

CORRECTION

Correction: Sensitive detection of protein ubiquitylation using a protein fragment complementation assay

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There was an error in *J. Cell Sci.* (2020) **133**, jcs240093 (doi:10.1242/jcs.240093).

In their article, the authors omitted to mention ubiquitin-mediated fluorescence complementation (UbFC), a method previously designed to probe the conjugation of ubiquitin or ubiquitin-like proteins (Ubls) to their substrates (Fang and Kerppola, 2004; Sung et al, 2013). UbFC and NUbICA are similar in that both methods rely on a protein-fragment complementation assay to detect Ubl conjugation, but the reporters (fluorescent proteins for UbFC, the NanoLuc luciferase for NUbICA) and assay conditions (live or fixed cells for UbFC, purified proteins for NUbICA) are different. UbFC is appealing since it enables direct visualization of the subcellular localization of Ubl conjugates in living cells, which is not possible with NUbICA. Yet, the irreversible assembly and slow maturation of fluorescent proteins (Hu et al., 2002; Kodama and Hu, 2012) precludes real-time monitoring of Ubl conjugation and deconjugation events, which may complicate the interpretation of UbFC results, especially for short-lived or dynamically modified conjugates. Other approaches based on FRET and BRET have also been devised to probe ubiquitylation of proteins in living cells, but their sensitivity has not yet been carefully assessed (Ganesan et al., 2006; Batters et al., 2010; Riching et al., 2018).

The authors apologise to readers for this omission, which does not impact the results or conclusions of the paper.

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TOOLS AND RESOURCES

Sensitive detection of protein ubiquitylation using a protein fragment complementation assay

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ABSTRACT

Ubiquitylation is a reversible post-translational protein modification that regulates a multitude of cellular processes. Detection of ubiquitylated proteins is often challenging because of their low abundance. Here, we present NUbICA, a sensitive protein-fragment complementation assay to facilitate the monitoring of ubiquitylation events in cultured cells and model organisms. Using yeast as a model system, we demonstrate that NUbICA enables accurate monitoring of mono- and polyubiquitylation of proteins expressed at endogenous levels. We also show that it can be applied to decipher the topology of ubiquitin conjugates. Moreover, we assembled a genome-wide collection of yeast strains ready to investigate the ubiquitylation of proteins with this new assay. This resource will facilitate the analysis of local or transient ubiquitylation events that are difficult to detect with current methods.

KEY WORDS: Ubiquitin, Ubiquitylation, PCA, UbiCREST, NanoLuc, NanoBiT

INTRODUCTION

Ubiquitylation is a prevalent posttranslational protein modification that plays a central role in the cell. It controls the homeostasis, turnover and activity of myriads of proteins. Defects in ubiquitylation are implicated in the etiology of numerous human diseases, including infection, neurodegenerative disorders and cancers (Popovic et al., 2014). Ubiquitylation is catalyzed by ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s) that act in concert to attach one or multiple ubiquitin moieties covalently onto their substrate proteins, generally on lysine residues. E2s and E3s can also target the seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) and the N-terminus of ubiquitin to assemble various types of polyubiquitin chains. Depending on their topology, these ubiquitin chains act as distinct molecular signals that can have different consequences for the ubiquitylated proteins (Komander and Rape, 2012; Yau and Rape, 2016). For instance, K48- and K11-linked ubiquitin chains typically target the modified protein for proteasomal degradation, whereas K63-linked chains are associated with lysosomal degradation or nonproteolytic regulatory mechanisms.

Elucidation of the functions and mechanisms of ubiquitylation demands sensitive tools to identify ubiquitylated proteins, monitor their modification and decipher ubiquitin linkages. Advances in mass spectrometry techniques and enrichment strategies now enable the identification of thousands of ubiquitylated proteins in cell extracts (Bennett et al., 2010; Udeshi et al., 2013). Such proteomic approaches are particularly effective for global analysis of the ubiquitylome but are costly and burdensome for investigating the ubiquitylation of one or a selected set of proteins. Multiple assays have been devised for monitoring and quantifying ubiquitylation reactions performed *in vitro* using recombinant proteins or extracts (Berndsen and Wolberger, 2011; Boisclair et al., 2000; Zuo et al., 2020; Gururaja et al., 2005; Mondal et al., 2016; Schneider et al., 2012), but they do not permit investigation of ubiquitylation events that take place in cells or tissues. This is generally achieved using conventional band shift assays and immunoblotting methods after isolation of ubiquitylated proteins with affinity reagents such as tandem ubiquitin-binding entities (TUBEs) (Hershko et al., 1982; Hjerpe et al., 2009; Hovsepian et al., 2016; Kaiser and Tagwerker, 2005). However, because of the low stoichiometry and unstable nature of many ubiquitin conjugates, these methods are often not sufficiently sensitive for robust assay of endogenous protein modification. Numerous studies thus rely upon overexpression of ubiquitin and/or substrate proteins, which may subvert endogenous ubiquitin conjugation pathways. There is therefore a need for alternative sensitive methods to probe the ubiquitylation of endogenously expressed proteins.

Protein-fragment complementation assays (PCAs) are a family of techniques devised to probe the proximity of proteins (Michnick et al., 2007). They rely on the use of complementary fragments of a reporter that are genetically fused to proteins of interest. These fragments can reconstitute the active reporter when brought into close proximity through association of their fusion partners. The activity of the reporter is thus an indirect measure of the association of proteins fused to the reporter fragments. We reasoned that, although generally used to probe noncovalent protein interactions, PCAs could also be utilized to demonstrate the conjugation of ubiquitin to its substrate proteins. In this work we describe a NanoBiT-based ubiquitin conjugation assay (NUbICA), a PCA derived from the NanoLuc luciferase and designed to probe the ubiquitylation of select proteins. Using budding yeast as a model system, we show that NUbICA is a sensitive method for examining mono- or polyubiquitin signals conjugated to proteins expressed at endogenous levels.

RESULTS

Design of a NanoBiT-based ubiquitin conjugation assay

NanoLuc is one of the smallest and brightest luciferases currently available (Hall et al., 2012). It has previously been engineered to develop NanoBiT, a protein complementation reporter consisting of two asymmetrically sized fragments (Dixon et al., 2016). The large

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fragment, termed LgBiT, remains folded and has been optimized to have a high thermal stability and slow turnover. The small fragment, termed SmBiT, is an 11 amino acid peptide that has been selected for its low intrinsic affinity for LgBiT (dissociation constant ~200 μM), but retains the ability to reconstitute a bright luciferase. NanoLuc and NanoBiT are also resistant to various environmental conditions; in particular, they can efficiently refold after denaturation (Hall et al., 2012). These properties make NanoBiT an excellent reporter for probing the conjugation of ubiquitin to cellular proteins.

To establish NUBiCA, we chose to fuse SmBiT to ubiquitin and LgBiT to ubiquitylation substrates (Fig. 1A). We attached SmBiT to the N-terminus of ubiquitin to preserve the C-terminal carboxyl group required for ubiquitin conjugation to its substrates. In contrast, LgBiT can be positioned at either extremities of substrate proteins, or even in an internal loop (see Cse4 example, described later). In addition, we appended a polyhistidine tag to the LgBiT sequence, which enables purification of the ubiquitylation substrates under fully denaturing conditions (Fig. 1B). This ensures the specificity of NUBiCA by eliminating interaction partners of the LgBiT/His-tagged proteins, which could themselves be ubiquitylated. After purification, the eluates are renatured to allow the LgBiT fragment to fold and reconstitute the NanoBiT reporter if the purified protein is ubiquitylated.

Importantly, the assay can be rigorously controlled (Fig. 1B,C). First, the total amount of purified LgBiT/His-tagged proteins is easily quantified using a SmBiT peptide variant termed HiBiT, which binds LgBiT with nanomolar affinity and reconstitutes an active luciferase (Schwinn et al., 2018a). Second, it is also possible to evaluate the expression level of SmBiT-ubiquitin. This is achieved by measuring the luminescence of total protein extracts in the presence of recombinant LgBiT (Fig. 1C; Fig. S1A; Materials and Methods). This control, which is rarely performed in conventional ubiquitylation assays, can serve to correct variations in SmBiT-ubiquitin expression in different genetic contexts. Thus, the ubiquitylation level of LgBiT/His-tagged proteins can be compared across different conditions using a normalized luminescence ratio (NLR; Fig. 1D). Conditions that impair ubiquitylation of a given protein result in NLR values below the control, whereas conditions that stimulate ubiquitylation increase NLR values.

