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REPORT

Efficient protein depletion by genetically controlled deprotection of a dormant N-degron

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Methods that allow for the manipulation of genes or their products have been highly fruitful for biomedical research. Here, we describe a method that allows the control of protein abundance by a genetically encoded regulatory system. We developed a dormant N-degron that can be attached to the N-terminus of a protein of interest. Upon expression of a site-specific protease, the dormant N-degron becomes deprotected. The N-degron then targets itself and the attached protein for rapid proteasomal degradation through the N-end rule pathway. We use an optimized tobacco etch virus (TEV) protease variant combined with selective target binding to achieve complete and rapid deprotection of the N-degron-tagged proteins. This method, termed *T*EV protease *i*nduced *p*rotein *i*nactivation (TIPI) of TIPI-degron (TDeg) modified target proteins is fast, reversible, and applicable to a broad range of proteins. TIPI of yeast proteins essential for vegetative growth causes phenotypes that are close to deletion mutants. The features of the TIPI system make it a versatile tool to study protein function in eukaryotes and to create new modules for synthetic or systems biology.
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Introduction

Regulation of protein activity by gene disruption/deletion, RNAi, promoter shut-off, temperature sensitive alleles, chemical inhibition/inactivation, constitutive protein destabilization, and heat or small molecule-regulated N-degrons for induced protein degradation (Dohmen *et al*, 1994; Stack *et al*, 2000; Mnaimneh *et al*, 2004; Dohmen and Varshavsky, 2005; Banaszynski *et al*, 2006; Suter *et al*, 2006; Boutros and Ahringer, 2008) are highly useful tools to study protein function. A restriction immanent to these methods is the difficulty to induce protein degradation/inactivation in a highly selective manner, for example, in a specific tissue or during a particular cell-cycle or developmental stage. Methods to overcome some of these limitations exist, for example, the Cre/lox system for the genetically encoded and regulated excision of a gene from the chromosome (Sauer, 2002). In this case, the time required to establish the phenotype is decided mainly by the stability of the protein. Another method relies on regulated cleavage of target proteins by the tobacco etch virus (TEV) protease inside living cells (Henrichs *et al*, 2005; Pauli *et al*, 2008; Satoh and Warren, 2008). This system requires *a priori* knowledge about the structure of a protein to be able to introduce the TEV protease cleavage site into a target protein and to render its function sensitive to site-directed proteolysis. Alternatively, proteolysis sensitive sites could be identified by means of functional assays. However, ectopically driven proteolytic fragmentation may only affect a specific subset of the functions of a protein.

Complete degradation of proteins has been achieved using N-degrons; this degradation mechanism is conserved from bacteria to higher eukaryotes (Varshavsky, 1997). N-degrons

constitute natural or artificial amino-terminal tags that are proteolytically processed, thereby leading to the exposure of an amino acid other than methionine at the amino terminus of a protein. The exposed amino acid serves as a recognition signal for poly-ubiquitylation and subsequent proteasomal degradation through the N-end rule pathway in eukaryotes (Bachmair *et al*, 1986). It determines the degradation rate of the protein with half-life times ranging between a few minutes (e.g. 2–3 min for arginine, phenylalanine, and aspartic acid) up to >20 h (e.g. serine or methionine) (Bachmair *et al*, 1986; Mogk *et al*, 2007).

