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Identification of Substances for Ubiquitin-Dependent Proteolysis During Breast Tumor Progression

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14. ABSTRACT

Ubiquitylation is post-translational modification in which a small and highly abundant protein called ubiquitin is attached to proteins. Ubiquitylation regulates several processes that are central to breast tumorigenesis, including cell division, inflammation, and angiogenesis. However, defining how ubiquitylation contributes to breast tumorigenesis has been technically limited. We developed an innovative methodology that utilizes protein microarrays as a platform to evaluate the ubiquitylation activity of breast tumor specimens on a proteome-wide scale. In this proposal, we utilized this methodology to define changes in ubiquitylation activity during breast tumor progression. Extracts from breast tumors of either low or high grade/stage were profiled and ubiquitylation activity (fluorescence intensity) quantified for >8,000 substrates on the protein microarray. Several distinct differences in ubiquitylation activity (>2-fold) were observed, with many of the substrates being involved in processes such as cell division, angiogenesis, and metastasis. Several targets of ubiquitylation were then validated. The results of this study show that distinct changes in ubiquitylation activity accompany the progression of breast tumors to more advanced disease. These activities likely drive breast tumor progression and could potentially represent novel targets for therapeutic intervention.

15. SUBJECT TERMS
ubiquitin, protein microarrays, breast tumor progression

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A. Introduction

Ubiquitylation is an enzymatic process of protein modification present in all cells in which a small and highly abundant protein called ubiquitin is physically attached to other proteins. The attachment of ubiquitin to proteins can have several consequences, including activating its function or targeting it for destruction. Ubiquitylation is an important regulatory process in cellular division. However, recent experimental evidence has shown that ubiquitination activity is altered in breast cancers and contributes directly to tumorigenesis.

Understanding how ubiquitination contributes to breast tumorigenesis has been problematic because no experimental tools existed that can identify which proteins are ubiquitylated in cells. To overcome this limitation, we have developed an innovative experimental assay that enables the identification of ubiquitylated proteins using protein microarrays, containing >8000 human proteins dotted onto glass slides. We have successfully used our assay to identify substrates of purified ubiquitylating enzymes and complex biological mixtures such as breast tumor extracts. The goal of this study is to identify changes in ubiquitylation activity associated with the transformation of normal breast epithelial cells into breast cancers.

B. Body

Establishment of a protein microarray-based ubiquitylation activity profiling assay. Our methodology for profiling ubiquitylation activity in breast tumor specimens is based on protein microarrays, glass microscope slides spotted with >8,000 human recombinant proteins, which serve as substrate for *in vitro* reactions (Fig. 1). Ubiquitylation activity is profiled by performing "on-chip" ubiquitylation reactions that include extract from a breast tumor specimen and biotin-labeled ubiquitin. The substrates of ubiquitin conjugation are then detected by incubation of the protein microarrays with fluorescein-labeled strepavidin and visualized using a fluorescence slide reader.

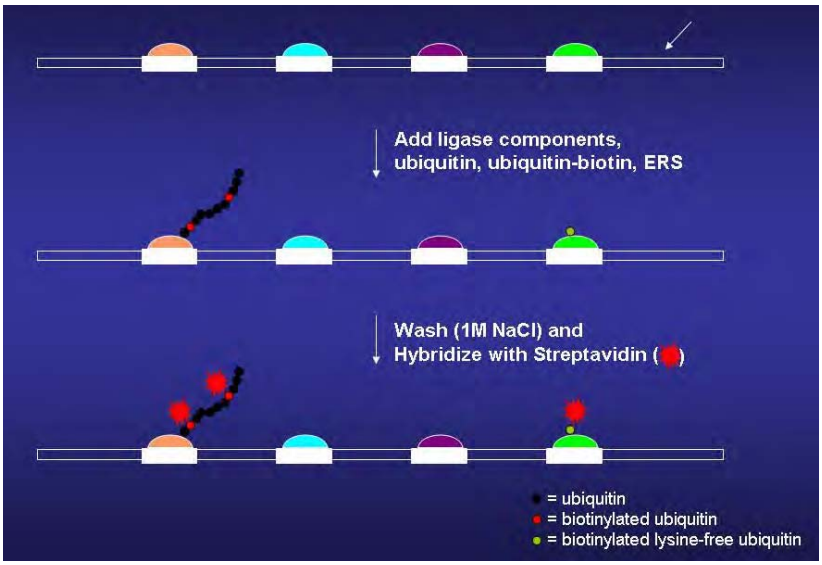

Figure 1. ***Overview of "on-chip" ubiquitylation reactions using protein microarrays as substrate.*** Protein microarrays containing >8,300 human recombinant proteins spotted onto glass microscope slides are used as substrates for *in vitro* ubiquitylation reactions containing biotin-labeled ubiquitin. Substrates are then detected using fluorescein-labeled strepavidin. We have successfully employed this methodology to profile the ubiquitin ligase activity of purified ubiquitin ligases and extracts prepared from human and mouse cell lines and primary tumors.

We first focused our efforts on refining our assay system to achieve maximal activity on the protein microarrays. We took this approach since the protein microarrays are expensive

($1,500/each) and believed that the assay should be well-defined before utilizing these materials. In collaboration with Invitrogen Corp., we first created custom protein microarrays which are spotted with ~20 different human recombinant proteins that we showed were ubiquitylated by the extracts of dividing cells. These custom arrays were created by a protein spotting machine at the Invitrogen facility. Using these custom protein microarrays, we established optimal conditions for profiling ubiquitylation activity for the breast tumor extracts (data not shown). Using these optimal conditions, we then performed test reactions on the full protein microarrays (Fig. 2). We tested extracts prepared from rabbit reticulocytes and S100 fractions of HeLa cells, both known to contain robust ubiquitin ligase activity. The results of these experiments showed that these extracts were capable of ubiquitylating >400 substrates on the protein microarray, including several substrates known to be regulated by the ubiquitin-proteasome system (UPS), as well as many novel substrates (Fig. 2).

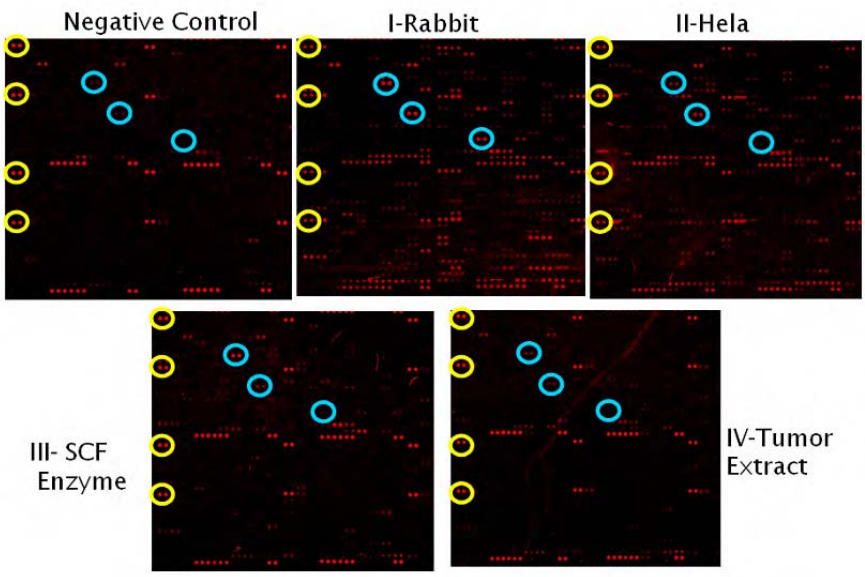

Figure 2. ***Protein microarray-based analysis of ubiquitylation activity in cell and tumor extracts.*** Ubiquitylation reactions were performed using extract prepared from rabbit reticulocytes (upper middle) and HeLa cells (upper right), whole breast tumor extract (lower right), and purified recombinant SCF^{Skp2} (lower left). Negative control includes E1, E2, ATP regeneration system, ubiquitin, and biotin-ubiquitin. Positive controls for fluorescence (yellow circles) and examples of ubiquitylated substrates (blue circles) are shown.

