Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60402R2
Full Title:	Profiling Ubiquitin and Ubiquitin-like Dependent Post-translational Modifications and Identification of Significant Alterations
Section/Category:	JoVE Biochemistry
Keywords:	post-translational modifications, ubiquitin family, proteome, profiling, alteration,pancreatic cancer
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Marseille, France

TITLE:

Profiling Ubiquitin and Ubiquitin-like Dependent Post-translational Modifications and Identification of Significant Alterations

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KEYWORDS:

post-translational modifications, ubiquitin, SUMO, Nedd8, proteome, profiling, alteration

SHORT ABSTRACT:

This protocol aims at establishing ubiquitin (Ub) and ubiquitin-likes (Ubls) specific proteomes in order to identify alterations of these kind of post-translational modifications (PTMs), associated with a specific condition such as a treatment or a phenotype.

LONG ABSTRACT:

Ubiquitin (ub) and ubiquitin-like (ubl) dependent post-translational modifications of proteins play fundamental biological regulatory roles within the cell by controlling protein stability, activity, interactions, and intracellular localization. They enable the cell to respond to signals and to adapt to changes in its environment. Alterations within these mechanisms can lead to severe pathological situations such as neurodegenerative diseases and cancers. The aim of the technique described here is to establish ub/ubls dependent PTMs profiles, rapidly and accurately, from cultured cell lines. The comparison of different profiles obtained from different conditions allows the identification of specific alterations, such as those induced by a treatment for example. Lentiviral mediated cell transduction is performed to create stable cell lines expressing a two-tags (6His and Flag) version of the modifier (ubiquitin or a ubl such as SUMO1 or Nedd8). These tags permit the purification of ubiquitin and therefore of ubiquitinated proteins from the cells. This is done through a two-step purification process: The first one is performed in denaturing conditions using the 6His tag, and the second one in native conditions using the Flag tag. This leads to a highly specific and pure isolation of modified proteins which are subsequently identified and semi-quantified by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) technology. Easy informatics analysis of MS data using Excel software enables the establishment of PTM profiles by eliminating background signals. These profiles are compared between each condition in order to identify specific alterations which will then be studied more specifically, starting with their validation by standard biochemistry techniques.

INTRODUCTION:

The method proposed here is dedicated to study PTMs mediated by the ubiquitin family members from cultured mammalian cells in order to identify potential alterations associated with a specific condition (treatment, differentiation, etc). PTMs represent the last step of regulation

of proteins' functions[1]. Indeed, once produced by the translational machinery, most if not all proteins undergo different kinds of PTMs that modulate their activity, molecular interactions, and intracellular location[1]. Among the plethora of PTMs are the ones mediated by the ubiquitin family of proteins, ubiquitin itself and all ubiquitin-likes, have the potential to regulate all intracellular or partially cytoplasmic proteins[2]. Because they are themselves proteins, they can be conjugated to each other, forming homogeneous and heterogeneous chains of diverse topologies, each associated with specific regulatory functions[2]. Tools are needed to try to decipher and understand this complex machinery. Many approaches were developed worldwide, having their own advantages and disadvantages, and here we propose one with high performance suitable for cultured cells.

The main advantage of this method is its accuracy. Indeed, the purity of isolated modified proteins is highly improved by the combinatorial use of the two tags (6His and Flag) and the two step-procedure and therefore it is much more selective than a single tag fusion Ub/Ubl[3,4]. The presence of the 6His tag enables a first step of purification in a fully denaturing condition thereby avoiding any co-purification of proteins containing ubiquitin binding domains or other proteins binding to the ubiquitinated ones. This is a technical problem encountered by several other approaches based on affinity purification of ubiquitinated proteomes using either specific antibodies[5] or tandem ubiquitin binding elements (TUBEs)[6]. Importantly, this technique is not biased in favor of purification of a certain type of ubiquitination, as it could be the case for some other approaches, since both mono and different kinds of polyubiquitinations were identified[7]. Consequently, once found, an alteration of ubiquitination will have to be studied in more details by standard biochemical approaches in order to identify the exact kind of ubiquitination involved. Finally, another technical advantage of this protocol is the use of lentiviruses, that easily and rapidly creates stable expressing cell lines with reasonable level of expressions of tagged modifier without interfering with the normal cellular behavior.

Whereas one important role of ubiquitination is to target proteins for proteasomal degradation, it is now known that it has many other regulatory properties for potentially most intracellular or partially intracellular proteins[1]. The number of these functions is further augmented by the existence of many ubiquitin like proteins, forming a family of proteins regulating almost every cell mechanisms[1]. Their alterations can have drastic impact on the cell biology and can lead or participate in pathological situations[8], such as cancer[9]. Hence, tools are needed to explore this vast landscape and identify the alterations associated with a pathological condition that could serve as novel therapeutic targets.

This protocol is dedicated to cells in culture since they need to be transduced to express exogenous tagged Ub/Ubl. Once created, these stable cell lines can be used to generate Ubl profiles from culture in 2D or 3D or xenografts, thereby extending the horizon of the different experimental models that can be applied to study PTMs profiles.

PROTOCOL:

1. Generation of stable cell lines expressing 6His-Flag-Ubl

NOTE: Co-transfection of HEK-293T cells with pCCL-6HF-Ubl, pVSVG and delta-Helper.

1.1. Day 0: Seed 293T cells in a 6-well plate to obtain 50-70% confluence the day after.

1.2. Day 1: Co-transfect 50-70% confluent cells with a mix of 1 µg of pCCL-6HF-Ubl or pCCL-GFP, 1 µg of pVSVG and 1 µg of delta-Helper vectors, using a transfection reagent and protocol for lentivirus production. After 6 h of transfection, change the medium to a fresh one corresponding to cells to be transduced. Seed the cells to be transduced in a 6 well plate in order to obtain a 10-20% confluence the day after (the day of starting the transduction).

1.3. Day 2: 24 h after transfection, recover the medium containing lentiviral particles and filter using 0.45 µm filters. If needed, add fresh medium at this point in order to produce a second batch of lentiviruses. Replace the medium of cells to be transduced (10-20% confluence) by the one containing lentiviruses.

NOTE: Lentiviral medium can be kept at +4 °C for several days before transduction or stored at -80 °C for months.

1.4. Incubate the cells with lentiviruses between 24 h to 72 h in a standard incubator (37 °C, 5% CO_2), and then change the medium for fresh standard one. If possible, check GFP expression using an inverted fluorescent microscope to evaluate efficiency of transduction: percentage of expressing cells and relative level of expression per cell. If no fluorescence is detected, wait for additional 2-3 days as expression may take longer depending on cells type to be transduced.

1.5. If GFP control is positive, grow all cells until having enough to perform an expression control of 6HF-Ubl by immunofluorescence and Western blot using anti-Flag antibody.

2. Double purification of modified proteins

NOTE: Buffer 1: 6 M Guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 0.5% Triton X-100.
Buffer 2: 50 mM NaH_2PO_4, 150 mM NaCl, 1% Tween20, 5% Glycerol, pH 8.0.
Buffer 3: 100 mM NH_4HCO_3, pH 8.0.