Results and discussion

To create an N-degron that is activated only upon the conditional expression of a specific activator, we developed a degron that is protected at its N-terminus by an attached peptide that can be removed by proteolysis using the site-specific TEV protease (Parks *et al*, 1994). The TEV protease has been used *in vivo* in many different organisms (bacteria, yeast cells, drosophila, and mammalian cell culture) without negative side effects (Uhlmann *et al*, 2000; Kapust *et al*, 2002; Wehr *et al*, 2006; Pauli *et al*, 2008). Initially, we generated a fusion of a seven amino acid long TEV protease recognition site to the N terminus of an earlier developed N-degron (Suzuki and Varshavsky, 1999). The TEV protease cleaves between positions 6 and 7 of the recognition site. The enzymatic activity of TEV is somewhat flexible towards changes in the sequence, especially at position 7 (Kapust *et al*, 2002), which becomes the new N-terminal amino acid (in the following termed residue X) after proteolytic cleavage. Destabilizing amino-acid residues at the amino terminus (position X) target a protein for rapid destruction if the N-degron contains a sequence that allows the attachment of ubiquitin (Varshavsky, 1997). To monitor the cleavage, we fused a fluorescent protein to the N terminus of the TEV protease recognition site. To improve the processivity of the TEV protease, we enhanced the binding of the TEV protease to its substrate. We fused the N-degron construct with the TEV protease recognition site to the SF3b155$^{381-424}$ protein domain. This domain is specifically recognized by the human spliceosome subunit p14 (Spadaccini *et al*, 2006), which we in turn fused to the TEV protease (named p14–TEV). Furthermore, we identified, by chance, a mutated allele of p14 (called p14*), which enhanced cleavage significantly. In summary, we have constructed a dormant N-degron that is constituted of a reporter, followed by a TEV protease recognition site (including residue X), an N-degron and SF3b155$^{381-424}$ (in the following termed Reporter–TDegX-tag, e.g. GFP–TDegF-tag). This dormant N-degron can be deprotected by the expression of the p14*–TEV fusion protein (pTEV). An overview of the TEV protease *i*nduced *p*rotein *i*nactivation (TIPI) system is shown in Figure 1. The mechanism underlying the enhanced activity of the p14*–TEV fusion versus p14–TEV is not clear, as the responsible mutation lies within a stretch of amino acids in p14 that is not involved in binding of p14 to SF3b155$^{381-424}$ (data not shown; Schellenberg *et al*, 2006; Spadaccini *et al*, 2006). For details on the development of the TIPI system, see Supplementary information.

