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abstract

Attachment of ubiquitin to proteins represents a central mechanism for the regulation of protein metabolism and function. In the NF-kappaB pathway, binding of NEMO to polyubiquitinated substrates initiates the pathway in response to cellular stimuli. Other polyubiquitin binding proteins can antagonize this pathway by competing with NEMO for polyubiquitin. We have used protein arrays to identify polyubiquitin binding proteins that regulate NF-kappaB activity. Using polyubiquitin as bait, protein arrays were screened and polyubiquitin binders identified. Novel polyubiquitin binders AWP1, CALCOCO2, N4BP1, RIO3, TEX27, TTC3, UBFD1 and ZNF313 were identified using this approach, while known NF-kappaB regulators including NEMO, A20, ABIN-1, ABIN-2, optineurin and p62 were also identified. Overexpressed AWP1 and RIO3 repressed NF-kappaB activity in a manner similar to optineurin, while siRNAs directed against AWP1 and RIO3 also reduced NF-κB activity. TNFalpha-dependent degradation of IkappaBalpha was also suppressed by overexpression of AWP1 and RIO3, possibly due to the polyubiquitin binding activity of these proteins. Protein array screening using polyubiquitin enabled rapid identification of many known and novel polyubiquitin binding proteins and the identification of novel NF-kappaB regulators.

1. Introduction

In recent years, the importance of polyubiquitin as a signaling molecule has come to the forefront of biochemical signaling research. The landmark discovery that the NF-κB signaling component NEMO (*NF-κB essential modifier*) is recruited to activated TNF (*Tumor Necrosis Factor*) receptor complexes by its interaction with K63-linked polyubiquitin chains [1] altered the perception that polyubiquitination was synonymous with proteosomal protein degradation. In the TNF pathway, ubiquitin monomers are ligated to form K63-linked polyubiquitin on the RIP (*receptor interacting protein*) scaffold protein, enabling NEMO to recruit IKK (*inhibitor of κB kinase*) kinases to the complex and initiate the NF-κB signaling cascade [2–4].

NEMO binds to polyubiquitin via a domain termed NUB (*NEMO ubiquitin binding*), which is located in the C-terminal region of the protein [2,5]. Unlike the common zinc finger ubiquitin binding domain [6], the NUB domain is rare among human proteins, and thus far has only been identified in NEMO and several functionally related proteins [7,8].

Recently, a number of works have demonstrated that the recruitment of NEMO to the TNF receptor complex is antagonized by other polyubiquitin binding proteins that modulate TNF signaling in response to secondary stimuli. One of the best examples of such a protein is optineurin, expressed in retinal ganglia and associated with glaucoma, which competes with NEMO to dampen NF-κB signaling [8]. Optineurin binds polyubiquitin via a domain very closely related to the NUB domain. The ABIN proteins also antagonize NF-κB signaling through polyubiquitin binding via a structurally similar domain [7]. These recent findings suggest that there may be other polyubiquitin binding proteins that regulate NF-κB signaling in response to different cellular and environmental cues.

In the current study, we have used membrane-based colony macroarrays to screen for human polyubiquitin binding proteins, with the aim of identifying novel proteins that possess new polyubiquitin binding domains and that can influence NF-κB signaling. In this screen we used human ubiquitin C (UbC), an ubiquitin precursor that contains nine head-to-tail repeats of ubiquitin, as a probe for polyubiquitin binding proteins. This approach enabled us to identify 31 polyubiquitin binding proteins, of which eight: AWP1, CALCOCO2, N4BP1, RIO3, TEX27, TTC3, UBFD1 and ZNF313, are novel interactors not previously known to bind polyubiquitin. Of these novel interactors, we show that AWP1 and RIO3 influence NF-κB-dependent transcriptional activation in human cells and thus serve as novel regulators of this pathway.

2. Materials and methods

2.1. Protein expression and purification

Human ubiquitin C and NEMO were expressed as soluble GST fusions from pGEX-4T-3 in *E. coli* BL21 (DE3) and purified using

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glutathione sepharose (GE Life Sciences) according to the manufacturer's instructions. Polyhistidine-tagged proteins were expressed from pQE30 in *E. coli* XL1 Blue cells and purified using nickel-NTA resin (Qiagen).

2.2. Colony array screening

Membrane-based colony macroarrays were obtained from ImaGenes (Berlin, Germany). Arrays consisted of cDNAs from human brain, colon, liver and lung expressed from pQE30 as His_6 fusions in PVDF membrane-immobilized *E. coli* XL Blue colonies. Array screening with GST, GST-UbC and anti-GST-HRP (GE Life Sciences) was performed as described previously [9].

2.3. Protein dot blots.

Solutions of purified GST, GST-UbC, monoubiquitin, K48-linked polyubiquitin and K63-linked polyubiquitin (Boston Biochem) were spotted in 0.1 μg (GST-UbC) or 1 μg (all other proteins) onto nitrocellulose membranes and dried at room temperature for 15 min. Membranes were blocked in PBS-T containing 5% BSA for 1 h, then probed with 20 μg of each purified protein and washed as described for the arrays. Binding was detected using anti-RGS-His (Qiagen) diluted 1:1000 followed by rabbit anti-mouse HRP conjugate (Millipore) diluted 1:1000, or anti-GST-HRP (GE Life Sciences) diluted 1:1000. Signals were detected as described for the arrays.

