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

Marie Le Boulch, Audrey Brossard, Gaëlle Le Dez, Sébastien Léon and Gwenaël Rabut
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**Original submission**

First decision letter

MS ID#: JOCES/2019/240093

MS TITLE: Sensitive detection of protein ubiquitylation using a protein-fragment complementation assay

AUTHORS: Marie Le Boulch, Audrey Brossard, Gaelle Le Dez, and Gwenael Rabut
ARTICLE TYPE: Tools and Resources

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

Le Boulch et al. describe a split-luciferase PTF assay for assessing protein ubiquitylation in yeast. They demonstrate that this system, which they name NanoLuc based ubiquitin conjugation assay

(NUbiCA), can be used to detected histone H2B mono-ubiquitylation, Stp2 (poly)ubiquitylation, and Cse4 (poly)ubiquitylation. The system has high sensitivity, although unlike gel-based and certain other assays, it does not provide a clear readout of ubiquitin chain lengths. It provides a quantitative measure of ubiquitylation, but given the altered nature of both substrate and ubiquitin, it is not clear whether rates of ubiquitylation and deubiquitylation will always be the same as with untagged proteins. Potentially the most useful aspect of the paper is that they also create a library of NUbiCA strains in which the large majority of yeast ORFs are tagged C-terminally with LgBiT and the strains also carry an integrated SmBiT-ubiquitin. This can in principle be used for measuring ubiquitylation of almost any desired protein. However, no suggested or proof-of-principle experiments are done with the library. Some of its possible uses should be elaborated in the Discussion.

*Comments for the author*

A sensitive and inexpensive assay for protein ubiquitylation is certainly welcome. The assay, as described, is a little cumbersome, but works with proteins of very low natural abundance. For a methods paper, details of the assays are a little hard to decipher: The level of ubiquitylation is measured after denaturing extracts, so the split luciferase parts presumably separate even though the ubiquitin is covalently linked to the LgBiT fusion, and the luciferase segments must reassociate upon renaturation. Correct? The measure of SmBiT-ubiquitin levels is unclear too. Extracts with SmBiT-ubiquitin are mixed with GST-LgBiT and luminescence is measured. Are these SmBiT-ubiquitin extracts from cells that also express LgBiT? How does SmBiT-ubiquitin give sufficient signal with GST-LgBiT (0.05 uM) if the Kd is 200 uM? I suspect I'm misunderstanding aspects of these assays, but I believe the authors can improve clarity.

The biggest problem with the paper in its current form is insufficient validation of important features of the new method:
(1) In the one case where NUbiCA is compared to gel-based assays using the H2B substrate (Fig. 2), it appears rad6D eliminates ubiquitylation by the gel assay but NUbiCA still shows ~30% of the WT signal in this mutant. Is this 'background' signal in NUbiCA or do the authors believe another E2 also contributes but is not detectable by gel assay?

(2) The chain-specific DUBs need to be validated in the yeast extract system where the differences in signal are often small (2x or less). The controls in supplementary Fig. 1 show weak cleavage activity under the conditions tested especially for Cezanne and AMSH, and the conditions used against the test substrates (Stp2, Cse4) might not be suitable. Substrates with KNOWN K11, K48, and K63 chains should be tested to show they are cleaved by the 'right' DUB. I know that Ubc6 is modified with K11 chains, and there are others, and there are quite a few known K48 and K63 modified substrates in yeast to test. This would also allow a little validation of the NUbiCA library.

(3) Another easy and helpful control would be to treat the free chains (Suppl. Fig. 1) with the same denaturing/renaturing treatments as the test conjugates in Fig. 3 and 4, and see if the chain specificity of the three DUBs is maintained. I worry that longer chains do not renature as readily and cleavage or chain specificity will be reduced.

(4) There is no statistical analysis of the differences in luminescence signals in any of the figures to judge their significance. Differences are often small; for one example, among several, in Fig. 4b, I'm not sure the psh1D-dependent reduction is statistically significant.

(5) I wonder how well NUbiCA performs compared to TUBE-based assays which can measure both ubiquitin chain linkage and length. This should at least be discussed or, better, experimentally compared.