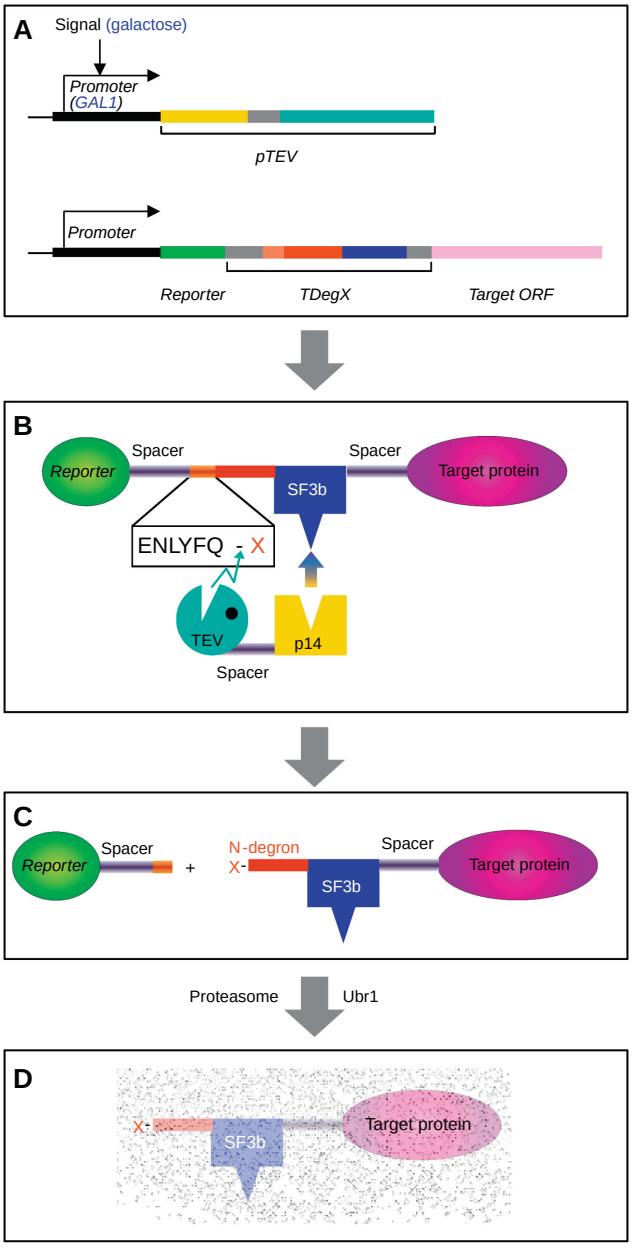

Figure 1 TEV protease induced protein instability (TIPI). The principle of TIPI, a method to genetically control abundance of proteins with an N terminus exposed to the cytoplasm or nucleus. (**A**) The GFP–TDegX-tag is fused to the 5′-end of the target open reading frame (target ORF), directly in front of the ATG. The gene for pTEV expression is regulated by a controllable promoter; in this study, we used the galactose responsive *GAL1*-promoter in yeast. (**B**) Upon expression of *pTEV*, the pTEV protease binds to the GFP–TDegX-target protein. Binding is mediated by interaction of p14 with SF3B155$^{381-424}$. This interaction directs efficient cleavage of the GFP–TDegX-tag by the TEV protease at its consensus site (ENLYFQ-X). (**C**) Cutting of the GFP–TDeg-tag leads to deprotection of the dormant N-degron that is part of the GFP–TDegX-tag. The N-degron is constituted by the new N-terminal amino acid X and a sequence that promotes efficient poly-ubiquitylation by Ubr1p (Suzuki and Varshavsky, 1999). The exposed amino acid X determines the fate of the protein. In yeast, X=A, C, G, M, P, S, T, and V lead to stable proteins, whereas X = D, E, F, H, I, K, L, N, Q, R, W, and Y render proteins instable (half lives=2–30 min) (Bachmair *et al*, 1986). (**D**) The target protein is poly-ubiquitylated by Ubr1p and degraded by the proteasome.

We used *Saccharomyces cerevisiae* as a model organism to develop and test the TIPI system. As a target protein, we used the non-essential, soluble, and freely diffusible protein Don1p (Maeder *et al*, 2007). Don1p is a protein with a role only in yeast sporulation, and it is absent in vegetatively growing cells (Knop and Strasser, 2000). We monitored the processing and degradation of the GFP–TDegX–Don1p fusion proteins as a function of *pTEV* expression (driven by the inducible *GAL1*-promoter) using western blotting and antibodies specific for GFP or Don1p. The amino acid at position X of the GFP–TDegX-tag is predicted to influences both, the cleavage efficiency of pTEV and the half-life of the target protein. We found that X=Phe (F; GFP–TDegF) and X=Asp (D; GFP–TDegD) provide optimal combinations of both, excellent cleavage followed by rapid protein degradation resulting in very low Don1p protein amounts upon *pTEV* expression (Figure 2A–C). Degradation is dependent on the E3 protein, which is encoded by the ubiquitin-protein ligase gene *UBR1* (Figure 2A), indicating proteasomal degradation by the N-end rule pathway (Bartel *et al*, 1990). Furthermore, repression of *pTEV* expression rapidly restores protein levels of the target protein (Figure 2B). The TEV protease cleaved target protein is not degraded in strains lacking Ubr1p or if the TDegM-tag is fused to the target protein (Figure 2A and B). This excludes that addition of the TDegF-tag or expression of the TEV protease caused side effects that act on target protein production. The use of different residues at position X enables specific modulation of the cleavage efficiency (e.g. TDegK) and the degradation rate (e.g. TDegH) (Figure 2C). In summary, TIPI is a new method suitable for the precise post-translational regulation of protein abundance.

The published crystal structure of the TEV protease (Phan *et al*, 2002; Nunn *et al*, 2005) allowed us to predict a TEV protease mutation, which improved its proteolytic activity by redefining the protein border at the C terminus. Using this modified pTEV, called pTEV^{+}, efficient cleavage of the protease was enhanced, as observed by improved processing of the GFP–TDegX–Don1p reporter in cells where its expression was driven by the very strong *GPD*-promoter (Figure 2D).

We used live cell imaging to obtain the kinetics of protein depletion by TIPI. We used the red fluorescent protein mKate as a target, N-terminally tagged with the CFP–TDegX-tag, yielding the construct CFP–TDegX–mKate. The pTEV^{+} protease (under control of the inducible *GAL1* promoter) was N-terminally tagged with the yellow fluorescent protein (YFP) citrine (YFP–pTEV^{+}). Using this setup, we observed rapid depletion of mKate fluorescence upon YFP–pTEV^{+} expression in wild type, but not in *ubr1*Δ cells or if a CFP–TDegM–mKate construct was used (Figure 2E). Quantification of the cellular fluorescence intensities in the yellow and red channel revealed rapid depletion of TDegF–mKate within the first hour of YFP–pTEV^{+} expression (Figure 2F). After 3–4 h of YFP–pTEV^{+} expression, we noticed some residual red fluorescence. This is because of the loss of YFP–pTEV^{+} encoding plasmids in a subpopulation of cells (as confirmed by microscopy) and, therefore, incomplete processing of CFP–TDegF–mKate. Such residual levels do not occur in the experiments where chromosomally integrated constructs were used (Figure 2A–C).