2.4. Pulldown of ubiquitin-binding proteins

Human embryonic kidney cells (HEK-293T) were transfected using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Plasmids encoding NEMO, N4BP1, TEX27 and ZNF313 were used for HEK-293T transfection, while endogenous AWP1, CALCOCO2, RIO3 and TTC3 were detected in HEK-293T cell extracts. GST pulldowns were performed as described previously [10] and immunoblots probed with anti-Xpress (Invitrogen), anti-Myc (Santa Cruz Biotech) or protein-specific antibodies (Santa Cruz Biotech).

2.5. NF-κB reporter gene assays

Transcriptional activation by NF-κB was monitored using a secreted alkaline phosphatase (SEAP) reporter plasmid, pNF-κB-

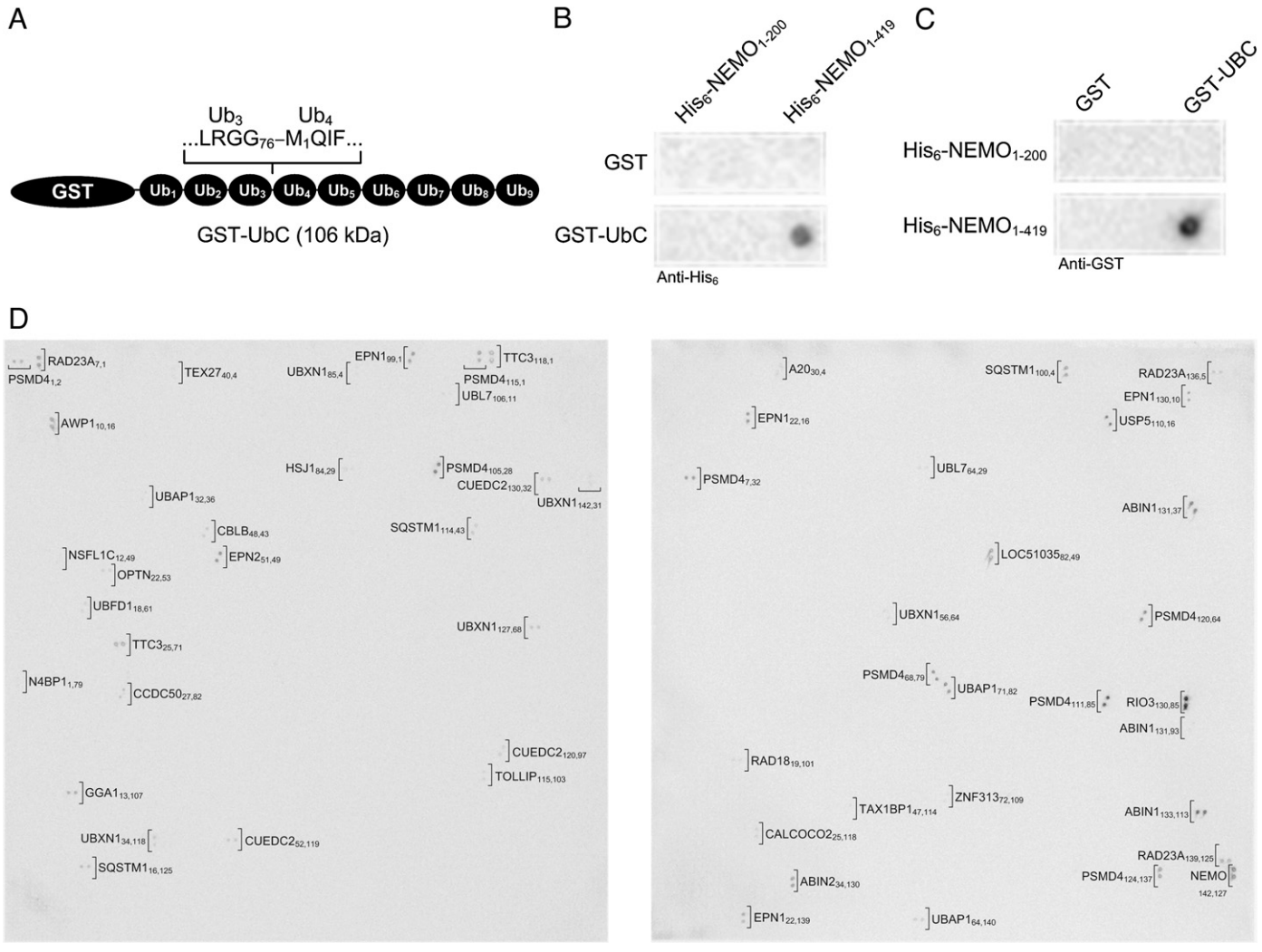

Fig. 1. Screening colony macroarrays with human ubiquitin precursor UbC, a 9-mer of ubiquitin, enables identification of polyubiquitin binding proteins. (A) Architecture of the recombinant GST-UbC protein used for array screening, showing the nine repeats of ubiquitin that form the UbC ubiquitin precursor. (B) Purified GST or GST-UbC (0.1 μg) were applied to membranes and overlaid with 10 μg of truncated (1–200) or full-length (1–419) His_6-NEMO. Binding was only observed between full-length NEMO and GST-UbC. (C) Truncated and full-length NEMO (0.1 μg) were immobilized on membranes and overlaid with 10 μg of GST or GST-UbC, followed by detection with anti-GST. Again, binding was only observed between GST-UbC and full-length NEMO. (D) Detection of polyubiquitin binding proteins following macroarray probing with GST-UbC and detection with anti-GST-HRP. Positive hits are visible as two duplicate spots on the array, labeled with the name of the expressed protein and the *x,y* coordinates used to identify the clones.

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SEAP (Clontech). HEK-293T cells in 24-well plates were transfected with plasmids encoding either AWP1 (in pCMV6-Entry; OriGene), RIO3 (in pCMV6; Imagenes) or optineurin (in pDEST26; [8]) and 400 ng of pNF-κB-SEAP for 24 h followed by treatment with TNFα (100 ng/ml). Alternatively, cells were transfected with pNF-κB-SEAP and 20 pmol of previously validated siRNAs against AWP1 (Santa Cruz Biotech), RIO3 (Ambion) or scrambled control siRNAs. SEAP activity was determined as described previously [11] using AttoPhos substrate (Roche) and a BioTek Synergy HT plate reader.

2.6. IκBα degradation assay

