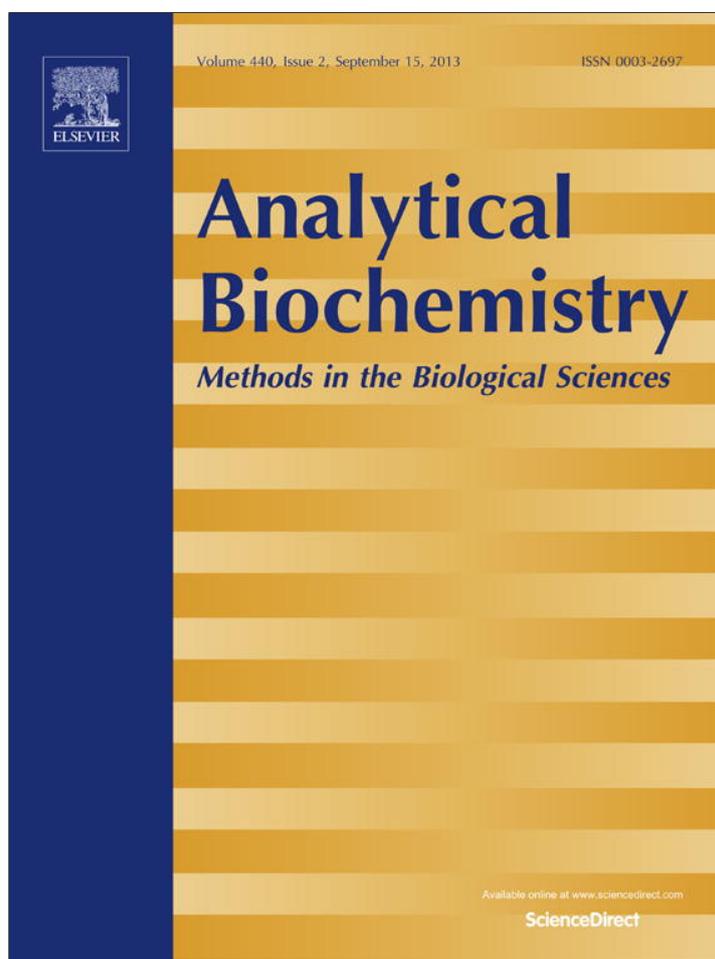


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## Analytical Biochemistry

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## An immunoaffinity purification method for the proteomic analysis of ubiquitinated protein complexes <sup>☆</sup>



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## ARTICLE INFO

## Article history:

Received 14 January 2013

Received in revised form 25 April 2013

Accepted 13 May 2013

Available online 3 June 2013

## Keywords:

Ubiquitin proteome

Protein isolation

FK2

Immunoprecipitation

Proteomics

Mass spectrometry

## ABSTRACT

Protein ubiquitination plays an important role in the regulation of many cellular processes, including protein degradation, cell cycle regulation, apoptosis, and DNA repair. To study the ubiquitin proteome we have established an immunoaffinity purification method for the proteomic analysis of endogenously ubiquitinated protein complexes. A strong, specific enrichment of ubiquitinated factors was achieved using the FK2 antibody bound to protein G-beaded agarose, which recognizes monoubiquitinated and polyubiquitinated conjugates. Mass spectrometric analysis of two FK2 immunoprecipitations (IPs) resulted in the identification of 296 FK2-specific proteins in both experiments. The isolation of ubiquitinated and ubiquitination-related proteins was confirmed by pathway analyses (using Ingenuity Pathway Analysis and Gene Ontology-annotation enrichment). Additionally, comparing the proteins that specifically came down in the FK2 IP with databases of ubiquitinated proteins showed that a high percentage of proteins in our enriched fraction was indeed ubiquitinated. Finally, assessment of protein–protein interactions revealed that significantly more FK2-specific proteins were residing in protein complexes than in random protein sets. This method, which is capable of isolating both endogenously ubiquitinated proteins and their interacting proteins, can be widely used for unraveling ubiquitin-mediated protein regulation in various cell systems and tissues when comparing different cellular states.

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The regulation of proteins involves posttranslational modifications (PTMs)—such as phosphorylation and ubiquitination—to control their activity, localization, stability, and assembly into protein complexes. Ubiquitination was shown to play an increasingly important role in many cellular processes, including protein degradation, cell-cycle regulation, apoptosis, and DNA repair [1–4]. The highly conserved 76-amino-acid protein ubiquitin can be covalently attached to a lysine residue in protein substrates via an E1–E2–E3 enzymatic cascade. The E1-activating enzyme uses an ATP molecule to form a high-energy thioester bond between the C-terminus of ubiquitin and an internal cysteine residue. Next, the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2). Finally, an E3 ubiquitin ligase is needed to transfer the ubiquitin to a lysine on the protein substrate [5]. Following addition of a single ubiquitin to a protein substrate

(monoubiquitination), further ubiquitin molecules can be conjugated to the first, resulting in a ubiquitin chain (polyubiquitination). Distinct chain linkages can be formed at all seven internal lysine residues of ubiquitin (K6, K11, K27, K29, K33, K48, and K63) and at its N-terminus (M1). Next to homotypic ubiquitin chains, which have a single linkage type, heterotypic chains exist containing mixed linkages within the same ubiquitin chain. The most abundant chain linkage, through K48, is primarily a signal for proteasomal degradation, while linkage through K63 has a well-established role in cell signaling. In comparison, relatively little is known about the other chain-linkage types [6,7]. The ubiquitination process is highly dynamic and reversible, as illustrated by the existence of at least 80 different deubiquitinating enzymes (DUBs), which can remove ubiquitin moieties from protein substrates [8–11].

The ubiquitination status of specific proteins can be studied by immunoblotting. To study the ubiquitin proteome, also known as the ubiquitinome, on a global scale, mass spectrometry (MS)-based proteomics is used. Since presumably not all proteins are ubiquitinated and as from those that are ubiquitinated only a fraction is usually modified at a given time, methods to enrich for these proteins are necessary to study them by MS [12].

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Initially, strategies for the isolation of ubiquitinated proteins were primarily based on the ectopic overexpression of tagged ubiquitin combined with a purification protocol incorporating at least one denaturing step to remove nonubiquitinated interactors [13–22]. Despite its proven use, it cannot be excluded that exogenous overexpression of tagged ubiquitin may lead to biased incorporation into monoubiquitination or certain ubiquitin chains and/or may interfere with, e.g., the stability, activity, and localization of ubiquitinated proteins. Additionally, tagged ubiquitin is not easily introduced into tissues and cell types that are difficult to transfect, such as primary and quiescent cells, limiting its applicability.

Alternatively, some studies have made use of ubiquitin-binding domains (UBDs) or anti-ubiquitin antibodies to isolate endogenously ubiquitinated proteins [13,23–30] or peptides [31–35], thereby overcoming the above-mentioned limitations.