In summary, the system shows promise, but there is still a good deal to do to validate NUbiCA as a way to measure protein-ubiquitin modification in vivo. Clarification of how the assays work and how the NUbiCA library can be used would help as well.

Reviewer 2

*Advance summary and potential significance to field*

The submitted manuscript by Le Boulch et al., describes a system (NUbiCA) which allows the use of luciferase to monitor the ubiquitylation of protein substrates in lysates. They have fused the LgBIT portion of the NanoBIT luciferase reporter to substrate proteins, and fused the SmBIT portion to ubiquitin in yeast. They have then shown that HT2B can be monoubiquiylated by ubiquitin-SmBIT and that this ubiquitylation can be monitored by luciferase activity. They then show that Stp2 and Cse4 ubiquitylation are indicated via this system and that it can be used to detect when Cse4 is ubiquitylated during the cell cycle. Finally, they have created a yeast library of proteins fused to LgBIT, creating 5,580 ready-to-use NUbiCA strains to potentially monitor the ubiquitylation of any of these fused proteins.
This was an interesting and novel study which appears to have significant potential for future studies of protein ubiquitylation in yeast.

*Comments for the author*

I have a number of questions and comments in regard to what has been presented in this manuscript.

Major points:

1] In Figure 3b the ubc7 mutant does not reduce the luciferase activity associated with Stp2 as much as the asi3 mutant. Should they not both have a similar impact?

2] In Figure 3c and 4c the authors use UbiCREST. However, they only use 4 of the DUBs from this system, and not the full 8 associated with the kit? Why did they not look at the others as well?

3] In Figures 3/4 it would be better to show again that the SmBIT can form polyubiquitin chains, at the least with Stp2, to demonstrate that the SmBIT ubiquitin can form these chains, and that the signal observed is not due to monoubiquitin. I appreciate this would be difficult with Cse4, and that the UbiCREST results match, but the DUBs could potentially still be removing the monoubiquitin, if there are no chains present.

4] In Figure 4b, why does the psh1 mutant not block Cse4 ubiquitylation?

5] In Figure 4b/c/d/e the error bars on the normalised luminescence ratios are very big in comparison with the other figures. Why is this?

6] In Figure 4d/e why does the scale go up to 4? I would assume this is due to what is being used to normalise it, but it would be good to explain why there is a difference!

7] As the SmBIT appears to be fused to the carboxy terminus of ubiquitin does this make it incompatible with examinations of linear ubiquitylation as the ubiquitin molecules in such a chain will be attached to each other via this region?

8] In the various figures the authors show impacts upon K11, K48 and K63 chains. Have they explored if the conjugation of SmBIT is compatible with all the other chain types?

9] The library created has all of the proteins fused to LgBIT at their carboxy termini. As many of these proteins may be reasonably large, and the ubiquitylation sites may not be close to this part of the protein in the final 3D structure, would the authors like to comment on how far the components of NanoBIT can be apart before this system will not work, and how often this is likely to be the case?

10] The authors state in the discussion that the use of endogenously tagged proteins is ‘of utmost important since overexpression can trigger protein quality control and ubiquitylation pathways that are not necessarily the ones one wants to investigate’. However, as they point out in their supplementary note, they are still having to express ubiquitin tagged with SmBIT from a construct

as it would be technically challenging to endogenously tag it. Would the addition of all this extra ubiquitin not also interfere with these systems? I would also assume this would have to happen in mammalian cells as well and could also cause issues there?

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**First revision**

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field...

Le Boulch et al. describe a split-luciferase PTF assay for assessing protein ubiquitylation in yeast. They demonstrate that this system, which they name NanoLuc based ubiquitin conjugation assay (NUbiCA), can be used to detected histone H2B mono-ubiquitylation, Stp2 (poly)ubiquitylation, and Cse4 (poly)ubiquitylation. The system has high sensitivity, although unlike gel-based and certain other assays, it does not provide a clear readout of ubiquitin chain lengths. It provides a quantitative measure of ubiquitylation, but given the altered nature of both substrate and ubiquitin, it is not clear whether rates of ubiquitylation and deubiquitylation will always be the same as with untagged proteins. Potentially the most useful aspect of the paper is that they also create a library of NUbiCA strains in which the large majority of yeast ORFs are tagged C-terminally with LgBiT and the strains also carry an integrated SmBiT-ubiquitin. This can in principle be used for measuring ubiquitylation of almost any desired protein. However, no suggested or proof-of-principle experiments are done with the library. Some of its possible uses should be elaborated in the Discussion.