2.1. Cell lysis: Once ready, wash culture dishes at least one time with phosphate-buffered saline (PBS) at room temperature (RT) and proceed to cell lysis or, alternatively, flash freeze in liquid N_2 and store at -80 °C. For lysis, add 2 mL of Buffer 1 per a 15 cm dish at RT. Use a cell scraper to recover all lysates in 50 mL conical centrifuge tubes (final volume about 20 mL).

2.2. Sonicate the lysates three times for 30 s separated by a 1 min pause.

2.3. Centrifuge the sonicated lysates at 15,000 x *g* for 15 min.

2.4. Transfer the supernatant to a new tube using a cell strainer (40 µm).

2.5. Determine samples' concentration and adjust, if necessary, to obtain the same amount of proteins and same volume. Use a total amount of protein between 50 and 100 mg (10 dishes with 15 cm diameter for MiaPaCa-2 cells).

2.6. Add Ni^{2+}-NTA beads, using 2 µL of beads per 1 mg of protein.

2.7. Rotate at 30 rpm during 2.5 h at RT.

2.8. Pellet the beads at 500 x *g* for 5 min.

2.9. Wash the beads with 1 mL of Buffer 1, transferring the samples to a 1.5 mL microcentrifuge tube, then transfer the tubes on ice. Perform all the next steps on ice or at 4 °C.

2.10. Wash two times with 1 mL of ice-cold Buffer 2 containing 10 mM imidazole.

2.11. To elute bound proteins, add 600 µL of Buffer 2 containing 250 mM imidazole and rotate for 2 h at 4 °C.

2.12. Pellet the beads by centrifugation at 500 x *g* for 1 min. Transfer the supernatants to new, pre-cooled, 1.5 mL tubes and add 50 µL of anti-Flag M2 antibody conjugated beads.

2.13. Rotate at 30 rpm for 2.5 h at 4 °C, then wash 2 times with 500 µL of Buffer 2, then 2 times with 500 µL of Buffer 3.

2.14. For the final elution, add 100 µL of Buffer 3 containing a Flag peptide at 0.1 µg/µL and rotate at 4 °C for 1.5 h.

2.15. Centrifuge at 500 x *g* for 1 min and transfer the supernatants to new pre-cooled tubes.

2.16. Take 10% (10 µL) to load on SDS-PAGE and perform a silver staining of the gel to control the purification quality. If the purification looks good, analyze the 90% left by LC-MS/MS.

3. Processing of mass spectrometry data to generate profiles of Ub/Ubls PTMs and to identify significant differences between them

NOTE: Results from MS analysis contain many information including the total number of peptides as well as the peak area values (mean of TOP 3 peptide area[10]) for each protein identified in each samples. These data can be processed using either the peptide count numbers or the peak area values, or both. For calculation with peak areas, because these values are usually in the range of 10^6, it is necessary to divide them by this order before applying the same formulas as below. The results obtained with both methodologies of counting should show a strong correlation as it usually does. For each identified protein, use the following formulas where:
v1 ⇔ peptides values in non-treated ubiquitin sample (e.g., Ub - drug)

v2 ⇔ peptides values in Gemcitabine treated ubiquitin sample (e.g., Ub + drug)
k1 ⇔ peptides values in non-treated control GFP sample (e.g., GFP - drug)
k2 ⇔ peptides values in Gemcitabine treated control GFP sample (e.g., GFP + drug).

3.1. Normalization: Normalize values between drug treated cells and untreated cells for Ubiquitin and GFP using the following formulas. Normalized v = V and normalized k = K.
$V1=v1.(\sum v1+ \sum v2) / (2. \sum v1)$; $V2=v2.(\sum v1+ \sum v2) / (2. \sum v2)$
$K1=k1.(\sum k1+ \sum k2) / (2. \sum k1)$; $K2=k2.(\sum k1+ \sum k2) / (2. \sum k2)$

3.2. Removal of background: Using the following formulas, subtract values in control sample (GFP) from values in the ubiquitin sample to obtain specific values (V'1 and V'2) for each identified protein in both conditions.
$V'1=V1-K1$ if $V1-K1 \geq 0$; $V'1=0$ if $V1-K1<0$
$V'2=V2-K2$ if $V2-K2 \geq 0$; $V'2=0$ if $V2-K2<0$

3.3. Variation (Var) of ubiquitination. To obtain a score (between -100 and +100) for positive and negative variations of PTMs induced by a drug, use the following formula in which the difference between specific values of treated and untreated samples are divided by the sum of all values, including those in control (to penalize proteins also identified in control GFP), and multiply by 100.
$Var = (V'2-V'1)/(V1+K1+V2+K2)*100$; $-100<Var<100$;
Variations below -50 (repression of PTM) or above 50 (induction of PTM) are usually considered as significant.

3.4. Confidence (Conf). Use the following formula to obtain a confidence value between 0 and 100%,:
$Conf = ((V1+V2)^2/(1+V1+V2+K1+K2)^2)*100 - 100/(1+V'1+V'2)$; =0 if <0
Values above 50 are usually considered to be confident.

3.5. To obtain a nicer distribution of induction/repression values and to consider both variation and confidence parameters, multiply Var and Conf values using the following formula where V ⇔ Var and C ⇔ Conf
=SI(V2>0;((V2*C2)^2)/(10^6);-((V2*C2)^2)/(10^6))

NOTE: As peak area values are usually more accurate than peptide counting, it is possible to use specific software which are dedicated to the interpretation of this kind of data such as Perseus (https://www.biochem.mpg.de/5111810/perseus), following recommendations of use.

REPRESENTATIVE RESULTS:

Transduction of culture mammalian cells to create GFP and 6HF-Ub expressing cells

To produce lentiviruses which will be used later to transduce MiaPaCa-2 cells, 70% confluent HEK-293T cells are co-transfected with an equal amount of the three vectors, pCCL-6HF-Ubiquitin or GFP/Delta-Helper/pvSvG. After 24 h of production, the medium containing lentiviral particles is recovered and filtered. It is possible at this point to control the efficiency of the transfection by

checking the green fluorescence of GFP expressing 293T cells on an inverted microscope. It should be near 100% of cells. MiaPaCa-2 cells are incubated with lentiviral supernatant for 1 to 3 days. The efficacy of lentiviral transduction is first controlled by looking at the GFP fluorescence of GFP transduced cells (**Figure 1A** upper panel). The GFP expression level may vary from one cell to another but 100% of them should be fluorescent. Once this control is done, the expression of Flag-ubiquitin will have to be also controlled. This is done by immunofluorescence staining using an anti-Flag antibody (usually M2 monoclonal) (**Figure 1A** lower panel). This will show the percentage of transduced cells, which should be 100% to guarantee a stable expression over future passages of cell culture. In order to control the expression level of exogenous Flag-ubiquitin, lysates from transduced cells are analyzed by SDS-PAGE followed by Western blot with anti-Flag antibody (**Figure 1B**). Both cell lines can be frozen.