Validation of NUBiCA as a probe for proteolytic and non-proteolytic ubiquitylation events

We used budding yeast as a model organism to evaluate NUBiCA. We first probed the ubiquitylation of histone H2B, one of the best characterized ubiquitin conjugates in cells. In yeast, H2B is mono-ubiquitylated on its lysine K124 by the ubiquitin ligase Bre1 and the ubiquitin-conjugating enzyme Rad6 (Hwang et al., 2003; Robzyk et al., 2000; Wood et al., 2003) (Fig. 2A). We endogenously fused *HTB2*, one of the two H2B-producing genes in yeast, with a C-terminal LgBiT/His tag and constructed wild-type and mutant strains expressing SmBiT-ubiquitin at near endogenous levels (Fig. S1B,C; Materials and Methods). We prepared protein extracts from these strains and purified Htb2-LgBiT/His under denaturing conditions. We first visualized Htb2 modification using a gel-based assay. The purified proteins were separated by gel electrophoresis, transferred onto a nitrocellulose membrane and renatured on the membrane. We observed a strong NanoBiT signal in wild-type cells, indicating that SmBiT-ubiquitin can be conjugated to LgBiT/His-tagged Htb2 (Fig. 2B). In contrast, only very weak NanoBiT signal was observed in *htb2(K124R)*, *bre1Δ* or *rad6Δ* mutants. The signal

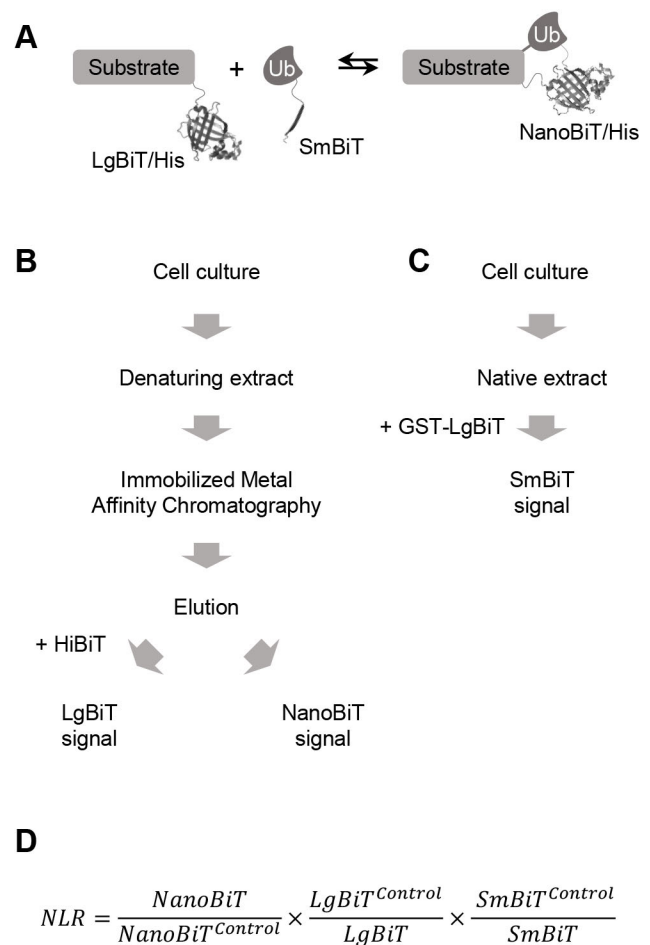


Fig. 1. Description of the NanoBiT-based ubiquitin conjugation assay. (A) Ubiquitin (Ub) and a substrate protein of interest are genetically fused to the SmBiT and LgBiT/His tags, respectively. Conjugation of ubiquitin to the protein of interest enables reconstitution of the NanoBiT reporter. (B) Main steps of the NUBiCA protocol. Cells are lysed in denaturing conditions. LgBiT/His-tagged ubiquitylation substrates are pulled-down using immobilized metal affinity chromatography. The resin is washed to eliminate any SmBiT-ubiquitin that is not conjugated to the substrate. The LgBiT/His-tagged proteins are eluted and diluted in a luciferase assay buffer to renature the LgBiT fragment. The eluates are distributed in a microtiter plate with or without the HiBiT peptide. The HiBiT peptide tightly binds to LgBiT and reconstitutes an active luciferase, which enables quantification of the total amount of LgBiT/His-tagged proteins present in the eluates (LgBiT signal). Without the HiBiT peptide, SmBiT-ubiquitin conjugated to LgBiT/His-tagged proteins can reconstitute the NanoBiT reporter (NanoBiT signal). The corresponding luminescence signals are recorded in presence of the NanoLuc substrate (furimazine) in a microtiter plate reader. (C) Main steps of SmBiT-ubiquitin quantification. Cells are lysed under native conditions. The extracts are supplemented with recombinant GST-LgBiT and distributed in a microtiter plate. The luminescence signals are recorded in the presence of furimazine with a luminometer (SmBiT signals). (D) The relative ubiquitylation level of the substrate is expressed as a normalized luminescence ratio (NLR). In each experiment, the NanoBiT and LgBiT signals are quantified under control and test conditions (e.g. wild-type and mutant cells). SmBiT signals can also be evaluated in order to control SmBiT-ubiquitin expression levels. LgBiT (and when necessary SmBiT) signals are used to correct NanoBiT signals. The NLR corresponds to the corrected NanoBiT signal of a test condition normalized by the corrected NanoBiT signal of the control condition.

detected in these strains at the molecular weight of ubiquitylated Htb2 was only visible in contrasted images and was in the same intensity range as the signal produced by the background luciferase activity of LgBiT fused to non-ubiquitylated Htb2 (Fig. 2B).

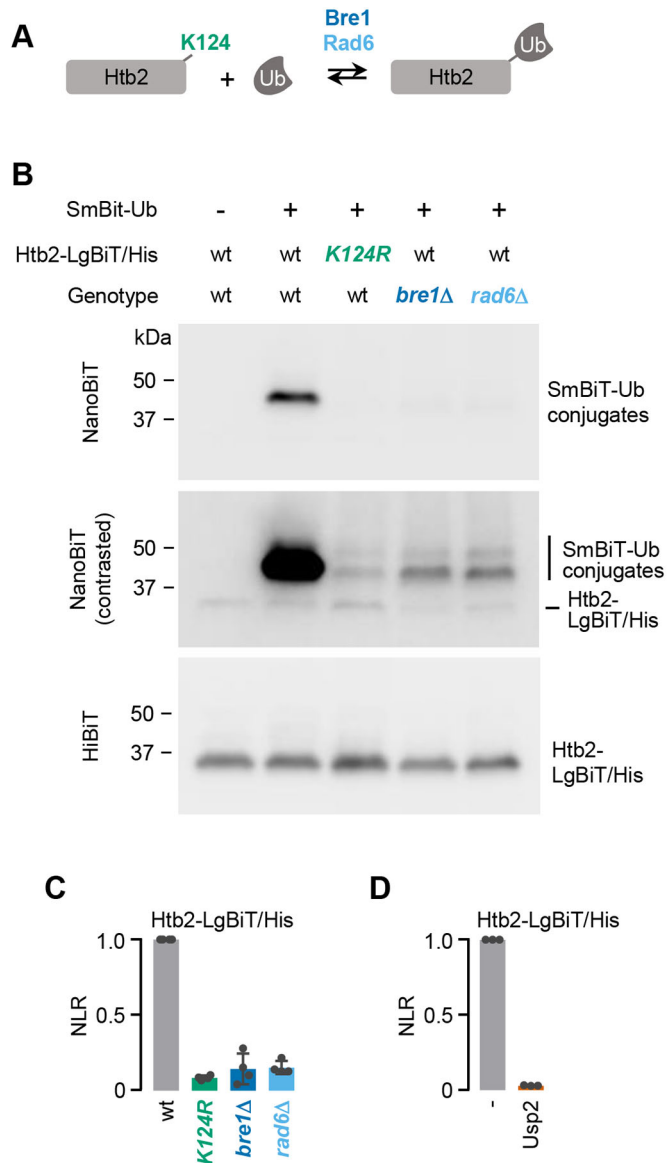


Fig. 2. Analysis of Htb2 mono-ubiquitylation. (A) Htb2 is ubiquitylated on K124 by the Bre1 ubiquitin ligase and the Rad6 ubiquitin-conjugating enzyme. (B) Conjugation of SmBiT-ubiquitin to Htb2-LgBiT/His is visualized after gel electrophoresis. LgBiT/His-tagged Htb2 purified from 2×10^8 cells of the indicated strains was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The NanoBIT signal was visualized after incubation of the membrane with the NanoLuc substrate furimazine (NanoBIT). To control the amount of Htb2-LgBiT/His purified from the different strains, the same membrane was subsequently imaged in the presence of the HiBiT peptide, which tightly interacts with LgBiT to reconstitute a functional luciferase (HiBiT). Images are representative of two independent experiments. (C) Relative ubiquitylation levels of Htb2-LgBiT/His purified from the indicated yeast strains ($n=4$). (D) Relative ubiquitylation levels of Htb2-LgBiT/His after Usp2 treatment. Htb2-LgBiT/His was purified from wild-type cells and treated for 2 h with or without Usp2 ($n=3$). Graphs display normalized luminescence ratios (NLR); data are expressed as mean \pm s.d.

We then measured the ubiquitylation signal produced by purified Htb2 without gel electrophoresis. Again, we observed that Htb2-LgBiT/His purified from wild-type cells produced a robust luminescent signal, which was largely reduced in *htb2(K124R)*, *bre1Δ* and *rad6Δ* mutants (Fig. 2C). This assay was particularly sensitive as 10^5 wild-type cells (i.e. 10 μ l of a culture with OD₆₀₀=0.5) were sufficient to produce a detectable NanoBIT signal

(Fig. S2A). Together, these results demonstrate that NUbICA can accurately report Htb2 ubiquitylation without the need for protein separation by gel electrophoresis.