Authors' response:
In the revised manuscript, we now provide two series of experiments using a subset of strains from the NUbiCA library. These experiments illustrate how this library will facilitate the exploration of ubiquitylation pathways and further uses are also discussed in the revised manuscript.

Reviewer 1 Comments for the Author...

A sensitive and inexpensive assay for protein ubiquitylation is certainly welcome. The assay, as described, is a little cumbersome, but works with proteins of very low natural abundance. For a methods paper, details of the assays are a little hard to decipher: The level of ubiquitylation is measured after denaturing extracts, so the split luciferase parts presumably separate even though the ubiquitin is covalently linked to the LgBiT fusion, and the luciferase segments must reassociate upon renaturation. Correct?

Authors' response:
We confirm that the NanoLuc fragments are denatured during the purification procedure and reassociate upon renaturation in the luciferase assay buffer. We have modified the text at several points to clarify this. Note that 100% NanoLuc renaturation after denaturation by chaotropic agents was reported previously (Hall et al., 2012).
Further, we acknowledge that NUbiCA may appear cumbersome at the first glance. This is however not the case. In practice, the NUbiCA protocol is faster than antibody-based methods (there's no blocking, washing or antibody incubation) and can be routinely performed in a single day.

The measure of SmBiT-ubiquitin levels is unclear too. Extracts with SmBiT-ubiquitin are mixed with GST-LgBiT and luminescence is measured. Are these SmBiT-ubiquitin extracts from cells that also express LgBiT?

Authors' response:
Yes, SmBiT-ubiquitin quantification can be performed in extracts prepared from cells that express LgBiT-tagged substrates. This is now explicitly explained in the manuscript (Method section, Supplementary note 1, Supplementary figure 1). In extracts prepared from most NUbiCA strains the luminescence signal produced after the addition of GST-LgBiT primarily originates from the interaction of GST-LgBiT and SmBiT-ubiquitin. This is because the concentration at which GST-LgBiT is added to

the extract is much higher than the concentration of the LgBiT-tagged protein in the same extract (see next point). In any case, the "background" luminescence produced by the same extracts without GST-LgBiT can (and should) be measured and subtracted from the measurement performed with GST-LgBiT. This is now discussed in the Supplementary note 1.

How does SmBiT-ubiquitin give sufficient signal with GST-LgBiT (0.05 uM) if the Kd is 200 uM? I suspect I'm misunderstanding aspects of these assays, but I believe the authors can improve clarity.

Authors' response:
We agree that using GST-LgBiT at a concentration of 50nM to quantify SmBiT-ubiquitin might be surprising and counter-intuitive. However, it can be demonstrated using the law of mass action that a wide range of GST-LgBiT concentration can be used to produce a luminescent signal which is linearly correlated to the concentration of SmBiT-ubiquitin present in the extracts. Higher concentrations of GST-LgBiT (e.g. 1µM) will produce a more intense signal, but this will also increase the level of background luminescence, due to weak luciferase activity of the LgBiT fragment in total protein extracts. Moreover, very high GST-LgBiT concentrations can produce a luminescence signal that saturates the detector. To clarify this, we added a new supplementary figure that illustrates the luminescence signals produced with a range of GST-LgBiT concentrations (Supplementary figure 1A). Moreover, the experiments that were previously performed with 50nM GST-LgBiT were repeated with a concentration of 1µM and the new data is shown in Supplementary figure 1C.

The biggest problem with the paper in its current form is insufficient validation of important features of the new method:

(1) In the one case where NUbiCA is compared to gel-based assays using the H2B substrate (Fig. 2), it appears rad6D eliminates ubiquitylation by the gel assay but NUbiCA still shows ~30% of the WT signal in this mutant. Is this 'background' signal in NUbiCA or do the authors believe another E2 also contributes but is not detectable by gel assay?