Proteolytic digestion of ubiquitinated proteins with trypsin generates a specific diglycine (Gly–Gly) remnant on the  $\epsilon$  amino group of the ubiquitinated lysine. This remnant causes a distinct mass shift of the peptide mass that can be used to precisely identify and localize the site of ubiquitination in the peptide. The recent development of specific antibodies directed against diglycine-modified peptides enables the efficient isolation of these peptides and the identification of ubiquitination sites by MS [31–35]. Because of the denaturing step that is necessary before trypsin digestion, the identification of nonubiquitinated interactors using this approach is minimal.

Most of the methods for endogenously ubiquitinated protein isolation were performed under nondenaturing conditions, which are necessary for efficient binding of the UBDs or antibodies to the ubiquitinated proteins. Although the ubiquitinated protein pool might be exposed to residual DUB and proteasome activity under these conditions, it has the additional advantage of allowing the study of protein complexes. Most biological processes mainly rely on intact, functional protein complexes [36], whose subunits, however, are not necessarily all modified by ubiquitin. Therefore, to study the biologically relevant protein modules, including the nonubiquitinated interactors, we started out by comparing three different methods for the isolation of endogenously ubiquitinated protein complexes under nondenaturing conditions. The most efficient approach of the three, a method based on FK2 antibody immunoprecipitation (IP), was further optimized and characterized for proteomic applications.

## Methods

### Cell culture

HeLa and XP2OS cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin (Gibco) at 37 °C and 5% CO<sub>2</sub> in a humidified cell culture incubator. Cells were grown to 90% confluence in 9-cm dishes for all experiments.

### Isolation of endogenously ubiquitinated protein complexes

Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and harvested by scraping in 500  $\mu$ l lysis buffer. Either RIPA lysis buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) or tandem ubiquitin-binding entities (TUBEs) lysis buffer (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol) was used, both supplemented with 15  $\mu$ M MG-132 (Enzo Life Sciences), 10 mM *N*-ethylmaleimide (Sigma), and Complete protease inhibitor cocktail (Roche). Lysates were incubated on ice for 10 min and centrifuged at 16,000g and 4 °C for 15 min to remove remaining cell debris and DNA. Cleared

lysates were added to the various purification resins: 100  $\mu$ l 50% slurry of agarose–TUBEs (LifeSensors), UbiQapture-Q matrix (Enzo Life Sciences), FK2 beads, or control beads. The FK2 beads and control beads were prepared by incubating 100  $\mu$ l 50% protein G-beaded agarose slurry (Pierce) with 87.5  $\mu$ g of FK2 antibody (Enzo Life Sciences) or random mouse IgG (Millipore) for 40 min at room temperature. For cross-linking FK2 to the protein G beads the Pierce Crosslink Immunoprecipitation Kit was used. All resins were washed two times with lysis buffer before use. After incubating with lysates for 4 or 16 h at 4 °C, the nonbound fractions were collected and the resins were washed four times with 10 bead volumes of lysis buffer. Bound protein complexes were eluted in 1 bead volume of 2 $\times$  Laemmli buffer for 5 min at 98 °C and loaded onto a 4–20% SDS–PAGE precast gradient gel (Invitrogen).

Three different elution buffers compatible with a concentration step using centrifugal filters (Amicon Ultra; Millipore) were tested for releasing ubiquitinated protein complexes from the FK2 beads: 8 M urea buffer (8 M urea, 300 mM NaCl, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5% NP-40; pH 8), 2% SDS, or 0.1 M glycine, pH 2. Proteins were eluted in four consecutive steps by shaking for 5 min at 1250 rpm in an Eppendorf Thermomixer in 2 bead volumes of elution buffer.

### Mass spectrometric analysis

Endogenously ubiquitinated protein complexes were isolated from one 90% confluent 9-cm dish of HeLa cells for each experiment using the FK2 beads as described above. SDS–PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol. Protein alkylation with iodoacetamide can produce a 2-acetamidoacetamide covalent adduct to lysine residues, which has an atomic composition and mass identical to that of the diglycine remnant present at ubiquitinated lysines after trypsin digestion [37]. To prevent false-positive identification of ubiquitinated peptides we used deuterium-labeled iodoacetamide (98%; D4; Cambridge Isotope Laboratories) for alkylation. Proteins were subsequently digested with trypsin (Promega; sequencing grade), as described previously [38]. Nano-flow liquid chromatography–tandem mass spectrometry (LC–MS/MS) was performed on an 1100 Series capillary LC system (Agilent Technologies) coupled to an LTQ–Orbitrap XL mass spectrometer (Thermo) operating in positive mode. Peptide mixtures were trapped on a ReproSil-C18 reversed-phase column (Dr Maisch GmbH; 1.5 cm  $\times$  100  $\mu$ m, packed in-house) at a flow rate of 8  $\mu$ l/min. Peptides were separated on a ReproSil-C18 reversed-phase column (Dr Maisch GmbH; 15 cm  $\times$  50  $\mu$ m, packed in-house) using a linear gradient of 0–80% acetonitrile (in 0.1% formic acid) for 170 min at a constant flow rate of 200 nl/min using a splitter. The elution was directly sprayed into the electrospray ionization source of the mass spectrometer. Spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode.

Raw mass spectrometry data were analyzed using the label-free algorithm of the MaxQuant software (version 1.3.0.5) with a 3-min time window for the match between runs option [39]. A false discovery rate (FDR) of 0.01 for proteins and peptides and a minimum peptide length of 6 amino acids were set. A site-specific FDR of 0.05 was applied separately. The Andromeda search engine [40] was used to search the MS/MS spectra against the UniProt human database (release April 2013) concatenated with the reversed versions of all sequences. A maximum of two missed cleavages was allowed. The precursor mass tolerance was set to 15 ppm, the fragment mass tolerance was set to 0.6 Da. The enzyme specificity was set to trypsin. Cysteine carbamidomethylation-2D was set as a fixed modification, whereas methionine oxidation and lysine ubiquitination were set as variable modifications. Before data analysis,

known contaminants and reverse hits were removed from the protein lists.

#### Data analysis

The Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, [www.ingenuity.com](http://www.ingenuity.com)) was used to identify canonical pathways associated with the FK2-specific proteins identified in the mass spectrometric analysis. Protein–protein interactions within the FK2-specific protein group were assessed and visualized using the GeneMANIA [41] plug-in of Cytoscape (version 2.7.0) [42]. Of the 296 FK2-specific proteins, 6 were not recognized by the GeneMANIA plug-in and were therefore excluded from this analysis. Protein interaction networks were built based on physical interactions only.

#### Protein concentration, immunoblotting, and silver staining

Protein concentration was determined using the Thermo Scientific Pierce BCA Protein Assay Kit according to the manufacturer's protocol.

