Ubiquitin and SUMO conjugation as biomarkers of Acute Myeloid Leukemias response to chemotherapies

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Summary blurb

This study describes the identification of a new class of biomarkers of cancer response to therapies based on protein modification by Ubiquitin and SUMO and provides the tools to analyze them in Acute Myeloid Leukemia patient samples.

Abstract

Ubiquitin and the ubiquitin-like SUMO are covalently conjugated to thousands of proteins to modulate their function and fate. Many of the enzymes involved in their conjugation are dysregulated in cancers and involved in cancer cells response to therapies. We describe here the identification of biomarkers of the activity of these enzymes and their use to predict Acute Myeloid Leukemias (AML) response to standard chemotherapy (daunorubicine-DNR and cytarabine-Ara-C). We compared the ability of extracts from chemosensitive and chemoresistant AML cells to conjugate ubiquitin or SUMO-1 on 9000 proteins spotted on protein-arrays. We identified 122 proteins whose conjugation by these post-translational modifiers marks AML resistance to DNR and/or Ara-C. Based on this modifomic signature, we defined a statistical score able to predict AML patient response to standard chemotherapy. We finally developed a miniaturized assay to easily assess the modification level of the selected biomarkers and validated it in patient cell extracts. Thus, our work identifies a new type of ubiquitin-based biomarkers that could be used to predict cancer patients response to treatments.

Introduction

Ubiquitin family proteins (collectively called UbL hereafter) are peptidic post-translational modifiers (Streich & Lima, 2014). The best-characterized ones are Ubiquitin and SUMO-1 to -3. SUMO-1 is 50% identical with SUMO-2 and -3, which are 97% identical. UbL are covalently and reversibly conjugated to the lateral chain of lysines from thousands of proteins. Their conjugation involves dedicated enzymatic cascades comprising E1 UbL-activating enzymes (2 for Ubiquitin, 1 for SUMO), E2 UbL-conjugating enzymes (46 for Ubiquitin, 1 for SUMO) and several E3 factors (~700 for Ubiquitin, ~15 for SUMO)(Streich & Lima, 2014). Ubiquitin can be conjugated to itself via the formation of isopeptide bonds between its C-terminal glycine and certain of its own lysines (K6, K11, K27, K29, K33, K48, K63)(Yau & Rape, 2016). SUMO-2 and SUMO-3 can also form chains via SUMOylation of a specific N-terminal lysine, which is absent in SUMO-1 (Tatham *et al*, 2001).

Due to the diversity of their target proteins, UbL control a large range of cellular functions. As most other post-translational modifiers, they can either hide or create interaction surfaces on the conjugated protein. The consequences of ubiquitylation also largely depend on the type of chains, K48-linked ubiquitin chains being mostly known to constitute a protein degradation signal recognized by the 26S proteasome (Chau *et al*, 1989; Glickman & Ciechanover, 2002; Ciechanover, 2017) whilst other types of chains, notably K63- and K11-linked chains, have been involved in protein-protein interactions, signaling, inflammatory response, DNA repair and ribosomal function (Haakonsen & Rape, 2019; Kwon & Ciechanover, 2017). SUMO is conjugated to more the 6000, mostly nuclear, proteins. In particular, many proteins involved in gene expression (transcription factors, transcription machinery, co-regulators, histones) are regulated by their SUMOylation (Neyret-Kahn *et al*, 2013; Cossec *et al*, 2018; Rosonina *et al*, 2017; Tempé *et al*, 2014; Chymkowitch *et al*, 2015). SUMOylation also plays key roles in DNA damage repair through the modification of many proteins involved in this process (Garvin & Morris, 2017).

Ubiquitin-like modifiers are critical players in the regulation of numerous cellular pathways and are involved in most, if not all, biological processes. As such dysregulation of the various enzymes involved in UbL conjugation are found in cancers and are involved in both tumorigenesis and cancer response to therapies (Mansour, 2018). This includes, among others, E3 ubiquitin ligases such as MDM2 (Carr & Jones, 2016), IAP (Mohamed *et al*, 2017) or F-box protein-containing SCF complexes (Uddin *et al*, 2016).

Overexpression/downregulation of SUMOylation enzymes has also been reported in many cancers (Seeler & Dejean, 2017), including various hematomalignancies (Boulanger *et al*, 2019). For instance, the SUMO E2 was shown to be overexpressed in hepatocellular carcinomas, where it participates to their resistance to doxorubicin (Fang *et al*, 2017) or in multiple myeloma, where it is a marker of bad prognosis (Driscoll *et al*, 2010). In addition, many components of the SUMO pathway, including the E1, E2 and various PIAS E3 proteins were shown to be highly expressed in B-cell lymphomas overexpressing c-Myc. This results in overall higher SUMO conjugation in these cells and makes them vulnerable to the inhibition of SUMOylation (Hoellein *et al*, 2014). Finally, we have recently shown that it plays an important role in the response of Acute Myeloid Leukemia (AML) to standard chemotherapies (Bossis *et al*, 2014) and in the resistance of non-promyelocytic AML to differentiation therapies using retinoic acid (Baik *et al*, 2018). UbLs, UbL-conjugating/deconjugating enzymes and UbL-conjugated substrates therefore constitute a potential new class of biomarkers to predict cancer response to therapies.

AML represent a very heterogeneous group of cancers affecting the myeloid lineage. They arise through the acquisition of oncogenic mutations or rearrangements by hematopoietic stem or -progenitor cells, which, instead of differentiating into normal leukocytes, proliferate and invade the bone marrow. The historical FAB (French American British) classification of AML was mostly based on the differentiation stage of the leukemic cells. It has now been replaced by the WHO classification, which is based on the number and nature of their genetic abnormalities (Döhner *et al*, 2017). It defines groups with favorable, intermediate or adverse prognosis (Estey, 2012; Dombret & Gardin, 2016). Except for the Acute Promyelocytic subtype (~10% of AML), which is the only one that can be effectively treated by a differentiation therapy (retinoic acid and arsenic trioxide)(Ng & Chng, 2017), patients diagnosed for all other AML subtypes are generally subjected to a chemotherapeutic treatment that has not significantly changed for the past 40 years (Dombret & Gardin, 2016). This standard therapy starts with a remission induction treatment combining two genotoxics, one anthracycline (daunorubicin-DNR or idarubicin-IDA) and cytarabine (Ara-C) (3+7 regimen), which is followed by a consolidation treatment using only Ara-C. However, a significant fraction of patients (20-30%) do not respond to the induction treatment and, among those achieving complete remission, relapse rates are high (~40% of 5-year survival of in patients below 60 and ~20% in older ones (Estey, 2012)).

As it is the case for most cancers, no rapidly implementable prognosis tool is currently available at diagnosis to predict the response of AML to standard chemotherapy. This is detrimental for at least two reasons: (i) unnecessary exposure of chemoresistant patients to severe side-effects-generating genotoxics (Minotti *et al*, 2004) and (ii) loss of time before redirecting refractory patients to novel therapies (targeted therapies, immunotherapies…) and/or enrolling them in clinical trials for innovative therapeutic strategies. Transcriptomic signatures are now used to better stratify patients and adapt treatments in several cancers. However, even though various transcriptomic signatures have been defined for AML, none of them is sufficiently reliable to be used in clinical practice. Proteomic signatures, in particular those based on mass spectrometry, are emerging as promising alternatives (Panis *et al*, 2016). Even closer to the biological functions dysregulated in cancer, « modifomic or PTMomic » analyses, which monitor the activity of enzymes involved in the post-translational modifications (PTM) of proteins, open new perspectives in cancer prognosis and diagnosis (Thygesen *et al*, 2018).

