

# Sensitive detection of protein ubiquitylation using a protein-fragment complementation assay

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**Keywords:** Ubiquitin, Ubiquitination, PCA, UbiCREST, NanoLuc, NanoBiT

**Summary statement:** We describe a sensitive protein-fragment complementation assay to facilitate the monitoring of ubiquitylation events that take place in cultured cells or model organisms.

## 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 to accurately monitor mono- and poly-ubiquitylation 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.

## 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 covalently attach one or multiple ubiquitin moieties onto their substrate proteins, generally on lysine residues. E2s and E3s can also target the 7 lysine residues (K6, K11, K27, K29, K33, K48 and K63) and the amino terminus of ubiquitin to assemble various types of poly-ubiquitin 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, while K63-linked chains are associated with lysosomal degradation or non-proteolytic regulatory mechanisms.

The 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 to globally analyze the ubiquitylome but are costly and burdensome to investigate the ubiquitylation of one or a selected set of proteins. Multiple assays have been devised to monitor and quantify ubiquitylation reactions performed *in vitro* using recombinant proteins or extracts (Berndsen and Wolberger, 2011; Boisclair et al., 2000; Chong et al., 2019; Gururaja et al., 2005; Mondal et al., 2016; Schneider et al., 2012), but they do not permit to investigate 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, due to the low stoichiometry and the

unstable nature of many ubiquitin conjugates, these methods are often not sufficiently sensitive to robustly assay the modification of endogenous proteins. 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 the 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 non covalent protein interactions, PCAs could also be utilized to demonstrate the conjugation of ubiquitin to its substrate proteins. In this manuscript, we describe 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 to examine mono- or poly-ubiquitin signals conjugated to proteins expressed at endogenous levels.

## RESULTS

### ***Design of a NanoBiT-based ubiquitin conjugation assay (NUbICA)***

NanoLuc is one of the smallest and brightest luciferase currently available (Hall et al., 2012). It has previously been further engineered to develop NanoBiT, a protein complementation reporter consisting of two asymmetrically sized fragments (Dixon et al., 2016). The large fragment, termed LgBiT, remains folded and has been optimized to have a high thermal stability and slow turnover. The small fragment, termed SmBiT, is a 11 amino acid peptide that has been selected for its low intrinsic affinity for LgBiT (their dissociation constant is  $\sim 200 \mu\text{M}$ ), while retaining the ability to reconstitute a bright luciferase. NanoLuc and NanoBiT are also robust to various environmental conditions, in particular they can efficiently refold after denaturation (Hall et al., 2012). These properties

make NanoBiT an excellent reporter to probe 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 hereafter). In addition, we appended a poly-histidine tag to the LgBiT sequence, which enables to purify 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 that 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 done 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 for instance serve to correct variations of SmBiT-ubiquitin expression in different genetic contexts. Altogether, 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 will result in NLR values below the control, while conditions that stimulate ubiquitylation will increase NLR values.

### ***Validation of NUBiCA to probe proteolytic and non-proteolytic ubiquitylation events***

We used budding yeast as a model organism to evaluate NUBiCA. We first probed the ubiquitylation of H2B, one of the best characterized ubiquitin conjugate in cells. In yeast, H2B is mono-ubiquitylated on its lysine K124 by the Bre1 ubiquitin ligase and the Rad6

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 condition. 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 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). 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 since  $10^5$  wild type cells (i.e.  $10\mu\text{L}$  of a  $\text{OD}=0.5$  culture) were sufficient to produce a detectable NanoBiT signal (Fig. S2A). Altogether, these results demonstrate that NUbICA can accurately report Htb2 ubiquitylation without the need for protein separation by gel electrophoresis.

Histone mono-ubiquitylation acts non-proteolytically to control gene activity (Weake and Workman, 2008). However, proteins modified by poly-ubiquitin 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 to detect the ubiquitylation of unstable proteins in endogenous conditions. We chose to probe the ubiquitylation of the N-terminal region of the transcription factor Stp2 (Stp2<sup>N</sup>), which comprises a strong degradation signal (Omnus and Ljungdahl, 2014). Using proteasome inhibition and classical immunoassays, we previously demonstrated that Stp2<sup>N</sup> is efficiently ubiquitylated, primarily by the Asi1/3 ubiquitin ligase complex and the Ubc7

conjugating enzyme (Khmelinskii et al., 2014) (Fig. 3A). We now used NUbICA to probe Stp2<sup>N</sup> ubiquitylation in the absence of proteasome inhibition. As for Htb2, we visualized the conjugation of SmBiT-ubiquitin to Stp2<sup>N</sup>-LgBiT/His in a gel-based assay (Fig. 3B). As expected (Khmelinskii et al., 2014), the modification of Stp2<sup>N</sup> 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 little as 10<sup>5</sup> cells (Fig. S2A). In comparison, we needed at least 10<sup>7</sup> cells to reveal Stp2<sup>N</sup> modification after enrichment of ubiquitylated proteins using TUBEs (Fig. S2B). Thus, NUbICA is suitable to probe the modification of a proteolytic ubiquitylation substrate without the need of proteasome inhibition.