Together, these results show that TIPI is a valuable method to induce the depletion of a protein. We used a controllable promoter to induce pTEV protease expression, but the method is easily adapted to a developmental process using distinct drivers for *pTEV* expression, which are only active at specific stages or in specific cell types. Protein depletion is easily followed in live cells; this allows correlating protein abundance with phenotype establishment.

To test whether TIPI is able to deplete *S. cerevisiae* proteins sufficiently to cause a phenotype similar to the corresponding gene-deletion, we fused the GFP–TDegF-tag to several soluble (nuclear and cytoplasmic) and membrane proteins, which are all essential for vegetative growth of *S. cerevisiae*. The amino terminus of all chosen proteins is either exposed to the cytoplasm or the nucleoplasm. The GFP–TDegX–tagged fusion proteins revealed localizations that were comparable to the corresponding C-terminally GFP-tagged proteins (Huh *et al*, 2003) (Supplementary Figure 2A). Expression of *pTEV* led to the cleavage of the fusion proteins (Supplementary Figure 2B) and to the inhibition of cell growth, which was either completely abolished (in 6/8 tested proteins) or reduced (Cdc15p and Nud1p) (Figure 3A). Growth was rescued by a *UBR1* deletion or by using GFP–TDegM that contains a stabilizing amino acid (Figure 3A and data not shown). Importantly, expression of *pTEV* or *pTEV*$^{+}$ protease alone did not affect growth of yeast cells (Figure 3A and B). Strong production of the target protein GFP–TDeg–Cdc14p using the *ADH1* promoter (Janke *et al*, 2004) required the presence of the more active pTEV^{+} protease to result in a growth phenotype (Figure 3B). The use of the pTEV protease was sufficient to abrogate Cdc14 function, if the target protein was expressed from the weaker *CYC1* promoter (Janke *et al*, 2004) (Figure 3A). Surprisingly, TIPI of the integral membrane proteins GFP–TDegF–Sec12p, GFP–TDegF–Pma1p, and GFP–TDegF–Alr1p resulted in non-viable cells. This indicates that these proteins are accessible to the degradation machinery, which may be the case prior or during their insertion into the membrane, or at their final localization. We analyzed whether pTEV is able to cut efficiently near the plasma membrane and found complete cutting within 3–4 h after induction of *pTEV* expression (Supplementary Figure 3). Up to now, there is no report of membrane proteins being degraded through the N-end rule pathway. It may be that degradation of these proteins is assisted by other ubiquitylation triggered degradation pathways, for example, through endocytosis and vacuolar degradation (Hicke, 1997; Hicke and Dunn, 2003).

TIPI of Cdc5p, Cdc14p, and Cdc48p, three proteins involved in cell-cycle regulation, led to cell-cycle defects: predominantly anaphase arrest in the case of GFP–TDegF–Cdc5p (55 ± 3% versus 3 ± 2% in the wild type) and increased frequency of metaphase arrested cells in the case of GFP–TDegF–Cdc14p (32 ± 10% versus 3 ± 3% in the wild type) and GFP–TDegF–Cdc48p (41 ± 21% versus 3 ± 3% in the wild type) (Figure 3C). The cell-cycle defects of GFP–TDegF–Cdc5p and GFP–TDegF–Cdc48p match the ones reported in the literature for temperature sensitive mutants (Hartwell *et al*, 1973; Frohlich *et al*, 1991). For GFP–TDegF–Cdc14p, we noticed a predominant arrest in metaphase (Figure 3C), whereas *cdc14* temperature sensitive mutants arrest in anaphase and exhibit defects in exit of mitosis (Charles *et al*, 1998). This phenotype