For immunoblotting we used a rabbit polyclonal against ubiquitin (Code Z0458; Dako) and a mouse monoclonal against monoubiquitinated and polyubiquitinated conjugates (FK2; Enzo Life Sciences). The anti-K48-linked polyubiquitin antibody (Apu2.07) and the anti-K63-linked polyubiquitin antibody (Apu3.A8) were kindly provided by Genentech and used according to their specifications [43]. Alexa Fluor 680 donkey anti-rabbit, Alexa Fluor 795 donkey anti-mouse, and Alexa Fluor 795 goat anti-human (Li-Cor Biosciences) were used to visualize the stained proteins using an infrared imaging system (Odyssey; Li-Cor Biosciences). Immunoblots were quantified with the Odyssey software (version 3.0.21). Data are expressed as integrated intensity of a specified area. The *in vitro* generated K48-linked and K63-linked ubiquitin chains were purchased from Enzo Life Sciences.

Total protein levels were visualized in-gel using a standard silver stain protocol. In short, gels were incubated for 16 h in 50% methanol, 12% acetic acid, 0.5 ml/L 37% HCOH. Subsequently, the gels were incubated 30 min in 50% EtOH and 1 min in 0.2 g/L Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. After three 30-s washes in dH<sub>2</sub>O the gels were incubated for 45 min in 2 g/L AgNO<sub>3</sub>, 0.75 ml/L 37% HCOH. Finally the gels were washed two times in dH<sub>2</sub>O for 30 s and incubated in 60 g/L Na<sub>2</sub>CO<sub>3</sub>, 4 mg/L Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 0.5 ml/L 37% HCOH until a desirable staining was achieved.

## Results

#### Isolation of endogenously ubiquitinated protein complexes

Our goal was to develop a method for the efficient isolation of endogenously ubiquitinated protein complexes suitable for MS-based proteomics (Fig. 1A). To this end, we compared three different affinity-based procedures under non-denaturing conditions: (1) agarose–TUBEs [44], a high-affinity ubiquitin trap based on UBDs that binds only polyubiquitinated conjugates; (2) UbiQapture–Q matrix, a high-affinity ubiquitin trap based on UBDs that binds both monoubiquitinated and polyubiquitinated proteins; and (3) the anti-ubiquitin antibody FK2 [45], which recognizes both monoubiquitinated and polyubiquitinated proteins. The three different enrichment strategies were performed in parallel using HeLa whole-cell extracts (WCEs) in RIPA lysis buffer. As a negative control—to determine nonspecific protein binding—random mouse IgGs bound to protein G beads were used. The isolation of endogenously ubiquitinated protein complexes was assessed on an immunoblot using a polyclonal  $\alpha$ -ubiquitin antibody (Fig. 1B)

and quantified using Odyssey software (Fig. 1C). In the negative control, the majority of ubiquitinated proteins (89%) remained present in the nonbound (NB) fraction and we observed virtually no nonspecific isolation in the IP fraction. In contrast, in the three different enrichment procedures we observed up to 80% depletion of ubiquitinated proteins in the NB fraction, which indicates efficient binding of ubiquitinated proteins to the various purification resins. The total yield of ubiquitinated proteins isolated was highest in the methods using TUBEs (41%) or FK2 antibody (46%).

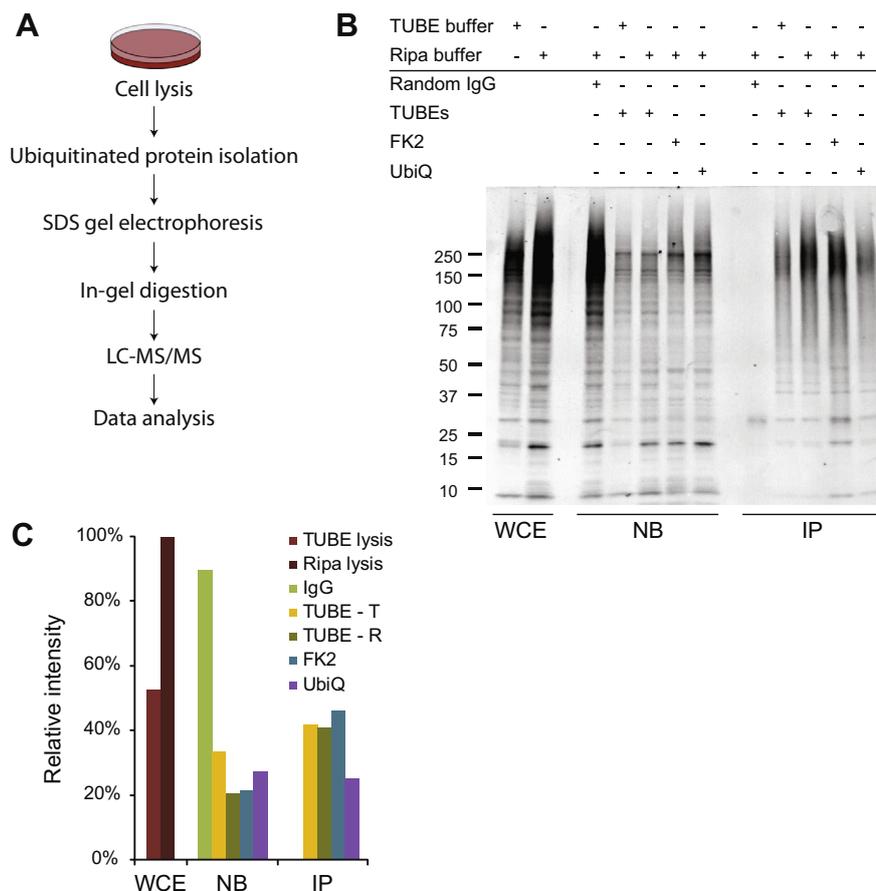
Since the manufacturer of the TUBEs has suggested that the inclusion of detergents such as SDS or deoxycholate—which are present in our RIPA lysis buffer—might have a negative impact on the overall yield of polyubiquitinated proteins, an enrichment experiment with the recommended TUBEs lysis buffer was performed in parallel. The relative efficiency of ubiquitinated protein isolation with TUBEs in TUBE buffer was comparable to those of the TUBEs in RIPA buffer and FK2 beads isolation procedures (Fig. 1C). However, quantification of the WCE shows that lysis in TUBE buffer led to an almost twofold less efficient extraction of ubiquitinated proteins from cells compared to when the cells were lysed in RIPA buffer. TUBE isolation using the TUBE buffer was therefore excluded from further experiments.

The efficiency of isolating ubiquitinated proteins was similar for the isolation procedures using TUBEs (in RIPA buffer) and FK2 antibody. However, the FK2 approach resulted in a slightly higher yield and has the further advantage of isolating monoubiquitinated proteins in addition to polyubiquitinated proteins [44,45]. This was also suggested by the stronger antibody staining for ubiquitinated proteins observed in the low-molecular-mass region (Fig. 1b). Therefore further experiments were performed with the FK2 antibody.

#### Optimization of the FK2 IP

Since DUBs and the 26S proteasome might retain some residual activity under non-denaturing conditions, despite the presence of specific inhibitors, a decrease in incubation time for the IP might result in less deubiquitination and protein degradation. Fig. 2A shows that a reduction in incubation time from 16 to 4 h did not change the total amount of ubiquitinated proteins isolated. Concomitantly, incubating HeLa WCE for up to 8 h at 4 °C did not decrease the total amount of ubiquitinated proteins (Supplementary Fig. 1A), indicating minimal loss of ubiquitinated proteins under the conditions used.

