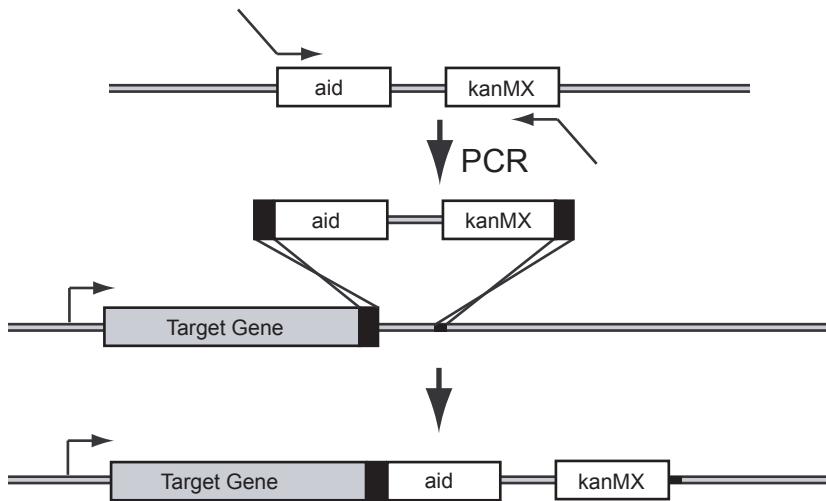
	Accession No.	Gene description	Function
1	CR385442	Gallus gallus finished cDNA, clone ChEST46211.	unknown
2	CV862621	gonad_EST10097 (Embryonic gonad cDNA Library)	unknown
3	gga-mir-206	microRNA	unknown
4	gga-mir-125b	microRNA	unknown
5	gga-mir-148a	microRNA	unknown
6	gga-mir-183	microRNA	unknown

Supplementary Figure 12: Auxin does not affect the global gene expression in DT40 cells. (a), Scattered plots of gene expression profile between a mock control cells (X axis) and cells cultured in the presence of 500 μ M IAA (Y axis). Data was analyzed on the GeneSpring GX10 software (Agilent Technologies). Obtained signals were normalized taking the total average signal from one array as 2500. Fold change value was set at 2.0. Among genes expressing relatively high level, 6 genes showed more than 2-fold differences in DT40 cells (left). Those genes were not affected in cells expressing OsTIR1 (right), suggesting that the observed difference in DT40 cells might not be significant. The complete data set is available at the Gene Expression Omnibus database in NCBI (accession no. GSE17523). (b), Differently expressed six genes found in DT40 cells. Two genes encode proteins of unknown function. The others are microRNAs of unknown function.

pMK38 (N-aid tagging vector)



pMK43 (C-aid tagging vector)



Supplementary Figure 13: PCR based ‘one-step tagging’ in budding yeast. pMK38, a plasmid to make PCR products for the amino-terminal aid tagging, is based on pKL187^{17, 18}. pMK43, a plasmid for carboxy-terminal tagging, is based on pYM18¹⁹. The same primers used for pKL187 or pYM18 can be used for aid tagging at the amino or carboxy terminus of target protein, respectively. Detailed sequence information and the plasmids can be available at the Yeast Genetic Resource Centre (http://yeast.lab.nig.ac.jp/nig/index_en.html).

Supplementary Table 1

Comparison of degradation based methods to control protein expression.

	Works in yeast?	Works in mammalian cells?	Ligand	Time required for depletion	Others
AID	Yes	Yes	Auxin	Less than 30 min	Auxin induces degradation
ddFKBP²⁰	Not known	Yes	Shield1	4 hours	Shield1 stabilizes the degron
ts-degron^{21, 22}	Yes	No	Non (temperature shift)	Less than 30 min	The degron can be placed only at the N terminus
Direct localization to the proteasome²³	Yes	No	Rapamycin	40-60 min	
The F-box fusion method²⁴⁻²⁷	Yes	Yes	Required a known binding domain for each target	30-60 min	
PROTACS^{28, 29}	Not known	Yes	Required a known binding chemical for each target	60 min	

Supplementary Table 2

All yeast strains used in this study.