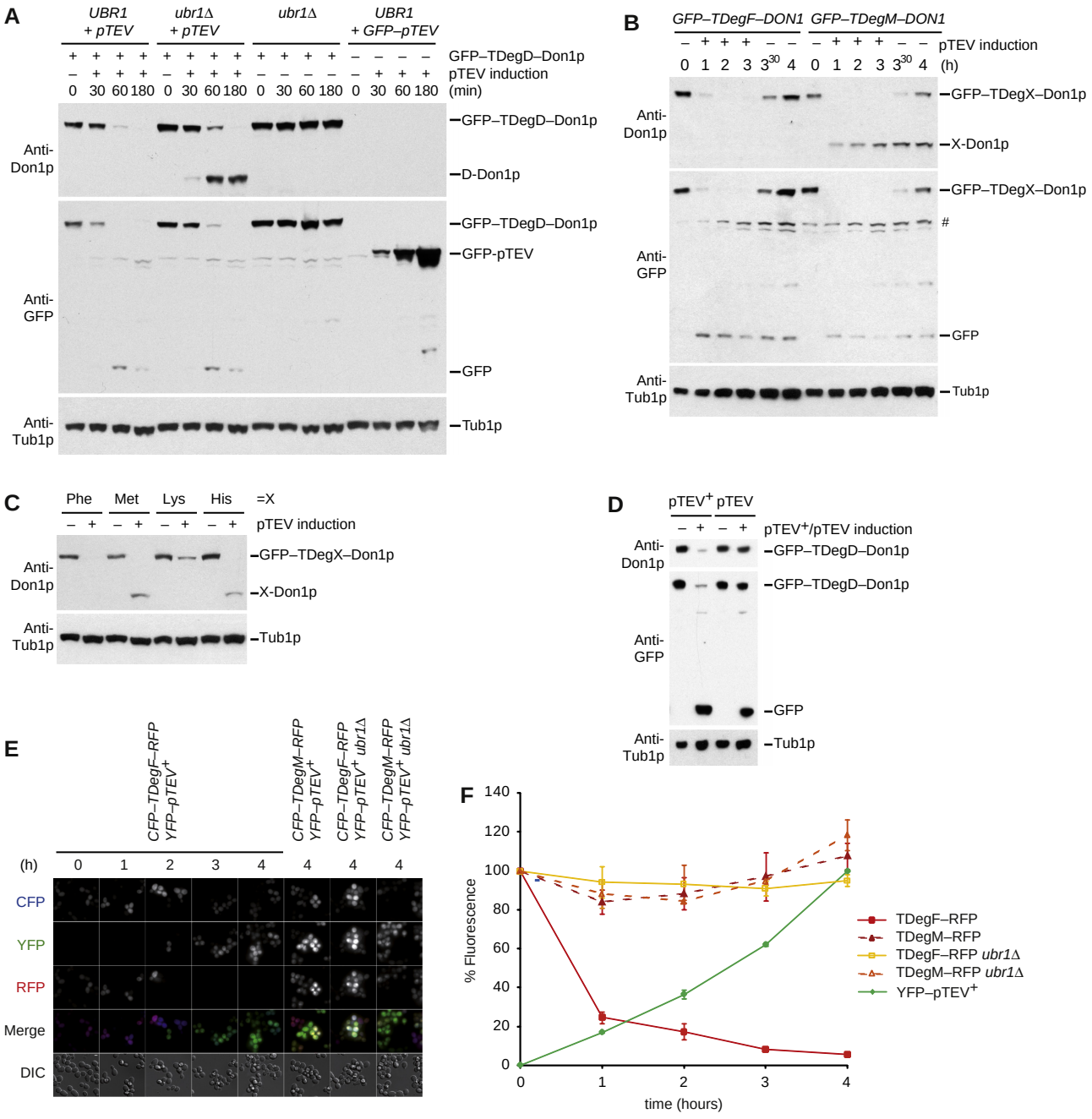

Figure 2 TIPI mediates rapid degradation of proteins in yeast. (**A**) TIPI leads to rapid degradation of GFP–TDegD-tagged proteins. *GFP–TDegD–DON1* was expressed chromosomally using the constitutive *ADH1* promoter. Expression of *pTEV* or *GFP–pTEV* was induced by the addition of galactose (2% final concentration) to the culture. Samples of logarithmically growing yeast cells were removed from the culture at the indicated time points and subjected to western blotting. For detection of reporter constructs, anti-GFP and anti-Don1p antibodies were used. Detection of tubulin was used as a loading control. Positions of cleaved and uncleaved species are indicated in the figure. Strains used were either wild type or deleted for the gene *UBR1* (*ubr1*Δ) as indicated. The strains we used in this experiment are described in Supplementary Table I, and their construction is indicated in Supplementary Table III. (**B**) Depletion of proteins by TIPI is reversible. *GFP–TDegF–DON1* and *GFP–TDegM–DON1* were expressed chromosomally using the constitutive *ADH1* promoter. To induce *pTEV* expression galactose was added (at time point 0 h), repression of *pTEV* expression was done by adding glucose (at time point 3 h). Western blotting was performed as described in panel A. A # indicates the position of a non-specific band. (**C**) Modulation of protein abundance using different versions of *GFP–TDegX*. Protein levels of cleaved and uncleaved GFP–TDegF-, GFP–TDegM-, GFP–TDegK-, or GFP–TDegH–Don1p were assessed in crude extracts of yeast cells before and after 3 h of *pTEV* expression. *GFP–TDegX* constructs were expressed chromosomally from the *ADH1*-promoter. Western blotting was performed as described in Figure 2A. (**D**) C-terminal truncation of pTEV protease enhances proteolytic activity. Protein levels of cleaved and uncleaved GFP–TDeg–Don1p were assessed before and after 3 h of *pTEV* or C-terminally truncated *pTEV*$^{+}$ expression. Strong overexpression of *GFP–TDegD–DON1* constructs was achieved using the strong *GPD*-promoter. Western blotting was performed as described in panel A. (**E**) Protein depletion by TIPI can be followed by live cell imaging. Plasmid encoded *CFP–TDegF–mKATE* and *CFP–TDegM–mKATE* were expressed constitutively under control of the *ADH1* promoter in wild-type cells and cells lacking *UBR1* (*ubr1*Δ). Expression of *pTEV*$^{+}$ (plasmid encoded) was induced by the addition of galactose (2% final concentration) to the cells. Images of the cells were taken at the indicated time points. (**F**) Quantification of the experiment shown in (E). Images from the cells used in (E) were recorded after induction of YFP–pTEV^{+}. Automated