To use the FK2 antibody more efficiently, we optimized the FK2/WCE ratio by performing FK2 IPs with increasing amounts of WCE. Proteins were allowed to bind to the FK2 beads for 4 h at 4 °C. Although the amount of unbound ubiquitinated proteins increased when more WCE was added to the same amount of FK2 beads, the amount of ubiquitinated proteins in the IP fractions increased as well, up to threefold (Fig. 2A and B and Supplementary Fig. 1D). This increase in the total amount of ubiquitinated proteins isolated extended linearly with the increase in WCE input up to the second highest WCE input. A maximum in the amount of recovered ubiquitinated proteins was reached for the two IPs with the highest amount of WCE input; however, at the same time the amount of ubiquitinated proteins in the corresponding NB fraction increased >50% for the highest amount of WCE (Fig. 2B). Together, this indicated that the maximum binding capacity of the beads was reached at the second highest amount of WCE. To exclude the possibility of a fraction of ubiquitinated proteins remaining bound to the beads after elution, a second elution from the FK2 beads was performed (Fig. 2A). Although additional FK2 antibody was eluted from the beads in the second elution step for all IPs, virtually all ubiquitinated proteins were recovered from the FK2 beads in the first elution step. Taken together, based on these results we



**Fig. 1.** Isolation of endogenously ubiquitinated protein complexes. (A) Work flow for isolating endogenously ubiquitinated protein complexes for proteomic analysis. (B) Ubiquitinated proteins were isolated from HeLa WCE using either agarose–TUBEs, UbiQapture–Q matrix, or FK2 beads. Protein samples were loaded on an SDS–PAGE gel in ratio and stained on an immunoblot with a polyclonal  $\alpha$ -ubiquitin antibody to determine enrichment efficiency. (C) Odyssey quantification of (B). NB and total yield of IP of the IgG, TUBE–R, FK2, and UbiQ IPs were determined by normalization to the total of ubiquitinated proteins as shown by the WCE in RIPA buffer. The TUBE–T IP was normalized to WCE in TUBE buffer. WCE, whole-cell extract. NB, nonbound fraction. IP, immunoprecipitated proteins.

conclude that the optimal ratio of WCE to FK2 beads is 5.3 mg of WCE protein for every 100  $\mu$ l of FK2 beads (50% slurry), which corresponds to 87.5  $\mu$ g of FK2 antibody.

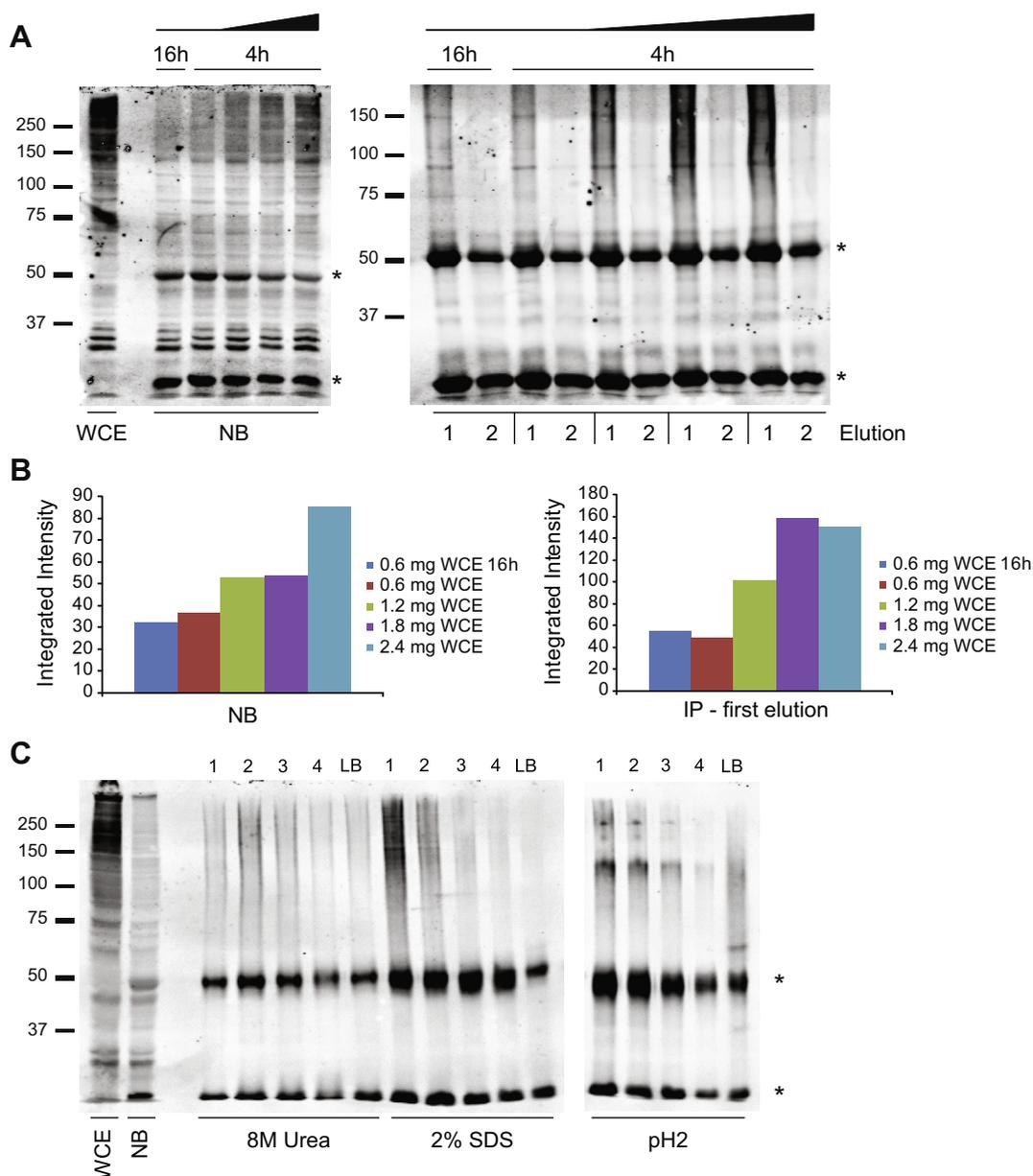
This immunoaffinity purification method has been developed for the proteomic analysis of ubiquitinated protein complexes. In such an analysis the presence of low-abundant proteins could be masked by large amounts of antibody in the IP fraction (as observed in Fig. 2A, see the asterisks). This occurs when antibodies are present in the sample, either during in-solution digestion or—if proteins have the same molecular weight as the antibody—during in-gel digestion for subsequent MS analysis. Although cross-linking of the FK2 antibody to the protein G beads greatly decreased the amount of antibody in the IP fraction, it also decreased the affinity of the antibody for ubiquitinated substrates (Supplementary Fig. 1B). Without cross-linking, the free antibody in the sample cannot be separated from the ubiquitinated proteins, rendering in-solution digestion unfavorable. Therefore, for future MS experiments we chose to perform FK2 IPs without cross-linking followed by in-gel digestion. The areas of the gel containing the bands corresponding to antibody chains were excised into separate gel slices to minimize the presence of antibody in the other gel slices.