HEK-293T cells in 6-well plates were transfected with 10 μg of His_6-optineurin (positive control), AWP1, RIO3 or empty expression vectors and incubated for 20 h. Cells were then treated with 5 ng/ml of TNFα for 15 min, placed on ice and rapidly scraped into 200 μl of cell lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% NP-40, protein inhibitor cocktail, pH 8.0). Following a 30 min incubation period with end-over-end turning at 4°C, the lysates were centrifuged, supernatants collected and mixed with equal volumes of Laemmli sample buffer. Proteins were resolved by SDS-PAGE and IκBα detected with anti-IκBα (Cell Signaling Technology).

3. Results

3.1. Ubiquitin C is a substrate for polyubiquitin binding proteins

To identify polyubiquitin binding proteins, we first overexpressed and purified human UbC, a ubiquitin precursor containing nine repeats of ubiquitin [12], as a 106 kDa GST fusion protein (Fig. 1A). To validate the usefulness of GST-UBC as an array probe for polyubiquitin binding proteins, we spotted full-length recombinant NEMO (419 aa) and a truncated variant (1–200 aa) lacking the coiled zipper required for ubiquitin binding [13] onto a membrane and probed with GST-UbC or GST, then detected binding using an anti-GST antibody. Binding occurred between full-length NEMO and GST-UbC (Fig. 1B), indicating that full-length NEMO bound the UbC moiety of the GST-UbC fusion. Similarly, when we spotted GST or GST-UbC onto the membranes and probed with full-length or truncated NEMO, the same interaction was observed (Fig. 1C). From this result we concluded that GST-UbC was a useful probe to search for polyubiquitin binding proteins.

3.2. Polyubiquitin binding proteins are detected by membrane array screening

We next used GST-UbC to probe a membrane-based colony macroarray for polyubiquitin binding proteins. The array used consists of a human cDNA library of approximately 8300 unique GenBank gene accession numbers from brain, colon, liver and lung in an *E. coli* protein expression host, and has been used to successfully identify interactions between other proteins [9,14]. From two independent screens of the array with GST-UbC and a control screen using GST, we arrived at a final dataset of 58 individual clones expressing 31 unique proteins (Table 1, Fig. 2). Of these proteins, 23 have previously been shown to bind polyubiquitin, including 10 proteins that are involved in the NF-κB pathway, namely NEMO, A20, ABIN-1, ABIN-2, Cbl-b, p62, optineurin, TOLLIP, TAX1BP1 and Ymer.

The majority of expressed UbC binding proteins were not full-length proteins, enabling us to use the screen to identify regions of these proteins responsible for UbC binding (Table 1). Inspection of the

Table 1
Polyubiquitin binding proteins identified using colony macroarrays.