Alterations in UbL modification being frequently found in AML, we have used AML as a model to address whether global analysis of UbL conjugation could define a new class modifomic biomarkers of patients' response to chemotherapeutic treatments. Using a large-scale protein array-based screening, we identified a UbL signature of AML chemoresistance composed of 122 proteins. We then defined a score based on selected proteins allowing to predict the response to chemotherapy of both AML cell lines and AML patient cells. Finally, we developed a miniaturized assay implementable in clinical routine to rapidly quantify these new biomarkers.

Results

Analysis of UbL-conjugating activities using Protein Arrays.

To identify potential UbL-modified biomarker proteins, we resorted to protein arrays (Protoarrays, Life Technologies). They display >9000 recombinant human proteins spotted in duplicate on a nitrocellulose-coated slide. Such arrays have already been used successfully to identify substrates of certain E3 Ubiquitin ligases using either total cell extracts(Merbl & Kirschner, 2009) or recombinant enzymes (Gupta *et al*, 2007; del Rincón *et al*, 2010). The arrays were incubated with cell extracts either from chemosensitive HL-60 or U937 reference AML cell lines, or from Ara-C- (ARA-R) or DNR-resistant (DNR-R) sublines that we generated from them (Figure 1A). As expected, the chemoresistant sublines showed significantly higher IC_{50} for Ara-C and DNR (Figure 1B). Our assumption was that any dysregulation in the activity of E1, E2 or E3 enzymes in chemoresistant cells would result in changes in UbL substrate modification levels as compared to parental cells. To maximize the reaction efficiency, extracts were complemented with, not only recombinant Ubiquitin or -SUMO-1 to avoid rate-limiting amounts of post-translational modifiers, but also vinyl-sulfone-coupled UbLs to block UbL deconjugation via inhibition of UbL-deconjugating enzymes. In each independent experiment, a control condition included a Protoarray incubated with extracts from parental cells treated with the alkylating agent N-ethylmaleimide (NEM) that inhibits all UbLs E1 and E2 enzymes. Identification of Ubiquitin and SUMO-1 substrates and quantification of their modifications were achieved by array scanning after incubation with, first, antibodies directed to either SUMO-1 or the Flag-tag epitope present on exogenous Ubiquitin and, then, fluorescent secondary antibodies (Figure 1A). Three independent experiments were performed for all cell lines (24 arrays in total). Signals were corrected for background, normalized using the PAA package (Turewicz *et al*, 2016) and analyzed using both the Welch and the Wilcoxon-Mann-Whitney statistical tests (see Methods for details). This led to the identification of 988 ubiquitylated- and 83 SUMOylated proteins, 72 proteins being both ubiquitylated and SUMOylated (Figure 1C and Supplementary Table 1).

Identification of a UbL signature of AML resistance to standard chemotherapy.

Among the proteins identified as robustly ubiquitylated or SUMOylated on the arrays, we then selected those that were differentially modified between cell extracts from chemoresistant and parental cell lines. A first analysis was performed by comparing the

pooled data obtained for all resistant cell lines to those from all sensitive ones. This led to the identification of 52 proteins differentially modified by Ubiquitin and 27 proteins differentially modified by SUMO-1 (Figure 2A and Supplementary Table 2), 4 of them being differentially modified by both UbLs. To identify biomarkers that might be specific for specific AML subtypes or for the resistance to one of the two drugs, we also performed a second analysis in which the data for each cell line (HL-60 and U937) and each resistance (DNR and Ara-C) were considered separately. Although the sample size for each condition was smaller than in the first analysis, -and consequently the statistical significances lower-, we identified 65 proteins for Ubiquitin and 12 for SUMO-1 that were differentially modified in at least one of the resistant cell lines as compared to its parental counterpart (Figure 2B and Supplementary Table 3). Among the 65 ubiquitylated proteins, 42 had not been identified in the global analysis. For SUMO-1 modified proteins, 8 new proteins were identified in the second analysis. Altogether, the compilation of all Protoarray data (global and separated analyses) identified a modifomic signature of AML chemoresistance comprising 94 ubiquitylated and 35 SUMOylated proteins, making a total of 122 individual proteins (Supplementary Table 4). An ontology analysis showed that these are principally involved in the Ubiquitin-Proteasome pathway, stress response, DNA damage repair and autophagy, which are all processes often dysregulated in chemoresistant cancer cells (Figure 2C).

Generation of a UbL score to predict patients' response to chemotherapeutic drugs.

To further validate the signature, in particular on patient samples, we selected 23 of the 122 proteins that showed both a high level of modification and the most robust signal differences between sensitive and resistant cell lines in the global or separated analysis (Supplementary Table 4). In a first step, we used them to generate a UbL score aiming at predicting patients' response to chemotherapy. To this aim, we applied a Genetic Algorithm (GA)(Scrucca, 2013) to select groups of proteins pertinent for predicting chemotherapy resistance in cell lines and in patients. We used 30 variables corresponding to the 23 selected proteins (7 SUMOylated + 9 ubiquitylated + 2x7 SUMOylated and ubiquitylated) (see Methods). The GA was run on the cell lines datasets multiple times with different parameter settings to obtain a total of 40 solutions (combinations of selected proteins). Out of the 30 variables, 25 were selected at least once in the 40 solutions obtained. They were ranked by their frequency of selection (Figure 3A), as frequently selected proteins are more likely to be predictive. We then divided this list in 5 embedded subsets containing an increasing number of selected variables/proteins (Figure 3A) for Linear Discriminant Analysis (LDA), which uses an optimized linear

combination of all variables to assign observations to target classes (here sensitive or resistant). As expected, we observed that the most frequently selected proteins were present in the most predictive selected solutions, which suggested that they were not random artefacts due to the genetic algorithm.

To validate the approach, we first used the LDA on cell lines with all the subsets and found that the solution containing the top7 proteins in the GA (subset 2) was the most efficient and could predict whether the cell line was sensitive or resistant to DNR or Ara-C in 16 cases out of 18 tested (6 cell lines, 3 replicates) (Figure 3B). To determine if it could also be used to predict AML patients' response to these drugs, we resorted to bone marrow aspirates from 4 patients, all of the rather immature M1 subtype in the FAB classification. Two of these patients were sensitive to induction chemotherapy (anthracycline + cytarabine) and two were refractory (Supplementary Table 5). These samples were used to prepare extracts and probe Protoarrays as described above for cell lines. Interestingly, the LDA containing “subset 2” proteins could predict the response to chemotherapy of all the 4 patients tested (Figure 3C). Although a higher number of patient samples still need to be tested for stronger validation of the approach, our data suggest that the modifomic biomarkers we identified might be used to predict AML patients' response to chemotherapies.