Histone mono-ubiquitylation acts nonproteolytically to control gene activity (Weake and Workman, 2008). However, proteins modified by polyubiquitin chains or multiple mono-ubiquitin are often rapidly degraded by the proteasome. Such proteolytic ubiquitylation events can be difficult to assay without using artificial conditions such as overexpression or proteasome inhibition. We therefore wished to test whether NUbICA enables detection of the ubiquitylation of unstable proteins in endogenous conditions. We chose to probe ubiquitylation of the N-terminal region of the transcription factor Stp2 (Stp2^N), which gives a strong degradation signal (Omnus and Ljungdahl, 2014). Using proteasome inhibition and classical immunoassays, we previously demonstrated that Stp2^N is efficiently ubiquitylated, primarily by the Asi1/3 ubiquitin ligase complex and the Ubc7 conjugating enzyme (Khmelninskii et al., 2014) (Fig. 3A). We now used NUbICA to probe Stp2^N ubiquitylation in the absence of proteasome inhibition. As for Htb2, we visualized the conjugation of SmBiT-ubiquitin to Stp2^N-LgBiT/His in a gel-based assay (Fig. 3B). As expected (Khmelninskii et al., 2014), the modification of Stp2^N was considerably decreased in *asi3Δ* cells and to a lesser extent in *ubc7Δ* cells. Identical results were obtained without separation of the purified proteins by gel electrophoresis (Fig. 3C). In this assay, the NanoBIT signal was detectable from as few as 10^5 cells (Fig. S2A). In comparison, we needed at least 10^7 cells to reveal Stp2^N modification after enrichment of ubiquitylated proteins using TUBEs (Fig. S2B). Thus, NUbICA is suitable for probing the modification of a proteolytic ubiquitylation substrate without the need for proteasome inhibition.

To further test the sensitivity of NUbICA, we investigated the ubiquitylation of the yeast histone variant Cse4. Cse4 is an essential protein that substitutes for histone H3 in centromeric nucleosomes (Meluh et al., 1998). With ~ 100 copies per cell, Cse4 is among the 20% least expressed proteins in yeast (Kulak et al., 2014). The ubiquitin ligase Psh1 controls Cse4 levels and prevents the mislocalization of overexpressed Cse4 (Hewawasam et al., 2010; Ranjitkar et al., 2010) (Fig. 3E), but whether and when it ubiquitylates endogenous Cse4 has not been directly demonstrated. We tagged endogenous Cse4 with LgBiT/His in cells expressing SmBiT-ubiquitin. The LgBiT/His tag was inserted at an internal position (between asparagine 80 and leucine 81), as this was shown to minimally perturb Cse4 function (Wisniewski et al., 2014). In contrast to Htb2 and Stp2^N, we could not detect Cse4 ubiquitylation after gel electrophoresis. However, when we purified Cse4-LgBiT/His^{intern} from at least 10^8 cells, we could detect a clear NanoBIT signal in the eluates (Fig. S2A). The intensity of this signal was significantly reduced in *psh1Δ* cells (Fig. 3F). This suggests that Psh1 can ubiquitylate Cse4 expressed at endogenous levels. To demonstrate further that the luminescence signal is a result of Cse4 ubiquitylation, we treated the purified proteins with Usp2, a nonspecific de-ubiquitylation enzyme (DUB) that hydrolyses all types of ubiquitin linkages (Hospenthal et al., 2015). As in the case of Htb2 and Stp2^N (Fig. 2D, Fig. 3D), this resulted in a large reduction in the luminescence signals produced by purified Cse4-LgBiT/His^{intern} (Fig. 3G). Interestingly, Usp2 treatment seemed to reduce the Cse4 ubiquitylation signal more strongly than *PSH1* deletion. It is thus possible that further ubiquitin ligases contribute to endogenous Cse4 regulation, as this has been proposed for overexpressed Cse4 (Cheng et al., 2016, 2017; Ohkuni et al., 2016). In conclusion, these results establish NUbICA as a

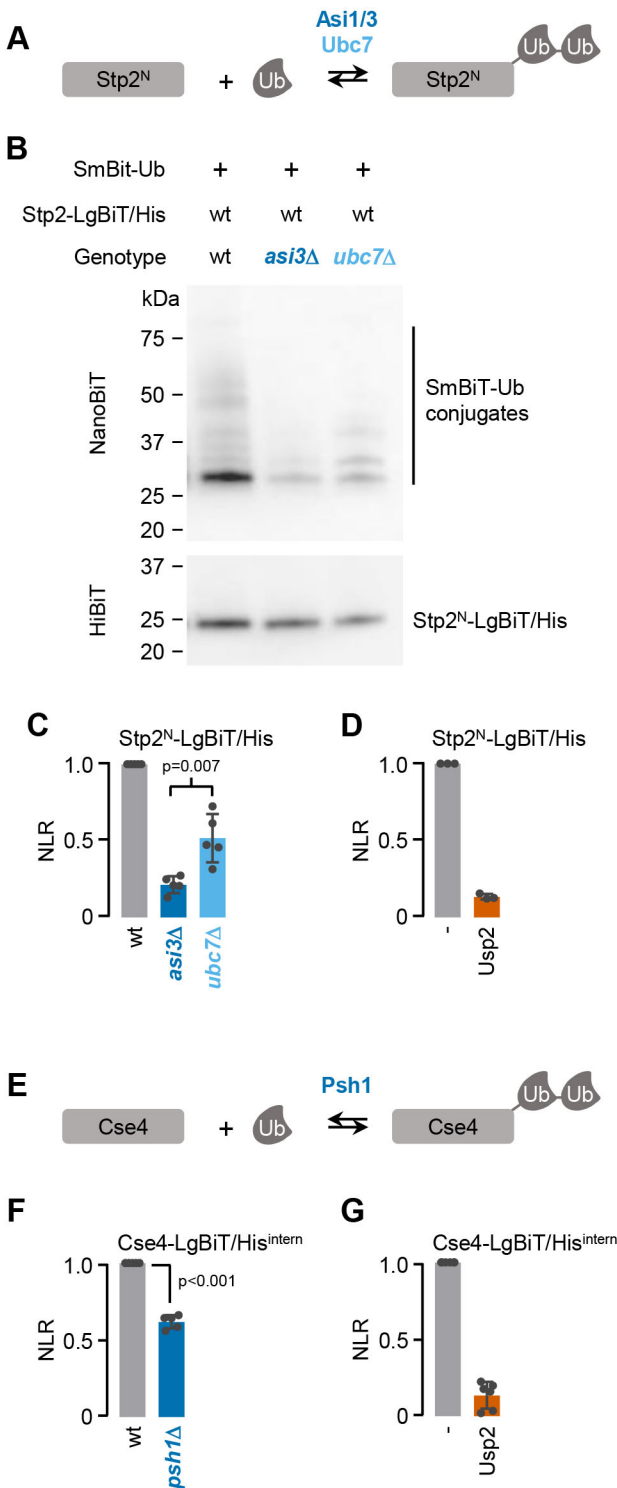


Fig. 3. Analysis of Stp2^N and Cse4 polyubiquitylation. (A) Stp2^N is primarily ubiquitylated by the Asi1/3 ubiquitin ligase complex and the Ubc7 ubiquitin-conjugating enzyme. (B) Conjugation of SmBiT-ubiquitin to Stp2^N-LgBiT/His visualized after gel electrophoresis. LgBiT/His-tagged Stp2^N purified from 2 × 10⁸ cells of the indicated strains was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The NanoBiT signal was visualized by incubating the membrane with the NanoLuc substrate furimazine (NanoBiT). To control the amount of Stp2^N-LgBiT/His purified from the different strains, the same membrane was subsequently imaged in the presence of the HiBiT peptide, which tightly interacts with LgBiT to reconstitute a functional luciferase (HiBiT). Images are representative of two independent experiments. (C) Relative ubiquitylation levels of Stp2^N-LgBiT/His purified from the indicated yeast strains (n=5). (D) Relative ubiquitylation levels of Stp2^N-LgBiT/His after Usp2 treatment. Stp2^N-LgBiT/His was purified from wild-type cells and incubated for 2 h with or without Usp2 (n=3). (E) Cse4 is ubiquitylated by the Psh1 ubiquitin ligase. (F) Relative ubiquitylation levels of Cse4-(LgBiT/His)^{intem} purified from wild-type (wt) and *psh1Δ* cells (n=5). (G) Relative ubiquitylation levels of Cse4-(LgBiT/His)^{intem} after Usp2 treatment. Cse4-(LgBiT/His)^{intem} was purified from wild-type cells and incubated for 2 h with or without Usp2 (n=3). Graphs display normalized luminescence ratios (NLR); data are expressed as mean ± s.d. P-values were calculated using a two-tailed paired t-test.

tag. The resulting strains were then crossed with a strain containing a chromosomally integrated SmBiT-ubiquitin expression cassette. The entire procedure was successful for more than 98% of the colonies from the original C-SWAT library, yielding a collection of 5580 ready-to-use NUBiCA strains (Table S1).