Gene name	Protein name	GenBank accession number	Amino acid sequence span (full-length)	Reference
Previously identified ubiquitin interactors				
CBLB	Cbl-b[a]	NP_733762	725–982 (982)	[24]
CUEDC2	CUE domain-containing 2	NP_076945	6–287 (287)	[25]
EPN1	Epsin 1	EAW72408	182–576 (576)	[26]
EPN2	Epsin 2	NP_683723	55–584 (584)	[26]
GGA1	GGA1	NP_037497	187–639 (639)	[27]
HSJ1	HSJ1 neuronal DnaJ (Hsp40) homolog	NP_006727	191–324 (324)	[28]
IKBKG	NEMO[a]	Q9Y6K9	190–419 (419)	[29]
NSFL1C	NSFL1 cofactor p47	Q9UNZ2	1–370 (370)	[30]
OPTN	Optineurin[a]	Q96CV9	309–577 (577)	[8]
PSMD4	26S proteasome regulatory subunit 4	P55036	221–377 (377)	[31]
hHR18	RAD18	AAF86618	152–495 (495)	[32]
RAD23A	RAD23	NP_005044	159–363 (363)	[33]
SQSTM1	Sequestosome 1/p62[a]	NP_003891	326–440 (440)	[34]
TAX1BP1	TAX1 binding protein 1[a]	NM_006024	219–498 (789)	[35]
TNFAIP3	A20[a]	NP_006281	705–790 (790)	[36]
TOLLIP	Toll interacting protein[a]	NP_061882	118–274 (274)	[37]
TNIP1	ABIN-1[a]	NP_006049	266–636 (636)	[7]
TNIP2	ABIN-2[a]	NP_077285	293–429 (429)	[7]
UBAP1	Ub-associated protein 1	Q9NZ09	399–502 (502)	[38]
UBL7	Ub-like protein 7	Q96S82	1–380 (380)	[39]
UBXN1	SAPK substrate protein 1	NP_056937	1–312 (312)	[40]
USP5	Ub-specific peptidase 5	NP_003472	623–835 (835)	[41]
CCDC50	Ymer[a]	AAH65004	1–306 (306)	[42]
Novel interactors				
AWP1	Associated with protein kinase C[a]	CAG38507	1–208 (208)	[43]
CALCOCO2	Calcium binding coiled-coil domain 2	NP_694574	118–446 (446)	[44]
N4BP1	NEDD4 binding protein 1	NP_694574	209–896 (896)	[45]
RIO3	RIO kinase 3	O14730	16–153 (519)	[46]
TEX27	Testis-expressed sequence 27	NP_068762	1–227 (227)	[47]
TTC3	Tetratricopeptide domain containing 3	NP_003307	1824–2025 (2025)	[48]
UBFD1	Ub-binding family domain containing 1	NP_061989	126–309 (309)	None available
ZNF313	Zinc finger protein 313	Q9Y508	1–228 (228)	[49]

A total of 31 UbC binding proteins were identified, with 23 known to have ubiquitin binding activity from previous work, and the remaining eight being novel binding proteins.
[a] Proteins known to associate with or influence the activity of the NF-κB pathway (see References).

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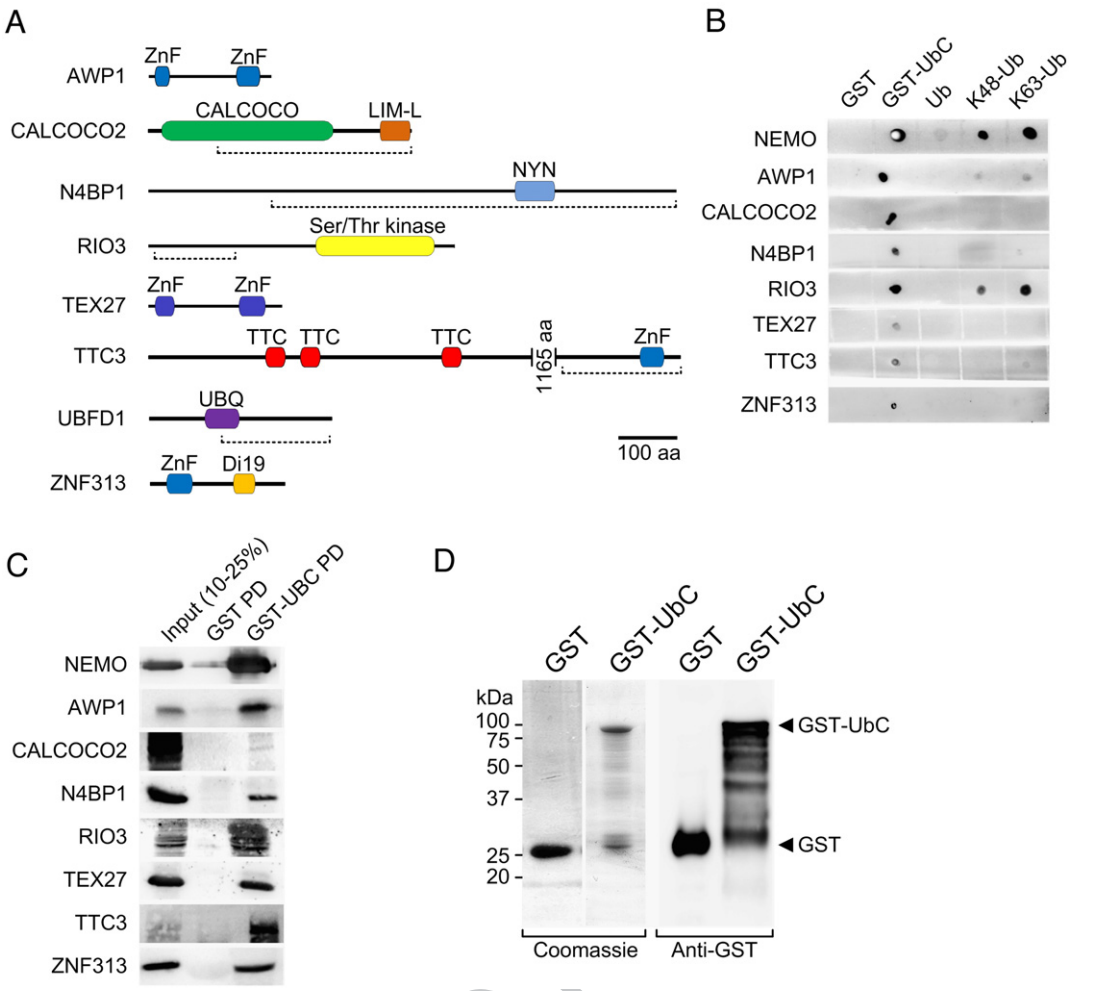