Since only a limited volume can be loaded into a slot of an SDS–PAGE gel lane, the volume into which bound proteins are eluted from the beads is limited as well when 2 $\times$  Laemmli buffer is used. For large-scale IP experiments, elution from the beads followed by a protein concentration step is therefore necessary. The compat-

ibility of three different elution strategies with a subsequent concentration step using centrifugal filters (Amicon Ultra, Millipore) was investigated by releasing ubiquitinated protein complexes from the FK2 beads into (1) 8 M urea buffer, (2) 2% SDS, or (3) 0.1 M glycine, pH 2 (Fig. 2C and Supplementary Fig. 1E). After elution the beads were boiled in 2 $\times$  Laemmli buffer to assess residual bound proteins for elution efficiency. Fig. 2C shows that elution with 2% SDS was the most efficient: not only was the majority (77%) of bound ubiquitinated proteins eluted within the first two rounds of elution, but also almost no residual proteins (3%) remained bound to the FK2 beads after four elution rounds, as illustrated by the absence of ubiquitinated protein signal in the LB lane (Fig. 2C and Supplementary Fig. 1C).

#### Characterization of the FK2 IP

The FK2 antibody is a mouse monoclonal recognizing both monoubiquitinated and polyubiquitinated conjugates. According to the manufacturer's data sheet the FK2 antibody can recognize various types of ubiquitin chains; however, it is unknown if it has equal affinities for all seven ubiquitin chain linkages and for different protein substrates. If the FK2 antibody does have such a bias, then a specific fraction of ubiquitinated proteins will not be immunodepleted. To investigate this, we compared by immunoblot analysis the FK2 antibody with a polyclonal antibody that recognizes both monoubiquitinated and polyubiquitinated conjugates as well as free ubiquitin [46]. Samples of small-scale FK2 IPs with

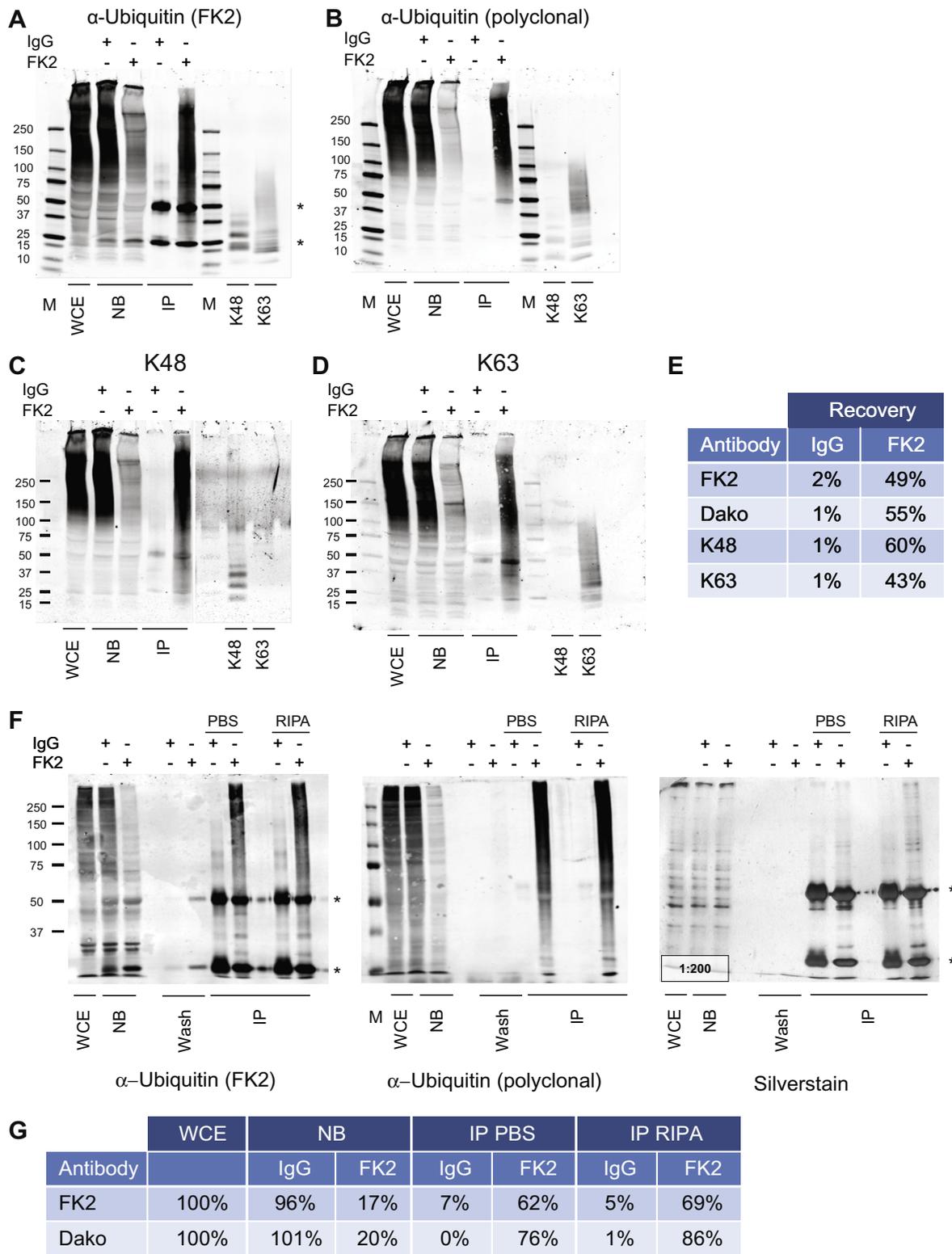


**Fig. 2.** Optimization and elution of the FK2 IP. (A) Endogenously ubiquitinated protein complexes were isolated using 40  $\mu$ l FK2 beads (50% slurry) and incubated for 4 or 16 h with an increasing amount of XP20S WCE (0.6–2.4 mg). Samples were loaded on SDS-PAGE gels. Immunoblots were stained with the FK2 antibody. The left immunoblot shows the WCE and NB fractions, the right immunoblot shows the eluted IP fractions. A representative blot of this IP is shown ( $n = 2$ ). (B) Odyssey quantification of (A). (C) The FK2 beads were incubated for 16 h with HeLa WCE. Bound proteins were eluted from the beads in four consecutive steps with 8 M urea buffer, 2% SDS, or 0.1 M glycine, pH 2. After elution the beads were boiled in 2 $\times$  Laemmli buffer to assess residual bound proteins for elution efficiency. Protein samples were loaded on an SDS-PAGE gel in ratio and stained on an immunoblot with the FK2 antibody. A representative blot of this IP is shown ( $n = 2$ ). WCE, whole-cell extract; NB, nonbound fraction; LB, 2 $\times$  Laemmli buffer. (\*) Heavy and light antibody chains.