Development of a miniaturized assay to measure signature protein modification by UbLs.
Protoarrays are useful to identify modifomic signatures. However, they are difficult to implement for routine clinical diagnosis due to the number of cells required, the challenge of their standardization and their high cost. To circumvent these difficulties, we developed a miniaturized flow cytometry assay to further evaluate the prognosis value of the biomarkers we identified. This assay is based on the use of Luminex XMap beads, which are color-coded magnetic beads. To validate it, we cloned the 23 selected proteins as fusion with glutathione-S-transferase (GST). Ten of them could be produced in *E. coli* and were coupled to differently colored XMap beads. The coupled beads were then multiplexed and incubated with cell extracts. They were then analyzed by flow cytometry using first anti-SUMO-1 or -Flag tag antibodies to quantify modification by SUMO-1 and Ubiquitin, respectively, and, then, specific secondary antibodies linked to two different fluorochromes (Figure 4A). If ubiquitylation was detected on most proteins tested, this was however not the case for SUMOylation. A most likely explanation for this discrepancy is that Ubiquitin can make long polyUbiquitin chains, facilitating their detection on protein substrates, whereas SUMO cannot. We consequently focused the rest of our study on ubiquitylated proteins. Out of the

ten proteins tested, three proteins (UBADC1, STAM and SQSTM1) showed significant differences in their ubiquitylation levels between parental, Ara-C- and/or DNR-resistant U937 (Figure 4B and 4C). Interestingly, although all proteins were incubated simultaneously in the same extracts, differences could be detected in their modification. For example, the highest ubiquitylation of STAM was obtained using Ara-C resistant extracts whilst UBADC1 showed increased modification only with DNR resistant extracts. Altogether, these data suggest that bead-based detection of ubiquitylation provides an easy and quantitative way to detect specific alterations in the ubiquitylation cascade and identify STAM, UBADC1 and SQSTM1 as potential Ub-modifiable biomarkers of AML chemoresistance.

Analysis of UbL signature proteins in primary AML cells using the miniaturized flow cytometry assay.

To further validate the use of the beads-based assay, we used cell extracts prepared from bone marrow aspirates from 17 patients who responded, or not, to induction chemotherapy. Interestingly, STAM, UBADC1 and SQSTM1 proteins showed a high level of ubiquitylation in 3/5 patients who did not respond to induction chemotherapy but only in 2/12 patients who responded (Figure 5 and Supplementary Table 5). Surprizingly, one of the patients (#16185), who responded to the induction chemotherapy, showed very high levels of ubiquitylation of all 3 proteins. Interestingly, this patient had a complex karyotype, which is a marker of bad prognosis. This patient relapsed rapidly after initial chemotherapy. Altogether, this suggested that the ubiquitylation signature proteins, among them STAM, UBADC1 and SQSTM1, might serve as a therapeutic response biomarker in AMLs.

Discussion

In summary, our work points to a new class of biomarkers based on ubiquitin and SUMO conjugation that might be used to predict the response of AML patients to standard chemotherapies. In addition, we have developed a miniaturized assay that allows the quantification of these biomarkers using low amounts of cells and amenable to the use of patient samples.

Dysregulations of the Ubiquitin and SUMO conjugated proteome in cancer cells can be linked to increased/decreased expression of their respective E1/E2/E3 conjugating enzymes, which could be (partly) monitored using transcriptomic approaches. However, in most cases, they are due to changes in their activities, which is more challenging to monitor, in particular in patient samples. Mass spectrometry has been widely used to analyze, at the proteome scale, cell protein SUMOylome and Ubiquitylome (Heap *et al*, 2017; Hendriks & Vertegaal, 2016). However, although the sensitivity of this technology increases rapidly, it still requires large amounts of material, which is generally not compatible with the use of patient samples as starting material. Moreover, the biological lability of the isopeptide bond linking UbLs to their substrates renders their mass-spectrometry identification particularly challenging. We used here protein arrays to monitor variations in the activity of UbL conjugating enzymes. The protein array technology is endowed with several advantages. Firstly, as it relies on enzymatic activities, it is amenable to relatively small numbers of cells as compared to mass spectrometry approaches. Secondly, it does not rely on the abundance of the spotted proteins since they are present in similar amounts on the arrays. Finally, it allows the analysis of two different UbLs in the same sample/experiment. We focused our study on ubiquitin and SUMO conjugation, as these modifiers are the best-characterized and best-studied UbLs. We identified 988 ubiquitylated and 83 SUMOylated proteins that are robustly modified on the protein arrays using cell extracts. These proteins can thus be used as biomarkers of the activity of the enzymes (E1, E2 and E3s) responsible for their Ubiquitin and/or SUMO modification *in vitro*. Of note, this assay does not monitor the activity of UbL deconjugating enzymes since they are inhibited with UbL-vinyl-sulfones. Many of the conjugated proteins we identified are themselves involved in UbL conjugation/deconjugation pathways. This probably reflects their higher ability to interact with the enzymes of the pathway, including via non-covalent interactions with ubiquitin or SUMO. It is however important to underline that the UbL-target proteins we identified are not necessarily genuine targets of the concerned

UbL-conjugating enzymes *in vivo*. At least one explanation for this is that many enzymes of the UbL cycle, as well as substrate proteins, are spatially partitioned in living cells and may never (or little) meet *in vivo*, a situation which is no longer preserved when resorting to cell extracts in an *in vitro* assay.

We show here that alterations of Ubiquitin and SUMO conjugation pathways are associated with AML resistance to DNR and Ara-C. We identified 122 proteins, the ubiquitylation/SUMOylation of which changes when using cell extracts from AML cell lines resistant to DNR or Ara-C comparatively to cell extracts from their chemotherapy-sensitive counterparts. In most cases, their ubiquitylation/SUMOylation is increased, suggesting an overactivation of specific UbL enzymes in chemoresistant AML cells. Only a small fraction (around 10%) of the Ubiquitin/SUMO-modifiable proteins we identified on the arrays are differentially modified between chemosensitive and chemoresistant cells. This suggests that, not the entire Ub/SUMO pathways, but only specific enzymes within these pathways are dysregulated in chemoresistant cells. These are most probably E3 enzymes since dysregulation of E1 or E2s would alter a much larger repertoire of substrates. Identifying, which are these dysregulated E3 enzymes could pave the way to the development of new therapeutic strategies to improve AML treatment.

Among the 122 UbL signature proteins, some are differentially modified only in DNR- or Ara-C-resistant cells or only in one of the two model AML cell lines. This suggests that some of the alterations resulting in changes in UbL enzymes activities are specific to the genotoxics they are resistant to and/or to the AML subtype. We then generated scores based on the most robust proteins of the signature in terms of level and differences of UbL modification between the parental and the resistant AML cell lines we used as models. These scores were used on protein array data obtained with 4 patient's samples and one of the scores could retrospectively predict the response of these 4 patients to the chemotherapy. Even if the number of patients' samples was too small to validate this assay on a definitive statistical basis, our work nevertheless provides a proof of concept that such an approach could be used to help predict AML patients' response to current standard chemotherapies.

To further validate the modifomic biomarkers we identified, in particular in patients' samples, it was important to develop a miniaturized assay that would require less cells and be easily and rapidly (few hours) implementable for future use in clinical practice. The assay is based on the Luminex technology, which uses multiplexable color-coded microbeads. This

technology is routinely used, including in clinical analysis services, to detect circulating hormones, chemokines, cytokines or auto-antigens using beads coupled with antibodies specific for these proteins. Here, instead of antibodies, we coupled purified recombinant proteins from the UbL-signature to the beads. The modified beads (one protein per bead color) were then multiplexed and incubated with cellular extracts, which were prepared under conditions preserving UbL-conjugating enzymes activities. This assay could precisely quantify the level of coupled protein modification by Ubiquitin and, thereby, precisely estimate the differences in their modification levels between samples. Among the proteins that we analyzed, we identified 3 proteins, STAM, UBADC1 and SQSTM1, which are differentially ubiquitylated with extracts from cell lines or patients that were resistant to chemotherapies compared to cells that are sensitive. It should however be noted that not all proteins identified on the Protoarrays could be validated in the XMap bead-based assay. At this stage of investigations, we do not exclude that this might be due to differences in their conformation and/or differences in pre-existing post-translational modifications, as they were produced in insect cells in the former case and in bacteria in the latter one. Along the same line, improvement of protein production and detection methods could also allow to better detect SUMOylation of the proteins we identified using the protein arrays and improve the prognosis efficiency of the assay.