Strain	Genotype
W303-1a	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</i>
YMK541	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i>
YMK539	<i>ura3-1::GAL-AtTIR1-9Myc (URA3), cdc53::CDC53-5FLAG (hphNT)</i>
YMK549	<i>trp1-1::ADH1- aid-EGFP-NLS (TRP1)</i>
YMK551	<i>ura3-1::GAL-AtTIR1-9Myc (URA3), trp1-1::ADH1- aid-EGFP-NLS (TRP1)</i>
YMK550	<i>trp1-1::ADH1-EGFP-aid-NLS (TRP1)</i>
YMK552	<i>ura3-1::GAL-AtTIR1-9Myc (URA3), trp1-1::ADH1-EGFP-aid-NLS (TRP1)</i>
YNK24	<i>trp1-1::ADH1-GFP-aid (TRP1)</i>
YMK26	<i>ura3-1::GAL-AtTIR1-9Myc (URA3), trp1-1::ADH1- EGFP-aid (TRP1)</i>
YNK77	<i>ura3-1::GAL-AtTIR1-9Myc (URA3), mcm4::aid-mcm4 (KanMX)</i>

YNK14	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i> , <i>mcm4::mcm4-aid (KanMX)</i>
YNK30	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i> , <i>cdc45::cdc45-aid (KanMX)</i>
YMK599	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i> , <i>ask1::ask1-aid (KanMX)</i>
YMK601	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i> , <i>myo2::myo2-aid (KanMX)</i>
YMK597	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i> , <i>myo1::myo1-aid (KanMX)</i>
YNK93	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i> , <i>alg14::aid-alg14 (kanMX)</i>
YNK99	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i> , <i>alg14::alg14-aid (kanMX)</i>
YNK95	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i> , <i>sam35::sam35-aid (kanMX)</i>
YNK97	<i>ura3-1::GAL-AtTIR1-9Myc (URA3)</i> , <i>sam50::sam50-aid (kanMX)</i>
YMK612	<i>ura3-1::ADH1-AtTIR1-9Myc (URA3)</i>
YMK614	<i>ura3-1::ADH1-AtTIR1-9Myc (URA3)</i> , <i>mcm4::mcm4-aid (kanMX)</i>
YMK688	<i>ura3-1::ADH1-GhTIR1-Myc (URA3)</i>
YMK690	<i>ura3-1::ADH1-GhTIR1-Myc (URA3)</i> , <i>mcm4::mcm4-aid (kanMX)</i>
YNK54	<i>ura3-1::ADH1-OsTIR1-9Myc (URA3)</i>
YMK683	<i>ura3-1::ADH1-OsTIR1-9Myc (URA3)</i> , <i>mcm4::mcm4-aid (kanMX)</i>
YMK675	<i>cdc34-2, ura3-1::GAL-OsTIR1-9Myc (URA3)</i> , <i>trp1-1::ADH1-EGFP-aid-NLS (TRP1)</i>
YMK676	<i>ura3-1::GAL-OsTIR1-9Myc (URA3)</i> , <i>trp1-1::ADH1-EGFP-aid-NLS (TRP1)</i>
YNK40	<i>ura3-1::GAL-OsTIR1-9myc (URA3)</i>
YNK41	<i>ura3-1::GAL-OsTIR1-9myc (URA3)</i> , <i>mcm4::mcm4-aid (kanMX)</i>

W303-1a (<http://wiki.yeastgenome.org/index.php/CommunityW303.html>) is the parental strain for all the others. All strains listed are available at the Yeast Genetic Resource Centre (http://yeast.lab.nig.ac.jp/nig/index_en.html).

Supplementary Table 3

DT40 strains used in this study.

Strain	Genotype
OsTIR1 clone 9	<i>CMV promoter-OsTIR1-9Myc transgene (pCMV-OsTIR1 containing neo')</i>
5-5 ³⁰	<i>Δ CENP-H (puro')</i> , <i>TRE promoter-CENP-H transgene (pUHD-CENP-H)</i> , <i>CMV promoter-tTA (pUHD15-1 containing zeo')</i>
N-aid	<i>Δ CENP-H (puro')</i> , <i>TRE promoter-CENP-H transgene (pUHD-CENP-H)</i> , <i>CMV promoter-tTA (pUHD15-1 containing zeo')</i> , <i>CMV promoter-OsTIR1-9Myc-IRES-aid-CENP-H (neo')</i>
C-aid	<i>Δ CENP-H (puro')</i> , <i>TRE promoter-CENP-H transgene (pUHD-CENP-H)</i> , <i>CMV promoter-tTA (pUHD15-1 containing zeo')</i> , <i>CMV promoter-OsTIR1-9Myc-IRES-CENP-H-aid (neo')</i>

All cell lines were derived from parental CL18 DT40 cell³¹.

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