Profiling ubiquitylation activity of breast tumor specimens. We next obtained fresh-frozen breast tumor specimens that were either of low or high pathological stage and grade from the Tumor Bank at the Sidney Kimmel Cancer Center, SKCC). Extracts were generated in ubiquitylation reaction buffer (20 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, 0.5 mM DTT) and protein concentration determined. We then performed ubiquitylation reactions by overlaying tumor extract (50 μg), E1 activating enzyme, ubiquitin, biotin-labeled ubiquitin, and an ATP regeneration system on the full protein microarrays (ProtoArrays, Invitrogen). Reaction mixtures (100 μl) were cover-slipped, and the slides incubated @ 37°C for 1hr in a humidified chamber. Reactions with no ATP regeneration system served as controls. Following a wash in 1M NaCl for 30 min to remove non-covalent interactions, the slides were incubated with fluorescein-labeled strepavidin for 1hr @ 25 °C with shaking. The slides were then analyzed using a Genepix 400B Scanner and fluorescence intensity of each spot quantified with Genepix

Pro 6.0 software. The data was then imported in Microsoft Excel and analyzed. Based on control reactions, we chose 500 fluorescence counts as an arbitrary cut off for ubiquitylation activity. We then analyzed the data sets for differences in ubiquitylation between the low and high grade/stage breast cancers. We chose a >2-fold difference in fluorescence intensity at any particular spot as a cut off for a difference between groups. Using these parameters, we found several differences in ubiquitylation activity between low and high grade/stage breast tumors (Table 1). We then performed a literature search of the differentially ubiquitylated substrates and found that many have defined roles in processes such as DNA repair, cell adhesion, apoptosis, and tumor invasion.

Table 1. Ubiquitylation changes associated with breast tumor progression

UP	FUNCTION	DOWN	FUNCTION
RAD23A	Involved in post-replication repair of UV-damaged DNA	FGFR2	Metastasis, EMT
TANK	TRAF-associated NF-kappaB activator	FLT3	RTK, AML
FES/FPS	protein tyrosine kinase	SULF1	Inhibits tumor growth
ZNF364	BCA E3 ligase	AIM2	Induces apoptosis
ABL1	Proto-oncogene, cell adhesion, cell division	PRRG1	SH3 recently shown to bind ubiquitin
ZAP70	TCR signaling and development	PLK3	Mitosis, DNA damage checkpoint activation
PELI1	Adaptor protein of IRAK1	UCHL3	DUB
WEE1	Cell cycle kinase	BC001294	unknown
TFPI2	tissue factor pathway inhibitor2		
BC050434	unknown		

Low vs. High Grade Tumors

Validation of differentially ubiquitylated substrates. We next determined the efficacy of our assay in detecting substrates of ubiquitin conjugation activity, we randomly selected 50 substrates identified on the protein microarrays as ubiquitylated and performed a literature search for evidence for their regulation by ubiquitylation. In 34 cases, the proteins were found to be either regulated by ubiquitin-dependent proteolysis or were known binders of ubiquitin. This represented a nearly 20-fold enrichment over a randomly selected proteins displayed on the protein microarray. We next selected several substrates identified as ubiquitylated on the protein microarrays but not reported in the literature and attempted to validate their ubiquitylation *in vivo*. We first cloned the cDNAs for the putative ubiquitylated substrates (obtained as Image clones from Open Biosystems) into the expression vector pFlag-CMV2 (Sigma). The substrate expression plasmids were then co-transfected with a HA-ubiquitin expression plasmid into HEK293T cells. After 48 hrs, the cells were incubated in media containing 10 μM of proteasome inhibitor MG132 for 4-6 hrs to stabilize ubiquitylated intermediates. The cells were then lysed in 1% SDS to dissociate protein complexes (denaturing immunopreciptation), the buffer reconstituted to 1x RIPA buffer and extracts immunoprecipitated with anti-Flag antibodies (Sigma). The immunoprecipitates were then separated on SDS-PAGE gels and blotted to nitrocellulose membranes. The membranes were then probed with anti-HA antibodies (Covance) to detect ubiquitylated species. An example of this analysis is shown in Fig. 3. In all cases tested (4/4), the substrate was found to be a target of modification activity *in vivo*.

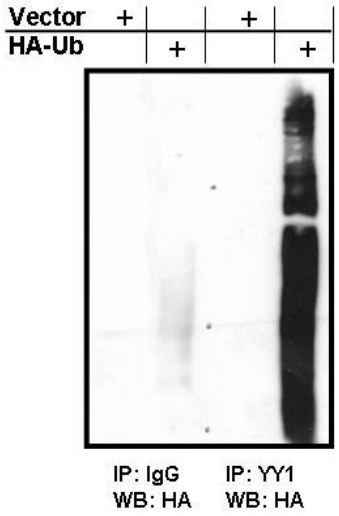

Figure 3. ***Validation of ubiquitin modifications in vivo.*** Ubiquitylation of YY1. HEK293T cells were transfected with HA-ubiquitin expression plasmids, extracts prepared, and YY1 immunoprecipitated. Western blot analysis using anti HA-antibodies show a smear of poly-ubiquitylated YY1.

Summary of SOW items completed

SOW #	Status	Comments
1	Completed	
2	Completed	
3	Completed	
4	Completed	
5	Completed	
6	Not-completed	Complemented by additional experiments in 8
7	Completed	
8	Completed	*In vivo* analysis performed
9	Completed	

C. Key research Accomplishments

1. Profiled ubiquitylation activity of primary breast tumor specimens on a proteome-wide scale on protein microarrays.
2. Identified differences in ubiquitylation activity associated with breast tumor progression.
3. Validated substrates that demonstrated differential ubiquitylation activity as ubiquitylated.

D. Reportable Outcomes

1. Methodology of protein ubiquitylation assay using protein microarrays as a substrate platform.
2. Characterization of differences in breast tumor ubiquitylation associated with breast cancer progression.

(See Appendix for submitted manuscript)

This methodology was presented at the 2008 Annual HUPO Meeting in Amsterdam, Netherlands.

E. Conclusions

We have found that key changes in ubiquitylation activity occur as breast tumors progress to advanced disease. The substrates of this activity include several proteins involved in key processes such as DNA repair, cell adhesion, apoptosis, and tumor invasion. The nature of these substrates together with our validation experiments adds confidence that this *in vitro* assay is faithfully identifying substrates of ubiquitylation activity *in vivo*. This assay could be applied to other significant problems in breast cancer, such as identify post-translational activity associated with breast cancer metastasis, the chemotherapeutic response, or associated with specific properties of the breast cancer.

G. References

None

H. Personnel Paid from Grant

Charles H. Spruck, Ph.D., Principle Investigator
Dahui Sun, Senior Research Associate

I. Appendices

Manuscript, Profiling Post-translational Modification Activity of Complex Biological Mixtures Using Protein Microarrays, by Sonia V. del Rincón, Jeff Rogers, and Charles Spruck.

J. Supporting Data

None

Profiling Post-translational Modification Activity of Complex Biological Mixtures Using Protein Microarrays

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Running Title: Post-translational modification profiling on protein microarrays

Abbreviations: PTM, post-translational modification; Ubl, ubiquitin-like; SUMO1, small ubiquitin-like modifier 1; NEDD8, neural precursor cell expressed and developmentally down-regulated 8.