Fig. 2. Novel polyubiquitin binding proteins possess conventional and novel polyubiquitin binding domains. (A) Each of the novel polyubiquitin binding proteins is represented with relevant domains and the regions expressed on the macroarrays. Abbreviations used are: ZnF, zinc finger; CALCOCO, calcium-binding coiled coil; LIM, Lin11, Isl-1 and Mec-3; NYN, NYN domain RNase; Di19, drought-induced protein 19; TTC, tetratricopeptide; UBQ, ubiquitin-like. The dotted lines indicate the region of the protein expressed by the vector when full-length proteins were not expressed. (B) Novel polyubiquitin interacting proteins AWP1, N4BP1, RIOK3 and TTC3 bind to physiologically relevant forms of polyubiquitin *in vitro*. Purified GST, GST-UbC, monoubiquitin and K48- and K63-linked polyubiquitin were spotted onto membranes and probed with purified RGS-His_6-tagged polyubiquitin binding proteins, followed by detection with anti-RGS-His_6 antibody. (C) Full-length polyubiquitin binding proteins expressed in human cells bind UbC. Purified GST or GST-UbC bound to glutathione sepharose beads were incubated with lysates from HEK-293T cells as indicated in the methods and bound protein detected with specific antibodies. (D) Protein staining and immunoblotting of the GST and GST-UbC proteins used as input for GST pulldown experiments. Proteins were resolved by SDS-PAGE and detected by Coomassie staining or immunoblotting with GST-HRP antibody.

expressed regions and comparison with available literature revealed that for each of the proteins the known polyubiquitin binding domains were localized within the expressed regions. Given the abundance of known polyubiquitin binding proteins that appeared in our screen, we concluded that this approach was useful for the detection of polyubiquitin binding proteins. We also found that this method is useful for the localization of binding domains within polyubiquitin binding proteins due to the fragmented nature of the arrayed proteins.

The group of novel interactors included AWP1, CALCOCO2, N4BP1, RIO3, TEX27, TTC3, UBFD1 and ZNF313. One of these proteins, AWP1 (*a*ssociated *w*ith *P*RK1) represses NF-κB-dependent transcriptional activation when overexpressed [15], while the remainder are not known to influence the NF-κB pathway.

3.3. Novel polyubiquitin binding proteins possess both typical and novel polyubiquitin binding domains

Sequence analysis of the novel putative polyubiquitin binding proteins revealed that AWP1, TEX27, TTC3 and ZNF313 possessed zinc finger motifs (Fig. 2A) which, given their location within the expressed protein fragments, may have mediated their binding to polyubiquitin substrates. AWP1 and TEX27 contain an N-terminal A20-like zinc finger and a C-terminal AN1-like finger, while TTC3 and ZNF313 contain RING-finger motifs. Of the remaining proteins, CALCOCO2 (calcium-binding coiled coil) possesses an N-terminal calcium-binding domain and a C-terminal region similar to the LIM zinc finger domain [16]. RIO3 possesses a C-terminal kinase domain, while UBFD1 contains the UBQ signature present in ubiquitin and related proteins (Fig. 2A). The absence of known polyubiquitin binding domains in these proteins suggests that they bind polyubiquitin through novel mechanisms.

3.4. Novel polyubiquitin binding proteins interact with K48- and K63-linked polyubiquitin

Under physiological conditions, UbC appears to be processed into ubiquitin monomers by a C-terminal hydrolase [17]. The monomers that arise are then used as substrates by E3 ligases for conjugation to proteins, typically as K48- or K63-linked polyubiquitin [18]. While other linkages, including K6, K11 and K27 do occur, to date only the linear, K48- and K63-linkage variants have been found in association with the NF-κB signaling pathway [19]. With this in mind, we next

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sought to determine if the novel polyubiquitin binding proteins could bind to these two different forms of ubiquitin. We thus spotted UbC, monoubiquitin, and either K48-linked or K63-linked polyubiquitin onto membranes and performed an overlay assay with each of the purified novel binders.