Authors' response:
We thank the reviewer for this observation. We addressed this point in two ways. First, we completely repeated the NUbiCA experiment, including the quantification of SmBiT-ubiquitin expression in the different strains. In this new experiment, we did not observe any statistically significant difference between the NUbiCA signals from *rad6*Δ and *bre1*Δ samples. Second to visualize the conjugation of SmBiT-ubiquitin to Htb2-LgBiT, we separated by gel electrophoresis the purification eluates rather than total extracts. This enabled us to load higher amounts samples and to directly visualize the NanoBiT signal produced by SmBiT-Ubiquitin conjugation to Htb2-LgBiT/His. In this way, we could obtain highly contrasted images that enabled us to observe that there is indeed a similar residual ubiquitylation level of Htb2 in *rad6*Δ and *bre1*Δ cells. We therefore do not think that another E2 contributes to Htb2 ubiquitylation. The figure 2 was corrected to include our new data.

(2) The chain-specific DUBs need to be validated in the yeast extract system where the differences in signal are often small (2x or less). The controls in supplementary Fig. 1 show weak cleavage activity under the conditions tested especially for Cezanne and AMSH, and the conditions used against the test substrates (Stp2, Cse4) might not be suitable. Substrates with KNOWN K11, K48, and K63 chains should be tested to show they are cleaved by the 'right' DUB. I know that Ubc6 is modified with K11 chains, and there are others, and there are quite a few known K48 and K63 modified substrates in yeast to test. This would also allow a little validation of the NUbiCA library.

Authors' response:
We agree with the reviewer that, under the conditions that we used, the cleavage activity of the chain specific DUBs was weak and not 100% specific. We tried to improve this but could not obtain entirely satisfying results. We therefore devised an alternative approach to probe ubiquitin chain topologies, using TUBEs to protect ubiquitin chains from deubiquitylation by Usp2. As suggested by the reviewer, we retrieved from the NUbiCA collection a series of strains expressing substrates with known chains. These substrates displayed clearly distinct deubiquitylation protection profiles, which were consistent with their known modification. This approach thus enables to qualitatively decipher ubiquitin conjugates and we used it to analyse the ubiquitin chains attached to Stp2 and Cse4. The results of these experiments are presented in the new figure 5. Further developments of this approach are mentioned in the discussion but are out of the scope of this manuscript.

(3) Another easy and helpful control would be to treat the free chains (Suppl. Fig. 1) with the same denaturing/renaturing treatments as the test conjugates in Fig. 3 and 4, and see if the chain specificity of the three DUBs is maintained. I worry that longer chains do not renature as readily and cleavage or chain specificity will be reduced.

Authors' response:
We agree with the reviewer that the renaturation may not be complete and could complicate the use of chain specific DUBs. Still, we are convinced that ubiquitin chains can at least partially refold after denaturation. Indeed, it is known that generic and chain specific TUBEs can be used to probe poly-ubiquitylated proteins after denaturing electrophoresis in far western experiments (see references in Mattern et al., 2019). Furthermore, our new results presented in figure 5 are fully consistent with (at least partial) refolding of ubiquitin chains after denaturation in guanidium chloride.

(4) There is no statistical analysis of the differences in luminescence signals in any of the figures to judge their significance. Differences are often small; for one example, among several, in Fig. 4b, I'm not sure the psh1D-dependent reduction is statistically significant.

Authors' response:
The results presented in the original figure 4B were statistically significant (two-tailed t-test, p=0.020). Yet, we agree that this experiment was not fully satisfying. To improve it, we improved our luminescence measurement conditions (we included 17.5 mM thiourea in the luciferase assay buffer, which reduced the spontaneous oxidation of furimazine and diminished the autoluminescence background). We repeated this experiment and obtained much more convincing results that are now presented in the new figure 3F. We also repeated the experiments originally presented in figures 4C-4E. We however had difficulties to convincingly reproduce the data obtained after cell cycle synchronisation. Although we believe this might be due to less efficiently cell cycle synchronization owing to difference in alpha-factor batches we decided not to include the data on Cse4 ubiquitylation after cell cycle synchronisation in the revised version of the manuscript. Furthermore, we now include statistical tests in all figures where it helps data interpretation.