Summary

Post-translational modifications (PTMs) can alter the expression, location, and function of proteins thereby achieving a greater functional diversity of the proteome. However, the evaluation of PTMs on a proteome-wide scale has proven technically difficult. To facilitate these analyses we have developed an assay system that uses protein microarrays as a platform to profile PTM activity in complex biological mixtures such as cellular extracts or pathological specimens. Reactions are performed 'on-chip' and modified substrates are identified via a fluorescent signal. Here, we apply this methodology to three molecularly complex PTMs; ubiquitylation, SUMOylation, and NEDDylation. We further validate this methodology by confirming the *in vivo* modification of several novel PTM substrates. This technique offers several advantages over currently used PTM detection methods, including ease of use, rapidity, scale, sample source diversity, and the ability to multiplex analyses. Furthermore, by allowing for the intrinsic enzymatic activities of cell populations or pathological states to be directly compared, this methodology has wide-spread applications for the study of PTMs in human diseases.

Introduction

Post-translational modifications (PTMs) are essential for the proper function of many proteins and dysregulation of this process plays a causative role in several human diseases (reviewed in (1)). Modifications ranging from the simple conjugation of a phosphate group to the complex addition of ubiquitin can drastically alter the function of a protein. For example, the conjugation of ubiquitin to a substrate can modulate its activity, target it for degradation, alter its cellular location, or determine its interaction with other proteins (2). However, despite the importance of these modifications in maintaining cellular homeostasis and promoting human diseases, identifying which proteins are modified by PTMs in mammalian cells on a proteome-wide scale has proven technically difficult.

To overcome these technical limitations, we explored the possibility of using protein microarrays as a platform to profile PTM activity. To date, the analysis of PTMs using protein microarrays has been limited to the phospho-proteome and profiling substrates of purified yeast enzymes (3-5). Phosphorylation is a 'simple' PTM compared to the complex enzymatic cascades required for many other modifications such as the conjugation of ubiquitin and ubiquitin-like (Ubl) proteins (*e.g*. SUMO1 and NEDD8). These modifications are mediated by multi-step enzymatic reactions involving an activating (E1), conjugating (E2), and ligase (E3) enzyme, that selectively transfers the PTM to substrates (1).

Experimental procedures

Extract Preparation. Cell (HeLa, mouse and human fibroblasts) and tumor (human breast) specimens were suspended in 20 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, 0.5 mM DTT, 5 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml pepsatin, and 1 μg/ml leupeptin, and the cells disrupted by brief sonication. The extracts were clarified by centrifugation for 15 min at 14,000 x *g* and then snap-frozen in liquid nitrogen until use. Rabbit reticulocyte lysate and HeLa S-100 fractions were purchased from Boston Biochem.

Recombinant Proteins. Human SCF^{Skp2} complexes were produced in Sf9 cells as described previously (6). Recombinant Cks1 was produced in bacteria and purified as described (7). Cyclin A-Cdk2 complexes were purchased from Invitrogen.

Antibodies. Antibodies used in substrate detection on protein microarrays included: anti-ubiquitin (Biomol, PW8805); anti-SUMO1 (Zymed, 33-2400); anti-NEDD8 (Zymed, 34-1400); and anti-phosphate (Zymed, 61-8300). For multiplex experiments, antibodies were labeled with Alexa Fluor NHS ester dyes AF488, AF532, AF594, and AF647 according to the manufacturer (Invitrogen). Antibodies used in substrate validation experiments included: anti-YY1 (Santa Cruz Biotechnology); anti-IGF-1R (Zymed); anti-HA (Covance); anti-Flag (Sigma); and anti-Myc (9E10, Santa Cruz Biotechnology).

PTM Profiling. Extracts (2-400 μg in a volume of 40 μl suspension buffer) were combined with 5 μM MG-132 and 4 μM of the relevant aldehyde (ubiquitin, SUMO1, or NEDD8; Boston Biochem), and incubated at 25 °C for 15 min. The reactions were then supplemented with modifier (1.25 μg/ml), biotin-labeled modifier (50 ng/ml), Tween-20 (0.1%), energy regenerating system (Boston Biochem), and 1 x reaction buffer (ubiquitylation, SUMOylation, NEDDylation buffers, Boston Biochem) in a 100 μl final volume. When antibodies were used for PTM detection, biotin-labeled modifiers were omitted from the reaction. For multiplex analysis of ubiquitin, SUMO1, NEDD8, and phosphorylation, 1 x ubiquitylation buffer was used. For SCF^{Skp2} experiments, reaction conditions were as described (6). The reaction mixtures were applied to Human ProtoArrays (Invitrogen), cover-slipped, and incubated at 37°C for 1 hr in a humidified chamber. The ProtoArrays were then washed for 10 min in PBS-Tween (0.1%, PBST) containing 1M NaCl, 2 x 10 min in PBST, and then incubated in Streptavidin Alexa Fluor 647 (100 ng/ml) for 1 hr at 25°C. For antibody detection experiments, the ProtoArrays were incubated with the relevant Alexa Fluor-labeled antibodies in PBST for 16 hrs at 4°C. The ProtoArrays were then washed 3 x 10 min in PBST and spun dry. Imaging was performed using a GenePix 4000B Slide Imager (Molecular Devices) and spots analyzed using GenePix Pro software. Gal files (which contain array production information–spot location, identification, and quantification) were downloaded from www.invitrogen.com and used with GenePix Pro software to analyze the median intensity of each spot.

Substrate Validation Experiments. HEK293T cells (ATCC) were either transfected with plasmids that express HA-tagged ubiquitin (ubiquitin), co-transfected with Myc-NEDD8 and Flag-PAK3 or Flag-MUSK (NEDD8), or non-transfected (SUMO1). Cells were then lysed in 1% SDS (containing 20 mM N-ethyl-maleimide (NEM)) to disrupt non-covalent interactions, and the buffer adjusted to 0.1% SDS, 0.5% NP40, 20 mM NEM, 50 mM Tris (pH 8.5), 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. For ubiquitin experiments, extracts were immunoprecipitated with anti-YY1 antibodies and ubiquitylation determined by Western blot analysis using anti-HA antibodies. For SUMO1, the extracts were immunoprecipitated with anti-IGF-1R antibodies and Western blots probed with anti-SUMO1 antibodies. For NEDD8, the extracts were immunoprecipitated with anti-Myc antibodies and Western blots probed with anti-Flag antibodies. In all cases, immunoprecipitation of extracts with IgG antibodies of the same species served as control.

Results

A schematic of our methodology is shown in Figure 1a. Reactions are performed 'on-chip' by overlaying the protein microarrays with a purified conjugation enzyme or extract prepared from a biological specimen (*e.g.* cell or pathological specimen) and any required factors. The protein microarrays are spotted with ~8,000 different human recombinant proteins in duplicate which serve as substrates for PTM conjugation. The substrates of PTMs are subsequently "tagged" by conjugation of a labeled-modifier (*e.g.* biotin-labeled) present in the reaction mixture. Following a stringent wash to remove non-covalent interactions, the conjugated substrates are then detected using 'binders' labeled with fluorescent dye (*e.g.* antibodies or strepavidin) and the fluorescent signals quantified using a standard slide reader.