In order 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 is described to control Cse4 levels and to prevent 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). Contrary to Htb2 and Stp2<sup>N</sup>, we could not detect Cse4 ubiquitylation after gel electrophoresis. However, when we purified Cse4-LgBiT/His<sup>intern</sup> from at least 10<sup>8</sup> 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 further demonstrate that the luminescence signal is due to Cse4 ubiquitylation, we treated the purified proteins with Usp2, a non-specific deubiquitylation enzymes (DUB) that hydrolyses all types of ubiquitin linkages (Hospenthal et al., 2015). As for Htb2 and Stp2<sup>N</sup> (Figs 2D, 3D), this resulted in a large reduction of the luminescence signals produced by purified Cse4-LgBiT/His<sup>intern</sup> (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; Cheng et al., 2017; Ohkuni et al., 2016). In conclusion, these results establish NubiCA as a sensitive method to probe 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 thought to construct 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 systematically fuse yeast open reading frames to the DNA sequence of the LgBiT/His 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 5,580 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 to validate the ubiquitylation of candidates identified by mass spectroscopy or to systematically analyze the ubiquitylation of protein families in 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 the 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 9 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,C,D,E,F,G,H,I). Treatment of the eluates with Usp2 confirmed that the signal was dependent on ubiquitin conjugation. As expected, we observed a significant increase of Art4/Rod1 and decrease of Art8/Csr2 ubiquitylation signals when cells were exposed to glucose (Fig. 4E,G). In contrast, the ubiquitylation level of the other ARTs remained unchanged upon glucose treatment. These results suggest that NUbICA allows to monitor changes in protein ubiquitylation in response to external stimuli and that among the ARTs, Art4/Rod1 and Art8/Csr2 are likely the main ARTs involved in plasma membrane protein composition remodeling in response to glucose.

### ***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. Since Usp2 recognizes folded ubiquitin (Renatus, 2006), the fact that Usp2 treatment resulted in a large reduction of NUbICA signals (Figs 2D, 3D,G and 4B,C,D,E,F,G,H,I) indicates that ubiquitin can be refolded after denaturing purification. If so, ubiquitin chains might be recognized and protected from deubiquitylation by generic and chain-specific ubiquitin binders such as TUBEs. We reasoned that this could enable to investigate ubiquitin chain topologies. To test this hypothesis, we took advantage of the NUbICA collection. We selected three non-proteolytic (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), while Sna3 is modified by K63-linked ubiquitin chains (Stawiecka-

Mirota et al., 2007). Sic1 and Cln2 are SCF substrates 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 deubiquitylation by Usp2 (Fig. 5A,B,C,D). We used 3 distinct TUBEs in this assay. The 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 deubiquitylation kinetics of mono-ubiquitylated Htb2 and Art9 (Fig. 5B). In contrast, the deubiquitylation of poly-ubiquitylated proteins was differentially impaired by the TUBEs. While the Ubiquitin1 TUBE protected all tested poly-ubiquitin substrates from deubiquitylation, the Rx3(A7) TUBE primarily impacted the deubiquitylation of Sna3 and the HR23A TUBE mostly inhibited the deubiquitylation 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 to qualitatively distinguish different types of ubiquitin conjugates. We applied this approach to examine the modification profile of Stp2<sup>N</sup> and Cse4 (Fig. 5E). The deubiquitylation kinetics of Stp2<sup>N</sup> and Cse4 purified from wild type cells matched the profiles previously observed for proteolytic ubiquitylation substrates. In contrast, the deubiquitylation of Stp2<sup>N</sup> purified from *asi3Δ* or *ubc7Δ* was only very weakly impaired by the TUBEs and resembled the profiles 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<sup>N</sup> 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 well 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 few experiences have been performed with these assays, it is difficult to evaluate their sensitivity and specificity for the detection of low abundance ubiquitylated proteins.

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 the ubiquitylation of the proteins they are fused to. Second, the LgBiT fragment efficiently refolds after denaturation, which enables to monitor NanoBiT signals after very stringent purification protocols or even gel electrophoresis. Last, NanoLuc is one of the brightest luciferase, 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 to investigate the ubiquitylation of most cellular proteins without overexpression. This is in our view of utmost importance since 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 to study ubiquitylation in tissue culture systems or genetically amenable model organisms. We tagged ubiquitin N-terminally since it was shown to 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). Except Cse4, all substrates investigated here were tagged C-terminally. This strategy will likely be 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 may 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 9 tested ARTs, the only one for which we could not detect NUbICA signal was Art7. These results suggest that in many instances, the position of the LgBiT/His tag will not prevent the functionality of NUbICA.

A central aspect of the NUbICA protocol described here is that the lysate preparation and substrate purification are done under highly denaturing conditions (Fig. 1B). This has the advantage to suppress DUB activity and, therefore, to preserve 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 to achieve a high sensitivity and specificity, which enables to directly measure the NanoBiT signal in the purification eluates. Moreover, when the ubiquitylated proteins are sufficiently abundant, the eluates can also be separated by gel electrophoresis to visualize the ubiquitin conjugates (Figs 2B, 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 since it would open the possibility to monitor ubiquitylation and deubiquitylation reactions in real time in live cells.

NUbiCA is simple to implement since 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 in 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 deubiquitylation reactions enables to qualitatively distinguish different types of ubiquitin conjugates. 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 deubiquitylation 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 deubiquitylation by a generic DUB (Fig. 5). Using a limited set of three ubiquitin binders we were able to clearly distinguish the deubiquitylation 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 is described to have a similar affinity for K48- and K63-linked ubiquitin chains (Hjerpe et al., 2009), did not efficiently protect K63-linked chains from deubiquitylation (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 to examine the properties of

ubiquitin conjugates. Besides, we are confident that the use of other ubiquitin binders or deubiquitylation enzymes will enable to reveal more deubiquitylation profiles and hence to more precisely decipher ubiquitin chain topologies.

In conclusion, we show that NUbICA can be used to probe the mono- and poly-ubiquitylation 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 posttranslational protein modifiers, such as SUMO or Nedd8, in any tissue culture system or genetically amenable model organism.