AML is a therapeutic emergency, as patients most often need to start chemotherapy in the next few days after diagnosis. It is however critical to rapidly determine the risk/benefit ratio before starting current standard chemotherapy to which severe side-effects are associated. Decision between intensive standard chemotherapy, epigenetic therapies (e.g. azacitidine) and best-supportive care is currently mostly based on the age, fitness, co-morbidities of the patient. Cytogenetics, in particular the number of genetic abnormalities, can also be used as a prognosis marker. However, it generally takes one or two weeks for the clinicians to get the results. Most of the time, the treatment needs to be started before having these results. We have established the basis of an assay, based on UbL modifications by patients' cell extracts, that could be performed at diagnosis in few hours together with flow-cytometry analyses routinely carried out to confirm AML diagnosis. Its results, combined with the above-mentioned criteria, might therefore help clinicians to propose the best therapeutic option to each patient, including redirection towards novel treatments or ongoing clinical trials, notably for emerging drugs targeting Ubiquitin or SUMO pathways.

Methods

Cell culture

HL-60 and U937 cells (DSMZ, Germany) were cultured at 37°C in RPMI medium supplemented with 10 % fetal bovine serum (FBS) and streptomycin/penicillin in the presence of 5 % CO_2. Both cell lines were authenticated by the ATCC using Short-Tandem-Repeat analysis. All cells were regularly tested negative for mycoplasma. After thawing, cells were passaged at a density of $3x10^5$/ml every 2-3 days for no more than 10 passages. U937 and HL-60 cells resistant to Ara-C (cytarabine) and DNR were generated by culture in the presence of increasing drug concentrations (up to 0.1 µM for Ara-C and 0.03 µM for DNR) for 2-3 months.

Patient samples

Bone marrow aspirates were collected after obtaining written informed consent from patients under the frame of the declaration of Helsinki and after approval by the institutional "Sud Méditerranée 1" Ethical Committee (ref 2013-A00260-45; HemoDiag collection). Fresh leukocytes were purified by density-based centrifugation using Histopaque 1077 (Sigma-Aldrich) and used immediately for extract preparation. Detailed characteristics and treatments of the patients involved in this study are provided in Supplementary Table 5.

IC_{50} measurement

Cells were seeded at a concentration of $3x10^5$/mL in RPMI medium complemented with 0.1, 1, 10, 50, 100 or 250 µM of Ara-C (Sigma-Aldrich) or 0.01, 0.05, 0.1, 0.5, 1 or 10 µM of daunorubicin (Sigma-Aldrich). Viability was measured 24 hrs later using the MTS assay from Promega following the manufacturer's protocol. IC_{50} were calculated using the GraphPad PRISM software.

Cellular extracts

Cell lines grown at a $5\text{-}8x10^5$/mL density or patient's bone marrow aspirates were spun down (300 g) at 4 °C for 5 min and washed once with PBS. After pellet resuspension in 1 mL of PBS, they were centrifuged again (16,000 g) at 4 °C for 5 min. Pellets were resuspended and incubated at 4 °C for 30 min in a hypotonic buffer (HEPES pH 7.5 20 mM, $MgCl_2$ 1.5 mM, KCl 5 mM, DTT 1 mM and 1 mg/L of aprotinine, leupeptin and pepstatin) in a volume of either (i) 100 µL per $50x10^6$ cells to generate concentrated extracts used for ProtoArray probing or (ii) 25 µL per $2x10^6$ cells in the case of extracts used in XMap bead-based flow

cytometry assays. Cell lysis was achieved through 4 freezing/thawing cycles using liquid nitrogen and DNA was sheared owing to 10 passages through a 20-1/2G needle. Extracts were finally centrifuged twice (16,000 g) at 4°C for 20 min and supernatants were aliquoted, flash-frozen and kept at -80 °C until use.

Protein arrays

Human protein arrays (ProtoArrays V5.0, Life Technologies) kept at -20°C were equilibrated at 4 °C for 15 min and, then, saturated with the Protoarray-Blocking Buffer (PA055, Life Technologies) supplemented with Synthetic Block (PA017, Life Technologies) and 1 mM DTT at 4 °C for 1 hr. To probe ProtoArrays for UbL protein modification, cell extracts were first supplemented with 5 µM Ubiquitin-vinyl sulfone, 2.5 µM SUMO1-vinyl sulfone and 2.5µM SUMO2-vinyl sulfone (Boston Biochem) to inhibit the corresponding UbL-deconjugating enzymes. Control extracts were also incubated with 50 mM N-Ethyl Maleimide (NEM; Sigma-Aldrich) to inhibit any UbL conjugation. The extracts were then supplemented with Tween-20 (0.1%), ATP (1 mM) and home-made recombinant SUMO-1 or SUMO-2 (15 µM, produced as previously described(Bossis *et al*, 2005)), or Flag-ubiquitin (30 µM, Boston Biochem) and were immediately laid on ProtoArray slides, which were then covered with a coverslip and incubated at 30 °C for 1 hr in a humidified atmosphere. Arrays were washed 3 times for 5 min with a washing buffer (PBS pH 7.4, Tween 20 0.1 %, 1X Synthetic Block) supplemented with 0.5 % SDS and, then, twice for 5 min with only the washing buffer. Next, they were incubated in the washing buffer under agitation (50 rpm) at 4°C for 1 hr with 1 µg/mL of rabbit anti-Flag- (SIGMA, F7425) or mouse anti-SUMO-1 (21C7 from the Developmental Studies Hybridoma Bank) antibodies. After 5 washes of 5 min in the washing buffer, they were incubated at 4 °C for 90 minutes with 0.5 µg/mL of Alexa Fluor 647-labelled anti-mouse- or Alexa Fluor 546-labelled anti-rabbit antibodies (Thermo Fisher). Arrays were washed 5 times for 5 min with the washing buffer, once with H_2O and, finally, dried by centrifugation before fluorescence scanning using the Innoscan 710 device from Innopsys.

Analysis of Protoarray data

Measured fluorescence intensities were associated to the corresponding protein ID according to their coordinates on the arrays using the Mapix software (Innopsys). Intensities were processed using the PAA R package(Turewicz *et al*, 2016). In brief, (i) duplicated protein spot intensities were averaged using the LoadGPR function and (ii) the background was corrected using the BackgroundCorrect function. Fluorescence intensities within the same

experiment were normalized by quantiles using the NormalizeArray function. Finally, intensities were normalized between the different experiments using the BatchAdjust function.

Data filtering

To identify the proteins modified by Ubiquitin or SUMO-1 on the ProtoArrays, we first had to filter proteins displaying signal significantly higher in UbL conjugation-permissive-conditions comparatively to non-permissive ones (i.e. control conditions using NEM-treated extracts). The classical Student t-test was not adapted to exploit our results, as variances could be very different between UbLs conjugation-permissive and non-permissive conditions. We therefore selected proteins with significant p-values (lower than 0.05) in both the parametrical Welch- and the non-parametrical Wilcoxon-Mann-Whitney (WMW) tests. Then, proteins having an averaged normalized fluorescence intensity value less than 800 (arbitrary threshold) were filtered out in order to obtain a list of robustly modified proteins.