This collection of yeast strains can in principle be used to study the ubiquitylation of almost any protein of interest. It will, for instance, be of great help in validating the ubiquitylation of candidates identified by mass spectroscopy or in systematically analyzing the ubiquitylation of protein families under various conditions. As a proof of principle, we examined the ubiquitylation of yeast arrestin-related trafficking adaptors (ARTs) (Fig. 4A). ARTs function with the Rsp5 ubiquitin ligase to promote plasma membrane protein ubiquitylation and endocytosis in response to changes in environmental conditions (Lin et al., 2008; Nikko and Pelham, 2009; Hatakeyama et al., 2010; O'donnell et al., 2010; MacGurn et al., 2011; Merhi and Andre, 2012; Becuwe et al., 2012b). ARTs are themselves ubiquitylated by Rsp5 and this modification regulates their activity. For instance, Art4/Rod1 ubiquitylation is stimulated by glucose, which promotes internalization of the monocarboxylate transporter Jen1 (Becuwe et al., 2012a). Conversely, ubiquitylation of Art8/Csr2 is inhibited in the presence of glucose in order to interrupt glucose transporter endocytosis (Hovsepian et al., 2017). To determine whether other ARTs are regulated by glucose, we retrieved nine of the corresponding strains from the NUBiCA collection. We grew these strains in rich medium using galactose as a carbon source and then exposed them to glucose. Conjugation of SmBiT-ubiquitin to LgBiT/His-tagged ARTs was then revealed using NUBiCA without gel electrophoresis. We detected a clear NanoBiT signal for all ARTs, except for Art7/Rog3 which was very poorly expressed (Fig. 4B-I). Treatment of the eluates with Usp2 confirmed that the signal was dependent on ubiquitin conjugation. As expected, we observed a significant increase in Art4/Rod1 and decrease in Art8/Csr2 ubiquitylation signals when cells were exposed to glucose (Fig. 4E,G). In contrast, the ubiquitylation levels of the other ARTs remained unchanged upon glucose treatment. These results suggest that NUBiCA allows monitoring of changes in protein ubiquitylation in response to external stimuli and that Art4/Rod1 and Art8/Csr2 are probably the main ARTs involved in plasma membrane protein composition remodeling in response to glucose.

sensitive method for probing the modification of scarce and short-lived ubiquitylated protein expressed at endogenous levels.

A genome-wide collection of yeast strains ready for NUBiCA

To facilitate the investigation of protein ubiquitylation using NUBiCA, we constructed a genome-wide collection of yeast strains expressing SmBiT-ubiquitin and proteins C-terminally tagged with LgBiT/His. We used the recently established C-SWAT yeast library (Meurer et al., 2018) to fuse yeast open reading frames systematically to the DNA sequence of the LgBiT/His

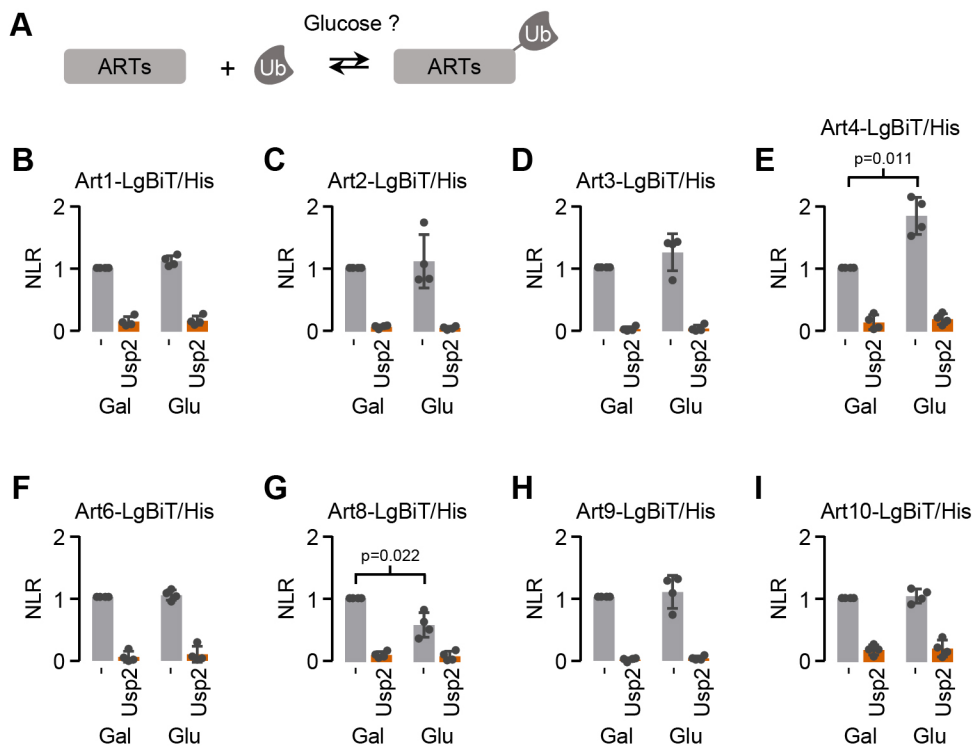


Fig. 4. Ubiquitylation of arrestin-related trafficking adaptors in response to glucose exposure. (A) The response of ART ubiquitylation to glucose was monitored. (B-I) Relative ubiquitylation levels of ARTs tagged with LgBiT/His in response to glucose exposure. The indicated ARTs were purified from 10^8 cells grown in galactose before (Gal) and after (Glu) 5 min exposure to 2% glucose. Purified proteins were incubated for 2 h with or without Usp2 ($n=4$). Graphs display normalized luminescence ratios (NLR); data are expressed as mean \pm s.d. *P*-values were calculated using a two-tailed paired *t*-test.

Use of NUBiCA to dissect ubiquitin conjugates

One of the most important challenges in the field of protein ubiquitylation is to decipher how ubiquitin signals regulate the fate and activity of cellular proteins. Multiple tools and methods have been devised to investigate ubiquitin chain topologies, including ubiquitin mutants, linkage-specific reagents and mass spectrometry (Hospenthal et al., 2015; Mattern et al., 2019; Meyer and Rape, 2014; Newton et al., 2008; Ordureau et al., 2015; Spence et al., 1995). We reasoned that NUBiCA could also be applied to examine the properties of ubiquitin conjugates. Because Usp2 recognizes folded ubiquitin (Renatus et al., 2006), the fact that Usp2 treatment resulted in a large reduction in NUBiCA signals (Fig. 2D, Fig. 3D,G, Fig. 4B-I) indicates that ubiquitin can be refolded after denaturing purification. If so, ubiquitin chains might be recognized and protected from de-ubiquitylation by generic and chain-specific ubiquitin binders such as TUBEs. We reasoned that this could enable investigation of ubiquitin chain topologies. To test this hypothesis, we took advantage of the NUBiCA collection. We selected three nonproteolytic (Htb2, Art9, Sna3) and four proteolytic (Cln2, Sic1, Clb2 and Clb3) ubiquitylation substrates. Like Htb2, Art9 is known to be primarily mono-ubiquitylated (Herrador et al., 2010), whereas Sna3 is modified by K63-linked ubiquitin chains (Stawiecka-Mirota et al., 2007). Sic1 and Cln2 are substrates of the SCF ubiquitin ligase complex and are modified by K48-linked ubiquitin chains (Skowyra et al., 1997; Kravtsova-Ivantsiv et al., 2009); Clb2 and Clb3 are substrates of APC/C, which assembles branched K48-linked chains (Rodrigo-Brenni, 2007). We purified these proteins from NUBiCA strains, incubated them with recombinant TUBEs and recorded the kinetics of their de-ubiquitylation by Usp2 (Fig. 5A-D). We used three distinct TUBEs in this assay. Ubiquitin1 and HR23A TUBEs bind both K48 and K63 tetra-ubiquitin chains with nanomolar affinities (Hjerpe et al., 2009). In contrast, the Rx3(A7) TUBE exclusively recognizes K63 chains (Sims et al., 2012). We observed that these TUBEs had little impact on the de-ubiquitylation kinetics of mono-ubiquitylated

Htb2 and Art9 (Fig. 5B). In contrast, the de-ubiquitylation of polyubiquitylated proteins was differentially impaired by the TUBEs. Although the ubiquitin1 TUBE protected all tested polyubiquitin substrates from de-ubiquitylation, the Rx3(A7) TUBE primarily impacted the de-ubiquitylation of Sna3 and the HR23A TUBE mostly inhibited the de-ubiquitylation of substrates modified by proteolytic K48-linked ubiquitin chains (Fig. 5C,D). These results indicate that NUBiCA combined with ubiquitin chain protection and digestion enables different types of ubiquitin conjugates to be qualitatively distinguished. We applied this approach to examine the modification profile of Stp2^N and Cse4 (Fig. 5E). The de-ubiquitylation kinetics of Stp2^N and Cse4 purified from wild-type cells matched the profiles previously observed for proteolytic ubiquitylation substrates. In contrast, the de-ubiquitylation of Stp2^N purified from *asi3Δ* or *ubc7Δ* was only very weakly impaired by the TUBEs and the profiles resembled those observed for mono-ubiquitylated proteins. These results are consistent with the roles of the Asi1/3 and Psh1 ubiquitin ligases in the degradation of Stp2^N and Cse4, respectively (Boban et al., 2006; Khmelinskii et al., 2014; Hewawasam et al., 2010; Ranjitkar et al., 2010).