## **MATERIALS AND METHODS**

### ***Yeast methods, plasmids***

Yeast genome manipulations (chromosomal gene tagging and gene deletion) were done 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 since yeast ubiquitin is encoded by 4 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 ~4 to 5 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 presence of the HiBiT peptide (VSGWRLFKKIS), which binds tightly to LgBiT ( $K_D = 0.7$  nM) and enables to reconstitute an active luciferase (Schwinn et al., 2018a). The lyophilized peptide was purchased from Proteogenix (Schiltigheim, France), solubilized at a concentration of 1 mM in ddH<sub>2</sub>O 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<sup>9</sup> exponentially growing yeast cells. Cell pellets were resuspended in 20% trichloroacetic acid 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 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 presence of protease inhibitors (cOmplete™ EDTA-free Cocktail, Roche, Basel, Switzerland) and lysed for 2 min with glass beads in a Disrupter Genie homogenizer (Scientific Industries, Bohemia, NY, USA). After clarification, the extracts were incubated for 2 h with 15  $\mu$ L TUBE2 agarose beads (LifeSensors, Malvern, PA) 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***

*E. coli* BL21(DE3)RIL cells were transformed with plasmids encoding GST (pGR0313), GST-Ubiquilin1-TUBE (pGEX6P1\_Ubiquilin1-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 addition of 1 mM IPTG during 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, 1 mM DTT, then with 50 mM Tris pH 7.8, 200 mM NaCl, 5 mM DTT. Purified proteins were eluted in 50 mM Tris pH 7.8, 200 mM NaCl, 5 mM DTT, 20mM reduced L-Glutathione and dialyzed against PBS, 10% glycerol, 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, Hercules, CA, USA). After transfer, the membranes were washed extensively in TBS-T (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% TWEEN® 20t) during at least 1h to allow the 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 were recorded for up to 20 minutes 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  $\mu$ M) and furimazine (1%).

### ***Anti-ubiquitin immunoblots***

Cell pellets were resuspended in 20% trichloroacetic acid and lysed for 2 min with glass beads in a Disrupter Genie homogenizer (Scientific Industries, Bohemia, NY, USA). Lysates were pelleted and resuspended in TCA sample buffer (15% Glycerol, 450mM Tris pH 8.8, 1% SDS, 2mM EDTA, 100mM DTT, 0.005% Bromophenol Blue), denatured 5 min at 95°C and separated by SDS-PAGE using 4-16% Mini-PROTEAN® TGX Stain-Free™ precast gels (Bio-Rad, Hercules, CA, USA). Proteins were then transferred on PVDF membranes with a Trans-Blot® Turbo™ semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). After transfer, the membranes were washed in TBS-T and probed a HRP-conjugated anti-ubiquitin antibody (1/1000, P4D1, sc-8017 HRP, Santa Cruz Biotechnology, Dallas, TX, USA) (antibody profile validation: Hovsepien et al., 2016) and imaged 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 a luciferase assay buffer (TBS-T, 17.5mM thiourea) containing furimazine (0.25% to 1%) and with or without HiBiT peptide (1  $\mu$ 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 crosstalk between neighboring samples. Luminescence was recorded with either 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 due to their low intrinsic affinity and produce a luminescent signal which 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 do 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 to 400 ng/ $\mu$ L in the luciferase assay buffer containing furimazine (0.25%) and GST-LgBiT (0.04-20  $\mu$ 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 presence of GST-LgBiT were subtracted by the luminescence values measured in the corresponding control samples.

### ***Endpoint deubiquitylation assays***

To confirm that the NanoBiT signal is dependent on ubiquitin conjugation, purified LgBiT/His-tagged protein were distributed in 96 half-well white microtiter polystyrene and diluted 10 times in TBS-T with or without Usp2 catalytic domain (0.5  $\mu$ M, Enzo Life Sciences, Farmingdale, New York, 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, 35 mM thiourea, 2% furimazine before recording their luminescence with a plate reader.

### ***Deubiquitylation kinetics***

Purified LgBiT/His-tagged proteins were diluted 5 fold in TBS-T and distributed in 384 shallow-well white polystyrene microtiter plates (Perkin Elmer, Waltham, MA, USA). Recombinant GST, GST-Ubiquilin1-TUBE, GST-HR23A-TUBE and GST-Rx3(A7)-TUBE were diluted in TBS-T at a concentration of 2  $\mu$ 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  $\mu$ 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) pre-warmed at 25°C and the luminescence signal of the samples was recorded during 60 min. The samples without Usp2 served to record the luminescence variations in the absence of deubiquitylation 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 et al. previously constructed a library of 5,661 yeast strains where an acceptor module has 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 Hygromycine 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 the exchange of the acceptor and donor modules and select the recombinant strains using the procedure described by Meurer et al. (Meurer et al., 2018). This produced a collection a MAT- $\alpha$  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 finally obtain MAT- $\alpha$  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 5,580 NUbICA strains.

## **ACKNOWLEDGEMENTS**

We thank Matthias Meurer and Michael Knop for discussions, reagent exchanges, the C-SWAT library and their help with the construction of the NUbiCA yeast strain collection. We also thank Thimo Kurz for TUBE expression plasmids, Rémy Le Guevel for the ImPACcell platform and the IGDR and Biosit for support and infrastructure.

## **AUTHOR CONTRIBUTIONS**

G.R. coordinated the project and designed the experiments. M.L.B., A.B. and G.L.D. performed the NanoBiT experiments. G.L.D. and A.B. constructed the NUbiCA yeast strain collection. S.L. suggested and helped with ARTs experiments. G.R. and M.L.B. prepared the figures and wrote the manuscript.

## **COMPETING INTERESTS**

The authors declare that they have no competing interests.

## **FUNDING**

This work was supported by the Centre National de la Recherche Scientifique [G.L.D., S.L. and grant PICS07394 to G.R.], the Institut National de la Santé Et de la Recherche Médicale [G.R.], the University of Rennes 1 [M.L.B], the Agence Nationale de la Recherche [grant ANR-16-CE11-0021-01 to G.R. and ANR-16-CE13-0002-02 to S.L.], La Ligue contre le cancer [G.R.] and Biosit [G.R.].

## **DATA AVAILABILITY**

Data generated during this study are included in this article and its additional files.