Full-length NEMO bound both K48- and K63-linked polyubiquitin (Fig. 2B), as demonstrated by other workers [1]. Among the novel interactors, AWP1 and RIO3, and to a lesser extent N4BP1 and TTC3, bound to K63-linked polyubiquitin. Only AWP1 and RIO3 displayed observable binding to K48-linked ubiquitin, similar to that observed for NEMO (Fig. 2B). A visible lack of K48- and K63-linked polyubiquitin binding by CALCOCO2, TEX27 and ZNF313 may be due to their affinity for other linkage variants of polyubiquitin, or possibly due to a low affinity for these forms of polyubiquitin which we were unable to detect. None of the novel interactors displayed affinity for purified GST, which confirmed that the interactions detected by array screening were not the result of binding to this tag. Additionally, we also noted that the yield of recombinant protein obtained from *E. coli* for each of the interactors corresponded closely with the spot intensities shown on the array, with highly expressed proteins (e.g., RIO3) having intense array spots and poorly expressed proteins (e.g., ZNF313) having relatively faint array spots. This finding suggests that the original array screen was limited by *E. coli* protein expression levels on the array rather than the amount of GST-UbC probe used for screening.

3.5. Cell line-expressed novel interactors bind to UbC

We next investigated whether the polyubiquitin binding proteins, expressed as full-length proteins in human cells, were able to bind to ubiquitin using a GST pulldown assay. Extracts from HEK-293T cells expressing the interactors were prepared and incubated with immobilized GST or GST-UbC, followed by SDS-PAGE and detection with specific antibodies. As shown in Fig. 2C, GST-UbC but not GST was able to bind each of the proteins which again confirmed that the interactions detected by the array screening were not the result of binding to the GST tag. Samples of lysate from HEK-293T cells expressing the interactors (input) were used as positive controls for the presence of the polyubiquitin binders in the cells. The lack of a commercially available antibody or full-length clone of UBFD1 prevented the investigation of this protein in these pulldown experiments. Validation of the input GST and GST-UbC proteins used for these pulldown experiments was performed by both Coomassie staining and immunoblotting. Both methods clearly revealed the presence of the expected 27 and 106 kDa protein bands corresponding to GST and GST-UbC, respectively (Fig. 2D).

3.6. AWP1 and RIO3 modulate NF-κB signaling in human cells

To investigate a potential role for the novel polyubiquitin interactors in NF-κB signaling, we overexpressed AWP1 and RIO3 in HEK-293T cells and monitored NF-κB activity in response to treatment with TNFα. The TNFα cytokine was chosen because of its well-characterized mode of action, which depends on polyubiquitination of RIP following activation of the TNF receptor. Over-expression of optineurin has previously been demonstrated to strongly suppress TNFα-induced NF-κB activation by competing with NEMO for polyubiquitinated RIP [8] and was used as a control in these experiments. Transfection of HEK-293T cells with plasmids encoding AWP1, RIO3 and optineurin yielded a clear dose-dependent increase in