Two steps purification and control by SDS PAGE and silver staining

Once the stable expressing cell lines have been validated, both GFP and 6HF-ubiquitin cells are amplified until enough material is obtained in order to proceed with the two-step purification. It is recommended to keep a backup of these cell lines as frozen stock in liquid nitrogen in order to be able to thaw them when needed. 36 h before processing, half of the cells (half GFP and half 6HF-Ubiquitin) are treated with 10 µM of Gemcitabine. When ready, ubiquitinated proteins are purified from 6HF-ubiquitin and from GFP control cells, using the two-step purification protocol (**Figure 2A**). 10% of the final elution is used to control the amount and integrity of purified material by SDS-PAGE and silver staining of the gel (**Figure 2B**). Molecular weight markers bands can be used to estimate the amount of purified ubiquitinated proteins. Alternatively, a known amount of BSA or any other protein can be loaded in a line of the gel to help quantifying the purified proteins. Once this verification is done, the remaining 90% of samples are analyzed by liquid chromatography coupled to tandem mass spectrometry to allow identification and semi-quantitation of purified proteins.

Identification of ubiquitinated proteins by background (GFP samples) subtraction

Data from LC-MS/MS analysis of the samples give the names and quantifications (peak areas and number of observed peptides) of each identified protein in GFP and 6HF-Ub samples. The proteins having the highest quantification in ubiquitin sample compared to GFP sample are most likely to be the ones really ubiquitinated and applying the formulas described in methods above allows their classification by giving a confidence score from 0 to 100%. Proteins identified with a score above 50% are considered ubiquitinated ones (**Figure 3A**).

This step which basically removes background proteins (GFP) from ubiquitin samples led to the identification of 364 proteins significantly ubiquitinated (**Figure 3B**)[7]. Note here that the proteins identified with the highest scores are mostly already known as main targets of ubiquitination which proves the efficacy of this purification method.

Then it is possible to perform a gene set enrichment analysis (GSEA) of these ubiquitinated proteins in order to highlight the biological processes in which they are involved (**Figure 3C**), their molecular functions, their cellular compartment, or any other gene ontology categorization. It is interesting to compare this ubiquitinated proteome with the full proteome of the cell when

possible. Indeed, this analysis reveals the real contribution of these ubiquitinated proteins in specific processes such as translation or proteolysis for example (**Figure 3C**).

The main purpose of this kind of experiment is to identify alterations within the ubiquitinated proteome induced by a treatment for example, here gemcitabine. By comparing the ubiquitinome (the ubiquitinated specific proteome) in treated and untreated cells using the specific formula yields a value from -100 (repression of ubiquitination) to +100 (induction of ubiquitination). Considering only the values below -50 or above +50 as significant, a total of 73 induced ubiquitinations and 29 repressed ubiquitinations have been identified (**Figure 4A**). GSEA analysis of these alterations of ubiquitination revealed specific enrichments in DNA repair processes or cell cycle, and important variations in translation and RNA metabolic processes (**Figure 4B**). This result is highly logical since gemcitabine is a base analogue that blocks DNA synthesis and provokes DNA damages.

To go further, it is also interesting to use databases of interacting proteins in order to explore and validate potential interacting networks formed by gemcitabine induced alterations of ubiquitination (**Figure 5**). This led to the identification of functional interacting networks strongly affected by increased or decreased ubiquitination of the involved proteins.

Finally, among the altered ubiquitination, some may involve proteins of highest interest, due to their known or potential functions according to literature. Hence, one important step is to validate that what is observed by mass spectrometry is real and trustworthy. PCNA was one of the most over-ubiquitinated protein upon gemcitabine treatment detected by mass spectrometry analysis (**Figure 4A**). To verify that gemcitabine indeed induces the ubiquitination of PCNA, MiaPaCa-2 cells expressing 6HF-Ub and GFP are grown, treated or not, and subject to either Ni-NTA purification in denaturing conditions or to anti-Flag immunoprecipitation followed by Flag peptide elution in native conditions, resolved on SDS PAGE followed by western blot with the corresponding antibody. The result shown in **Figure 6** confirmed that indeed PCNA is strongly ubiquitinated in response to gemcitabine treatment in MiaPaCa-2 cells.

FIGURE AND TABLE LEGENDS:

Figure 1: Establishment of stable cell lines expressing 6His-Flag-Ubl. (A) Control of GFP expression in GFP transduced cells (upper panel) and of 6His-Flag-Ubiquitin by immunofluorescence using anti-Flag (M2) antibody as primary and alexa-567 anti-mouse secondary antibody. DAPI was used to stain nuclei. Scale bar: 50 µm. **(B)** Control of 6His-Flag-Ubiquitin expression in cell lysates by Western blot using anti-Flag antibody (Alternatively, an anti-6his antibody can be used) on the left, and anti-ubiquitin antibody, on the right, to compare the expression of 6His-Flag-Ubiquitin (6HF-Ubiq) with endogenous Ubiquitin (Endo. Ubiq).

Figure 2: Two step purification of ubiquitinated proteins. (A) Schematic representation of the procedure. **(B)** 10% of the final elution was subjected to SDS PAGE followed by silver staining of the gel in order to estimate the quantity and purity (compared to GFP) of isolated proteins.

Figure 3: Identification of ubiquitinated proteins (adapted from Bonacci et al.[7]). (A) Relative

amount of specific (ubiquitin sample) and non-specific (GFP sample) peptides for each identified protein were plotted in function of their confident scores. As shown, the proportion of non-specific over ubiquitinated proteins becomes too important below the score of 50. Hence, only proteins identified with a score superior to 50 are considered significant. **(B)** Table showing the 20 best ubiquitinated proteins among the 364-total identified. **(C)** Repartition of ubiquitinated proteins and total proteins of MiaPaCa-2 cells within biological processes (Values > 1.5% were considered only).

Figure 4: Gemcitabine induced alterations of PTM profiles (adapted from Bonacci et al.[7]). (A) Listing of the 20 proteins with highest increased (total 73) or decreased (total 29) ubiquitination upon gemcitabine treatment (Conf: confidence; Ind: induction; Rep: repression). **(B)** Repartition of Gemcitabine induced altered ubiquitination within biological processes and comparison with non-treated.

Figure 5: Functional interactomes of gemcitabine induced altered ubiquitination. Potential interactions between all proteins with gemcitabine induced alteration of ubiquitination are identified using a protein-protein interactions database (STRING: string-db.org).

Figure 6: Biochemical validations of interesting gemcitabine induced alterations of ubiquitination. In order to validate the increased ubiquitination of PCNA after gemcitabine treatment, lysates of cells expressing 6HF-Ubiquitin, treated or not with gemcitabine, were subjected to Nickel pull-down (Ni-NTA) followed by anti-PCNA Western blot.