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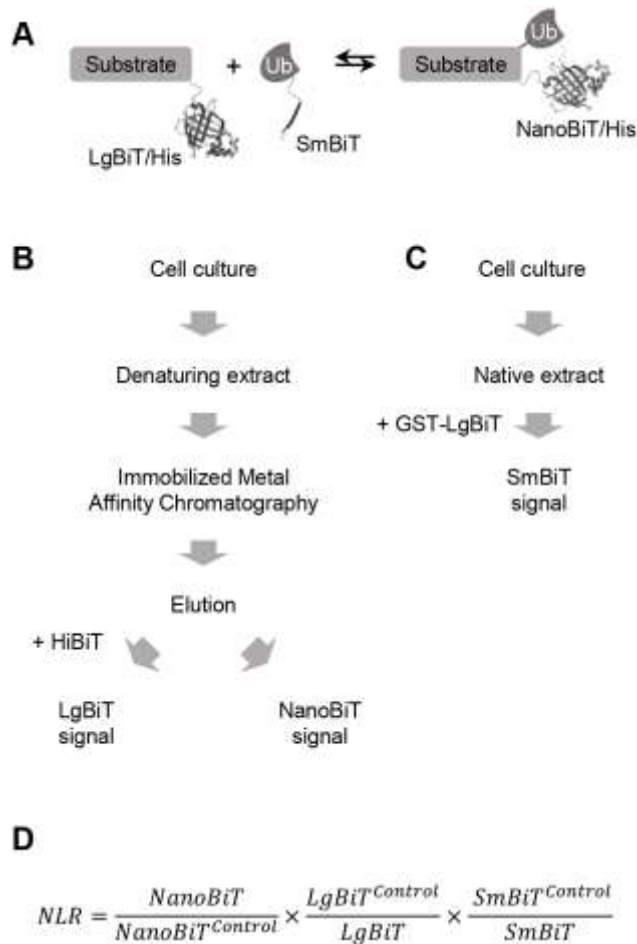
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## Figures



**Fig. 1: Description of the NanoLuc-based Ubiquitin Conjugation Assay (NUbiCA).**

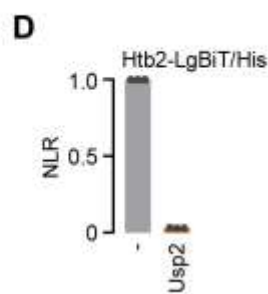
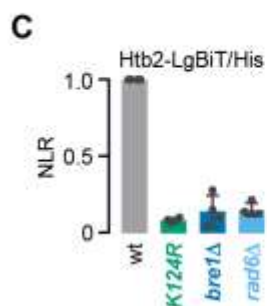
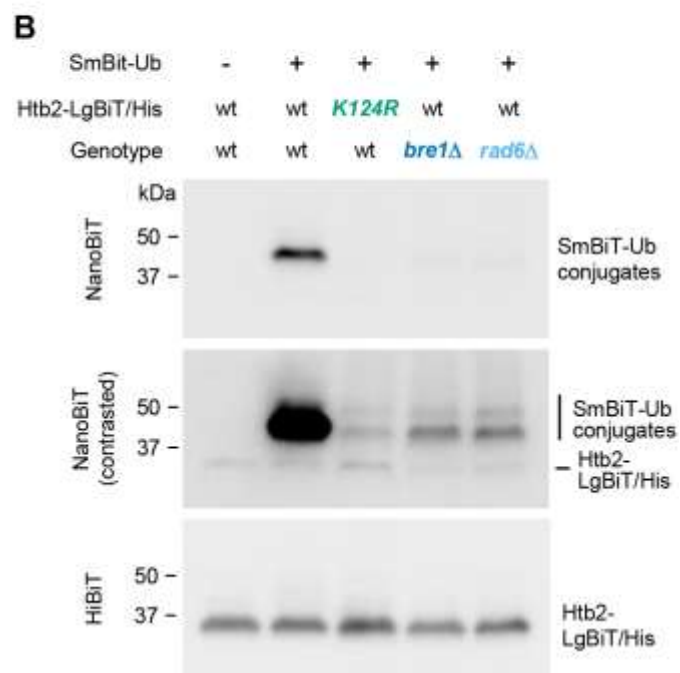
(A) Ubiquitin (Ub) and a substrate protein of interest are genetically fused to the SmBiT and LgBiT/His tags, respectively. Conjugation of ubiquitin to the protein of interest enables the reconstitution of the NanoBiT reporter.

(B) Main steps of the NUbICA protocol. Cells are lysed in denaturing conditions. LgBiT/His-tagged ubiquitylation substrates are pulled-down with 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 to quantify the total amount of LgiT/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 in native conditions. The extracts are supplemented with recombinant GST-LgBiT and distributed in a microtiter plate. The luminescence signals are recorded in 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 in control and test conditions (e.g. wild type and mutant cells). SmBiT signals may also be evaluated to control SmBiT-ubiquitin expression level. 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.