quantitative image analysis was used to measure the cellular fluorescence of the different fluorescent protein reporters in 1000 to 3000 cells per strain (error bars represent the standard error of the mean). The yeast strains that were used to perform the experiments (A–F) are listed in Supplementary information. The genotypes are given in Supplementary Table I, the plasmids are described in Supplementary Table II.

may indicate that deprotection of the degron in GFP–TDegF–Cdc14p leads to a Cdc14p species that interferes with an early mitotic function (Bloom and Cross, 2007) or that the commonly used *cdc14* temperature sensitive mutants do not block this function completely. In the absence of TEV protease, no difference between GFP–TDegF-tagged and control cells was found (data not shown). TIPI of Sec12p, a membrane protein involved in ER to Golgi transport caused a prominent enrichment of the high molecular weight precursor forms of carboxypeptidase Y (CPY) (Figure 3D), indicative for impaired transport of proCPY to the vacuole where it is proteolytically processed (Kaiser and Schekman, 1990).

Our experiments demonstrate that TIPI enables the construction of conditional mutants: the regulated induction of *pTEV* expression enables the depletion of proteins from cells by targeted degradation. In conclusion, our yeast work demonstrates that protein depletion by TIPI is very quick; another advantage is that live cell imaging can be used to follow protein inactivation. This allows the comparison of protein abundance to phenotype establishment.

The high conservation of the N-end rule pathway in eukaryotic organisms (Mogk *et al*, 2007) suggests that TIPI could be useful in many different cell types and model organisms. Alternative implementations of TIPI could be developed, for example, using small molecule-regulated binding (Bayle *et al*, 2006) of the TEV protease to the N-degron. This, or the use of another site-specific protease for which a specific inhibitor is available, may provide additional ways to control the deprotection of the N-degron. Instead of using the TEV protease to deprotect the N-degron, one could also use the split-ubiquitin-system. This system was originally developed to detect protein–protein interactions (Wittke *et al*, 2000). Here, conditional expression of one half of ubiquitin and its specific targeting to a substrate carrying the other half, for example, using the p14–SF3b155 interaction, would trigger the removal of the reconstituted ubiquitin by the ubiquitin proteases (Johnsson and Varshavsky, 1994) and would lead to target protein destabilization.

One attractive application of TIPI is to use it for developmental studies in higher eukaryotes. Distinct drivers or