Identification of differentially UbL-modified proteins between chemosensitive and chemoresistant cells

The signal intensity ratios between the parental and the drug resistant cells for the proteins selected after data filtering (see above) were calculated using both a Wilcoxon signed-rank test and a one sample *t*-test. This analysis was first performed on all arrays (parental, DNR- or ARA-c-resistant U937 and HL60 cell lines). In this case, the sample size being large, we considered as differentially modified all proteins showing p-values<0.05 in both tests. For the analysis on the individual cell lines (U937 and HL-60) and drugs (Ara-C and DNR), the sample size being smaller, we considered as differentially modified the proteins showing p-values<0.05 in the one sample t-test.

Ontology analysis

Ontology analyses were performed using the Panther software.

Genetic Algorithm and Linear Discriminant Analysis

The R package GA, providing a genetic algorithm (GA)(Scrucca, 2013), was used. The aim was to determine the right number of variables in order to create a parsimonious predictive model. GAs are mathematical models inspired by Charles Darwin's model of natural selection. The natural selection preserves only the fittest individuals over the different generations. An evolutionary algorithm improves the selection over time and allows the best

solution to emerge from the best of prior solutions. The selected features were then tested with linear discriminant analysis (LDA) using the R package MASS(Venables & Ripley, 2002; Ripley, 1996). This mathematical method uses a linear combination of all variables to assign observations to target classes. To this aim, it creates a decision rule based on the n available variables (score = $\alpha*R_1$ + $\beta*R_2$ + …. + $\omega*R_n$) by maximizing the between-class variability and minimizing the within-class one. A cross-validation step that separates the observations in two groups (training dataset on which the model is established and test dataset on which the model is validated) was performed to get more robust results.

Production of recombinant proteins

cDNAs encoding the proteins of interest were recovered from the Ultimate ORF library (Thermofisher) and cloned in the bacterial expression vector pGGWA(Busso *et al*, 2005) vector using the Gateway technology according to the manufacturer's protocol (Life Technologies). Constructs were then transformed in the BL21 (DE3) *E. coli* strain. Protein production was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 hrs in bacteria exponentially growing at 25°C. Bacterial pellets were resuspended in Tris-HCl pH 8.6 50 mM containing NaCl 500 mM and $MgSO_4$ 50 mM and flash-frozen in liquid N_2. After thawing, bacterial suspensions were supplemented with 1 mg/mL lysozyme (Sigma-Aldrich), 8 mM β-mercaptoethanol and 1 mg/L of aprotinin, leupeptin and pepstatin and incubated at 4°C for 1 hr. Bacterial debris were spun down at 100,000 g for 1 hr. The extract was then bound to glutathione agarose beads (Generon) equilibrated in buffer A (Tris-HCl pH 8.6 50 mM, NaCl 150 mM, $MgSO_4$ 50 mM, β-mercaptoethanol 8 mM and 1 µg/L of aprotinin, leupeptin and pepstatin). The column was then extensively washed with buffer A and eluted by addition of 20 mM reduced glutathione (Sigma-Aldrich).

Protein coupling to XMap

$2x10^5$ magnetic XMap beads (low concentration) from Luminex were transferred to a low binding microtube (Eppendorf) and washed using NaCl 500 mM. They were then resuspended in 50 µL of MES (2-ethanesulfonic acid) pH 6.1 50 mM and incubated in the presence of 5 µg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Pierce) and 5 µg/mL Sulfo-NHS (Pierce) at room temperature for 20 min under agitation. Beads were then washed in PBS containing 500 mM NaCl and incubated with 7 µg of recombinant protein to be coupled in 100 µL PBS at room temperature for 2 hrs. They were then washed twice with PBS containing 0.1 % BSA, 0.02 % Tween 20, 0.05 % sodium azide

and 500 mM NaCl and stored at 4 °C in PBS containing 0.1 % BSA, 0.02 % Tween-20, 0.05 % sodium azide.

UbLs conjugation to proteins coupled to XMap beads

SUMO-1-, SUMO-2- and Ubiquitin-vinyl sulfones (0.5 µM each) were added to diluted cellular extracts (10 µL), which were incubated at 4 °C for 15 min. Control extracts were also incubated with 100 mM NEM. We then added to the extract 10^3 protein-coupled XMap beads contained in 10 µL of a reaction buffer containing 20 mM HEPES pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 0.05 % Tween-20, 0.5 mM EGTA, 0.2 mg/mL ovalbumine, 1 mM DTT, 1 mg/L aprotinin, leupeptin and pepstatin, 1 mM ATP, 30 µM Flag-ubiquitin, 15 µM SUMO-1 and 15 µM SUMO-2. Reaction were performed at 30 °C for 45 min. Beads were washed twice for 5 min with PBS containing 0.05 % Tween-20 and 0.5 % SDS and 3 times for 5 min with PBS containing 0.05 % Tween-20. They were then incubated with 1 µg/mL of mouse anti-SUMO-1 (21C7) and rabbit anti-Flag antibodies for 1 hr under agitation at room temperature. After washing in PBS containing 0.05% Tween-20 for 5 min, they were incubated for 30 min at room temperature with anti-mouse Alexa Fluor 488- and anti-rabbit Alexa Fluor 405 antibodies in 100 µL of PBS containing 0.05 % Tween-20. Beads were again washed for 5 min with PBS containing 0.05 % Tween-20. They were then resuspended in 200 µL PBS and flow-cytometry-analysed using the LSR Fortessa device from BD Biosciences. Results were analysed using the FlowJow software.

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Authors contribution

PG and FB performed and analyzed the experiments. CR and MP performed the statistical analyses. YH and GC provided patient samples and associated clinical data. MP and GB supervised the study and wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest

Figure Legends

Figure 1: Measuring UbL conjugating activities in AML cell extracts using protoarrays. (A) *Flowchart followed for UbL signature characterization*. Extracts from parental and chemoresistant HL-60 or U937 cells were first supplemented with recombinant UbLs (to avoid rate-limiting amounts of the modifiers) and UbL-vinyl-sulfones (to inhibit UbL deconjugating activities). They were then incubated with protein Protoarrays. After extensive washes, the arrays were incubated with, first, a primary mouse anti-SUMO-1 antibody and a rabbit anti-Flag antiserum recognizing the Flag-tag present on the recombinant Ubiquitin added to the reaction and, then, appropriate fluorescent secondary antibodies. Fluorescence signals were processed using the PAA R package. The statistical analysis was performed to identify a UbL signature of chemoresistance, as described in Methods. Three independent experiments were performed for each cell line. **(**B) *IC_{50} of chemosensitive and chemoresistant AML cell lines*. IC_{50} of chemosensitive parental HL-60 and U937 (wt) cells and of their resistant counterparts (see Materials and Methods) (ARA-R and DNR-R) were assayed after 24 hrs of exposure to drugs. n=3, Mean +/- SEM with * corresponding to $p<0.05$. (C) *Identification of ubiquitylated- and SUMOylated proteins*. Normalized Ub and SUMO-1 fluorescence data obtained on all arrays incubated with extracts were compared to averaged signals on control arrays (extracts supplemented with NEM to inhibit UbL conjugation activities) to identify robustly UbL-conjugated proteins. Proteins showing significant differences between the 2 groups when using both the Welch- and the Wilcoxon-Mann-Whitney statistical tests and having mean fluorescence intensities values higher than 800 (arbitrary threshold) on Protoarrays were selected for further analysis. The Venn diagram shows the number or proteins identified as modified by SUMO-1 and/or ubiquitin.