As proof of principle, we first sought to determine whether complex PTMs could be profiled using protein microarrays as a platform. Using ubiquitylation as a model system, we profiled substrates of the ubiquitin ligase SCF^{Skp2}, which has a well-defined role in human tumorigenesis (5). SCF^{Skp2} is known to ubiquitylate several substrates including the cyclin dependent kinase inhibitor $p27^{Kip1}$ (6, 8). This reaction is molecularly complex and requires: *1*) substrate phosphorylation; *2*) association of the substrate with cyclin A-Cdk2 complexes; and *3*) the co-factor Cks1. We performed ubiquitylation reactions that included recombinant human SCF^{Skp2}, E1 and E2 enzymes, ATP regeneration system, ubiquitin, and biotin-labeled ubiquitin. The results of these experiments showed that $p27^{Kip1}$ could be efficiently ubiquitylated on the protein microarray by SCF^{Skp2}, and conjugation activity was only present when all the required

components were added to the reaction (Fig. 1b). In addition to $p27^{Kip1}$, several novel substrates of SCF^{Skp2} were also identified (Fig. 1b).

We next tried various perturbations of the ubiquitylation reaction to determine the optimal assay conditions. We evaluated different slide surface chemistries, reaction buffers, assay conditions, and detection methods. PATH slides (glass slides coated with nitrocellulose) proved to be superior to epoxy or hydragel slides in reducing background (data not shown). The addition of 0.1% Tween-20 to both the reaction and wash buffers also significantly limited background and had little effect on PTM conjugation activity. Furthermore, the addition of inhibitors of de-conjugating enzymes (*e.g*. ubiquitin-aldehyde) was found to increase PTM conjugation activity (data not shown).

Using optimized conditions, we next sought to determine whether this methodology could be used to profile the PTM activity of complex biological mixtures, such as cellular extracts or pathological specimens. We first tested rabbit reticulocyte lysates and S-100 fractions from human HeLa cells, both known to contain robust ubiquitylation activity. These extracts were found to efficiently ubiquitylate many substrates on the protein microarrays (reticulocytes- 249; S-100 fraction- 119 on the protein microarrays (Fig. 1c, Table 1). Summarized in Table 2 are 66 substrates of ubiquitylation activity detected with either rabbit reticulocyte lysate or HeLa S-100 fractions. Several of these substrates were previously shown to either bind ubiquitin (*e.g.* LIVIN (9), RNF4 (10), ZNF364 (11)), contain ubiquitin binding domains (*e.g.* CUED1C (12), RAD23A (13)), or are known substrates of ubiquitylation activity (*e.g.* FLT1 (14), JAK2 (15), INSR (16)), lending strong support that this methodology can

detect true substrates of ubiquitin conjugation activity. Similarly, whole-cell extracts prepared from various cultured cell lines of both human and mouse origin were also found to efficiently ubiquitylate many (~120) substrates on the protein microarrays (Table 1). Approximately half of these substrates were found to be consistently ubiquitylated by all the cellular extracts profiled. Collectively, these results demonstrate that this methodology could be used to profile the PTM activity of complex biological specimens of various species origin. Of note, the frequency of ubiquitylated substrates detected on the protein microarrays compares favorably to several proteome-wide analyses of ubiquitylation using mass spectrometry as a platform (17, 18).

A clinically relevant application of this methodology is comparative profiling, wherein disease-associated changes in PTM activity are compared, for example, in normal versus diseased state tissues or during disease progression. To this end, we next profiled the ubiquitylation activity of extracts prepared from human pathological breast tumor specimens that had been kept frozen at -80°C for >10 years. Remarkably, these extracts were found to contain robust ubiquitin-conjugation activity (Table 1); comparable to that observed for cellular fractions or whole-cell extracts prepared from cultured cells. Next, we pooled extracts from 5 low-grade and 5 high-grade tumors and applied to the protein microarrays. The results of this analysis showed that differences in ubiquitin conjugation activity occurred as tumors progressed to more advanced stages (Table 3). Interestingly, several of the differentially modified substrates were found to have defined roles in processes that are central to tumor progression (e.g. growth control, DNA repair, angiogenesis).

We next determined whether this methodology could be easily adapted to other complex PTMs, such as SUMO1 (small ubiquitin-like modifier 1) and NEDD8 (neural precursor cell expressed and developmentally down-regulated 8). SUMO1 and NEDD8 are conjugated to substrates in complex multi-step enzymatic cascades similar to but distinct from ubiquitylation (19). Reaction conditions used were similar to those used for ubiquitin (described above) except for the substitution of the relevant reaction buffer, E1 enzyme, aldehyde derivative, and biotin-labeled modifier (see Methods). The results of these experiments showed that HeLa cell extracts were capable of conjugating SUMO1 and NEDD8 to many substrates on the protein microarrays (Table 2). Of the SUMOylated substrates identified, HIPK3 (20) and RNF4 (10) were previously shown to bind SUMO1, and the majority of the remainder of substrates contained consensus SUMO targeting sequences (yKxE/D) (21). Although few substrates of NEDDylation have been identified (22-24), our screen did detect one putative NEDD8 target, LGALS3, that was previously identified using an alternative proteomic approach (24).

We next explored whether multiple PTM activities could be simultaneously analyzed on a single protein microarray. For these multiplex experiments, antibodies specific for ubiquitin, SUMO1, NEDD8, and phosphorylated amino acids were labeled with different fluorescent dyes of non-overlapping excitation and emission wavelengths (see Methods). The protein microarrays were first incubated with HeLa cell extracts supplemented with an ATP regenerating system, the various E1 enzymes, aldehydes, and modifiers, and subsequently probed with a mixture of the four PTM detection antibodies. The results of these experiments showed that multiple PTM activities could

be simultaneously profiled on the same protein microarray, with some substrates conjugated to multiple modifiers (Fig. 1d).

To determine the efficacy of this methodology in profiling PTM activity, we selected several PTM substrates that were identified on the protein microarrays but had not been previously reported in the literature and determined whether they were indeed modified *in vivo*. HEK293T cells were transfected with plasmids that express a tagged-modifier, the putative substrates then immunoprecipitated from the extracts, and PTM conjugation determined by Western blot analysis. YY1, a regulator of the ubiquitin ligase MDM2 that controls the ubiquitin-dependent proteolysis of p53 (25), was found to be poly-ubiquitylated in HEK293T cells (Fig. 2a). Additionally, insulin-like growth factor 1 (IGF-1R), a receptor tyrosine kinase that mediates IGF1 signaling (25), was found to be conjugated to SUMO1 *in vivo* (Fig. 2b). Furthermore, $p21^{Cip1}$-activated kinase 3 (PAK3), which is associated with non-syndromic mental retardation in humans (26), and MUSK, a receptor tyrosine kinase that plays a role in neuromuscular junction organization (27), were both found to be conjugated to NEDD8 (Fig. 2c). These results confirm that the PTM activity detected on the protein microarrays was indeed present *in vivo*.

Discussion

Currently used techniques for profiling PTM activity on a proteome-wide scale have included two-hybrid and high-copy suppressor screens in yeast, and mass spectrometry (24, 28-30). However, several limitations exist with these techniques. For example, mass spectrometry-based analyses of PTMs is often complicated by: *1*) low substrate abundance, a characteristic of many ubiquitylated proteins, and/or a sub-stoichiometric level of the PTM; *2*) the labile nature of many PTMs, making their preservation through biochemical purification, separation techniques, fragmentation, and analysis problematic, especially if native conditions are required leaving substrates vulnerable to de-conjugating enzymes; *3*) the adverse effects of certain PTMs on proteases, ionization, and detection efficiency; and *4*) multi-site or multi-species modifications, which could make data interpretation problematic.