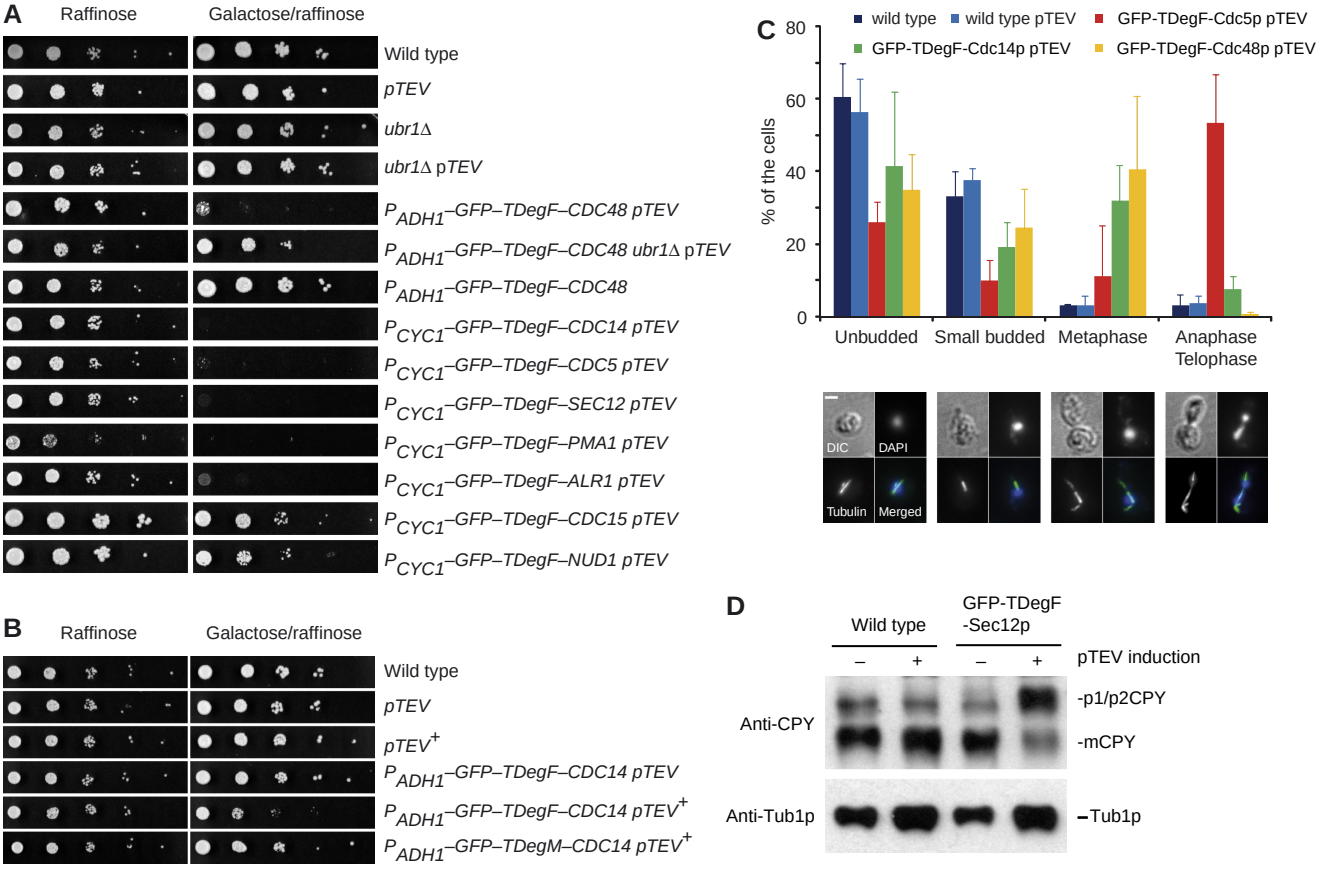

Figure 3 TIPI of essential yeast proteins causes lethal phenotypes. (**A**) TIPI of essential proteins leads to impaired growth phenotypes. Serial dilutions (1:10) of yeast cultures (genotypes of yeast strains are indicated) were spotted on synthetic complete media containing either raffinose or galactose/raffinose and incubated at 30°C for 3 days. GFP–TDegF fusions were expressed either from the *ADH1* (P_{ADH1}) or the *CYC1* (P_{CYC1}) promoter (as indicated). (**B**) pTEV$^+$ protease exhibits increased activity as compared with pTEV. Experimental conditions were the same as described in (A) using strains that express the indicated constructs. (**C**) TIPI of Cdc5p, Cdc14p or Cdc48p leads to cell-cycle defects. Cell-cycle phenotypes were assessed after 3 h of *pTEV* expression in *GFP–TDegF–CDC5*, *GFP–TDegF–CDC14* and *GFP–TDegF–CDC48* expressing strains. Wild-type cells with and without expression of *pTEV* were used as controls. Samples were fixed and cell-cycle stages assessed based on bud size, spindle morphology, and DNA segregation. (**D**) TIPI of Sec12p leads to impaired secretion. Samples of control cells and TDegF–Sec12p expressing cells were taken before (−) and after 3 h (+) of pTEV protease induction and subjected to western blotting. The secretory marker protein carboxypeptidase Y (CPY) was detected. mCPY, mature, vacuolar form of CPY; p1 + p2CPY, ER and Golgi glycosylated forms of CPY. The yeast strains that were used to perform the experiments (A–D) are listed in Supplementary information. The genotypes of these strains are given in Supplementary Table I.