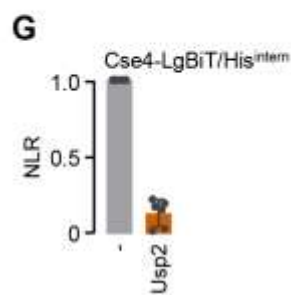
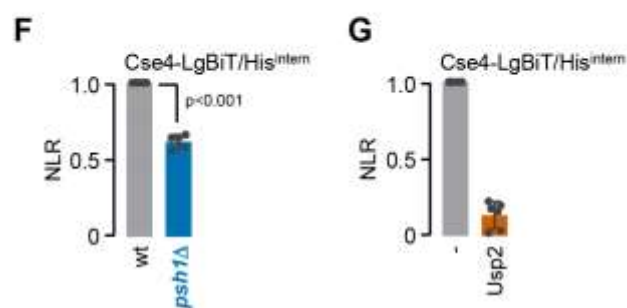
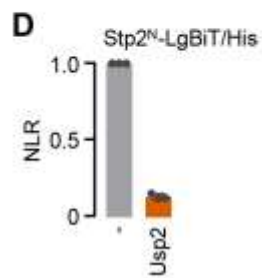
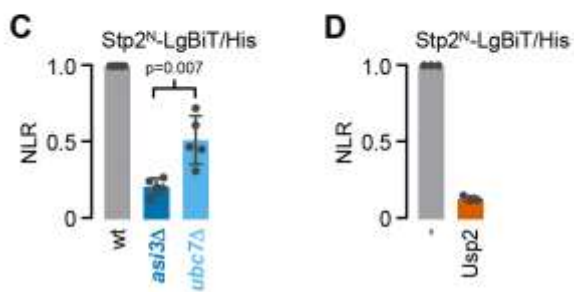
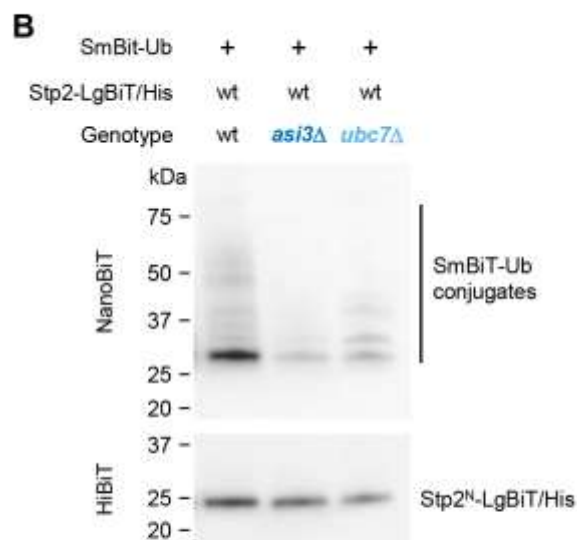
**Fig. 2: Analysis of Htb2 mono-ubiquitylation.**

(A) Htb2 is ubiquitylated on its lysine K124 by the Bre1 ubiquitin ligase and the Rad6 ubiquitin conjugating enzyme.

(B) Conjugation of SmBiT-Ubiquitin to Htb2-LgBiT/His visualized after gel electrophoresis. LgBiT/His-tagged Htb2 purified from  $2 \cdot 10^8$  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 presence of the HiBiT peptide, which tightly interacts with LgBiT to reconstitute a functional luciferase (HiBiT). Representative of 2 independent experiments.

(C) Relative ubiquitylation levels of Htb2-LgBiT/His purified from the indicated yeast strains. The graph displays normalized luminescence ratios (NLR) (mean  $\pm$  s.d., 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 2h with or without Usp2. The graph displays normalized luminescence ratios (NLR) (mean  $\pm$  s.d., n=3).



### Fig. 3: Analysis of Stp2<sup>N</sup> and Cse4 poly-ubiquitylation.

(A) Stp2<sup>N</sup> is primarily ubiquitylated by the Asi1/3 ubiquitin ligase complex and the Ubc7 ubiquitin conjugating enzyme.

(B) Conjugation of SmBiT-Ubiquitin to Stp2<sup>N</sup>-LgBiT/His visualized after gel electrophoresis. LgBiT/His-tagged Stp2<sup>N</sup> purified from  $2 \cdot 10^8$  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<sup>N</sup>-LgBiT/His purified from the different strains, the same membrane was subsequently imaged in presence of the HiBiT peptide, which tightly interacts with LgBiT to reconstitute a functional luciferase (HiBiT). Representative of 2 independent experiments.

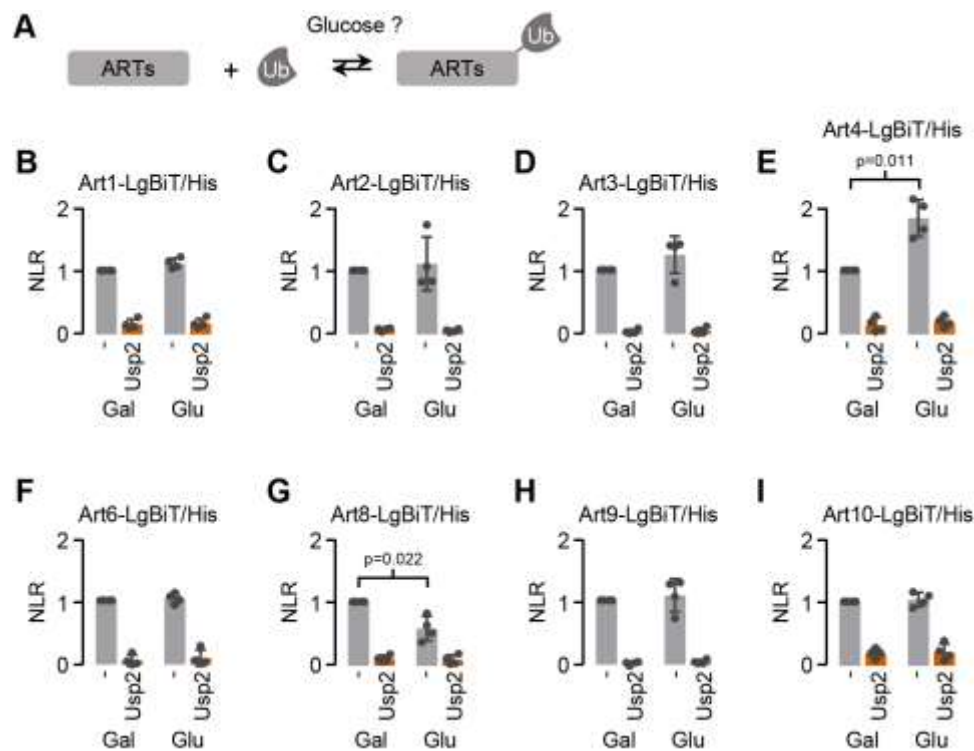
(C) Relative ubiquitylation levels of Stp2<sup>N</sup>-LgBiT/His purified from the indicated yeast strains. The graph displays normalized luminescence ratios (NLR) (mean  $\pm$  s.d., n=5, p-value: two-tailed paired t-test).