DISCUSSION

The methods most commonly used to demonstrate the ubiquitylation of a protein of interest rely on the separation of the ubiquitylated and unmodified protein forms by gel electrophoresis. However, these methods are not always sufficiently sensitive to detect the ubiquitylation of proteins expressed at endogenous levels. They are also difficult to quantify and not very amenable to large scale studies. Recently, alternative assays based on ELISA and FRET approaches have been established to quantify ubiquitylated proteins in cell or tissue extracts (Foote et al., 2018; Guven et al., 2019). Because only a few experiments have been performed with these assays, it is difficult to evaluate their sensitivity and specificity for the detection of low abundance ubiquitylated proteins.

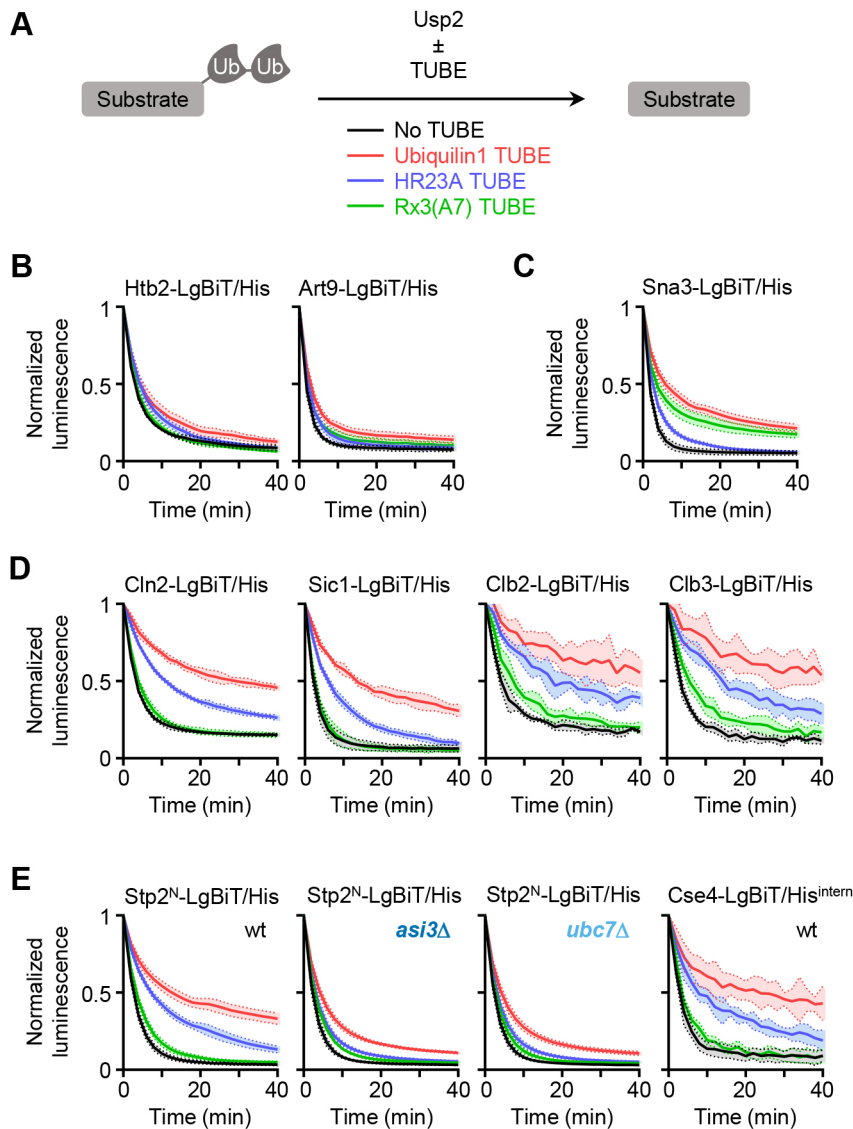


Fig. 5. De-ubiquitylation kinetics in the presence of tandem ubiquitin-binding entities. (A) De-ubiquitylation reactions were performed in the presence of Usp2 using substrates previously incubated with ubiquitin1, HR23A or Rx3(A7) TUBEs. (B-D) De-ubiquitylation profiles of substrates known to be modified with mono-ubiquitin (B), K63-linked chains (C) or K48-linked chains (D). (E) De-ubiquitylation profiles of Stp2^N-LgBiT/His and Cse4-LgBiT/His^{intern} in the indicated yeast strains. Graphs display mean luminescence values normalized to the initial time point. Standard deviations are drawn as shaded areas ($n \geq 4$).

In the present study, we demonstrate that the conjugation of ubiquitin to proteins can also be monitored using a protein complementation assay. We chose NanoBiT as a complementation reporter for multiple reasons. First, the NanoBiT fragments, LgBiT and SmBiT, are small, stable and have very low intrinsic affinity (Dixon et al., 2016). They are thus less likely to perturb the function and ubiquitylation of the proteins they are fused to. Second, the LgBiT fragment efficiently refolds after denaturation, which allows the NanoBiT signal to be monitored after very stringent purification protocols or even gel electrophoresis. Third, NanoLuc is one of the brightest luciferases, with a detection limit of less than 1 amol (Hall et al., 2012; Schwinn et al., 2018a). We reasoned that this exquisite sensitivity should enable the detection of scarce ubiquitylated proteins. Indeed, we successfully detected the modification of several proteolytic ubiquitylation targets expressed from their endogenous chromosomal locus, including the low abundance histone Cse4. This suggests that NUbICA will be applicable for investigating the ubiquitylation of most cellular proteins without overexpression. This is, in our view, of utmost importance because overexpression can trigger quality control ubiquitylation pathways that are not necessarily the ones one wants to investigate. For instance, overexpressed Cse4 is massively ubiquitylated to prevent its

accumulation in euchromatin (Ranjitkar et al., 2010), which precludes the investigation of endogenous low abundance regulatory ubiquitylation events.

One of the limitations of NUbICA is that the proteins of interest and ubiquitin have to be tagged with LgBiT/His and SmBiT, respectively. Hence, this method is only applicable for the study of ubiquitylation in tissue culture systems or genetically amenable model organisms. We tagged ubiquitin N-terminally because it can functionally replace endogenous ubiquitin in yeast (Ling et al., 2000). Yet, specific ubiquitylation pathways such as linear ubiquitylation may not be compatible with this form of ubiquitin and could require the use of internally tagged ubiquitin (Kliza et al., 2017). Apart from Cse4, all substrates investigated here were tagged C-terminally. This strategy is probably effective for many substrates, but will need to be adapted for proteins that cannot tolerate a modification at their C terminus, such as many endomembrane proteins (Yofe et al., 2016). Besides its impact on functionality, the position of the LgBiT/His tag might affect the efficiency of NanoBiT reconstitution, in particular when the ubiquitylation sites are distant to the position of the LgBiT fragment. Yet, we successfully probed the ubiquitylation of 15 distinct C-terminally tagged proteins. Among the nine tested ARTs,

the only one for which we could not detect a NUbICA signal was Art7. These results suggest that, in many instances, the position of the LgBiT/His tag does not prevent the functionality of NUbICA.

A central aspect of the NUbICA protocol described here is that the lysate preparation and substrate purification are carried out under highly denaturing conditions (Fig. 1B). This has the advantage of suppressing DUB activity and, therefore, preserving ubiquitin conjugates. It also ensures that the luminescence signal originates from ubiquitin conjugated to the LgBiT/His-tagged protein and not to one of its interaction partners. Thus, the denaturing purification is important in achieving high sensitivity and specificity, enabling direct measurement of the NanoBiT signal in purification eluates. Moreover, when the ubiquitylated proteins are sufficiently abundant, the eluates can also be separated by gel electrophoresis to visualize the ubiquitin conjugates (Fig. 2B, Fig. 3B). Although we have not investigated this possibility, it is worthy of note that the denaturing purification step could be omitted for certain proteins. The ubiquitylation of these proteins could then be monitored directly in extracts or even in intact cells. Indeed, NanoBiT has recently been used to monitor the modification of Cullin1 by the ubiquitin-like protein NEDD8 in mammalian cells (Schwinn et al., 2018b). It will be important to determine to what extent this approach can be applied to other proteins as it opens the possibility of monitoring ubiquitylation and de-ubiquitylation reactions in real time in live cells.