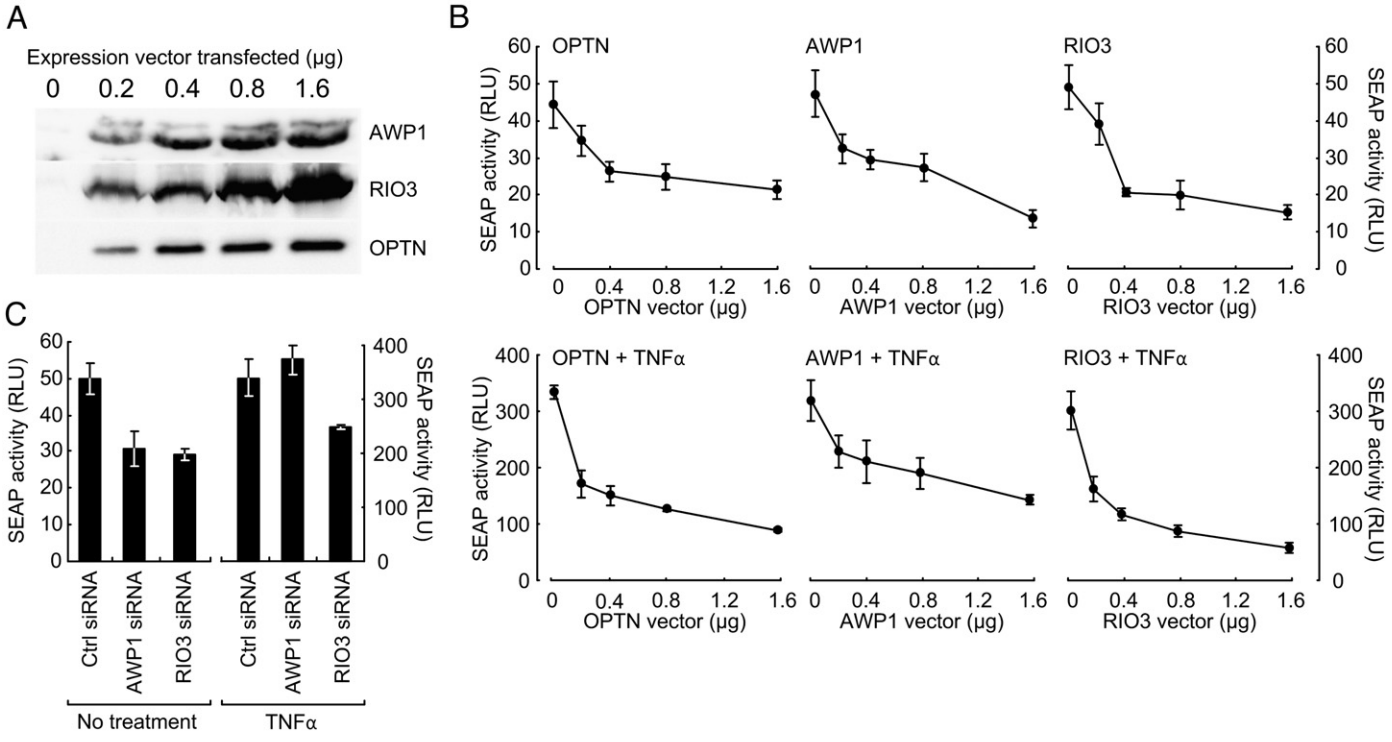

Fig. 3. AWP1 and RIO3 modulate NF-κB activity. (A) HEK-293T cells were transfected with pNF-κB-SEAP and expression vectors for AWP1, RIO3 or His_6-tagged optineurin (control), with empty vector being added to equalize the amount of DNA used in transfection. Overexpression was confirmed by immunoblotting cell lysates at 24 h post-transfection using anti-AWP1, anti-RIO3 or anti-His_6. (B) Determination of SEAP activity in cell culture supernatants following transfection. HEK-293T cells were transfected for 24 h prior to treatment with TNFα (100 ng/ml) for a further 24 h. SEAP activity was measured and expressed as relative light units (RLU). Error bars represent standard deviation of at least three independent determinations. Treatment with TNFα did not alter levels of transfected protein expression as determined by immunoblot (data not shown). (C) AWP1 and RIO3 are required for maximal NF-κB transcriptional activation. HEK-293T cells were transfected with pNF-κB-SEAP and 20 pmol of AWP1, RIO3 or scrambled (ctrl) siRNA, followed by addition of TNFα at 24 h post-transfection. SEAP activities are expressed as a percentage of the scrambled siRNA control. Values represent the means of at least four determinations, with error bars representing the standard deviations.

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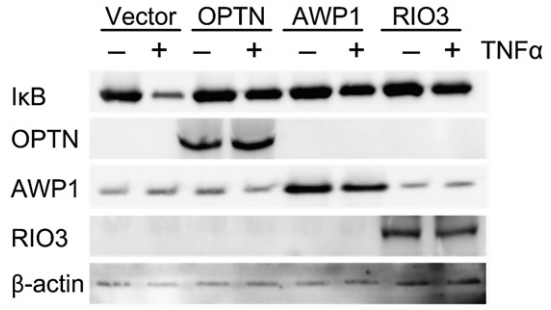

Fig. 4. Overexpression of AWP1 and RIO3 suppresses TNFα-dependent degradation of IκBα. HEK-293T cells in 6-well plates were transfected with His_6-optineurin (positive control), AWP1, RIO3 or empty expression vectors for 20 h and treated with TNFα (5 ng/ml) or vehicle for 15 min. Proteins were detected by immunoblotting as described in the methods, with β-actin being used as a loading control.

protein expression (Fig. 3A) that remained unaffected by the addition of TNFα (data not shown). Overexpression of both AWP1 and RIO3 substantially reduced reporter activity in both the presence and absence of TNFα, similar to the repression seen when optineurin was overexpressed (Fig. 3B).

While AWP1 and RIO3 negatively affected NF-κB transcriptional activation when overexpressed, we were also interested to determine if these proteins affected NF-κB signaling at physiological levels. We therefore cotransfected HEK-293T cells with pNF-κB-SEAP and synthetic siRNAs directed against either AWP1 or RIO3. In the absence of TNFα, both the AWP1 and RIO3 siRNAs reduced reporter activity by approximately 40% compared to scrambled control siRNAs (Fig. 3D). In the presence of TNFα, AWP1 siRNA had little impact on NF-κB reporter activity, while RIO3 siRNA reduced the reporter activity by almost 30% compared to the control siRNA (Fig. 3D). Surprisingly, these results indicated that both AWP1 and RIO3 are positive regulators of NF-κB activity, despite our finding that both proteins repress NF-κB activity when overexpressed.