(D) Relative ubiquitylation levels of Stp2<sup>N</sup>-LgBiT/His after Usp2 treatment. Stp2<sup>N</sup>-LgBiT/His was purified from wild type cells and incubated for 2 h with or without Usp2. The graph displays normalized luminescence ratios (NLR) (mean  $\pm$  s.d., n=3).

(E) Cse4 is ubiquitylated by the Psh1 ubiquitin ligase.

(F) Relative ubiquitylation levels of Cse4-(LgBiT/His)<sup>intern</sup> purified from wild type (wt) and *psh1* $\Delta$  cells. The graph displays normalized luminescence ratios (NLR) (mean  $\pm$  s.d., n=5, p-value: two-tailed t-test).

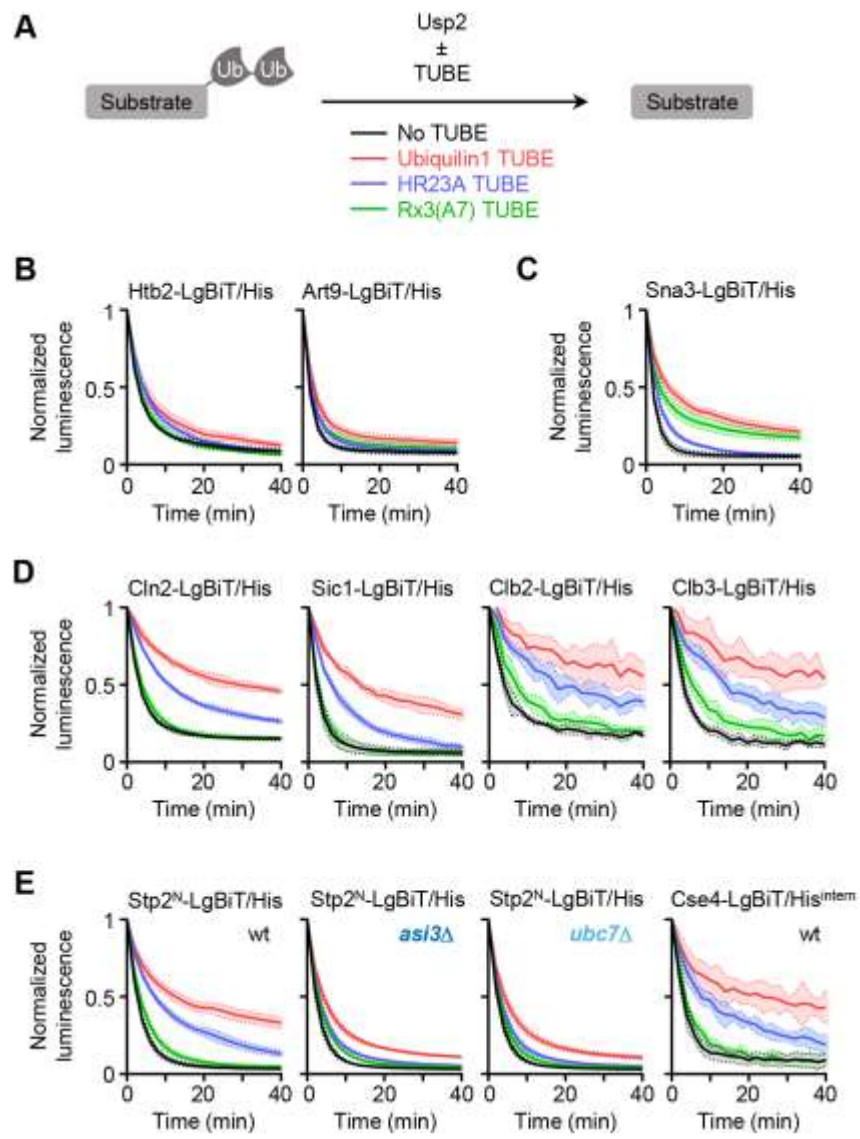
(G) Relative ubiquitylation levels of Cse4-(LgBiT/His)<sup>intern</sup> after Usp2 treatment. Cse4-(LgBiT/His)<sup>intern</sup> was purified from wild type cells and incubated for 2 h with or without Usp2. The graph displays normalized luminescence ratios (NLR) (mean  $\pm$  s.d., n=3).



**Fig. 4: Ubiquitylation of Arrestin-related trafficking adaptors (ARTs) in response to glucose exposure.**

(A) ART ubiquitylation was monitored in response to glucose.

(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 5 min exposure to 2% glucose (Glu). Purified proteins were incubated for 2 h with or without Usp2. The graphs display normalized luminescence ratios (NLR) (mean  $\pm$  s.d.,  $n=4$ ,  $p$ -values: two-tailed  $t$ -tests).