NUbICA is simple to implement because it does not require gel electrophoresis or antibody-based detection methods. Moreover, the protocol is generic and can be similarly applied to any protein of interest. It will thus be easier to perform larger scale studies with NUbICA than with other currently available assays. In this respect, the genome-wide collection of NUbICA yeast strains that we constructed will be especially useful. These strains can be used directly to characterize the ubiquitylation of protein families under different conditions, as exemplified here with ARTs (Fig. 4). Importantly, the collection is compatible with high-throughput genetic approaches. Hence, individual NUbICA strains or the entire collection can be easily crossed to one or multiple mutants of interest to investigate their impact on the ubiquitylation of a selected range of proteins. We expect that this will greatly ease the validation and characterization of ubiquitylation candidates identified by mass spectrometry. More generally, the NUbICA collection will facilitate systematic studies of ubiquitylation pathways.

We also show that NUbICA combined with de-ubiquitylation reactions qualitatively enables different types of ubiquitin conjugates to be distinguished. Initially, we thought to combine NUbICA with UbiCRest, a method that uses chain-specific DUBs to distinguish ubiquitin linkages (Hospenthal et al., 2015). However, the de-ubiquitylation kinetics with such DUBs were slow and it was difficult to ensure their specificity. We therefore devised a novel strategy that takes advantage of ubiquitin binders to protect ubiquitin chains from de-ubiquitylation by a generic DUB (Fig. 5). Using a limited set of three ubiquitin binders, we were able to distinguish clearly the de-ubiquitylation profiles of substrates modified by single ubiquitin moieties or by K48- and K63-linked ubiquitin chains. This indicates that SmBiT-ubiquitin can be incorporated in both types of chains. Interestingly, the HR23A TUBE, which has a similar affinity for K48- and K63-linked ubiquitin chains (Hjerpe et al., 2009), did not efficiently protect K63-linked chains from de-ubiquitylation (Fig. 5C). Hence, the protective effect of a given ubiquitin binder does not simply depend on its capacity to bind ubiquitin moieties but, more likely, on how it binds them. Although qualitative, this approach will be helpful in

examining the properties of ubiquitin conjugates. Besides, we are confident that the use of other ubiquitin binders or de-ubiquitylation enzymes will enable more de-ubiquitylation profiles to be revealed and, hence, ubiquitin chain topologies to be deciphered more precisely.

In conclusion, we show that NUbICA can be used to probe the mono- and polyubiquitylation of a wide range of proteins. Although we limited our study to ubiquitylation in yeast, the principles of NUbICA can be generalized to monitor other post-translational protein modifiers, such as SUMO or NEDD8, in any tissue culture system or genetically amenable model organism.

MATERIALS AND METHODS

Yeast methods and plasmids

Yeast genome manipulations (chromosomal gene tagging and gene deletion) were carried out using conventional procedures based on PCR targeting and plasmid integration. Cassettes for PCR targeting were amplified with the Phusion DNA polymerase (New England Biolabs, Ipswich, MA, USA). Gene deletion and tagging were validated by PCR. Yeast strains and plasmids used in this study are listed in Tables S2 and S3, respectively.

Expression of LgBiT-tagged proteins and SmBiT-ubiquitin

All LgBiT-fusion proteins presented in this manuscript were expressed from their endogenous chromosomal locus. In contrast, endogenous tagging of ubiquitin was not easily achievable because yeast ubiquitin is encoded by four distinct genes. Hence, SmBiT-ubiquitin was expressed ectopically, either from a shuttle plasmid (pGR892) or from a chromosomally integrated expression cassette. SmBiT-ubiquitin produced from pGR892 was expressed at a level similar to that of endogenous ubiquitin (Fig. S1B) and was used to probe the ubiquitylation of Htb2 (Fig. 2). The level of SmBiT-ubiquitin expressed from the chromosomal cassette was about four to five times lower (Fig. S1B,C), which is unlikely to perturb the homeostasis of ubiquitin. Moreover, the level of SmBiT-ubiquitin expressed from the chromosomal cassette was more reproducible than from pGR892 (Fig. S1C). This cassette was used to probe the ubiquitylation of proteins other than Htb2 and to construct the collection of NUbICA yeast strains.

Luciferase substrate and HiBiT peptide

Luminescence measurements were performed with the NanoLuc substrate furimazine, which was purchased from Promega (Madison, WI, USA) as a ready to use stock solution (Nano-Glo Luciferase Assay Substrate). To visualize or quantify LgBiT/His-tagged proteins, luminescence measurements were performed in the presence of the HiBiT peptide (VSGWRLFKKIS), which binds tightly to LgBiT ($K_D=0.7$ nM) and enables reconstitution of an active luciferase (Schwinn et al., 2018a). The lyophilized peptide was purchased from Proteogenix (Schiltigheim, France), solubilized at a concentration of 1 mM in ddH₂O and kept at -80°C for long term storage.

Purification of LgBiT/His-tagged proteins in denaturing conditions

LgBiT/His-tagged proteins were purified from up to 10^9 exponentially growing yeast cells. Cell pellets were resuspended in 20% trichloroacetic acid (TCA) and lysed for 2 min with glass beads in a Disrupter Genie homogenizer (Scientific Industries, Bohemia, NY, USA). After precipitation, proteins were resuspended in a denaturing extraction buffer (6 M guanidinium chloride, 100 mM Tris, pH 9, 300 mM NaCl and 0.2% Triton X-100), clarified at $30,000\times g$ and incubated for at least 90 min at room temperature with TALON Metal Affinity Resin (Clontech, Mountain View, CA, USA). The beads were then washed three times with the extraction buffer and twice with a wash buffer (2 M urea, 100 mM sodium phosphate pH 7.0, 300 mM NaCl). LgBiT/His-tagged proteins were finally eluted with an elution buffer (2 M urea, 100 mM sodium phosphate pH 7.0, 300 mM NaCl, 250 mM imidazole).

Purification of ubiquitylated proteins using TUBEs

Cell pellets were prepared from up to 10^8 exponentially growing yeast cells. The pellets were resuspended in an extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20, 1 mM EDTA, 10 mM chloroacetamide) in the presence of protease inhibitors (cOmplete EDTA-free cocktail, Roche, Basel, Switzerland) and lysed for 2 min with glass beads in a Disrupter Genie homogenizer. After clarification, the extracts were incubated for 2 h with 15 μ l TUBE2 agarose beads (LifeSensors, Malvern, PA, USA) previously washed with the extraction buffer. The beads were then washed five times with the extraction buffer before elution of the bound proteins with Laemmli sample buffer containing 100 mM DTT.

Recombinant protein expression and purification

Escherichia coli BL21(DE3)RIL cells were transformed with plasmids encoding GST (pGR0313), GST-ubiquitin1-TUBE (pGEX6P1_ubiquitin1-TUBE), GST-HR23A-TUBE (pGEX6P1_HR23A-TUBE), GST-Rx3(A7)-TUBE (pGR0691) or GST-LgBiT (pGR0890) and were cultivated in LB medium. Protein expression was induced by incubation with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 4 h at 23.5°C. Cells were pelleted, resuspended in a lysis buffer (PBS, 0.05% lysozyme, 1 mM DTT) and lysed by sonication. Clarified lysates were rotated with glutathione beads (GE Healthcare, Chicago, IL, USA) for 45 min at 4°C. Beads were washed, first with PBS containing 1 mM DTT, then with 50 mM Tris pH 7.8, 200 mM NaCl and 5 mM DTT. Purified proteins were eluted in 50 mM Tris pH 7.8, 200 mM NaCl, 5 mM DTT and 20 mM reduced L-glutathione and dialyzed against PBS containing 10% glycerol and 1 mM DTT. Protein purity was tested using Stain-Free imaging (Bio-Rad, Hercules, CA, USA). Protein concentration was estimated by absorbance at 280 nm.

NanoBiT and HiBiT blots

Protein samples were denatured in Laemmli sample buffer containing 100 mM DTT and separated by SDS-PAGE using 4-20% Mini-PROTEAN TGX Stain-Free precast gels (Bio-Rad, Hercules, CA, USA). Proteins were then transferred on nitrocellulose membranes with a Trans-Blot Turbo semi-dry transfer apparatus (Bio-Rad). After transfer, the membranes were washed extensively in TBS-T (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20) for at least 1 h to allow renaturation of the LgBiT fragment. To visualize the NanoBiT signal produced by the conjugation of SmBiT-ubiquitin to LgBiT/His-tagged proteins, the membranes were incubated in TBS-T supplemented with furimazine (1%) and the luminescence signals recorded for up to 20 min using a gel and blot imaging system (Imager 680, Amersham, Little Chalfont, UK). To reveal total LgBiT/His-tagged proteins, the same membranes were imaged after incubation with TBS-T supplemented with HiBiT peptide (1 μ M) and furimazine (1%).

Anti-ubiquitin immunoblots

Cell pellets were resuspended in 20% TCA and lysed for 2 min with glass beads in a Disrupter Genie homogenizer. Lysates were pelleted and resuspended in TCA sample buffer (15% glycerol, 450 mM Tris pH 8.8, 1% SDS, 2 mM EDTA, 100 mM DTT, 0.005% Bromophenol Blue), denatured for 5 min at 95°C and separated by SDS-PAGE using 4-16% Mini-PROTEAN TGX Stain-Free precast gels (Bio-Rad). Proteins were then transferred on PVDF membranes with a Trans-Blot Turbo semi-dry transfer apparatus (Bio-Rad). After transfer, the membranes were washed in TBS-T and probed with a HRP-conjugated anti-ubiquitin antibody (1:1000; P4D1, sc-8017 HRP, Santa Cruz Biotechnology, Dallas, TX, USA) (for antibody profile validation, see Hovsepian et al., 2016) and imaged using a gel and blot imaging system (Imager 680, Amersham, Little Chalfont, UK).