random IgG beads as negative control were separated by SDS-PAGE and immunoblotted with FK2 and the polyclonal  $\alpha$ -ubiquitin antibody. The staining patterns for the IP samples were similar for both antibodies: we observed a strong depletion of ubiquitinated proteins in the NB fraction and a specific enrichment in the FK2 IP fraction (Fig. 3A and B). The relative signal intensities in the IP fractions were also similar for both antibodies (Fig. 3E), suggesting that FK2 has no bias for binding specific types of ubiquitin chain linkages or protein substrates. To further support the notion that proteins with all-ubiquitin chain linkages may be isolated using the FK2 antibody, the immunoblots were also stained with two available chain-specific antibodies that specifically recognize K48-linked and K63-linked ubiquitin chains. The chain specificity

of the antibodies was confirmed by also loading K48-linked and K63-linked ubiquitin chains that were generated *in vitro* onto the gels. The staining patterns and relative signal intensities of ubiquitinated proteins in the IP fractions were again very similar for the antibodies used (Fig. 3A–E).

Proteolytic digestion of ubiquitinated proteins with trypsin generates a specific diglycine (Gly–Gly) remnant on the ubiquitinated lysine. This remnant causes a distinct mass shift on the peptide mass that can be used to precisely identify and localize ubiquitination sites by MS. We performed *in-gel* digestion on an FK2 IP fraction with trypsin and identified ubiquitin-modified peptides on positions K6, K11, K27, K29, K48, and K63 of ubiquitin, representing six of the seven possible chain linkages of ubiquitin



**Fig. 3.** Characterization of the FK2 IP. (A–D) Endogenously ubiquitinated protein complexes were isolated from HeLa WCE. Samples were loaded twice on SDS–PAGE gels in equal amounts compared to the WCE; IP sample was loaded at twice the amount. A representative blot of this IP is shown ( $n > 3$ ). The immunoblots were stained with (A) FK2, (B) polyclonal  $\alpha$ -ubiquitin, (C)  $\alpha$ -K48-linked polyubiquitin (Apu2.07), and (D)  $\alpha$ -K63-linked polyubiquitin (Apu3.A8). As specificity control for the chain-linkage-specific antibodies, in vitro generated K48 and K63 ubiquitin chains (4  $\mu$ g) were loaded. The last three lanes of the immunoblot in (C) were scanned at a higher intensity. M, molecular weight marker; WCE, whole-cell extract; NB, nonbound fraction; IP, immunoprecipitated proteins. (E) Odyssey quantification of (A–D). Recovery efficiency was determined by normalization to the total of ubiquitinated proteins as shown by the WCE. (F) Endogenously ubiquitinated protein complexes were isolated from HeLa WCE in two independent experiments. FK2 beads and control beads were washed 4 $\times$  with either PBS or RIPA buffer before elution of bound proteins in 2 $\times$  Laemmli buffer. The immunoblot was stained with FK2 and a polyclonal  $\alpha$ -ubiquitin antibody. Total proteins levels were assessed with silver staining, WCE and NB were diluted 200 $\times$  compared to the IP fractions. Wash, final wash fraction before protein elution. (G) Odyssey quantification of (F). Recovery efficiency was determined by normalization to the total of ubiquitinated proteins as shown by the WCE.

(Supplementary Table 1). Together with the immunoblot results this suggests that there is no bias for FK2 in binding specific types of ubiquitin chains or protein substrates under the conditions used.

To further evaluate the application of the FK2 antibody for our immunoaffinity purification method (intended for the proteomic analysis of ubiquitinated protein complexes), we performed two additional independent FK2 IP experiments with random IgG beads as a negative control for MS analysis. This replicate experiment differed only in the final washing step: beads were washed with either PBS or RIPA buffer four times. We observed a strong, specific, and reproducible enrichment for ubiquitinated proteins, as shown by immunoblot analysis using two different antibodies (Fig. 3F). Additionally, analysis of total protein levels—as visualized in-gel by silver staining—showed an enormous decrease (>200-fold) in total amount of protein in the IP fraction (Fig. 3F), while most of the total ubiquitinated protein signal (Fig. 3G) was recovered as shown by the immunoblot analysis. These results illustrate a high degree of specific enrichment for ubiquitinated factors.

#### MS analysis

To further confirm the specificity and reproducibility of our enrichment strategy, the IP fractions of the two independently executed FK2 IPs (Fig. 3F) were in-gel digested and run on an LTQ-Orbitrap XL mass spectrometer. Raw mass spectrometry data were analyzed using the label-free quantitation (LFQ) algorithm of the MaxQuant software (version 1.3.0.5).

The proteins identified were considered true FK2 antibody interactors when an LFQ intensity (the total signal intensities of the peptides identifying each protein) was listed in the FK2 IP and there was either no LFQ intensity observed in the control IP or the FK2/control LFQ intensity ratio was >2. Of the identified and validated 951 true interactors, 296 proteins were identified in both experiments (Fig. 4A and Supplementary Table 2A).

To confirm that the presented enrichment method indeed isolates ubiquitinated and ubiquitination-related proteins, we performed three further analyses. First, we performed a functional pathway analysis of the 296 FK2-specific proteins found in both experiments. The IPA software (Ingenuity Systems, [www.ingenuity.com](http://www.ingenuity.com)) identified 32 canonical pathways ( $p < 0.05$ , Fisher's exact test; Supplementary Table 3) with the protein ubiquitination pathway as most significantly present (Fig. 4B), indicating an enrichment for ubiquitinated and ubiquitination-related proteins. As expected, a wide variety of other pathways was also identified, indicative of the importance of ubiquitination in different pathways. In line with this, when the 296 FK2-specific proteins were subjected to a Gene Ontology (GO) enrichment analysis using the functional annotation tool DAVID [47], proteins associated with ubiquitination in the Biological Processes term were highly enriched for (Fig. 4C and Supplementary Table 4). Finally, the FK2-specific protein list was compared with two large datasets of ubiquitinated proteins that were recently identified in peptide screens using antibodies recognizing the specific diglycine remnant on the ubiquitinated lysine [32,33]. There was a 62% overlap with the dataset of Kim et al. [32] and a 70% overlap with that of Wagner et al. [33], indicating that a high percentage of the isolated proteins were indeed ubiquitinated (Supplementary Table 2B).