**Fig. 5: Deubiquitylation kinetics in the presence of Tandem Ubiquitin Binding Entities (TUBEs).**

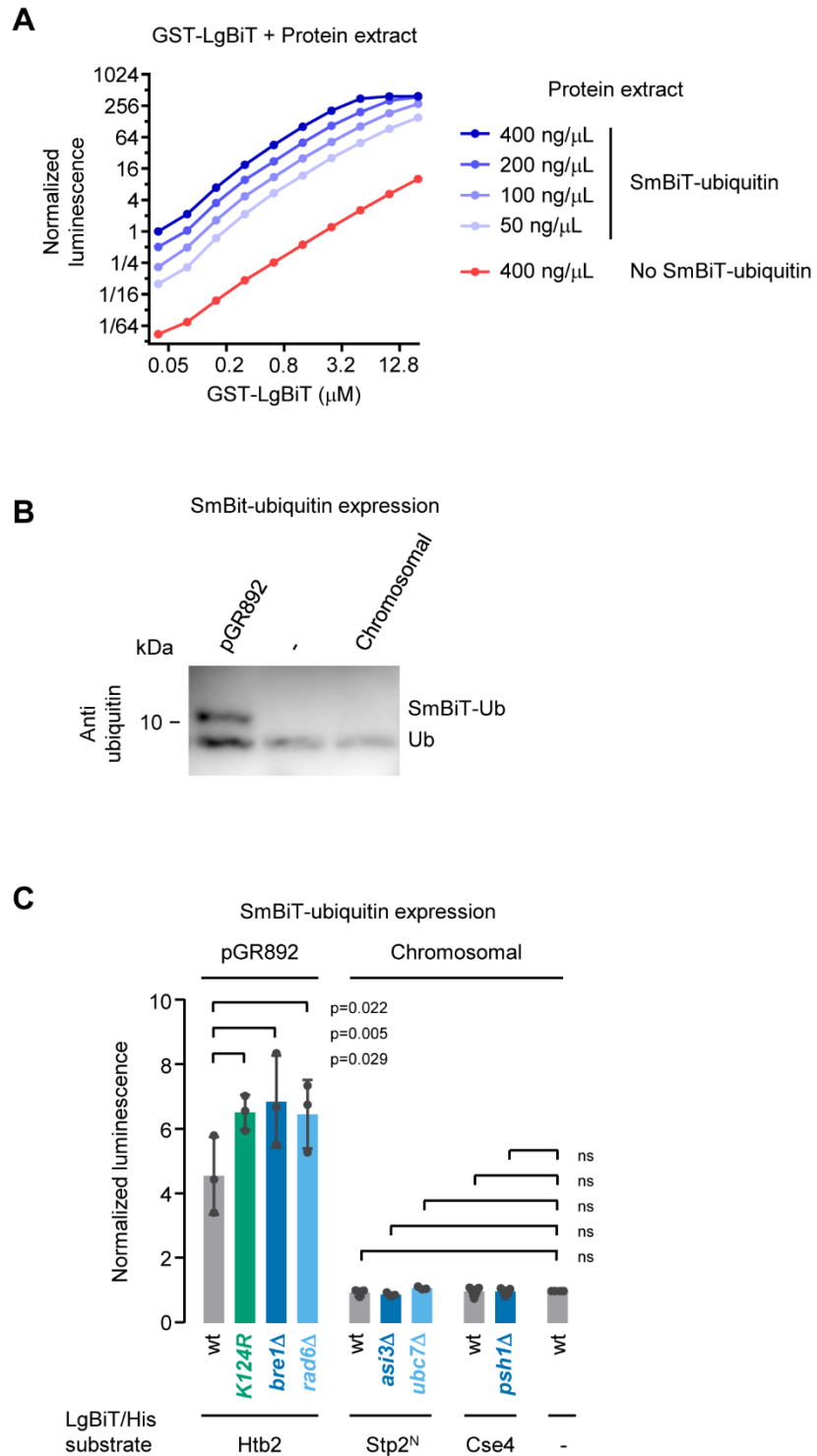
(A) Deubiquitylation reactions were performed in the presence of Usp2 using substrates previously incubated with Ubiquitin1, HR23A or Rx3(A7) TUBEs.

(B-D) Deubiquitylation profiles of substrates known to be modified with mono-ubiquitin (B), K63-linked chains (C) or K48-linked chains (D).

(E) Deubiquitylation profiles of Stp2<sup>N</sup>-LgBiT/His and Cse4-LgBiT/His<sup>intem</sup> in the indicated yeast strains.

The graphs display mean luminescence values normalized to the initial time point. Standard deviations are drawn as shaded areas (n≥4).

# 1. Supplementary Figures



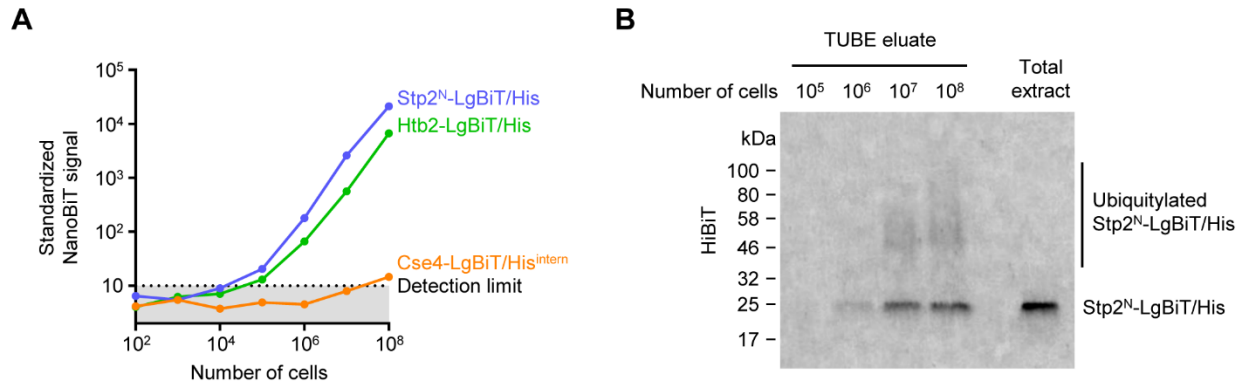
### **Fig. S1: Analysis of SmBiT-ubiquitin expression.**

**(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 log<sub>2</sub> 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  $\mu$ 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<sup>N</sup> and Cse4. LgBiT/His-tagged proteins were purified from extracts prepared from 10<sup>2</sup> to 10<sup>8</sup> 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 log<sub>10</sub> scale.