Measurement of NanoBiT signals and quantification of LgBiT/HIS-tagged proteins

To measure the NanoBiT signal and quantify the amount of purified LgBiT/His-tagged proteins, the purification eluates were diluted 20 times in luciferase assay buffer (TBS-T, 17.5 mM thiourea) containing furimazine (0.25-1%) and with or without HiBiT peptide (1 μ M). The samples were distributed in 96 half-well (Greiner Bio-One, Kremsmünster, Austria) or 384 shallow-well (Perkin Elmer, Waltham, MA, USA) white polystyrene microtiter plates and, if necessary, spaced with blank wells to avoid signal

cross-talk between neighboring samples. Luminescence was recorded with a Xenius XL (SAFAS, Monaco), a FLUOstar Omega (BMG Labtech, Ortenberg, Germany) or an EnSight (Perkin Elmer, Waltham, MA, USA) plate reader.

Quantification of SmBiT-ubiquitin expression level

The expression level of SmBiT-ubiquitin was measured in native protein extracts supplemented with recombinant GST-LgBiT. Under such conditions, a small fraction of SmBiT and LgBiT fragments associate because of their low intrinsic affinity and produce a luminescent signal that can be used to derive the amount of SmBiT-ubiquitin present in the extract. (Fig. S1A). The native extracts were prepared from NUBiCA strains and from control strains that did not express SmBiT-ubiquitin or LgBiT/His-tagged proteins. Cell pellets were resuspended in PBS and lysed with glass beads in a Disrupter Genie homogenizer. After clarification, the total protein concentration of the extracts was evaluated with a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The extracts were then diluted to a final concentration of 50-400 ng/ μ l in luciferase assay buffer containing furimazine (0.25%) and GST-LgBiT (0.04-20 μ M). Control samples without GST-LgBiT were also prepared in parallel and all samples were distributed in 96 half-well white polystyrene microtiter plates for luminescence measurement. To derive the relative amount of SmBiT-ubiquitin in the NUBiCA strains, the luminescence values measured in the corresponding control samples were subtracted from the luminescence values measured in the presence of GST-LgBiT.

Endpoint de-ubiquitylation assays

To confirm that the NanoBiT signal is dependent on ubiquitin conjugation, purified LgBiT/His-tagged protein samples were distributed in 96 half-well white polystyrene microtiter plates and diluted ten times in TBS-T with or without the Usp2 catalytic domain (0.5 μ M; Enzo Life Sciences, Farmingdale, NY, USA). The samples were incubated for 2 h at 37°C and then mixed in a 1:1 ratio with a solution of TBS-T containing 35 mM thiourea and 2% furimazine before recording the luminescence with a plate reader.

De-ubiquitylation kinetics

Purified LgBiT/His-tagged proteins were diluted fivefold in TBS-T and distributed in 384 shallow-well white polystyrene microtiter plates (Perkin Elmer, Waltham, MA, USA). Recombinant GST, GST-ubiquitin1-TUBE, GST-HR23A-TUBE and GST-Rx3(A7)-TUBE were diluted in TBS-T at a concentration of 2 μ M, mixed in a 1:1 ratio with the LgBiT/His-tagged protein samples and incubated for 10 min at 25°C. The samples were then mixed simultaneously in a 1:1 ratio with a solution of TBS-T, 35 mM thiourea, 2% furimazine and with or without 0.25 μ M Usp2 catalytic domain (Enzo Life Sciences, Farmingdale, New York, USA). The plates were immediately placed in a luminometer (EnSight, Perkin Elmer, Waltham, MA, USA) prewarmed to 25°C and the luminescence signal of the samples recorded over 60 min. The samples without Usp2 served to record the luminescence variations in the absence of de-ubiquitylation and were used to normalize the luminescence variations of the samples containing Usp2.

Construction of a collection of NUBiCA yeast strains

The SWAP-Tag approach was used to assemble a genome-wide collection of yeast strains coexpressing SmBiT-ubiquitin and proteins C-terminally tagged with LgBiT/His. Meurer and colleagues had previously constructed a library of 5661 yeast strains where an acceptor module had been integrated before the stop codon of individual ORFs (Meurer et al., 2018). This C-SWAT acceptor module can be efficiently exchanged with a donor module provided by a plasmid. The plasmid pAB0010 (which provides a donor module containing a LgBiT/His tag followed by a heterologous terminator and a truncated Hygromycin B resistance marker) was transformed into the yMAM1205 strain. The transformed strain was crossed with the full collection of C-SWAT strains arrayed in a 384-colony format using a ROTOR HDA pinning robot (Singer Instruments, Watchet, UK). The colonies were sequentially pinned on appropriate media to select diploids, sporulate, select haploids, induce exchange of the acceptor and

donor modules and select the recombinant strains using the procedure described (Meurer et al., 2018). This produced a collection of MAT- α haploid strains expressing proteins C-terminally tagged with LgBiT/His. This collection was then crossed with the scGLD0122 strain, which contains an SmBiT-ubiquitin expression cassette inserted at the *MET17* locus flanked with a Nourseothricin resistance marker. Again, the colonies were pinned on appropriate selection media to obtain MAT- α haploid strains coexpressing SmBiT-ubiquitin and proteins C-terminally tagged with LgBiT/His. The entire procedure was successful for more than 98% of the colonies, yielding a collection of 5580 NubiCA strains.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.L.B., S.L., G.R.; Methodology: M.L.B., A.B., G.L.D., G.R.; Validation: M.L.B., A.B., G.L.D., G.R.; Formal analysis: M.L.B., G.R.; Investigation: M.L.B., A.B., G.L.D., G.R.; Resources: M.L.B., A.B., G.L.D.; Data curation: G.R.; Writing - original draft: M.L.B., G.R.; Writing - review & editing: S.L.; Supervision: G.R.; Project administration: G.R.; Funding acquisition: G.R., S.L.

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1. Supplementary Figures

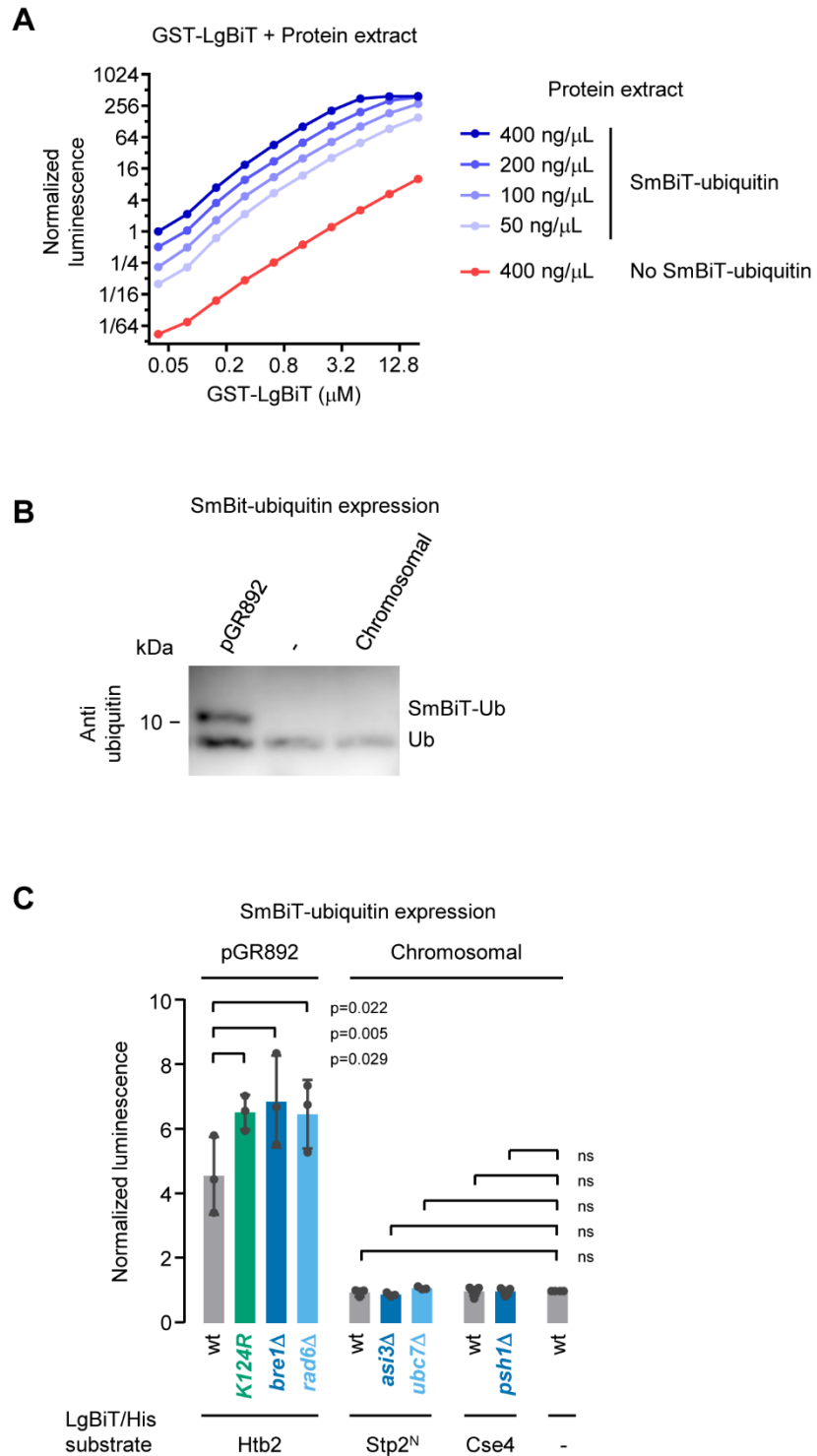


Fig. S1: Analysis of SmBiT-ubiquitin expression.