To more clearly define how AWP1 and RIO3 influence NF-κB activity, we next looked at whether these proteins could interfere with the TNFα-dependent degradation of IκBα. As before, we used optineurin, a known suppressor of IκBα degradation [8], as a control protein. In the presence of the empty control vector, addition of TNFα resulted in a clear reduction of IκBα (Fig. 4), while overexpression of optineurin clearly suppressed this degradation as described previously [8]. Similarly, AWP1 and RIO3 overexpression had an obvious effect of IκBα levels, reducing its TNFα-dependent degradation almost as efficiently as optineurin. Thus, overexpression of both AWP1 and RIO3 appears to influence IκBα activity in a similar way to a known pUb-binding NF-κB suppressor.

4. Discussion

This study represents the first use of protein arrays for the rapid identification of polyubiquitin binding proteins. Our screen using UbC revealed 31 different polyubiquitin binding proteins, of which eight were novel interactors. The majority of the identified interactors are known from previous work to bind polyubiquitin, including proteins known to modulate NF-κB signaling through their polyubiquitin binding such as NEMO, optineurin, ABIN-1, ABIN-2 and A20, which validates the usefulness of our screening method.

Among the novel polyubiquitin interactors, AWP1, TEX27, CALCOCO2, TTC3, UBFD1 and ZNF313 all possess domains that suggest an interaction with ubiquitin, including zinc finger domains in AWP1, TTC3 and ZNF313, and the LIM domain in CALCOCO2. Of particular interest was the finding that RIO3, the third member of the RIO kinase family, bound ubiquitin via a domain in the N-terminus of the protein. Among the three known RIO kinases, the N-terminus of RIO3 is the only one of previously unknown function [20]. RIO1 and RIO2 kinases possess N-terminal helix-turn-helix domains that appear to mediate DNA binding and play a role in regulating ribosome biogenesis and the cell cycle [20]. Our finding that the N-terminal region of RIO3 mediates polyubiquitin binding suggests a distinct function for this protein. The observation that RIO3 also binds K48- and K63-linked polyubiquitin and is required for maximal NF-κB activity suggests that this protein is an accessory of the NF-κB signaling pathway. RIO3 is ubiquitously expressed [21] and hence may represent an important new modulator of NF-κB signaling.

Previous work has indicated that overexpressed AWP1 represses NF-κB mediated transcriptional activation [15], and an analysis of AWP1 domain structure provides obvious hints as to how this might occur. Of the zinc finger domains in AWP1 (see Fig. 2), the N-terminal domain belongs to the A20 family of zinc fingers, of which the A20 protein functions as a deubiquitinating enzyme that removes K63-linked polyubiquitin from RIP to suppress TNFα signaling. While AWP1 does not possess the cysteine protease domain that enables A20 to deubiquitinate RIP, overexpressed AWP1 might instead compete with NEMO for K63-linked polyubiquitin, similar to optineurin [8]. However, our finding that siRNA directed at endogenous AWP1 mRNA also reduced NF-κB activity suggests that AWP1 is a positive regulator of the NF-κB pathway under physiological conditions. The apparent contradiction in results for both RIO3 and AWP1 when comparing overexpression with siRNA knockdown is difficult to explain as the biological functions of these proteins remain unknown. However, it is interesting to note that NEMO overexpression leads to dramatic repression of NF-κB signaling [22,23], which is counterintuitive given that siRNA knockdown or genetic knockout of NEMO completely ablates NF-κB activity. Previous workers have suggested that this paradox is due to misregulation [22,23] that occurs following overexpression, which may explain our results. One conclusion we can make, however, is that AWP1 and RIO3 do not function in the same way as optineurin, whose knockdown by siRNA leads to dramatically enhanced NF-κB activity [8], while AWP1 and RIO3 knockdown reduces NF-κB activity somewhat. Future work will be required to determine at what step in the NF-κB signaling pathway AWP1 and RIO3 exert their effects.

While numerous known polyubiquitin binding proteins were identified in this screen, it is likely that many other binders were not identified in this screen due to a poor or reversible affinity for polyubiquitin or UbC in particular, or incomplete and insoluble expression in *E. coli* prior to screening of the arrays. Additionally, the arrayed represent only a fraction of the human proteome and we thus cannot conclude that the identified binders represent the bulk of such proteins in human cells. However, the identification of such a large number of known NF-κB regulators is quite compelling in terms of the usefulness of this screen for this subgroup of polyubiquitin binding proteins. Moreover, the recent discovery by Tokunaga et al. [19] that conjugation of linear polyubiquitin chains to NEMO regulates canonical NF-κB signaling appears to increase the relevance of our screen using UbC, a linear form of polyubiquitin. Proteins such as RIO3 and AWP1 would, given their UbC binding activity, be able to interact with these linear polyubiquitin chains linked to NEMO and may influence NF-κB signaling via this interaction.

In short, we have demonstrated here that UbC is a useful bait protein for the rapid detection of polyubiquitin binding proteins using protein arrays and has enabled the identification of both known and novel regulators of NF-κB activity, which is a key to the control of immune responses, differentiation and survival of most eukaryotic cells.

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