(B) Sensitivity of the detection of the ubiquitylation of Stp2<sup>N</sup> after TUBE pull-down. Protein extracts were prepared from 10<sup>5</sup> to 10<sup>8</sup> 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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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

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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

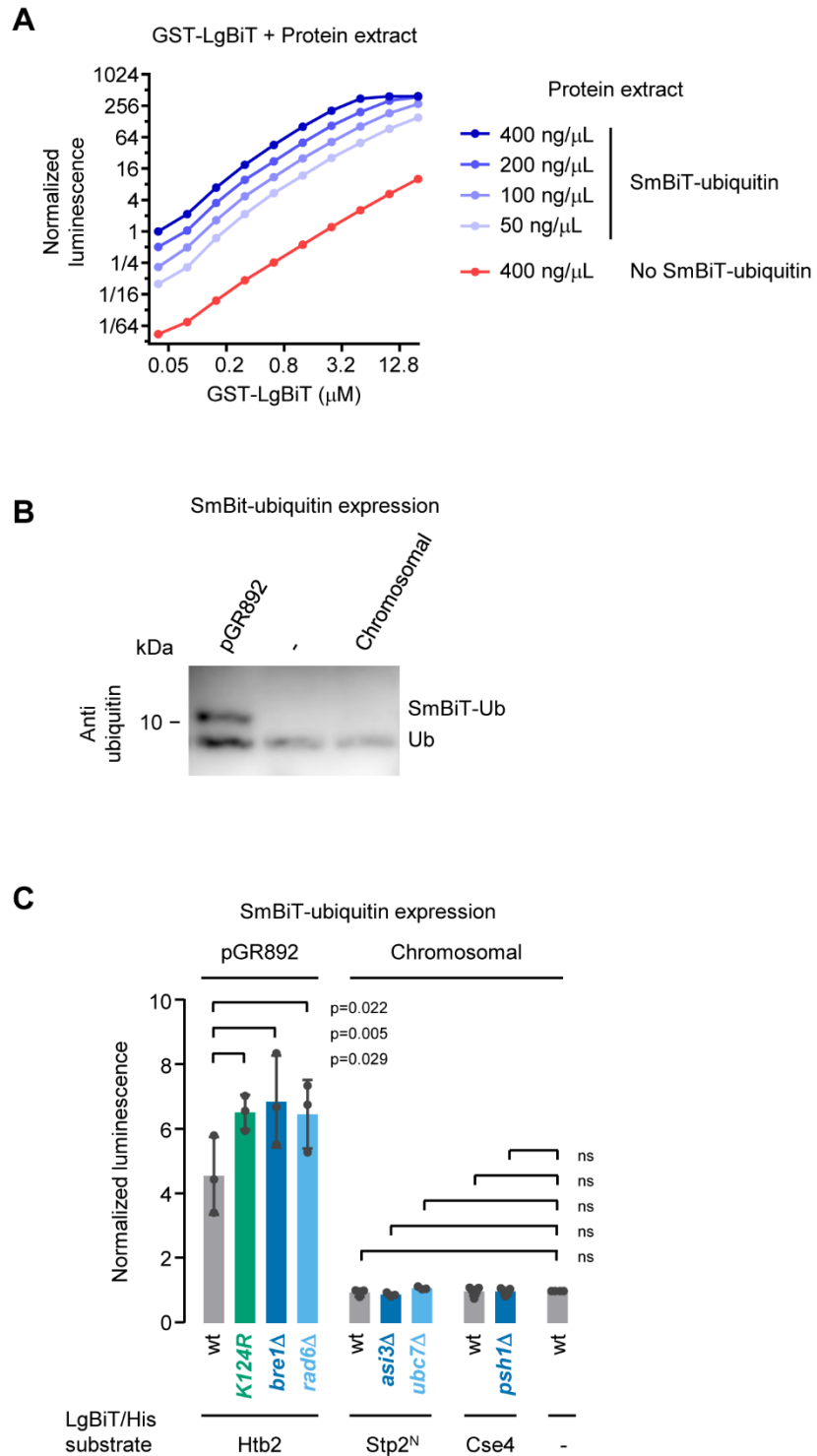
### Table References

**Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P. and Boeke, J. D.** (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**, 115–132.

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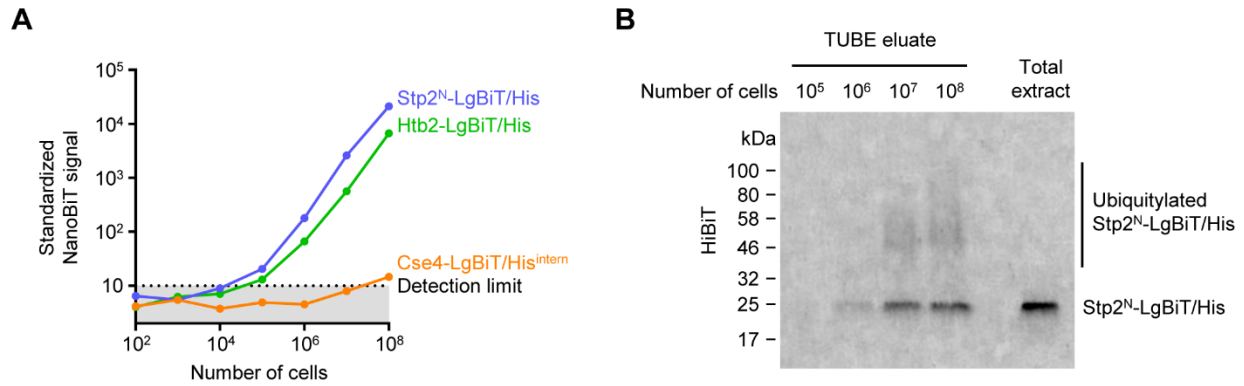


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scMLB0191	BY4741	<i>cse4::CSE4-LgBit-10xHis-CSE4-URA</i> <i>lyp1::pPDC1-SmBit-UBQ-Leu</i> <i>can1::STE2pr-Sphis5</i> <i>ura3Δ::KAN</i>	This study
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