One of the advantages of our approach is that ubiquitinated protein complexes can be isolated. To demonstrate that many of the proteins present in the FK2 IP fraction are part of protein complexes we evaluated their protein–protein interactions. Functional association data were obtained and visualized using the GeneMANIA plug-in in Cytoscape. The resulting protein network showed that 53% of the FK2-specific proteins interacted with at least one other protein within the FK2-specific protein group (Fig. 4D). In contrast, when four sets of 290 proteins were randomly chosen

from the Ensembl database, a significantly smaller percentage of proteins interacted (on average 15%,  $p = 3.3 \times 10^{-23}$ , Fisher's exact test; Fig. 4D). Since a low percentage of interacting proteins in random protein sets could be explained by low-abundant, tissue-specific, and/or badly annotated proteins, we also determined the percentage of interacting proteins in four random sets of proteins that were extracted from the two large datasets of ubiquitinated proteins recently identified in peptide screens by Kim et al. [32] and Wagner et al. [33]. Because the enrichment step in both of these screens was performed at the peptide level—after a denaturing and digestion step—protein complexes were not isolated and all information about protein–protein associations was lost. For the random sets extracted from Kim et al. [32] and from Wagner et al. [33], the percentages of interacting proteins were on average 25% and 29%, respectively (Supplementary Fig. 2). Although these percentages were higher than those found for the random sets collected from the Ensembl database, the percentages of interacting proteins for both datasets were similar and significantly lower than the 53% interacting proteins in the FK2-specific protein dataset ( $p = 1.8 \times 10^{-12}$  for the Kim et al. dataset and  $1.4 \times 10^{-9}$  for the Wagner et al. dataset, Fisher's exact test). This clearly indicates that the optimized enrichment method described here indeed isolates endogenously ubiquitinated protein complexes.

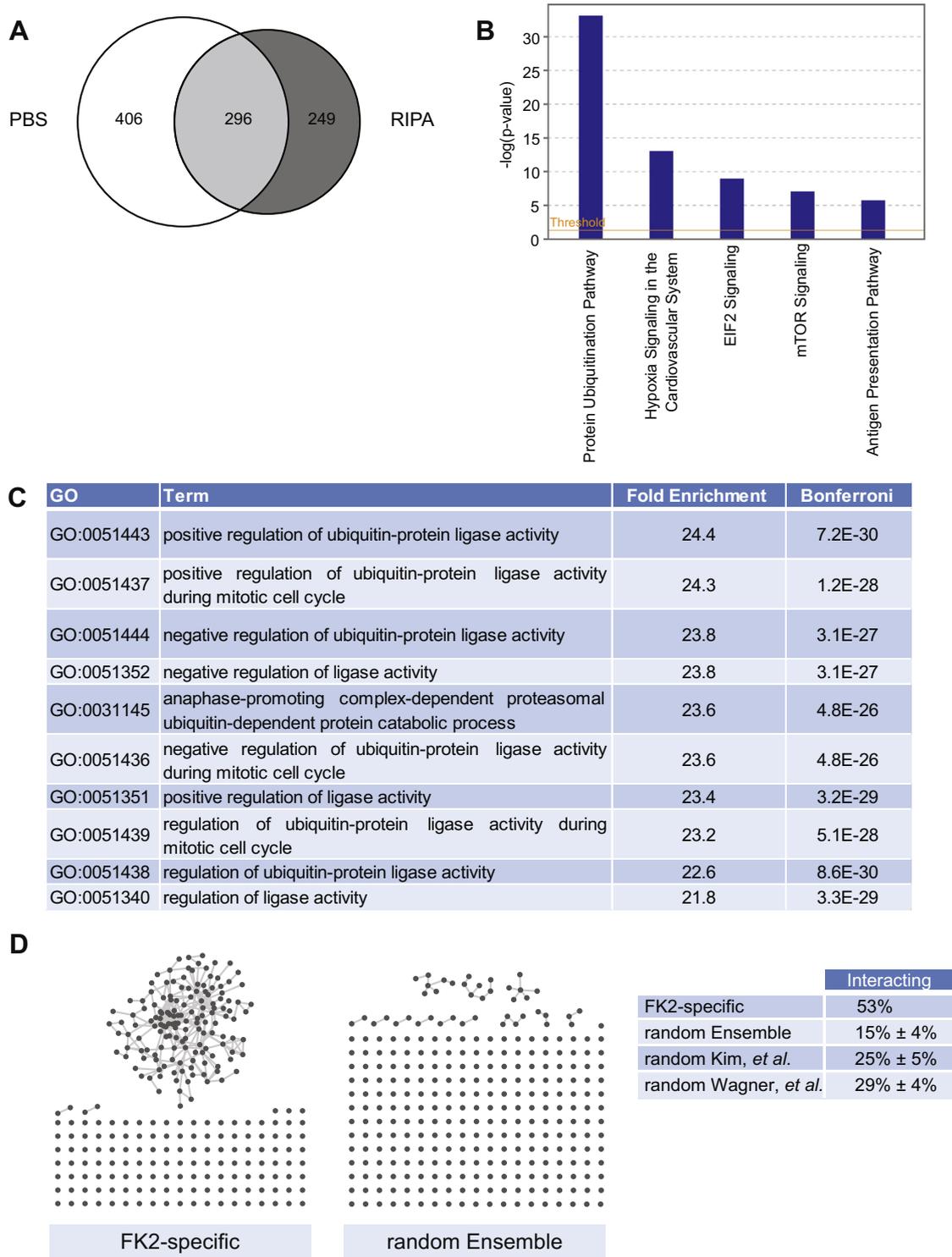
#### Discussion

We present here an immunoaffinity purification method for the proteomic analysis of endogenously ubiquitinated protein complexes. Proteins were successfully isolated using the FK2 antibody bound to protein G-beaded agarose, which recognizes monoubiquitinated and polyubiquitinated conjugates (Figs. 1 and 3F). An optimal WCE/FK2 ratio was determined for the efficient isolation of ubiquitinated proteins and an IP incubation time of 4 h was shown to be sufficient for immunodepletion of ubiquitinated proteins from WCE of human cells (Fig. 2A). A high degree of specific enrichment for ubiquitinated factors was achieved, as shown by the high recovery of ubiquitinated proteins (up to 86%, Fig. 3G), while the total amount of proteins decreased strongly in the IP fraction (>200-fold, as assessed on a silver-stained gel in Fig. 3F). Finally, the efficient elution of bound proteins with 2% SDS from the FK2 beads (Fig. 2C) illustrates the compatibility of this method with large-scale proteomic assays.

The MS analysis of two small-scale FK2 IPs resulted in the identification of 296 FK2-specific proteins in both experiments (Fig. 4A). The isolation of ubiquitinated proteins and ubiquitination-related proteins was confirmed by pathway analyses using IPA and GO-annotation enrichment (Fig. 4B and C). Additionally, comparing the FK2-specific proteins with databases of ubiquitinated proteins in the literature indicated that a high percentage of proteins in the enriched fraction in our assay was indeed ubiquitinated (Supplementary Table 2B).

Further characterization of the FK2 IP, with immunoblot analysis using chain-linkage-specific antibodies and two different  $\alpha$ -ubiquitin antibodies, suggested that there was no detectable bias for the FK2 antibody in binding specific types of ubiquitin chains or protein substrates under the conditions used (Fig. 3A–D). This was further supported by MS analysis of FK2-enriched proteins, which identified six (of seven possible) specific polyubiquitin chain linkages. The fact that the K33 ubiquitin-modified peptide was not identified could be explained by the fact that it is the least abundant chain-linkage type in unperturbed cells [22] and that these experiments were performed on a small scale (IPs were performed on WCE from a single 9-cm dish).