(5) I wonder how well NUbiCA performs compared to TUBE-based assays which can measure both ubiquitin chain linkage and length. This should at least be discussed or, better, experimentally compared.

Authors' response:
To better illustrate the sensitivity of NUbiCA, we present data showing NUbiCA sensitivity limit for the detection of Htb2, Stp2 and Cse4 ubiquitylation (new supplementary figure 2A). We also used a TUBE pull-down experiment to probe the poly-ubiquityation of Spt2 in the NUbiCA strain. The results we obtained indicate that for this substrate NUbiCA is way more sensitive than TUBE-based assays. Furthermore, the TUBE pull-down was less specific (a fraction of unmodified Stp2 was recovered in the eluate) and was not able to reveal Stp2 modification with one or few ubiquitin moieties.

In summary, the system shows promise, but there is still a good deal to do to validate NUbiCA as a way to measure protein-ubiquitin modification in vivo. Clarification of how the assays work and how the NUbiCA library can be used would help as well.

Authors' response:
As described in the previous answers, we include in the revised version of the manuscript a number of new data and controls which further validate the use of NUbiCA to investigate ubiquitylation of endogenously expressed proteins. We also clarified the text and provided examples of the use of the NUbiCA collection.

Reviewer 2 Advance Summary and Potential Significance to Field...

The submitted manuscript by Le Boulch et al., describes a system (NUbiCA) which allows the use of luciferase to monitor the ubiquitylation of protein substrates in lysates. They have fused the LgBIT portion of the NanoBIT luciferase reporter to substrate proteins, and fused the SmBIT portion to ubiquitin in yeast. They have then shown that HT2B can be monoubiquiylated by ubiquitin-SmBIT and

that this ubiquitylation can be monitored by luciferase activity. They then show that Stp2 and Cse4 ubiquitylation are indicated via this system and that it can be used to detect when Cse4 is ubiquitylated during the cell cycle. Finally, they have created a yeast library of proteins fused to LgBIT, creating 5,580 ready-to-use NUbiCA strains to potentially monitor the ubiquitylation of any of these fused proteins. This was an interesting and novel study which appears to have significant potential for future studies of protein ubiquitylation in yeast.

Reviewer 2 Comments for the Author...

I have a number of questions and comments in regard to what has been presented in this manuscript.

Major points:

1] In Figure 3b the ubc7 mutant does not reduce the luciferase activity associated with Stp2 as much as the asi3 mutant. Should they not both have a similar impact?

Authors' response:
The reviewer is correct: NUbiCA shows a statistically significant difference in the ubiquitylation level of Stp2 between *ubc7*Δ and *asi3*Δ mutants. We were not surprised by this result since it matched our previous observations (Khmelinskii et al., 2014). To further substantiate this observation, we visualized SmBiT-ubiquitin conjugation to Stp2-LgBiT/His after gel electrophoresis (new figure 3B). The results clearly confirm that there is more Stp2 conjugates in *ubc7*Δ cells than in *asi3*Δ cells. One explanation could be that in the absence of Ubc7 other ubiquitin conjugating enzymes could function with the Asi1/3 complex to ubiquitylate Stp2.

2] In Figure 3c and 4c the authors use UbiCREST. However, they only use 4 of the DUBs from this system, and not the full 8 associated with the kit? Why did they not look at the others as well?

Authors' response:
We used the chain specific DUBs Cezanne, AMSH and OTUB1 because they are the only ones displaying single chain specificity. As pointed by reviewer 1 the validation of UbiCREST experiments demands careful controls and we did not wish to use less specific DUBs.

3] In Figures 3/4 it would be better to show again that the SmBIT can form polyubiquitin chains, at the least with Stp2, to demonstrate that the SmBIT ubiquitin can form these chains, and that the signal observed is not due to monoubiquitin. I appreciate this would be difficult with Cse4, and that the UbiCREST results match, but the DUBs could potentially still be removing the monoubiquitin, if there are no chains present.