DISCUSSION:

We have developed a robust and reliable methodology to generate profiles of proteins modified by the main ubiquitin family members. Indeed, we have successfully applied this protocol to generate profiles of PTMs by ubiquitin, and also by SUMO and Nedd8, and to detect alterations associated with a treatment[7], in response to the over expression or knockdown of a certain gene (data not shown) and in cells that acquired a resistant phenotype to diverse chemotherapeutic drugs.

There are only few critical steps during the procedure that the manipulator should be careful with. Upon pipetting of beads (Ni-NTA or anti-Flag coupled), it is important to resuspend them thoroughly, especially the anti-Flag beads since are suspended in a viscous glycerol based buffer, and to cut a bit the end of the tip to increase the section. Another precaution should be followed is to pipette slowly in order to avoid stacking of beads. Additional critical steps are the elution with imidazole and then with Flag peptide. A clean Hamilton syringe must be used to recover the supernatant after elution to avoid, as much as possible pipetting of the beads on which nonspecific proteins remain.

Depending on the cell type and the amount of required final material for mass spectrometry, the starting material may be increased or decreased accordingly. Then, the only important adjustment resides in the volume of nickel beads as it should be of 2 µL per 1 mg of proteins in lysate. It may happen that the amount of purified proteins is too low. The expression level of

tagged Ub/Ubl should be controlled and, if it is too low, cells can be re-transduced using the same lentiviruses. Alternatively, it is possible to increase the amount of starting material. Sometimes, the amount of nonspecific purified material in GFP or parental cells is too high. One solution to overcome this problem is increasing the volume and the number of washes at both Ni-NTA and Flag purification steps. It is also possible to increase the concentration of imidazole in washes with guanidine buffer, up to 20 mM. Inversely, it is not necessary to increase the concentration of Flag peptide in the final elution step as it will not increase the elution. It is more important to use fresh peptide as over time, even if stored at -20 °C, the efficacy of the peptide may decrease.

The main limitation of this protocol is that it is only suitable for cultured cells because they have to be transduced to express the desired tagged Ubl. Hence, any study on tissues or tumor samples has to be performed using alternative approaches such as diGly peptides enrichments after trypsin digestion[11], immunoprecipitation with antibodies specific to the Ub/Ubl of interest[5], or use of TUBEs[6]. All these alternative methods are also suitable for their application in cultured cells. They have the advantage of using the endogenous Ub/Ubls machinery. However, several drawbacks exist. Immunoprecipitations are difficult to be performed with a low background and, like TUBEs approaches, proteins interacting with modified proteins, and even the modifier itself, cannot be eliminated, even though practical improvements have been obtained using more stringent conditions. DiGly peptides enrichment is a very powerful technic but may correspond to different kind of PTMs. For example, diGly enrichment from a trypsin digested lysate will identify mainly ubiquitinated proteins but also neddylated ones, as Nedd8 leaves the same remnant diGly signature as ubiquitin does[11].

Another limitation of this protocol is that the presence of the 6His-Flag tag may alter the normal function of Ub/Ubl and the overexpression by itself could alter the machinery. This could for example impede the generation of polyubiquitin chains and favor monoubiquitination. However, since both mono multi and polyubiquitin chains have been identified using this protocol, it seems that it is not the case[7]. The presence of the tag at the N-terminus also prevents the formation of linear ubiquitination, where ubiquitin moieties are linked together via the N-terminal methionine of ubiquitin. However, even though 6HF-udiquitin cannot be ubiquitinated on its first Met residue, it still has the ability to end this kind of chain.

Also, whereas it is a substantial advantage to use lentiviruses which enables the creation of stable cells lines in one or two weeks, it is possible that not all cells would express the desired construct. This may be not a real problem for the purification procedure as long as enough cells express the exogenous Ub/ubl, but the problem may come with long term culture as over several passages there could be a clonal variation with enrichment in cells with less or no expression. This drawback, however, can be easily bypassed by using lentiviruses containing either a resistance selection gene or a fluorescent protein which can be use in flow cytometry to select only positive cells.

ACKNOWLEDGMENTS:
This work was supported by La Ligue Contre le Cancer to HV and MS, and the ARC (association pour la recherche sur le cancer) to PS, INCa (institute national du cancer) and Canceropole PACA

to JI. The mass spectrometry facility of Marseille Proteomics (marseille-proteomique.univ-amu.fr) supported by IBISA (Infrastructures Biologie Santé et Agronomie), Plateforme Technologique Aix-Marseille, the Cancéropôle PACA, the Provence-Alpes-Côte d'Azur Région, the Institut Paoli-Calmettes and the Centre de Recherche en Cancérologie de Marseille.

DISCLOSURES:

The authors have nothing to disclose.

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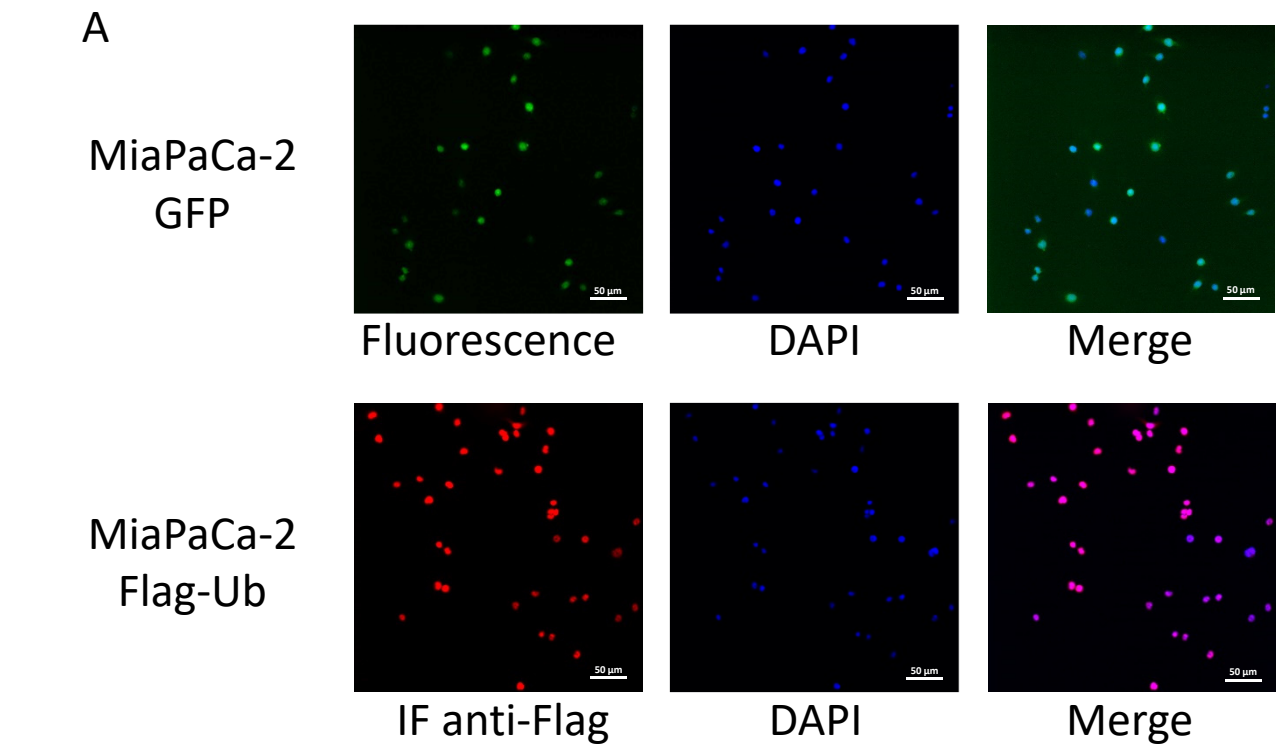
Figure 1