The FK2 IP was performed under non-denaturing conditions, which allows the preservation of protein complexes. Most biolog-



**Fig.4.** Mass spectrometric data analysis. (A) Venn diagram of the FK2-specific MS-identified proteins in two independently executed FK2 IPs. (B) Functional annotation of the FK2-specific proteins identified in both experiments into canonical pathways using IPA. The five most significant pathways are shown. The significance of the pathways was determined using Fisher's exact test. (C) GO annotation enrichment analysis using DAVID. The 10 most enriched biological processes with  $p < 0.005$  are shown. The  $p$  values were corrected for multiple hypotheses testing using Bonferroni FDR. (D) Protein interaction networks were built using association data based on physical interactions, which were obtained through the GeneMANIA database and visualized using Cytoscape. Every dot represents a protein and connecting lines represent a physical interaction. Percentages of interacting proteins for the random protein sets are averages of  $n = 5$ . For the random protein set from the Ensembl database a representative protein interaction network is shown.

ical processes mainly rely on functional protein modules [36]; however, within complexes not all interactors are necessarily ubiquitin modified. Evaluation of protein–protein interactions within the FK2-specific protein group showed that a significantly higher

percentage (53%) of the FK2-specific proteins are interacting among one another compared to proteins in random datasets from the Ensembl database (15%) or datasets of ubiquitinated-peptide IPs from Kim et al. (25% [32]) and Wagner et al. (29% [33])

(Fig. 4D). This indicates that in our assay intact ubiquitinated protein complexes are indeed isolated. MS analysis identified 296 FK2-specific proteins in two FK2 IPs using PBS or RIPA buffer as the final wash buffer. Additionally, 655 proteins were found in only one of these IPs. Interestingly, 62% of the additional identified proteins were specific for the PBS-washed IP (Fig. 4A and Supplementary Table 2A). Since PBS is a much milder buffer than RIPA buffer in terms of salt and detergent concentrations, our data suggest that changing the wash buffer stringency can result in a different amount of proteins isolated. It will be of interest to study whether changes in wash buffer stringency will result in the identification of additional ubiquitinated proteins or proteins that are transiently or weakly bound to ubiquitinated protein complexes.

We showed that the FK2 IP efficiently enriched for ubiquitinated proteins of different chain linkages; however, we cannot be certain that all ubiquitinated factors were equally efficient isolated. For this reason we performed an isolation of endogenously ubiquitinated protein complexes using the UbiQapture-Q matrix under the same conditions as for the FK2 IP. MS analysis identified a total of 735 UbiQapture-Q-specific proteins (Supplementary Table 2A). From this set 351 overlapped with the FK2-specific proteins (Supplementary Fig. 2B). This indicates that while these methods isolate identical proteins, a considerable amount of additional method-specific proteins was also isolated. This suggests that the FK2 isolation method can be combined with other isolation procedures for ubiquitinated proteins, to broaden the pool of proteins isolated and to overcome a putative bias in the isolation procedure.

A limitation of the FK2 isolation method presented here is that, apart from sites on ubiquitin itself, we identified only a few ubiquitination sites on substrate proteins (Supplementary Table 1). It is therefore not possible to differentiate with certainty between ubiquitinated and nonubiquitinated proteins based on the results of the MS analysis alone. The low number of ubiquitination sites identified could be explained by the absence of an enrichment protocol for ubiquitinated peptides in our assay. The presence of ubiquitinated peptides is probably masked by large amounts of nonubiquitinated peptides from the same protein and from nonubiquitinated interactors of the ubiquitinated proteins. Enrichment strategies for the detection of ubiquitination sites are available and would be an ideal tool to complement the data generated with this FK2-enrichment strategy. The recently developed method for the immunoenrichment of ubiquitinated peptides using a diglycine-specific antibody [31–35] is highly efficient and could be implemented to discriminate between ubiquitinated proteins and interacting nonubiquitinated proteins in our FK2 samples containing ubiquitinated protein complexes.

In conclusion, the use of the FK2 antibody for protein isolation enables the enrichment of endogenously ubiquitinated proteins. The advantage of this method is that possible negative side effects of introducing tagged ubiquitin into the cells—such as a biased incorporation into monoubiquitination or certain ubiquitin chains and/or interference with, e.g., the stability, activity, and localization of protein substrates—are therefore prevented. Additionally, the FK2 isolation method described here is broadly applicable as it can also be used to isolate proteins from tissues and cell types that are usually difficult to transfect.

In recent years, mass spectrometry has become the method of choice for studying the proteome and, more specifically, PTMs. It can be used not only to analyze proteins and protein complexes, but also to dissect biological pathways and identify proteins not previously known to be involved in specific processes. Moreover, various quantitative mass spectrometry strategies are available for detecting and quantifying the effects of a specific stimulus in a proteome-wide fashion [48]. Label-based quantification methods—such as stable isotope labeling by amino acids in cell culture (SILAC) and isobaric tags for relative and absolute quantitation

[48]—are very suitable for comparing the abundance of proteins on a proteome-wide scale since they can provide a quantitative ratio for a large number of proteins. Combining the method we describe for the isolation of endogenously ubiquitinated protein complexes using the FK2 IP with such quantitative proteomic techniques would therefore generate a powerful tool to study dynamic changes in the ubiquitinome following, for example, environmental stresses, drug treatment, or knockdown of proteins or when comparing two disease states.

Recently we showed a clear example of the successful application of this approach [49]. Combining the FK2 IP described here with SILAC-based proteomics identified several differentially ubiquitinated proteins in HeLa cells following UV irradiation. The most prominent factors were DNA repair proteins that are involved in nucleotide excision repair (NER) and that are known to be ubiquitinated. Importantly, it also resulted in the identification of UVSSA (UV-stimulated scaffold protein A) as the causative gene for UV-sensitive syndrome, a previously unresolved NER deficiency disorder [49–51]. Follow-up experiments showed that the ubiquitination status of UVSSA remained unchanged after UV and that this protein was copurified as part of a UV-induced ubiquitinated protein complex [49]. These data illustrate the value and advantage of our non-denaturing immunoaffinity purification method, which is capable of isolating both ubiquitinated proteins and their interacting proteins, for the proteomic analysis of endogenously ubiquitinated protein complexes.

## Acknowledgments

We thank Genentech for providing the chain-linkage-specific antibodies and we are grateful to K. Derks for his help in generating the random proteins lists. This work was funded by The Netherlands Genomics Initiative NPCII (to P.S.), 935.19.021 and 935.11.042 (to W.V., C.L., and J.A.M.), the Dutch Organization for Scientific Research ZonMW Veni Grant (917.96.120 to J.A.M.) and TOP Grant (912.08.031 to W.V.), and the Association for International Cancer Research (10-594 to W.V.).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ab.2013.05.020>.

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