Figure 2: UbL-conjugated protein signature of chemoresistance. (A) Identification of UbL-modified biomarkers of AML chemoresistance. Modification levels of the proteins modified by Ubiquitin (left panel) or SUMO-1 (central panel) selected in Figure 1C were compared between all parental (U937 and HL-60) and drug-resistant (ARA-R or DNR-R) sublines. Differentially modified proteins with significant p-values in both Wilcoxon signed-rank- and one sample t-test and with a drug-resistant vs parental cell ratio higher than 1.25 or lower than 0.8 are indicated in red for ubiquitylated proteins and in blue for SUMOylated ones. The Venn diagram shows the overlap between differentially ubiquitylated- and

SUMOylated proteins. (B) *Identification of UbL-conjugated biomarkers specific for HL-60 and U937 cell resistance to Ara-C or DNR*. Statistical analyses between drug-resistant and parental cells were performed separately for U937 and HL-60 cell lines and for each drug resistance. The number of proteins showing a significant p-value in one sample *t*-test and a ratio between drug-resistant and parental cells higher than 1.5, or lower than 0.66, are shown. (C) *Ontology analysis of the UbL signature.* An ontology analysis of the 122 proteins of the UbL signature was performed using the Panther software.

Figure 3: Generation of a UbL-predictive score of AML response to chemotherapies. (A) *Selection of the best predictive proteins*. A list of 30 predictors among the 122 signature proteins was chosen to run a genetic algorithm (GA). These predictors corresponded to 23 proteins modified either by Ubiquitin (Ub), SUMO (Su) or both UbLs. The rate of selection of each of these proteins and its associated UbL (Ub or Su) is indicated on the graph. The proteins were separated in 5 subsets for LDA analysis. (B,C) *Probability of AML sensitivity/resistance using the subset 2 solution.* The 7 proteins showing the highest rate of selection in the GA (subset 2) were used for Linear Discriminant Analysis (LDA) to predict the probability of resistance for the U937 and HL60 cell lines (B) or patient samples (C). Cells were considered sensitive to the chemotherapy if the probability to belong to the group of resistant cells was below 50% and resistant if over 50%.

Figure 4: Flow cytometry assay for the detection of UbL-conjugated chemoresistance biomarkers. (A) *Principle of the assay.* Recombinant signature proteins are produced in *E. coli* and coupled to differently colored XMap beads. Coupled beads are then mixed and incubated with cell extracts supplemented with recombinant UbL, in the presence of UbL-vinyl-sulfones to inhibit deconjugating activities. Protein modification is quantified by flow cytometry using a combination of primary antibodies directed to UbLs and fluorescent secondary antibodies. (B, C) *Analysis of STAM, SQSTM1 and UBADC1 ubiquitylation.* Extracts from parental, ARA-R or DNR-R U937 cells were used as described in A with beads coupled to STAM, SQSTM1 or UBADC1. Representative flow-cytometry profiles for their ubiquitylation are shown in B. Quantification are presented in C. For quantification, background (cell extracts supplemented with NEM to inihibit UbL conjugation activities) was

substracted and ratio between resistant and parental cell lines were shown. (n=6 for STAM, n=3 for SQSTM1 and UBADC1). Mean +/- SEM. Paired student t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns not significant. MFI: median fluorescence intensity.

Figure 5: Ubiquitin-conjugation to chemoresistance signature biomarkers in patient samples using the miniaturized flow cytometry assay. Extracts from bone marrow aspirates from 17 patients at diagnosis were used in a multiplexed flow cytometry analysis using XMap beads coupled to STAM, UBADC1 and SQSTM1. Twelve patients responded to induction chemotherapy (blue: <10% of blasts in bone marrow 30 days after the beginning of chemotherapy) and 5 did not (red: >10% of blasts in bone marrow 30 days after the beginning of chemotherapy). The average intensity of ubiquitylation is presented as a line for both responder and non-responder patients.

Supplementary Table 1: List of proteins identified on arrays as ubiquitylated or SUMOylated. The normalized fluorescence values are given for every selected protein and for each experiment. Proteins are ranked by their mean intensities of fluorescence (most modified proteins are on the top). The p-values (Welch and MannWhitney) are obtained by the comparison of the control slides (NEM, 6 arrays) and the other arrays (18 arrays).

Supplementary Table 2: Comparison of the levels of UbL modification between parental and chemoresistant cell lines. The ratio of modification for all the ubiquitylated and SUMOylated proteins was obtained by averaging the normalized fluorescence signals obtained for all chemoresistant cells (HL60 and U937, resistant to ARA-C or DNR, 12 arrays) and comparing them to those from the parental cells (6 arrays). Only those showing p-values <0.05 for both Student and Wilcoxon statistical tests were selected.

Supplementary Table 3: Comparison of the levels of UbL modification between parental and chemoresistant cell lines. The ratio of modification for the ubiquitylated and SUMOylated proteins was obtained by separately averaging the signals obtained for HL60 or U937 cells resistant to ARA-C or DNR (3 arrays for each condition) and comparing them to those from the parental cells (3 arrays). Only those showing p-values <0.05 in Student t-test for one of the two resistance were selected.

Supplementary Table 4: UbL signature of AML chemoresistance. List of the 122 proteins selected in the global analysis (HL60 and U937 cells combined; see Supplementary Table 2) and the cell-specific analysis (HL60 and U937 cells separately; see Supplementary Table 3) for Ubiquitin and SUMO. Among these proteins, 23 were selected for GA/LDA analysis (Figure 3) and 11 were tested in the XMap Luminex assay (Figure 4).

Supplementary Table 5: Clinical data associated with all patient samples used in this study. In addition to cytogenetic characteristics and treatment, the percentage of blasts is given at diagnosis and after 30 days. Patients with less than 10% of leukemic blasts after 30 days of treatment were considered as responsive to treatment and the others as refractory.