Our methodology overcomes many of these limitations and provides several distinct advantages. Since the assay measures the intrinsic enzymatic activity of a specimen, it is less sensitive to substrate concentration and sub-stoichiometric modifications are easily detected. The reactions can be performed using crude extracts, eliminating elaborate purification protocols that may promote PTM de-conjugation. The methodology can also be easily adapted to the analysis of other PTMs, and multiplexing PTM analyses on the same protein microarray is possible and interpretation of data is not overly complicated. Additionally, the assay can be performed with “natural” modifiers (if antibodies are used for detection) circumventing any potential biases caused by labeled-modifiers. Furthermore, the reactions are

sensitive (can be performed with as little as 2 μg of extract) and analysis completed in a single day.

However, there are several potential limitations with our assay. Firstly, the latest generation protein microarrays display ~8,000 human proteins, representing only 1/3 of the proteome. Secondly, a portion of the substrates on the protein microarrays could be misfolded. Thirdly, being an *in vitro* assay, various *in vivo* regulations (*e.g.* temporal or spatial regulations) will likely be lost during extract preparation. Finally, information regarding the type or site of PTM attachment to substrates cannot be ascertained however, this information could possibly be obtained by coupling the analysis with mass spectrometry.

An important potential application of this methodology is the ability to compare PTM activities between different cell populations or pathological states. In combination with genetic mutants, small molecule perturbants, or RNAi technology, this methodology could help to define both substrate-specific and global aspects of PTMs. Considering that dysfunction of PTMs play a critical role in the initiation and progression of a number of human pathological states, this methodology is an important step forward in the field of proteomics because it allows for the identification of aberrant PTM patterns in human diseases using cell extracts, specific organelles, or fresh or archival tissue specimens. Modified cell lines, disease model systems and specialized tissues all lend themselves well to PTM profiling using this methodology, with the ultimate goal of furthering our understanding of disease states and identifying novel therapeutic targets for their treatment.

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Figure Legends

Figure 1. ***PTM profiling on protein microarrays. A, Schematic of protein microarray-based profiling of PTM activity.*** Protein microarrays containing ~8,000 recombinant human proteins spotted onto glass slides in duplicate provide a platform for assessing PTM activity. Reactions are performed 'on-chip' using purified enzymes or extracts prepared from cell or pathological specimens, ATP regenerating system, modifier, and labeled-modifier (*e.g.* biotin-ubiquitin). PTM conjugation is then detected by incubating the slide with fluorescent-labeled 'binders' (*e.g.* streptavidin or antibodies) and quantified using a fluorescent slide reader. ***B, Profiling substrates of the SCF^{Skp2} ubiquitin ligase.*** Purified recombinant SCF^{Skp2} was applied to the protein microarrays in the presence of required co-factors (cyclin A-Cdk2 and Cks1). Insets show ubiquitylation of known SCF^{Skp2} substrate p27^{Kip1} on the protein microarray (red circle) and novel substrates (blue circles). SCF core complex (minus substrate recognition component Skp2) was used as control. ***C, Profiling ubiquitin conjugation activity of cellular extracts.*** Rabbit reticulocyte lysate or S-100 fractions from HeLa cells were applied to protein microarrays in the presence of ATP regeneration system, ubiquitin, and biotin-labeled ubiquitin. Ubiquitylated substrates (blue circles) and fiducials (positive controls for fluorescence, yellow circles) are highlighted. Similar results were obtained using whole cell extracts from mouse and human cell lines, and human tumor specimens. ***D, Multiplex PTM analysis.*** Reactions were performed with HeLa cell extract that supported the simultaneous conjugation of ubiquitin, SUMO1, NEDD8, and phosphate to substrates on the protein microarray. The four modifications were then

detected by probing the protein microarrays with antibodies specific for each modification that had been labeled with various fluorescent dyes of non-overlapping excitation and emission wavelengths (see Methods).

Figure 2. ***In vivo confirmation of novel PTM substrates identified on protein microarrays. A, Ubiquitylation of YY1.*** HEK293T cells were transfected with plasmids that express HA-ubiquitin, endogenous YY1 protein then immunoprecipitated from the extracts, and conjugation to ubiquitin determined by Western blot analysis with anti-HA antibodies (left). Immunoprecipitation efficiency was determined by probing blots with anti-YY1 antibodies (right). Immunoprecipitation with IgG antibodies of the same species was used as control. ***B*, *SUMOylation of IGF-1R.*** Endogenous IGF-1R was immunoprecipitated from extracts prepared from HEK293T cells and conjugation to SUMO1 determined by Western blot analysis with anti-SUMO1 antibodies. Immunoprecipitation efficiency was determined with anti-IGF-1R antibodies (right). ***C*, NEDDylation of MUSK and PAK3.** HEK293T cells were transfected with plasmids that express Flag-MUSK or Flag-PAK3 with or without Myc-NEDD8. Extracts were immunoprecipitated with anti-Myc or IgG antibodies of the same species (control) and conjugation to NEDD8 determined by Western blot analysis with anti-Flag antibodies.

Table 1. Ubiquitylated substrates profiled using cell extracts and tumor samples.