promoters specific to developmental stages, tissues, and cell types are available for model organisms like worm, *Drosophila*, or mice. They should be applicable for selective induction of *pTEV* or $pTEV^{+}$ protease expression. The specificity of the expression system used will decide how precisely the effects of TIPI-mediated protein inactivation can be linked to the process under investigation. In addition, it is essential to conduct the experiments in animals that lack the functional wild-type protein (e.g. by using a mutant, gene knock-out animal, or gene downregulation by RNAi-based methods). Control experiments without expressed TEV protease will report whether the TIPI-tag constricts the function of the target protein. Overproduction of the target protein should be avoided, as it may interfere with the function of the protein and with its downregulation by the TIPI system. The use of stable variants of the TIPI tag, for example, TDegM, provide further controls for the effect of the N-degron.

In conclusion, the TIPI system provides a method for efficient regulation of protein abundance in functional studies and for the creation of regulatory modules in synthetic biology.

Materials and methods

Yeast strains, plasmids, and growth conditions

All yeast strains used in this study were derived from the S288C strain ESM356-1 (Pereira *et al*, 2001). Genotypes are listed in Supplementary Table I. Manipulation of yeast strains using PCR targeting was performed as described (Janke *et al*, 2004). Standard methods for yeast strain construction were used otherwise (Sherman, 2002). Supplementary Table II lists the plasmids used to construct the yeast strains (Supplementary Table I), as indicated in Supplementary Table III. The gene encoding the TEV protease was isolated from TEV-infected tobacco leaves by PCR. The mutation S219V that inhibits autoproteolysis (Kapust *et al*, 2001) was introduced along with mutations that increase the solubility of the TEV protease (van den Berg *et al*, 2006). The codons for leucine and arginine were exchanged to optimize expression in yeast cells. The amino-acid sequences of the GFP–TDegX-tag and p14–TEV protease are provided in Supplementary Figure 4. Cloning details and nucleotide sequences are available upon request.

Standard preparations of growth media were used as described (Sherman, 2002). Growth tests were performed on synthetic complete media plates supplemented either with 2% raffinose or with 2% raffinose and 2% galactose. Cells used for immunodetection of tagged proteins by western blotting were grown in liquid synthetic complete media supplemented with 2% raffinose. TEV protease expression was induced by adding 2% galactose. TEV protease expression was repressed by the addition of 2% glucose. Cells used for fluorescence microscopy were grown in low-fluorescence media (Sheff and Thorn, 2004) supplemented with 2% raffinose.

Western blotting, antibodies, and immunofluorescence

Aliquots of cells from growing cultures were taken for crude protein extract preparation and western blotting using the protocol described in Janke *et al* (2004). Polyclonal rabbit anti-CPY, rabbit anti-tubulin, and rabbit anti-GFP antibodies were used to detect CPY, tubulin, and GFP (Finger *et al*, 1993; Maier *et al*, 2008). Immunofluorescence microscopy was performed as described in Maier *et al*, 2008.

Light microscopy and quantification

Live cell imaging was performed as described earlier (Taxis *et al*, 2006). Briefly, the cells were grown to logarithmic growth phase, adhered to concanavalin A coated glass-bottom-dishes (MaTek Corp.) and imaged in bright field and green fluorescence (Supplementary Figures 2A and 3) or bright field, cyan, yellow, and red fluorescence (Figure 2E). For quantification, cells were segmented from image backgrounds using yellow fluorescence images. Subsequently, cell outlines were transformed into masks used to measure pixel intensities of all fluorescent channels. Images of yeast cells expressing no fluorescent proteins were used to measure background fluorescence. Quantification data shown in Figure 2E were obtained by imaging between 1000 and 3000 cells for each time point and each yeast strain. Image processing, segmentation, and quantification were performed using the software imageJ. Background subtraction, normalization (time point 0 h for RFP, time point 4 h for YFP), calculation of the mean fluorescence and the standard error of the mean were done using the software Excel (Microsoft Corp.).

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

Conflict of interest

The authors declare that they have no conflict of interest.

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