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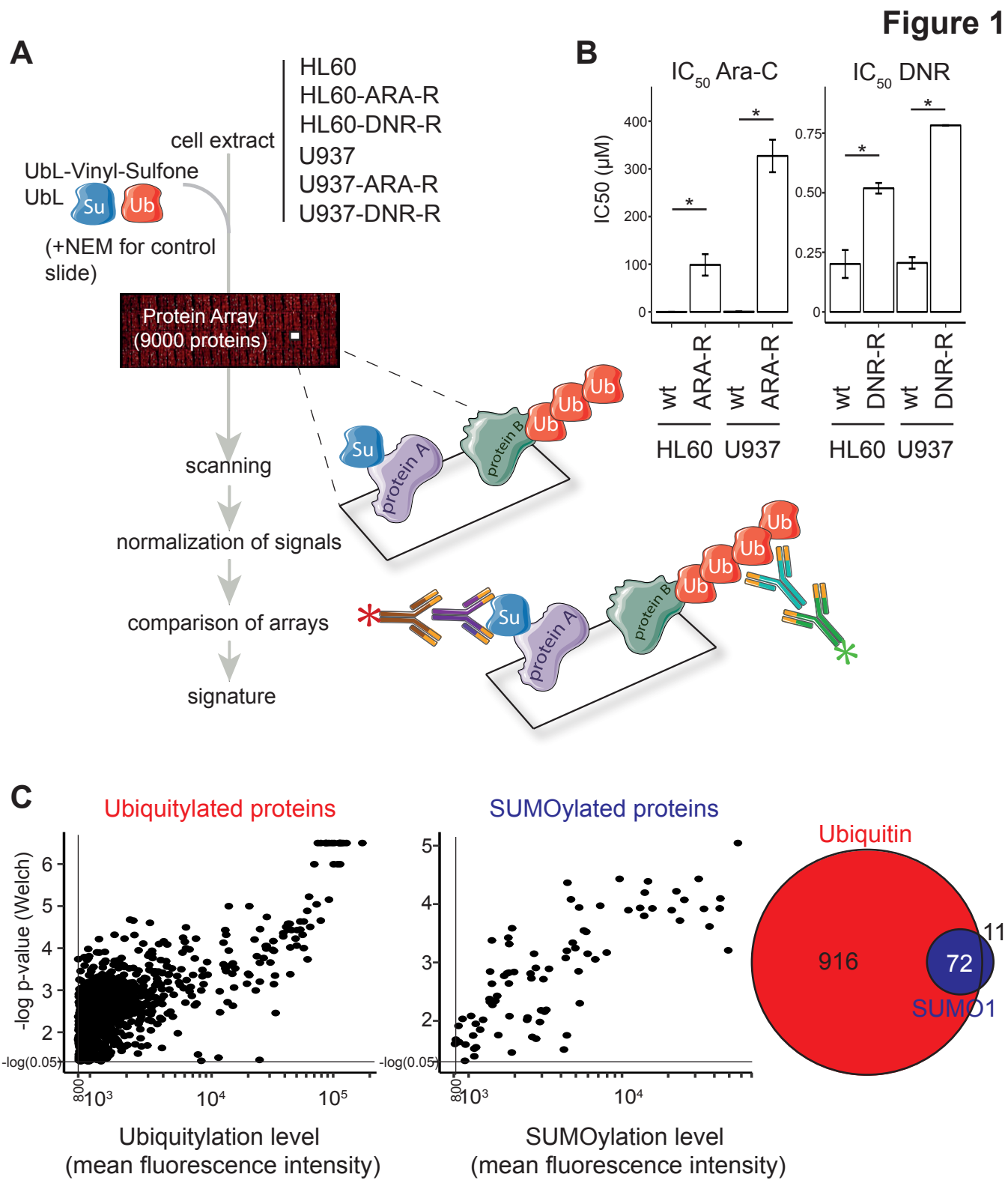
Figure 1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cell extract
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UbL
Su
Ub
(+NEM for control slide)
Protein Array (9000 proteins)
scanning
normalization of signals
comparison of arrays
signature
protein A
protein B
B
IC50 Ara-C
IC50 DNR
IC50 (μM)
wt
ARA-R
DNR-R
HL60
U937
C
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-log p-value (Welch)
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Ubiquitin
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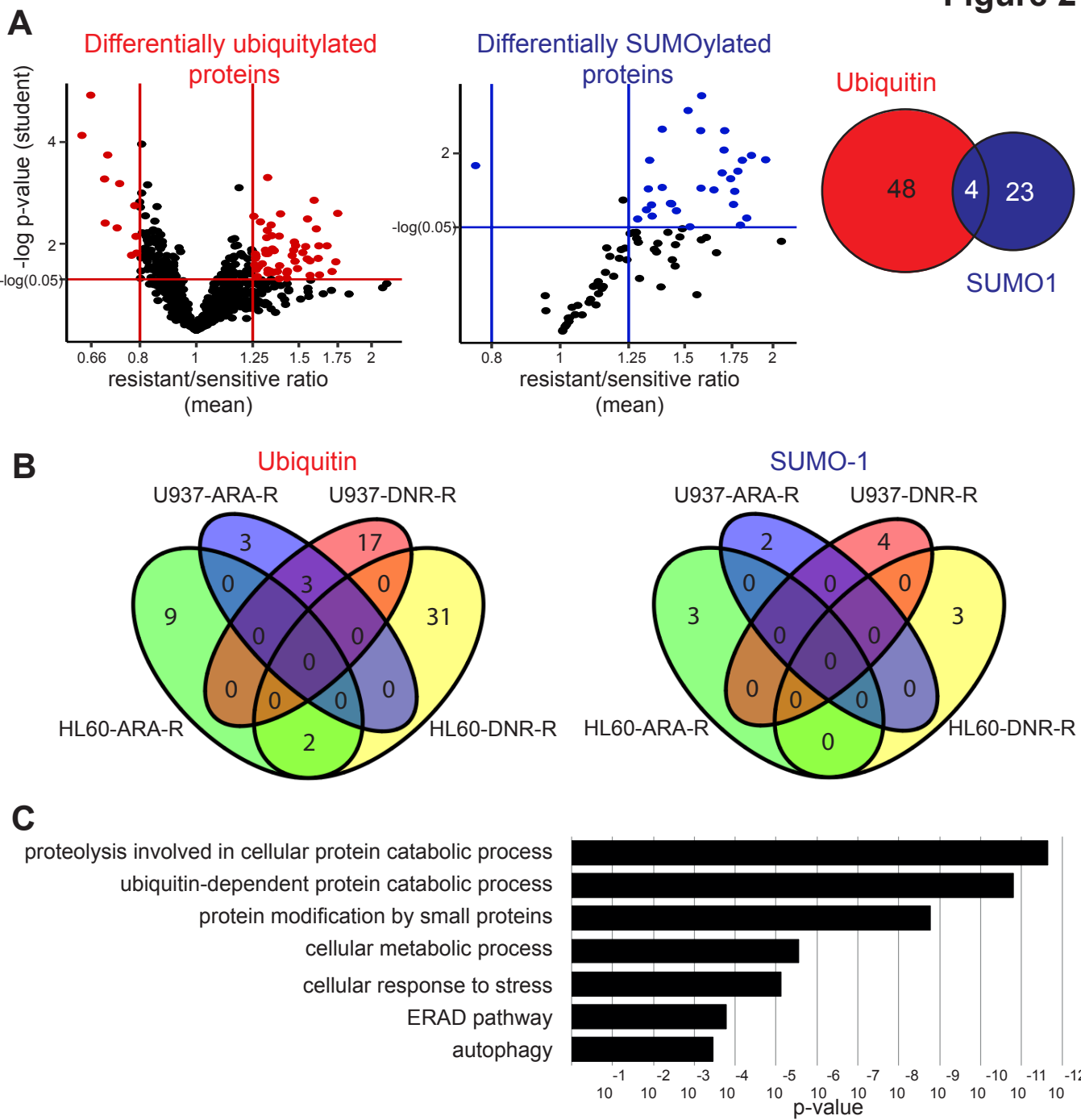
72
11
SUMO1