BC066929[12]	CCDC55[1]	FGFR3[2]	LOC370014[1235]	OR1Q1[1]	RPL41[1]	TSPAN17[12345]
XM_375359[1]	CCDC97[1]	FGFR4[1]	LOC440295[1]	PAK1[4]	RPS6KA1[2]	TSPO[4]
ABI1[1]	CDC2[1]	FGR[123]	LOC51491[1]	PAK3[1]	RPS6KA4[145]	TTK[14]
ABL1[12]	CDIPT[1]	FLT1[14]	LOC51765[45]	PBK[1]	RPS6KA5[145]	TYRO3[14]
ACBD6[12]	CDK2/cyclinA[1]	FLT3[1234]	LOC55319[1]	PDAP1[1]	RPS6KB1[4]	UBADC1[12345]
ACVR1B[14]	CDK9/cyclinT1[1]	FLT4[14]	LOC645591[4]	PDCL[13]	SCGB1C1[4]	UBE2C[1]
AHCYL1[2]	CETN3[12]	FRK[14]	LOC83786[1]	PDGFRA[1234]	SCYE1[1]	UBE2E2[15]
ADRBK2[14]	CHEK1[1]	G3BP1[1]	LOC84714[4]	PELI1[1]	SDCCAG3[1]	UBE2H[25]
AFF4[1]	CHERP[4]	GABRA3[1]	LYN[123]	PFDN5[45]	SEPT1[1]	UBE2O[124]
AIM2[4]	CHKA[4]	GADD45G[12]	MAGEB1[1]	PIM1[125]	SEPT5[1]	UBE2S[125]
AKT1[2]	CHUK[1]	GBA[4]	MAP2[1]	PIM2[145]	SERPINA3[1]	UBE3A[1235]
ANKHD1[1]	CLK3[4]	GMNN[1]	MAP2K2[1]	PKN2[14]	SGK[45]	UBQLN2[2]
ANKRD13A[1235]	CNOT7[12]	GNGT1[4]	MAP2K3[145]	PLK1[14]	SGK3[4]	UBXD1[13]
ANKRD13D[12345]	COPE[2]	GRK4[145]	MAP2K6[1]	PLK3[14]	SGPL1[1]	UBXD8[1]
ANKS4B[1]	COPZ1[15]	GRK6[14]	MAP3K2[14]	POMZP3[1]	SH3BP5[1]	VRK3[1]
APOBEC4[1]	CSAG1[1]	GSDMDC1[12]	MAP3K9[1]	PRKCA[145]	SIP1[4]	WDFY1[4]
ARL6IP4[1]	CSF1R[123]	GSK3B[14]	MAP4K5[145]	PRKCG[1]	SLAIN2[3]	WDR1[1]
ASCC2[1]	CSNK1D[14]	GYG2[1]	MAPK11[12]	PRKCH[1]	SLC6A13[1]	WEE1[1]
ASMTL[4]	CSNK1E[1]	HCK[12]	MAPKAPK3[1]	PRKCI[1]	SMCR7[15]	WIBG[2]
ATF6[1]	CSNK1G1[14]	HGS[2]	MAPKAPK5[12]	PRKG2[14]	SPATS2[1]	YES1[123]
ATP6V1G1[1]	CSNK1G3[4]	HOMER2[13]	MARK2[1]	PRKX[14]	SPDEF[1]	YY1[2]
ATXN3[12345]	CSNK2A1[1]	HPCAL1[4]	MATK[1]	PRRG1[1235]	SRMS[4]	ZAP70[1]
AURKB[1]	CSNK2A2[14]	HPGD[1]	MERTK[14]	PSMD4[12345]	SRPK1[1]	ZMYM5[123]
BIN1[4]	CUEDC1[12345]	IFI44L[4]	MET[1]	PSRC1[1]	SRPK2[1]	ZNF313[1]
BIRC7[124]	CXorf48[2]	IGF1R[123]	MINK1[4]	PTK2[1]	SRPK3[1]	ZNF364[12345]
BLK[1]	DAPK1[4]	IKBKB[1]	MPG[1]	PTPN5[1]	STIP1[1]	ZNF434[4]
BMX[1]	DAPK2[1]	ING5[1]	MSRB3[4]	RAB20[1]	STK17A[14]	
BRAF[4]	DHX32[1]	INSR[14]	MST1R[14]	RABEP2[25]	STK22D[1]	
BTK[14]	DNAJB2[2]	INSRR[145]	MYL5[14]	RAD23A[12345]	STK25[1]	
C10orf97[123]	DNAJC8[14]	IRAK4[13]	MYLK2[14]	RAF1[4]	STK3[145]	
C11orf52[1]	DYRK3[14]	IRF3[1]	NAP1L2[1]	RASGRP3[12]	STK4[14]	
C11orf53[1]	EIF5[1]	IRS1[2]	NBPF1[4]	RASL11B[2]	STRAP[1]	
C1orf165[1]	EPHA1[12345]	ITK[1]	NDUFB6[4]	RBCK1[1]	SULF1[45]	
C1orf91[1]	EPHA2[4]	JAK2[145]	NECAP1[1]	RBM34[1]	TAOK2[145]	
C20orf11[1]	EPHA5[14]	JAK3[14]	NECAP2[1]	RET[14]	TAOK3[145]	
C2orf13[45]	EPHA8[14]	KDR[1234]	NEK1[14]	RHBDD1[2]	TARBP2[1]	
C9orf78[1]	EPHB3[4]	KIAA1900[1]	NEK2[1]	RIOK3[12]	TBK1[1]	
CACNB1[1]	EPHB4[13]	KIF2C[1]	NEK4[1]	RNF34[1345]	TCP11[45]	
CALCOCO1[2]	ERBB2[125]	KIF3B[1]	NEK6[1]	RNF111[12345]	TCP11L1[15]	
CAMK1[123]	ERBB4[4]	KIT[1]	NEK9[145]	RNF126 [235]	TEC[1]	
CAMK1D[1]	FAM126B[2]	LCK[1]	NFKBIB[1]	RNF128 [2]	TEK[14]	
CAMK2N1[1]	FAM112B[1]	LMNA[1]	NGLY1[2]	RNF130 [2]	TMEM139[2]	
CAMK2N2[12]	FAM50A[1]	LOC10572[2]	NMT1[1]	RNF185[1235]	TNIK[1]	
CAMKIIalpha[1]	FES[1]	LOC112860[4]	NR4A1[1]	RNF4[1245]	TNIP2[125]	
CAMKIIdelta[1]	FER[4]	LOC115460[1]	NTRK1[1]	ROR1[1]	TOM1[125]	
CASQ2[1]	FGF21[2]	LOC120376[1]	NTRK2[1]	ROR2[45]	TOM1L2[12345]	
CAT[1]	FGFR1[12]	LOC121457[4]	NTRK3[1]	ROS1[1]	TRIM44[1]	
CCDC12[1]	FGFR2[1]	LOC284440[4]	NUAK1[1]	RPAIN[12]	TRIM52[12345]	

[1]Rabbit reticulocyte lysates, [2]Mouse embryonic fibroblasts, [3]Human foreskin fibroblasts, [4]HeLa cell S-100 fractions, [5]Breast tumor specimens.

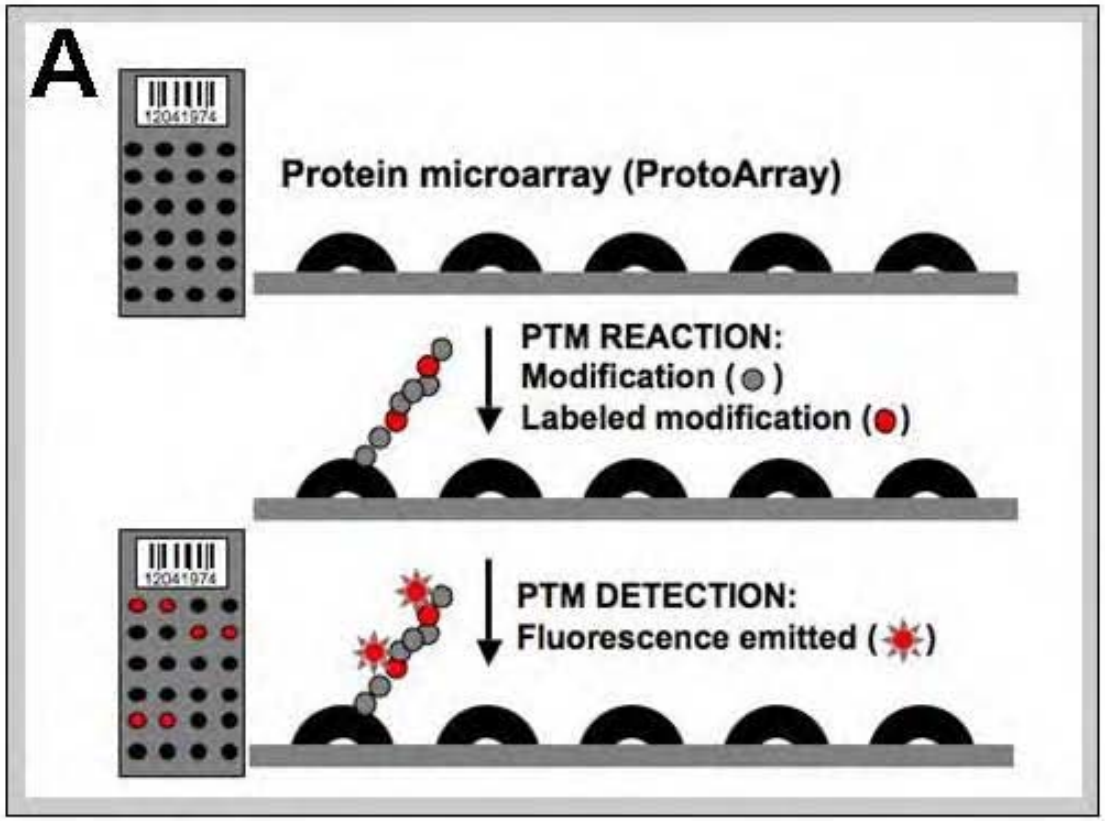
TABLE 2. Ubiquitin, NEDD8, or SUMO1 conjugated proteins identified on protein microarrays.