Authors' response:
As already mentioned, we now include a new panel showing Stp2 ubiquitin conjugates after gel electrophoresis. We were however not able to achieve this for Cse4 because the ubiquitylated forms of Cse4 are several orders of magnitude less abundant than those of Stp2 or Htb2 (see new supplementary figure 2A).

4] In Figure 4b, why does the psh1 mutant not block Cse4 ubiquitylation?

Authors' response:
The fact that *PSH1* deletion does not fully abolish Cse4 NUbiCA signal probably reflects the fact that other ubiquitin ligases are involved in the regulation of Cse4. Indeed, several other ubiquitin ligases have been proposed to regulate overexpressed Cse4 (see Cheng et al., 2016; Cheng et al., 2017; Ohkuni et al., 2016). This point is now mentioned in the revised manuscript (see page 7).

5] In Figure 4b/c/d/e the error bars on the normalised luminescence ratios are very big in comparison with the other figures. Why is this?

Authors' response:
The ubiquitylated forms of Cse4 are several orders of magnitude less abundant than those of Htb2 or Stp2 (see new supplementary figure 2A). Hence the NUbiCA experiments performed with Cse4 have a lower signal to noise ratio. Yet, we improved our luciferase assay buffer to reduce the

autoluminescence background (see previous response to reviewer 1). This enabled us to more convincingly demonstrate the effect of *PSH1* deletion on Cse4 ubiquitylation (see new figure 3F).

6] In Figure 4d/e why does the scale go up to 4? I would assume this is due to what is being used to normalise it, but it would be good to explain why there is a difference!

Authors' response:
The normalized luminescence ratio (NLR) enables to compare the ubiquitylation level of a given substrate across multiple conditions. Throughout our manuscript, we arbitrarily set the NLR value of control conditions to 1. Conditions or treatments that decrease ubiquitylation will reduce NLR values. Conversely, conditions that increase ubiquitylation will increase NLR values. For instance, in the new figure 4E, ubiquitylation of Art4 is stimulated by glucose treatment which results in an NLR value of ~1.8. The interpretation of NLR values is now more clearly explained in the revised manuscript (page 5).

7] As the SmBIT appears to be fused to the carboxy terminus of ubiquitin, does this make it incompatible with examinations of linear ubiquitylation, as the ubiquitin molecules in such a chain will be attached to each other via this region?

Authors' response:
This is correct, an N-terminal SmBiT is not compatible with linear ubiquitylation. This is not an issue in yeast where linear ubiquitylation has not been described but could be problematic in other model systems. However, this limitation could be overcome using an internally tagged ubiquitin as described by Kliza et al., 2017. This limitation is now discussed in the revised manuscript (page 12).

8] In the various figures the authors show impacts upon K11, K48 and K63 chains. Have they explored if the conjugation of SmBIT is compatible with all the other chain types?

Authors' response:
In yeast, current data indicate that K48- and K63-linkages represent the vast majority (~90%) of all ubiquitin-ubiquitin linkages (Tsuchiya 2017, Mol Cell; Ziv 2011 Mol Cell Proteomics). The data presented in the revised manuscript confirms that SmBiT-ubiquitin is compatible with both these linkages. NUbiCA will thus be suitable to investigate a wide range of ubiquitylation reactions in this organism. We have not investigated the capacity of SmBiT-ubiquitin to assemble other types of linkages, but consider this possibility unlikely given the low expression level of the chromosomally integrated cassette (see new supplementary figure 1B, 1C). Furthermore, we consider that addressing it experimentally is out of the scope of the manuscript since it would require the characterization of substrates with such linkages and probably the development of new ubiquitin binders able to protect these linkages from deubiquitylation reaction.

9] The library created has all of the proteins fused to LgBIT at their carboxy termini. As many of these proteins may be reasonably large, and the ubiquitylation sites may not be close to this part of the protein in the final 3D structure, would the authors like to comment on how far the components of NanoBIT can be apart before this system will not work, and how often this is likely to be the case?