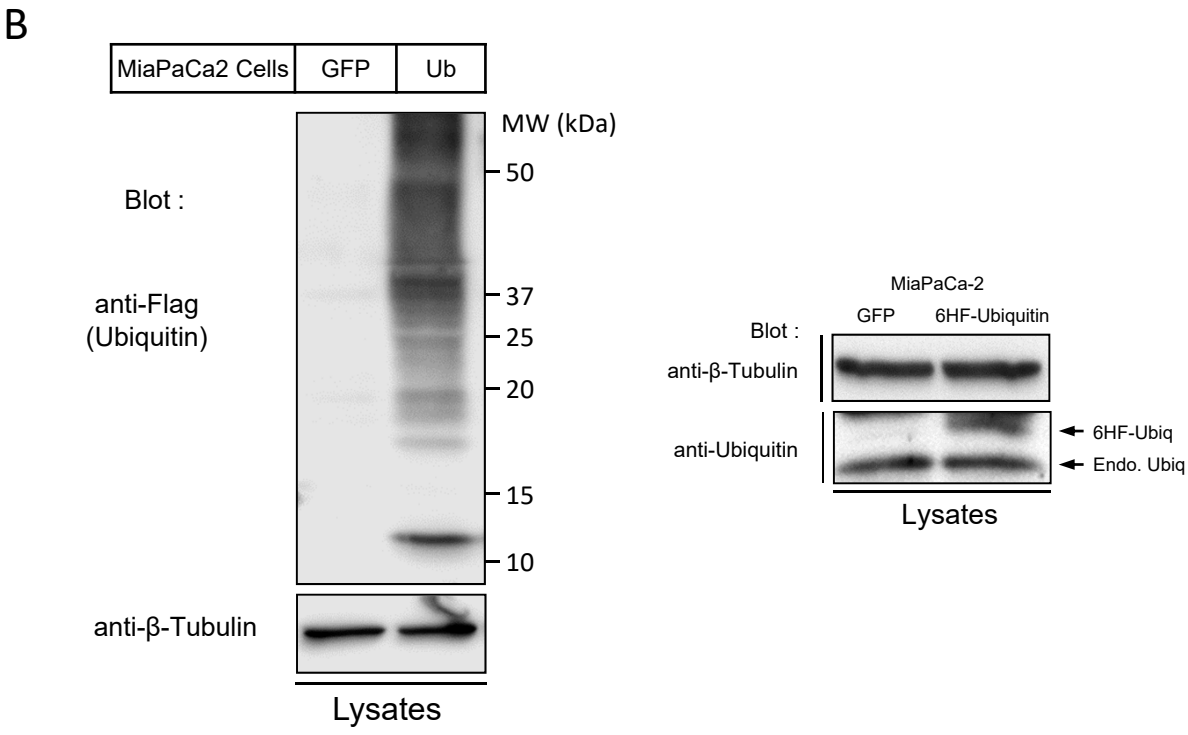
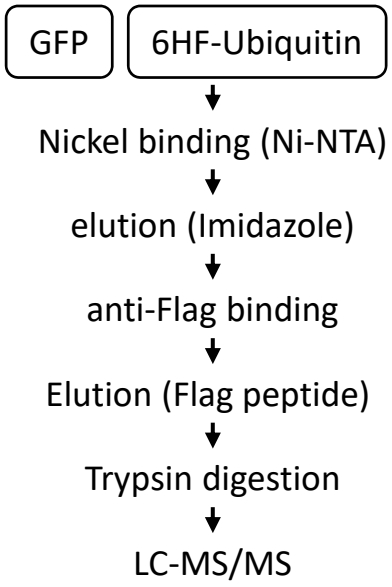


Figure 2

A



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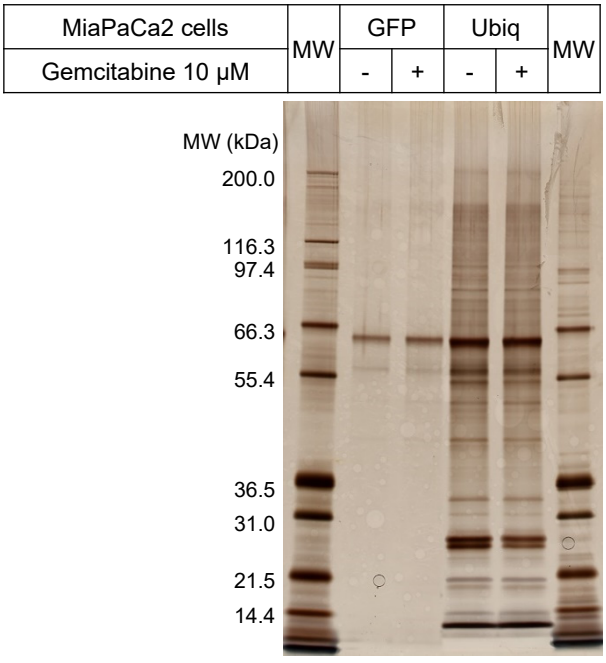
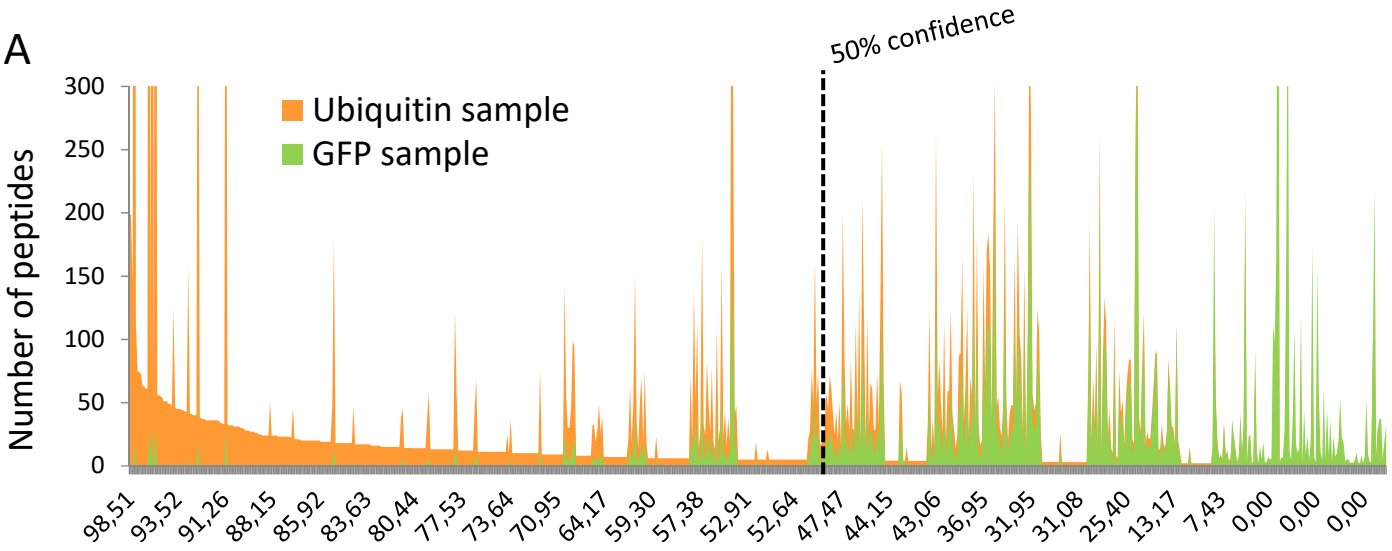