Figure 2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4
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SUMO1
B
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U937-ARA-R
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HL60-ARA-R
HL60-DNR-R
SUMO-1
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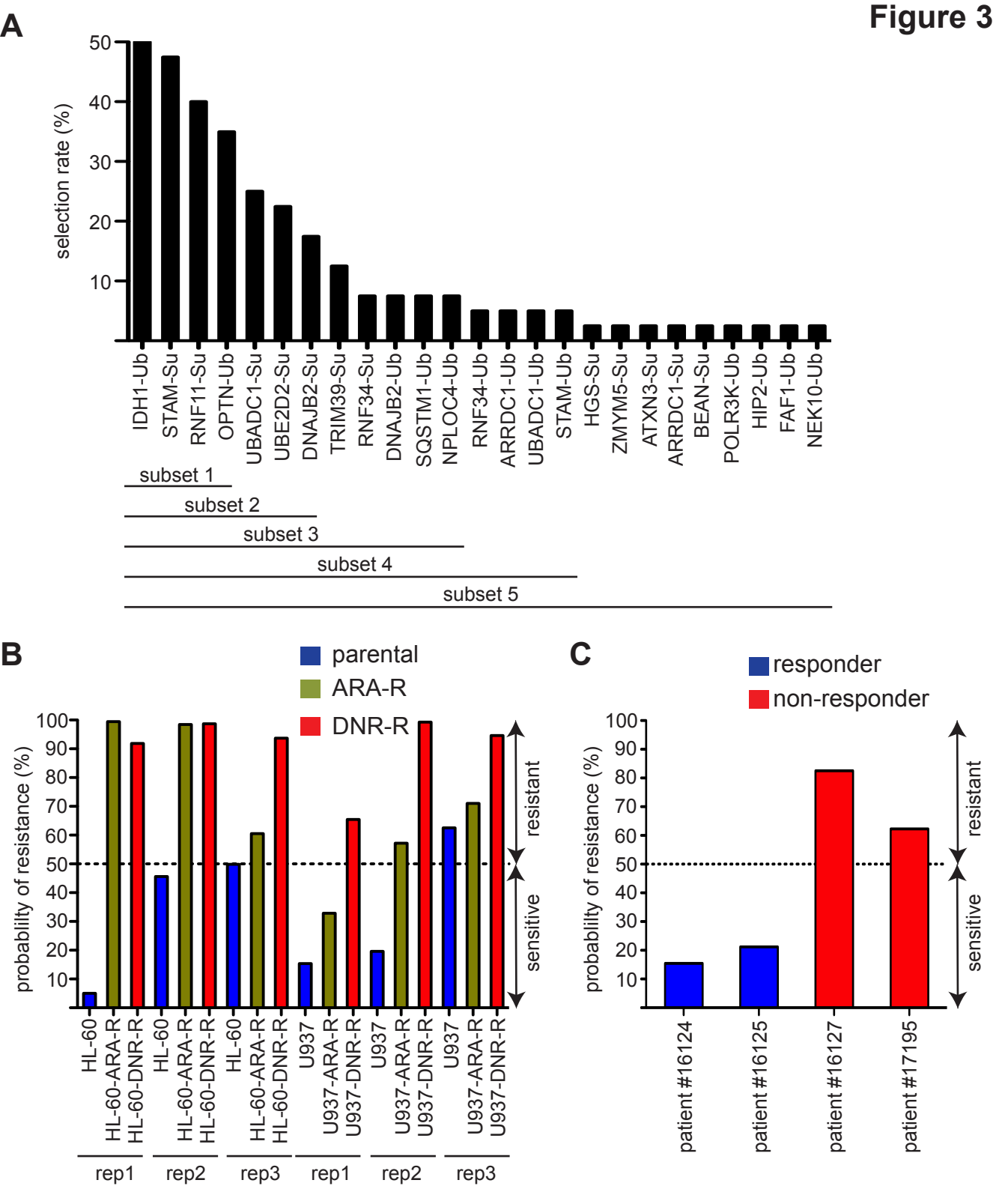
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40
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20
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OPTN-Ub
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DNAJB2-Su
TRIM39-Su
RNF34-Su
DNAJB2-Ub
SQSTM1-Ub
NPLOC4-Ub
RNF34-Ub
ARRDC1-Ub
UBADC1-Ub
STAM-Ub
HGS-Su
ZMYM5-Su
ATXN3-Su
ARRDC1-Su
BEAN-Su
POLR3K-Ub
HIP2-Ub
FAF1-Ub
NEK10-Ub
subset 1
subset 2
subset 3
subset 4
subset 5
B
parental
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HL-60-ARA-R
HL-60-DNR-R
U937
U937-ARA-R
U937-DNR-R
rep1
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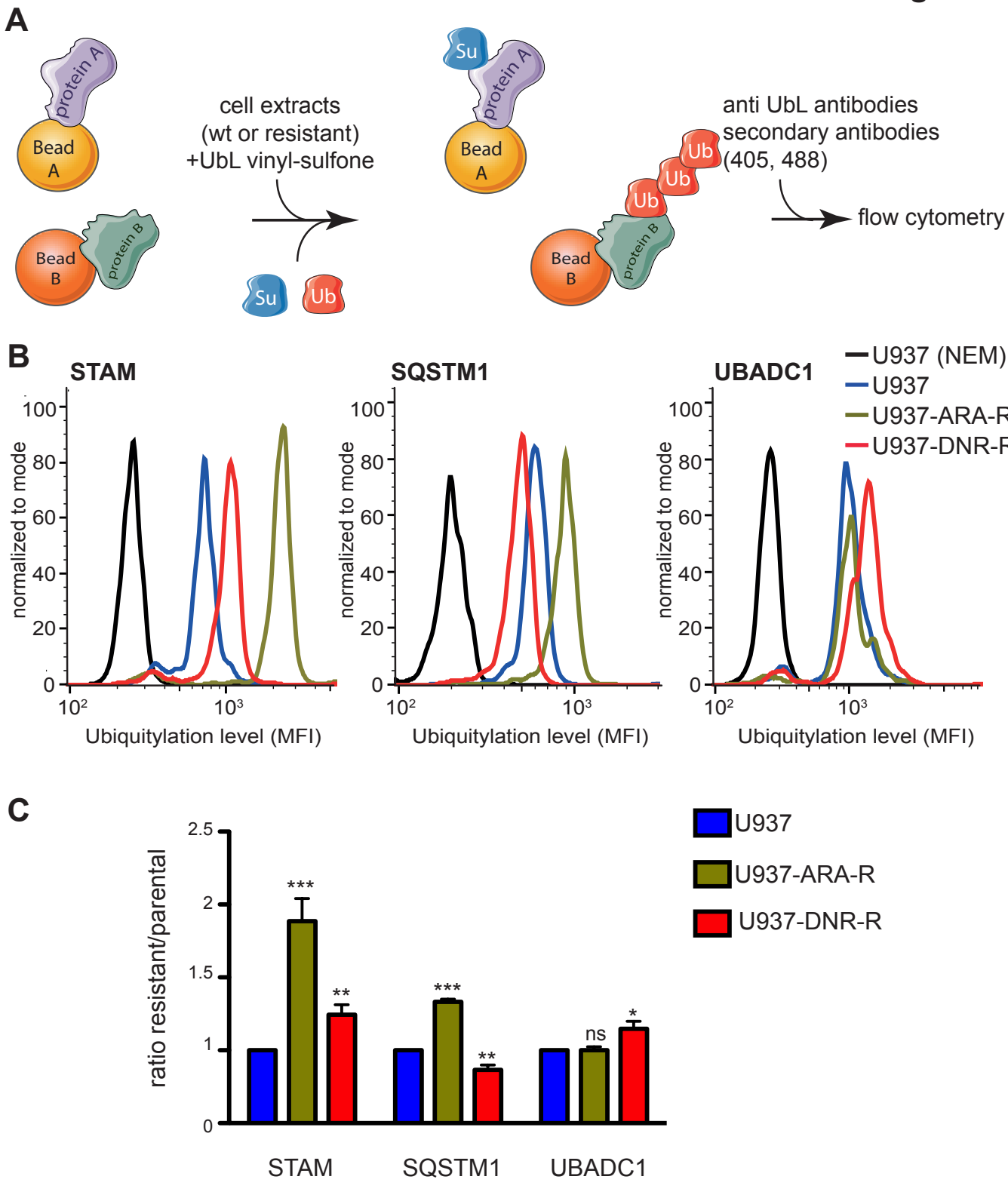
Figure 4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B
STAM
SQSTM1
UBADC1
U937 (NEM)
U937
U937-ARA-R
U937-DNR-R
normalized to mode
Ubiquitylation level (MFI)
C
ratio resistant/parental
U937
U937-ARA-R
U937-DNR-R
STAM
SQSTM1
UBADC1
ns

responder
non-responder

Figure 5