Authors' response:
As pointed by the reviewer the way the LgBiT fragment is fused to a given substrate protein might influence its capacity to reconstitute the NanoBiT reporter. Using another PCA assay Tarrasov et al. reported that the PCA fragments could efficiently reconstitute the reporter when they were within a distance of 8nm (Tarassov et al., 2009). Since the average protein diameter is in the range of 4 nm (BNID 101827 and 100018) this suggests that positioning the LgBiT at the C-terminus should be suitable to detect the ubiquitylation of a majority of proteins. Indeed, in the revised version of the manuscript we were able to probe the ubiquitylation of 15 distinct C-terminally tagged proteins (Htb2, Stp2, Art1, Art2, Art3, Art4, Art6, Art8, Art9, Art10, Sna3, Cln2, Sic1, Clb2 and Clb3). Out of 9 tested ARTs, the sole protein for which we could not detect NUbiCA signal was Art7, but this protein was very poorly expressed in the condition we used. This confirms that C-terminal tagging is compatible with NanoBiT reconstitution for a wide array if not the majority of proteins. This is discussed in the revised manuscript (page 12).

10] The authors state in the discussion that the use of endogenously tagged proteins is 'of utmost

important since overexpression can trigger protein quality control and ubiquitylation pathways that are not necessarily the ones one wants to investigate'. However, as they point out in their supplementary note, they are still having to express ubiquitin tagged with SmBIT from a construct as it would be technically challenging to endogenously tag it. Would the addition of all this extra ubiquitin not also interfere with these systems? I would also assume this would have to happen in mammalian cells as well and could also cause issues there?

Authors' response:
As explained in the supplementary note 2, tagging endogenous ubiquitin in yeast and other model organisms is not trivial since it is expressed from multiple genes as fusion proteins or tandem head-to-tail precursors. We therefore decided to express SmBiT-ubiquitin ectopically, either from the plasmid pGR0892 or from a chromosomally integrated expression cassette. While pGR0892 yields a level of expression of SmBiT-ubiquitin close to the level of endogenous ubiquitin, SmBiT-ubiquitin expressed from the chromosomally integrated expression cassette is 4-5 fold lower (see new Supplementary figure 1B,1C). Hence this cassette produces a moderate overexpression and was used to assay the ubiquitylation of all proteins studied in this manuscript, except Htb2. Regarding the use of NUbiCA in mammalian cells, we indeed consider that it will be important to construct and select stable cell lines that express a modest level of SmBiT-ubiquitin.

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Second decision letter

MS ID#: JOCES/2019/240093

MS TITLE: Sensitive detection of protein ubiquitylation using a protein-fragment complementation assay

AUTHORS: Marie Le Boulch, Audrey Brossard, Gaelle Le Dez, Sebastien Leon, and Gwenael Rabut
ARTICLE TYPE: Tools and Resources

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

*Advance summary and potential significance to field*

This is a vastly improved revision of the original submission. There are much clear descriptions of the methods used, and excellent validation assays now as well. I especially like the addition of the proof-of-principle analysis of the ART adaptor proteinsÂ' ubiquitination and its regulation by glucose using the NUbiCa yeast strains, as well as the ubiquitin chain-specific protection analysis (from Usp2) by various chain-specific TUBEs.

At this point, I am comfortable recommending the paper for publication. There are still some typos and the like, but science-wise, I think this ready to go.

*Comments for the author*

Only minor writing details left to fix. The authors and editors will have no trouble with these.

Reviewer 2

*Advance summary and potential significance to field*

The submitted manuscript by Le Boulch et al., describes a system (NUbiCA)
which allows the use of luciferase to monitor the ubiquitylation of protein substrates in lysates. They have fused the LgBIT portion of the NanoBIT luciferase reporter to substrate proteins, and

fused the SmBIT portion to ubiquitin in yeast. They have then shown that HT2B can be monoubiquiylated by ubiquitin-SmBIT and that this ubiquitylation can be monitored by luciferase activity. They then show that Stp2 and Cse4 ubiquitylation are indicated via this system and that it can be used to detect when Cse4 is ubiquitylated during the cell cycle. In addition, they have demonstrated a range of other proteins can be shown to be ubiquitinated (Art1-4,6, 8-10).
This is an interesting and novel study which appears to have significant potential for future studies of protein ubiquitylation in yeast.

*Comments for the author*

The authors appear to have addressed all of my comments and I am happy for this manuscript to be published as long as it addresses any other comments from the other reviewer and editor.