(A) Quantification of SmBiT-ubiquitin in protein extracts. Protein extracts were prepared from control cells or from cells expressing SmBiT-ubiquitin from the pGR892 plasmid. Extracts were diluted at the indicated concentrations and supplemented with varying amounts of GST-LgBiT before measuring their luminescence. Luminescence values were normalized to 1 in the sample having the highest concentration of SmBiT-ubiquitin and the lowest concentration of GST-LgBiT. A wide range of GST-LgBiT concentrations produce luminescence signals that are linearly related to the concentration of SmBiT-ubiquitin in the extract. Higher GST-LgBiT concentrations yield higher luminescence signals and higher background levels. Detector saturation was observed in the samples with the highest GST-LgBiT concentration. Axis are displayed in log₂ scale. Mean of 3 independent experiments.

(B) Comparison of the expression level of SmBiT-ubiquitin and endogenous ubiquitin. Protein extracts were prepared from the indicated strains. The proteins were separated by SDS-PAGE, transferred on a PVDF membrane and revealed with an anti-ubiquitin antibody (P4D1, Santa-Cruz). Representative of 3 experiments.

(C) Relative expression level of SmBiT-ubiquitin in NUbICA strains. Protein extracts were prepared from the indicated strains and supplemented with 1 μ M GST-LgBiT before measuring their luminescence. Htb2 strains express SmBiT-ubiquitin from the pGR892 plasmid, while the other strains express SmBiT-ubiquitin from a chromosomally integrated expression cassette. Background subtracted luminescence values were normalized to 1 in the control strain expressing SmBiT-ubiquitin from the chromosomally integrated expression cassette. Mean \pm s.d., $n \geq 3$, p-value: One-way ANOVA with Tukey's correction for multiple comparison, ns: not significant.

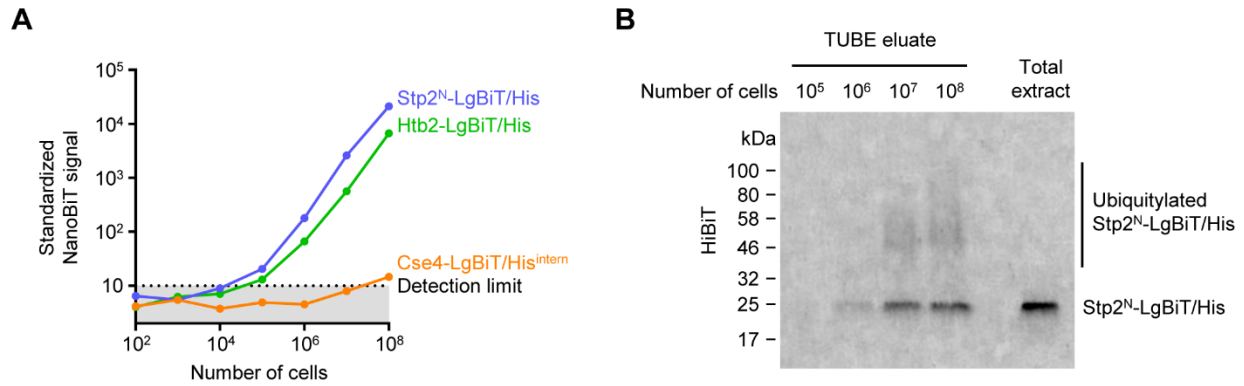


Fig. S2: Sensitivity of NUbICA.

(A) NUbICA sensitivity for the detection of the ubiquitylation of Htb2, Stp2^N and Cse4. LgBiT/His-tagged proteins were purified from extracts prepared from 10² to 10⁸ cells. The recorded NanoBIT signals were normalized and adjusted so that background measurements have identical standard deviations and means. The dotted line and shaded area delimit a detection limit corresponding to 5 times the standard deviation of background measurements (Detection limit). Axis are displayed in log₁₀ scale.

(B) Sensitivity of the detection of the ubiquitylation of Stp2^N after TUBE pull-down. Protein extracts were prepared from 10⁵ to 10⁸ cells expressing Stp2-LgBiT/His. Poly-ubiquitylated proteins were then pulled-down using TUBE2-agarose, separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was imaged in presence of the HiBiT peptide to reveal the migration pattern of Stp2-LgBiT/His. Representative of 2 independent experiments.

2. Supplementary Tables

Table S1: List of strains in the NUBiCA collection

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Table S2: Yeast strains used in this study

Strain	Background	Genotype	Source
BY4741	S288c	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Brachman et al., 1998
BY4742	S288c	<i>MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Brachman et al., 1998
BY4745	S288c	<i>MATalpha his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Brachman et al., 1998
C-SWAT library	BY4741	<i>ORF::C-SWAT-acceptor (L3-CYC1term-ScURA3-hphΔN-ALG9term-L4)</i>	Meurer et al., 2018
NUBiCA library	BY4745	<i>ORF::LgbiT-HIS-HPH met17::PDC1pr-SmBiT-Ub-NAT can1Δ::STE3pr-LEU2-Gal1pr-NLS-I-SCEI lyp1Δ</i>	This study
scAB0178	BY4742	<i>htb2::HTB2-LgBit/His-URA3</i>	This study
scAB0179	BY4742	<i>htb2::htb2(K124R)-LgBit/His-URA3</i>	This study
scAB0180	BY4742	<i>htb2::HTB2-LgBit/His-URA3 bre1Δ::HPH</i>	This study
scAB0181	BY4742	<i>htb2::HTB2-LgBit/His-URA3 rad6Δ::HPH</i>	This study
scAB0190	BY4741	<i>stp2::STP2(1-45)-yLgBiT/His-URA lyp1Δ::PDC1pr-SmBiT-Ub-LEU can1Δ::STE2pr-SpHis5</i>	This study
scAB0191	BY4741	<i>stp2::STP2(1-45)-yLgBiT/His-URA asi3::HPH lyp1Δ::PDC1pr-SmBiT-Ub-LEU can1Δ::STE2pr-SpHis5</i>	This study
scAB0195	BY4741	<i>stp2::STP2(1-45)-yLgBiT/His-URA ubc7::KAN lyp1Δ::PDC1pr-SmBiT-Ub-LEU can1Δ::STE2pr-SpHis5</i>	This study
scGLD0122	BY4741	<i>met17::PDC1pr-SmBiT-Ub-NAT</i>	This study
scGLD0153	BY4741	<i>cse4::CSE4-LgBit-10xHis-CSE4-URA psh1Δ::HPH lyp1::pPDC1-SmBiT-UBQ-Leu</i>	This study

		<i>can1::STE2pr-Sphis5</i> <i>ura3Δ::KAN</i>	
scMLB0191	BY4741	<i>cse4::CSE4-LgBit-10xHis-CSE4-URA</i> <i>lyp1::pPDC1-SmBit-UBQ-Leu</i> <i>can1::STE2pr-Sphis5</i> <i>ura3Δ::KAN</i>	This study
YMaM1205	BY4745	<i>can1Δ::STE3pr-LEU2-Gal1pr-NLS-I-SCEI</i> <i>lyp1Δ</i>	Meurer et al., 2018

Table S3: Plasmids used in this study

Plasmid	Description	Source
pAB0010	Yeast/ <i>E. coli</i> shuttle plasmid, KanR, L3-yLgBiT/His-ADH1term-TEF1pr-hphΔC-L4	This study
pGEX6P1_HR23A-TUBE	<i>E. coli</i> expression plasmid, GST-HR23A-TUBE	Thimo Kurz lab
pGEX6P1_ubiquilin1-TUBE	<i>E. coli</i> expression plasmid, GST-ubiquilin1-TUBE	Thimo Kurz lab
pGR0135	Yeast/ <i>E. coli</i> shuttle plasmid, LEU2	This study
pGR0313	<i>E. coli</i> expression plasmid, GST	Rabut et al., 2011
pGR0691	<i>E. coli</i> expression plasmid, GST-Rx3(A7)-TUBE	This study
pGR0890	<i>E. coli</i> expression plasmid, GST-yLgBiT	This study
pGR0892	Yeast/ <i>E. coli</i> shuttle plasmid, LEU2, TEFpr-SmBiT-Ub	This study

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