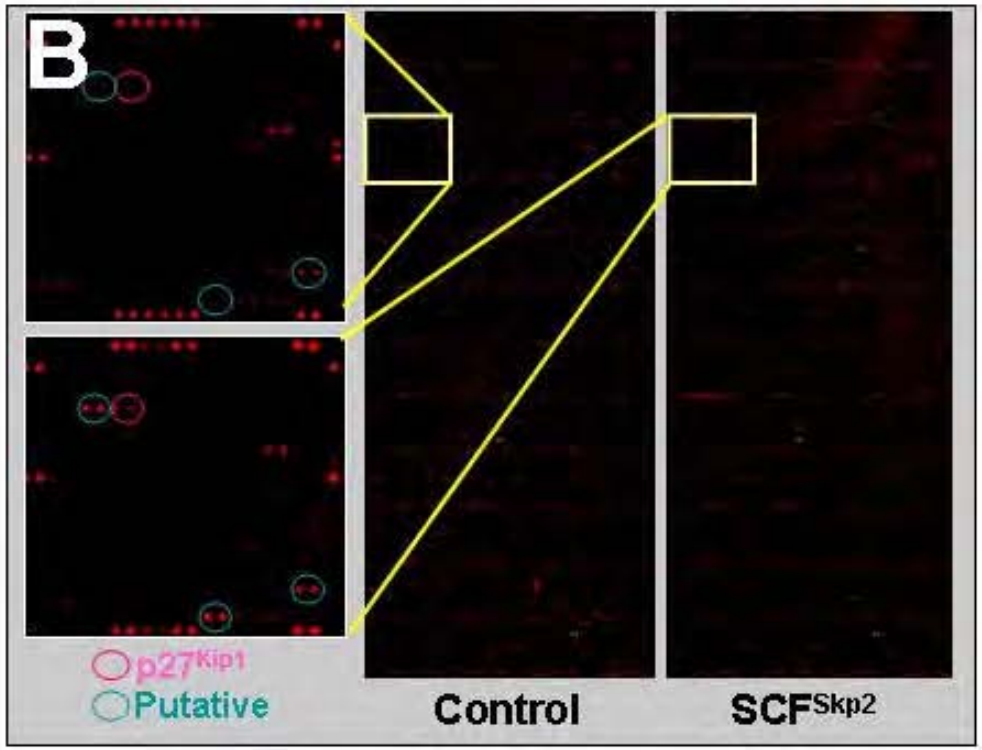
UBIQUITYLATION				NEDDYLATION		SUMOYLATION
UPS-Associated		UBIQUITIN SUBSTRATES		NEDD8 SUBSTRATES		SUMO1 SUBSTRATES
ACVR1B*	MST1R*	ADRBK2	MYLK2	ANKHD1	LSM3	*ADRBK1*
ATXN3	PDGFRa*	ANKRD13D	NEK1	ANKRD13D	MAP3K10	*AKT2*
BTK*	PLK1*	CSNK1D	NEK9	ANKRD17	MAP3K11	CDK5
CAT*	PLK3*	CSNK1G1	**PIM2**	ANKRD39	MAP3K9	*CENPB*
CUEDC1	PRKCa*	CSNK2A2	**PKN2**	ANKS4B	MATK	*COPE*
FLT1*	PRKCg*	DYRK3	PRKX	BTK	MCC	*FES*
FLT3*	PSMD4	EPHA1	**ROS1**	CCDC69	MINK1	FGFR3
GSK3-beta*	RAD23A	EPHA5	**RPS6KA4**	CENPB	MST1R	*FGR*
INSR*	RET*	**FLT4**	**RPS6KA5**	CETN3	NAP1L1	*FYN*
ITK*	RNF4	FRK	STK3	CHEK1	NFKBIB	*HIPK3*
JAK2*	RNF111	GRK4	STK4	CSNK2A1	OTUD6B	HK1
JAK3*	TTK*	GRK6	STK17A	CUEDC1	PAIP2	*ING3*
LIVIN	UBADC1	INSRR	**TAOK2**	CXorf48	PAK1	JAK3
MAP3K2*	UBE2O	KIAA1900	**TAOK3**	DIXDC1	PAK3	*LCK*
MAP4K5*	ZNF364	MAP2K3	TEK	EIF2B2	PBK	LENG4
		MCAK	**TRIM52**	EPHA1	PDCL	*MAPKAPK5*
		MERTK	**TSPAN17**	EPHB4	PEX19	*MERTK*
		MYL5	TYRO3	FAIM	PIM1	*PAK3*
				FGR	PRKCA	*PBK*
				GCC1	PRKCE	*RBCK1*
				GOPC	PSCD1	*RIPK2*
				GSDMDC1	RAD23A	RNF4
				LCK	RGS20	*RPS6KA3*
				LGALS3	RPS6KB1	STK3
				LMNA	TOM1L2	*VPS29*
				LOC126382	TRIM44	ZMYM5
				LOC57596	UBOX5	

Substrates shown for ubiquitin are common to both rabbit reticulocyte lysate and HeLa S-100 fractions. Underlined, E3-associated; *, known substrate of ubiquitylation; **Bold**, high homology to proteins known to be ubiquitylated; Highlighted, substrates also common to human fibroblasts; *Italics*, SUMO1 substrates containing SUMO consensus sequences (yKxE/D). UPS, ubiquitin proteasome system.

Table 3. Changes in protein ubiquitylation associated with human tumor progression.

INCREASED	PROTEIN FUNCTION	DECREASED	PROTEIN FUNCTION
RAD23A	Involved in post-replication repair of UV-damaged DNA	ATXN3	Machado-Joseph disease gene product, nucleotide excision repair
MARK2	Microtubule binding protein, Ser/Thr kinase	FLT4	VEGFR3, angiogenesis, RTK
TRIM52	E3 ligase activity	SULF1	Inhibits tumor growth
ZNF364	BCA E3 ligase	PIM2	Oncogene, Ser/Thr kinase
TSPAN17	FBXO23, cell adhesion	PRRG1	SH3, binds ubiquitin
DDR2	RTK, metastasis	NEK9	Maintains proper G_1 and S progression
MYLK2	Cytoskeletal regulation, cell motility, Ser/Thr kinase	FGF21	Growth factor activity
ALK	Anaplastic lymphoma kinase, RTK, tumor aggressiveness	SLAIN2	Unclassified
TFPI2	Tissue factor pathway inhibitor 2	CAMKK2	Calcium signaling
BC050434	Unknown	PLK3	Mitosis, DNA damage checkpoint activation