Figure 3



B

Ubiquitylation profile NT only (364 proteins)
Description
40S ribosomal protein S7
Histone H2A type 2-B
40S ribosomal protein S10
Ubiquitin-B
Core histone macro-H2A.1
Transcription elongation factor A protein-like 4
Ubiquitin-conjugating enzyme E2 N
14-3-3 protein beta/alpha
Polymerase I and transcript release factor
14-3-3 protein zeta/delta
Histone H2B type 2-B
Proliferating cell nuclear antigen
NSFL1 cofactor p47
Histone H2A type 2-A
Histone H2A type 1-H
14-3-3 protein epsilon
Histone H2A type 1-B/E
Histone H1.2
Histone H2B type 2-E
Queuine tRNA-ribosyltransferase subunit
…

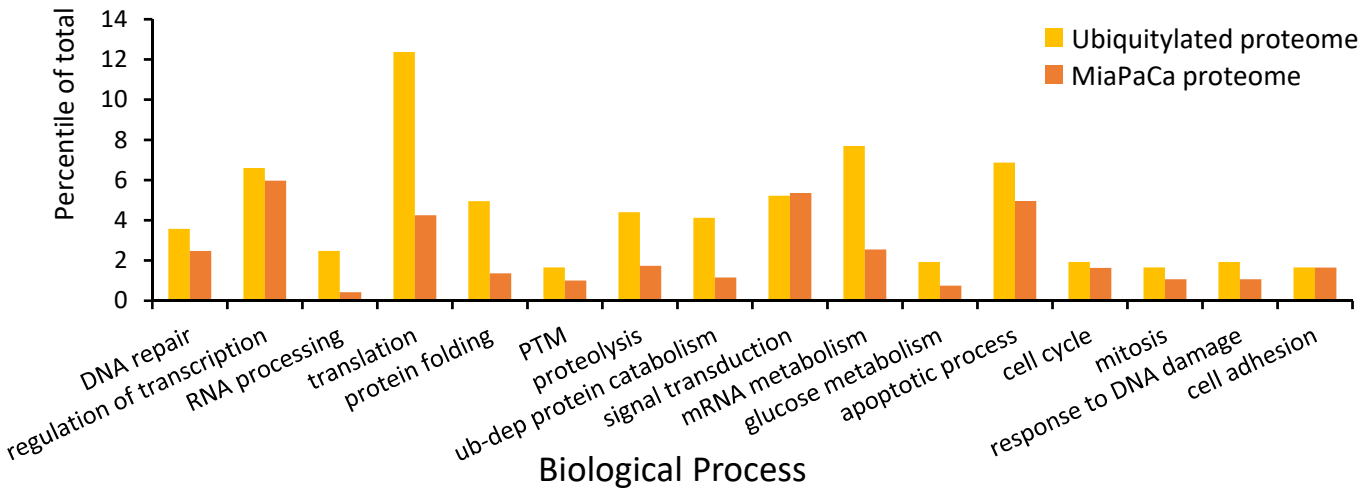


Figure 4

A

Ubiquitylation induced by Gemcitabine (n=73)			Ubiquitylation repressed by Gemcitabine (n=29)		
Gene name	Conf (>50)	Ind (>50)	Gene name	Conf (>50)	Rep (<50)
PCNA	95.2	53.8	SCLY	85.3	-58.1
UBE2C	91.2	63.4	PAF15	80.5	-71.6
CLK3	86.5	52.1	ACO2	80.5	-57.4
COPA	85.2	100.0	APMAP	77.6	-50.3
PSMD2	84.5	55.3	CTSC	75.8	-100.0
RAN	84.5	66.5	ABHD11	73.6	-100.0
SMPD4	83.6	100.0	HK1	71.1	-100.0
XRCC5	79.0	69.0	RPL29	71.1	-100.0
DYRK1A	79.0	100.0	PLAU	68.0	-100.0
EZR	77.4	100.0	SQSTM1	64.2	-100.0
CDC20	75.6	100.0	SEPT2	64.2	-100.0
RPL23A	75.6	100.0	C8orf38	64.2	-100.0
PRPS2	73.5	100.0	TNPO1	64.2	-100.0
HDAC6	73.5	100.0	VDAC1	59.3	-100.0
USP22	70.9	55.3	FHIT	59.3	-100.0
DNAJB6	70.9	100.0	PSME1	59.3	-100.0
ZWINT	70.9	100.0	SMAD2	59.3	-100.0
EPS15	70.9	100.0	RPS16	59.3	-100.0
H2AFY2	70.9	100.0	DDX1	52.9	-100.0
USP11	67.8	100.0	REPIN1	52.9	-100.0
…			…		

B

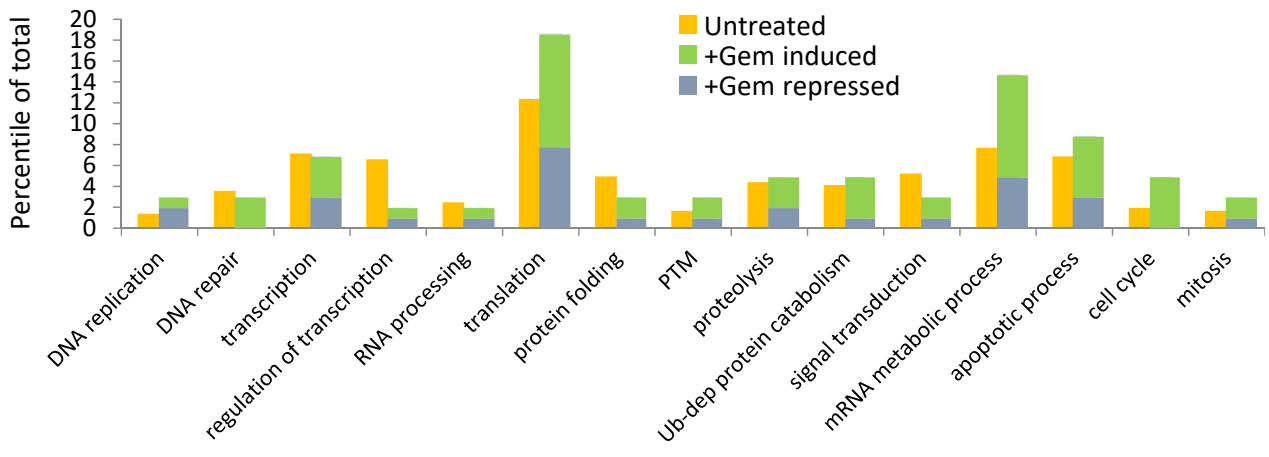


Figure 5

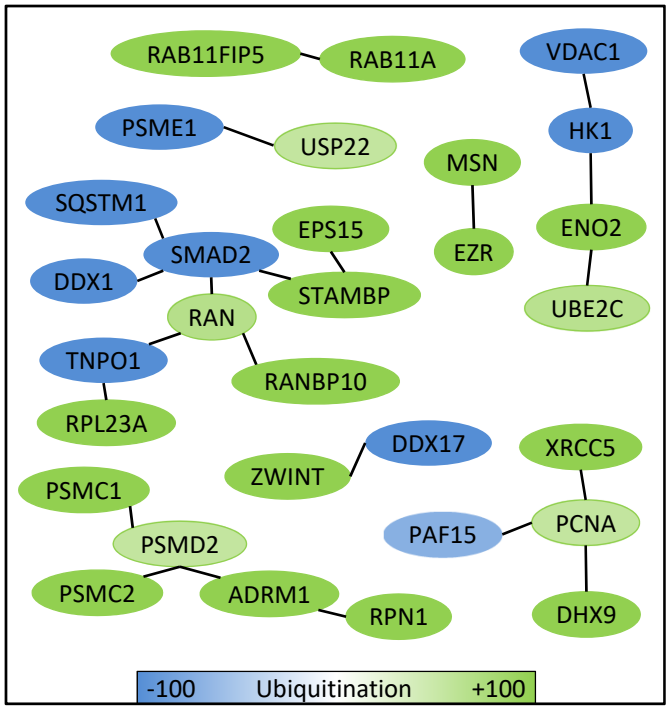
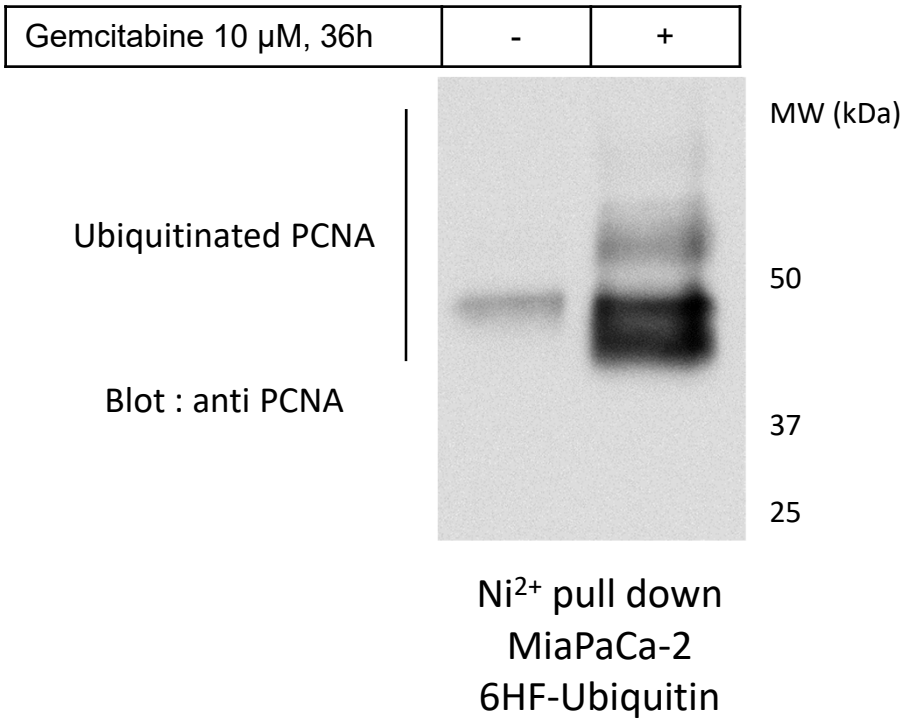


Figure 6



Name of Material/ Equipment	Company	Catalog Number
ANTI-FLAG M2 Affinity Gel	Sigma-Aldrich	A2220-5ML
anti-Flag M2 antibody	Sigma-Aldrich	F3165
Cell strainer 40 µm	Falcon	352350
Flag peptide	Sigma-Aldrich	F3290
Guanidine hydrochloride	Sigma-Aldrich	50933
Imidazole	Sigma-Aldrich	I5513
Lipofectamine 3000	ThermoFisher	L3000015
Lobind tubes	Sigma-Aldrich	Z666491
Membrane Filter, 0.45 µm	Millipore	HAWP04700F1
Ni-NTA	Qiagen	30210

Comments/Description

binds all Flag tagged proteins

to detect 6His-Flag tagged expression of ub/ubl

to remove floating pellet from guanidine lysed cells

elute flag tagged proteins from anti-flag beads

chaotropic agent used to denature all proteins in cell lysate

eluates 6His bond proteins from Ni-NTA beads

to transfect HEK-293T cells to produce lentiviruses

avoids absorption of precious material

to filter the lentiviral supernantant

purification of the 6His tag

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Author(s):	Mirna Swayden, Aurélie Dobric, Yolande Berthois, Hugo Villalba, Stéphane Audebert, Luc Camoin, Juan Iovanna, Philippe Soubeyran

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CORRESPONDING AUTHOR

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Department:	Pancreatic cancer team
Institution:	CRCM INSERM U1068
Title:	Dr.
Signature:	
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3. Please provide at least 6 keywords or phrases.
Done.

4. Please use a single space between numerical values and their units.
Done.

5. Please use h, min, s for time units.
Done.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
Done.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). Any text that cannot be written in the imperative tense may be added as a "Note."
Done.

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9. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file.
Done.

10. Please sort the items in alphabetical order according to the name of material/equipment.
Done.

11. Figure 1B: Please add molecular size marker.
Done.

12. Figure 2B: Please use a single space between numerical values and their units. Please add a unit for molecular weight.
Done.

13. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please check the iThenticateReport attached to this email.
Technical descriptions and some figures legends were modified accordingly. However, for some parts, such as acknowledgments part, it is impossible to change the names of organisms and facilities.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
In this manuscript, the authors introduced a new two-step purification method (1st: His-tag and 2nd: Flag-tag) into ubiquitinated proteome analysis to improve its quality. For this approach, they used a conventional lentivirus-mediated transduction and generated stable cell lines which expressed 6XHis- and Flag-tagged ubiquitin.