A
Protein microarray (ProtoArray)
PTM REACTION:
Modification ( )
Labeled modification ( )
PTM DETECTION:
Fluorescence emitted ( )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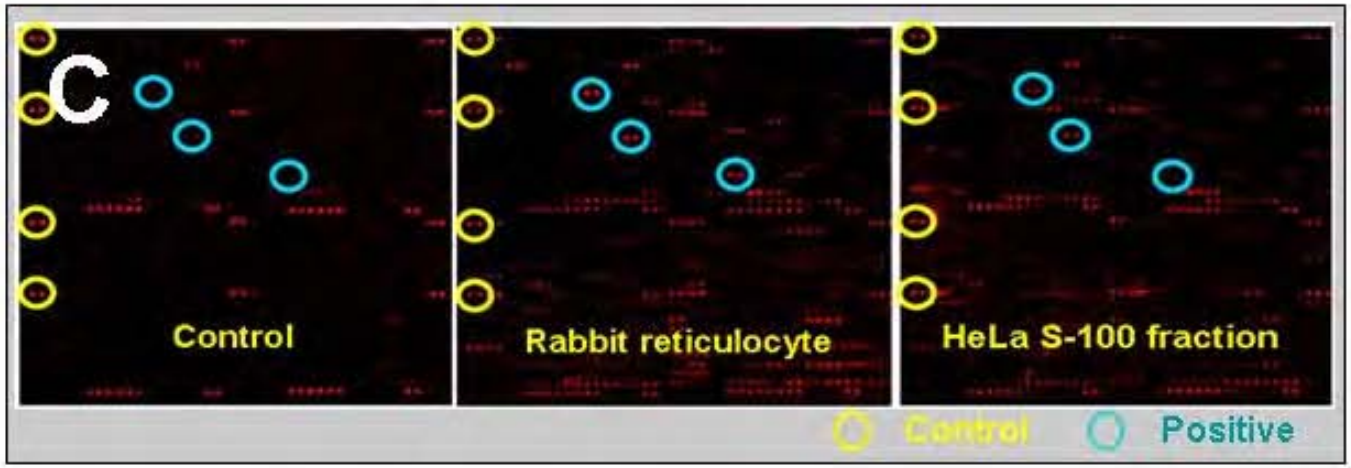
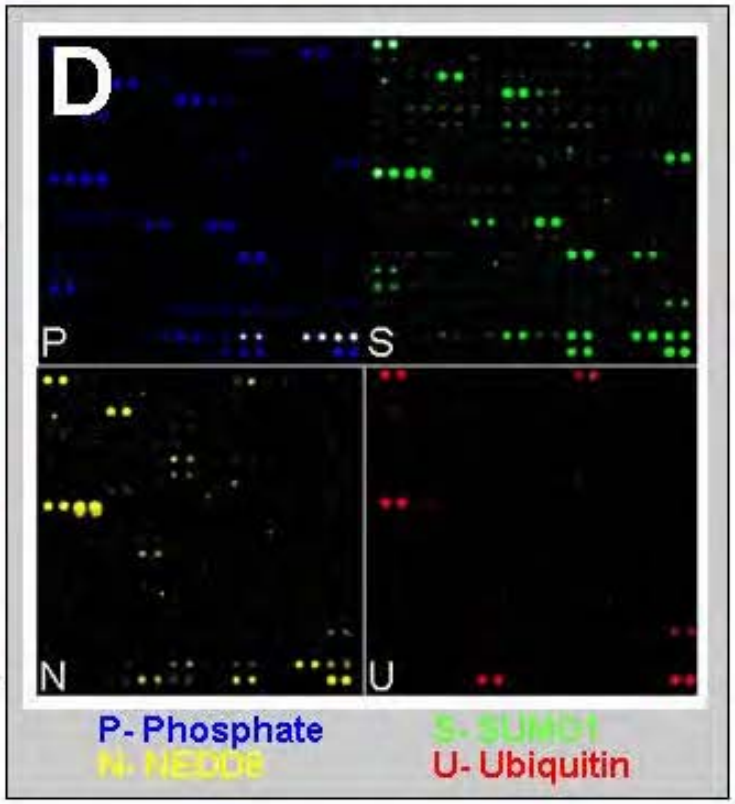
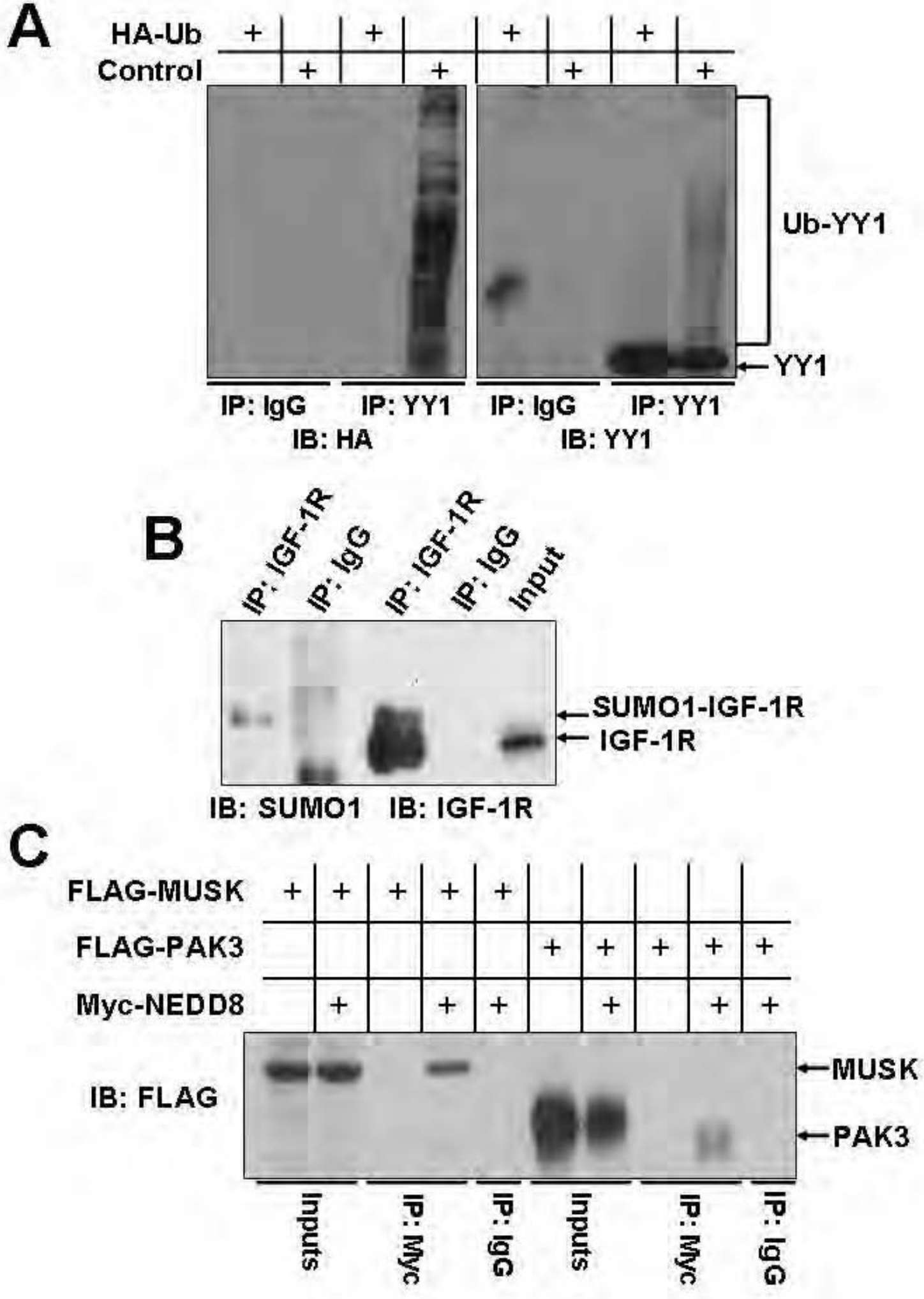
Putative
Control
SCFSkp2


C
Control
Rabbit reticulocyte
HeLa S-100 fraction
Control
Positive


D
P
S
N
U
P- Phosphate
S- SUMO1
N- NEDD8
U- Ubiquitin


A
HA-Ub
Control
Ub-YY1
YY1
IP: IgG
IP: YY1
IP: IgG
IP: YY1
IB: HA
IB: YY1
B
IP: IGF-1R
IP: IgG
IP: IGF-1R
IP: IgG
Input
SUMO1-IGF-1R
IGF-1R
IB: SUMO1
IB: IGF-1R
C
FLAG-MUSK
FLAG-PAK3
Myc-NEDD8
IB: FLAG
MUSK
PAK3
Inputs
IP: Myc
IP: IgG
Inputs
IP: Myc
IP: IgG