Major Concerns:

(1) Although the authors validated transduction efficiency by monitoring the fluorescence, there may be some cells that were not infected by lentivirus. The authors need to discuss the advantage of this method compared to generating stable cell line by traditional methods such as drug-selection or fluorescence-mediated cell sorting.
This has been added to the discussion.

(2) Furthermore, this method provides the information about the ubiquitination of targets, but does not provide the information about the types of ubiquitin chains. The authors need to discuss the limitation of this approach.
This limitation has been added in the manuscript.

Minor Concerns:

(1) In Figure 1(A), the authors mentioned that the efficiency of transduction was need to be about 100%. However, it is difficult to determine the transduction efficiency without seeing the merged images. The authors need to present the merged images.
Merge images are now shown in Figure 1(A).

(2) In Figure 1(B), the upper band of Endo SUMO1 in GFP lane should be clearly shown and need to be explained in the figure.
There was a mislabeling as this was the control of ubiquitin expression. This has been corrected and it is now clearly explain in the figure legend what is the endogenous and the 6His-Flag ubiquitin bands.

(3) In Figure 3(B), the authors suggested ubiquitinated proteins by LC-MS/MS. However, this reviewer cannot understand the meaning of Ubiquitin-B because there are no proteins named Ubiquitin-B. Is it possible that the authors confused the protein and gene nomenclatures of ubiquitin?
There are 4 genes in human genome which code for ubiquitin among which UBB (Ubiquitin-B). When the mass spectrometry analysis software (MAxquant) identify peptides belonging to ubiquitin, it choses one of those genes. In that particular case it was UBB, but sometimes it attributes the score to UBC or UBA52 or RPS27A.

(4) The authors mentioned that the accuracy is a main advantage of this method. Can the authors compare the results of this two-step method from those of one-step only (e.g., Ni-NTA alone)?
While developing this method, we started with 6His and even 8His constructs. We chose this tag because we wanted a purification in denaturing condition to remove all interacting proteins and it was also previously used in other works, such as the two papers we referred to. Unfortunately, this always gave many contaminant proteins, mainly the ones naturally rich in histidine residues or containing histidine stretches within their sequence. At the end, the addition of a second tag, Flag, demonstrated the best improvement, even though this second step is realized in non-denaturing condition.

(5) The followings need to be corrected. In line 96, PCCL -> pCCL; in line 124, pH8 -> pH8.0; and in Figure 1(A), scale bars need to be included.
These typo mistakes were corrected.

Reviewer #2:

The paper describes a method to study PTMs, in particular ubiquitin, at a proteome-wide level. For this, the authors create a stable cell line expressing a two tags version of ubiquitin to purify modified proteins and identify them by mass spectrometry. Simple bioinformatics tools could then tell which proteins were ubiquitinated under the imposed conditions (DNA damage in this case).

I have several questions and comments and I hope the authors could clarify these.

- In the Introduction section, the authors state that ubiquitin and ubl's have the potential to regulate "all intracellular or partially cytoplasmic proteins". Although it is tempting to speculate this, there is no evidence that "all" proteins could be targets for ubiquitin. The authors should slightly downplay their statement.

According to UbiNet resource, 14692 unique human proteins were found as ubiquitin substrate with 43948 ubiquitin sites. Also, one recent publication, Akimov et al (https://doi.org/10.1038/s41594-018-0084-y) identified more than 63000 unique ubiquitination sites within 9200 human proteins. Hence, though this is not yet proved, and maybe not all cytoplasmic or partially cytoplasmic proteins can be ubiquitinated, recent big

scale screenings tend to show it. However, according to this comment the sentence was changed to be less affirmative.

- The authors state that the combinatorial use of two tags is more efficient that single tag versions of the method. I would say it is probably more selective, not more efficient.
Indeed, this is right. Hence we changed the text accordingly.

- The protocol steps 1 and 2 are described in detail. However, section 3 is bothering me: while mass spectrometry is the central, crucial tool in this method, there is no description whatsoever about the details of the mass spectrometry methods. I think this is a void that ought to be filled: which instruments were used, which LC-MS configuration, and, most importantly, which software tools were used for both identification and (semi-)quantitation of proteins?
We kept to the description of what would be the movie about. We can add these details regarding the mass spectrometry settings and software used with parameters. They can also be found in refs such as Bonacci et al 2014. Alternatively, we can add them in this manuscript and then we should go to film part of the experiment at the proteomic platform. This is possible and will depend on the Editor.

- In addition, the data interpretation needs to be revised. The idea that "[…] proteomic platforms usually send mass spectrometry data as Excel files […] is slightly outdated: there are better tools in protein identification software these days. The authors have chosen to base their comparative method on peptide counting as "[…] the results obtained with peptide count numbers and peak area values should be similar […]". I do not agree with this statement: peptide and protein quantitation based on peak area values is more accurate than just counting peptides. Peptide counting as a method for protein quantitation (e.g. emPAI, etc.) has become an obsolete tool and there are plenty of methods available which are based on peak area values. For abundant proteins the difference between both methods may not seem spectacular, but for lower abundant proteins there are substantial differences.
Manuscript was changed accordingly to these comments. The formulas that we provide here are dedicated to simple and rapid exploration of data from mass spectrometry which usually contain both peptide numbering and peak areas. These formulas can be easily applied to both type of values. Furthermore, we now also mention that dedicated software exist, such as Perseus, which can be used to give a better and more accurate result.

- Also, the scoring cutoff of 50% seems like a relatively non-stringent cutoff to me. If I understand it correctly, proteins that have a peptide count that is at least two times higher than in the GFP control, are considered ubiquitinated in this method. I would like to see a similar analysis based on peak area numbers and see whether the threshold value can be set more stringently then.
Figure 3(A) shows the repartition of signals over nonspecific GFP background. There, we can easily see that below this 50% value the amount of nonspecific signals becomes major. Hence it sounded logical to fix the limit at this value. It is true that, however, one can chose more or less stringent values, depending on what is expected.

- Finally, the limitations of the method are clearly described in the Discussion section.

Like any method this one has its advantages and limitations. With the help of both reviewers, this listing in now more complete.

Point by point reply to editorial comments:

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This was done one more time.

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Commercial language was removed.

3. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.
It is unfortunately impossible to detail all the data manipulations using perseus software since there are a lot, they may vary depending on the format of the data, and most of these are found within the help menu or on the web site. We have to mention the possibility of using this kind of software to study MS peak areas data following second reviewers' recommendation, but this is not the purpose of this manuscript to explain how to use them.

4. Step 1.1: What's the cell density?
This information was added.

5. Step 1.2: Please write this step in the imperative tense.
The sentence was corrected.

6. Step 1.4: What's the temperature for incubation?
This information is now added.

7. Step 2.7: What's rate for rotation? Is it 2 h 30 min?
These information were added.

8. Please do not use more than 1 note for each step. Please avoid long notes (more than 4 lines).
This point was corrected.

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