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RESEARCH ARTICLE

Modification of small ubiquitin-related modifier 2 (SUMO2) by phosphoubiquitin in HEK293T cells

Julius T. Dongdem[1,2] | **Simon P. Dawson**[1] | **Robert Layfield**[1]

[1] School of Life Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, UK

[2] Department of Biochemistry and Molecular Medicine, School of Medicine, University for Development Studies, Tamale, Ghana

Correspondence
Julius T. Dongdem, School of Life Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH, Nottinghamshire, England. UK.
Email: mbajd2@nottingham.ac.uk, julius.dongdem@uds.edu.gh

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Abstract

Additional complexity in the post-translational modification of proteins by ubiquitin is achieved by ubiquitin phosphorylation, for example within PINK1-parkin mediated mitophagy. We performed a preliminary proteomic analysis to identify proteins differentially modified by ubiquitin in HEK293T, compared to phosphomimetic ubiquitin (Ser65Asp), and identified small ubiquitin-related modifier 2 (SUMO2) as a candidate. By transfecting SUMO2 and its *C*-terminal−GG deletion mutant, along with phosphomimetic ubiquitin, we confirm that ubiquitin modifies SUMO2, rather than vice versa. Further investigations revealed that transfected SUMO2 can also be conjugated by endogenous phospho-Ser65-(poly)ubiquitin in HEK293T cells, pointing to a previously unappreciated level of complexity in SUMO2 modification, and that unanchored (substrate-free) polyubiquitin chains may also be subject to phosphorylation.

KEYWORDS
HEK293T, phosphorylation, post-translational modification, Ser65, SUMO2, ubiquitin, unanchored polyubiquitin

1 | INTRODUCTION

Ubiquitin is a small (8.6 kDa) 76-residue regulatory protein which is found 'ubiquitously' in the cytoplasm and nucleus of eukaryotic cells and is a post-translational modifier of other proteins [1, 2]. Protein ubiquitylation is a process in which ubiquitin is covalently attached to a specific protein target in a three-step enzymatic cascade involving the action of an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-ligase enzyme [3]. The functional consequences of ubiquitylation vary as a result of recognition by different ubiquitin-binding modules which are able to distinguish different (poly)ubiquitin modifications [4]. For example, monoubiquitylation has been implicated in the endocytic trafficking of certain cargo proteins for example, GTPases and receptors for example, epidermal growth factor receptor, to specific cellular compartments at different stages of the endocytic pathway. Lys48-linked polyubiquitylation that is, polyubiquitin chains linked *via* Lys48 of ubiquitin, have classically been linked to targeting substrates for proteasomal degradation [5]. In contrast, Lys63-linked polyubiquitin chains function as scaffolds to assemble signalling complexes including activation of the transcription factor NF-κB, involved in inflammatory and immune response.

Abbreviations: CCCP, carbonyl cyanide m-chlorophenyl hydrazine; DLB, denaturing lysis buffer; DUB, deubiquitinase; E2, E2 ubiquitin conjugating enzyme; E3, E3 ubiquitin ligase; EV, empty vector; GFP-SUMO2, Green fluorescent protein tagged SUMO2; HF, His FLAG-tag; HF-SUMO2, His FLAG-tagged SUMO2; HF-UbS65D, His FLAG-tagged ubiquitin Serine65Aspartate phosphomimetic mutant; HF-UbWT, His FLAG tagged ubiquitin wild-type; IMAC, immobilised metal affinity chromatography; MuBiSiDa, mammalian ubiquitylation site database software; NETN, Sodium EDTA Tris NP-40 buffer; PEI, polyethylenimine; pkk223-3, pKK223-3 vector, GenBank: M77749; pSer65, phospho-Ser65; RUBI, Rapid ubiquitylation software; S65D_UbpKK223-3, Human ubiquitin Ser65Asp mutant cloned into a pKK223-3 vector; SMART, simple modular architectural research tool; SUMO2ΔGG, *C*-terminal −GG deleted SUMO2 variant incapable of substrate conjugation but able to accept post-translational modifications; Ub, ubiquitin; UbiSite, ubiquitylation site prediction software; Ubpkk233-3, human ubiquitin wild-type cloned into a pKK223-3 vector; UbS65D, ubiquitin Serine65Aspartate phosphomimetic mutant; UbWT, ubiquitin wild-type; ZnF-UBP, zinc finger ubiquitin binding domain; ZnF-UBP-Sepharose, zinc finger ubiquitin binding domain coupled to Sepharose

Ubiquitylation of ULK1 with Lys63-linked polyubiquitin by TRAF6 activity, stabilises ULK1 thereby promoting the initiation of autophagy and autophagosome formation. In addition, ligation of Lys63-linked polyubiquitin to BECLIN 1 by TRAF6 (E3), promotes BECLIN 1-mediated induction of autophagy [6]. Parkin-mediated Lys63-linked polyubiquitylation is also a signal that promotes the sequestration of misfolded proteins into aggresomes via HDAC6-dynein motor complex for subsequent autophagic clearance [7]. Activation of the NF-κB pathway via activation of TAK1 which in turn phosphorylates and activates IκB kinase (IKK) by unanchored (substrate-free) Lys63-linked polyubiquitin chain assembled via UBE2N/UBE2V1 (E2) and TRAF6 enzymes has also been demonstrated [8]. Dysfunction of ubiquitin signalling has been implicated in a wide range of diseases including cancer [9], neurodegeneration [10], cardiovascular [11] and metabolic disorders [12].

More recently, the demonstration that ubiquitin itself can be modified through phosphorylation by kinases including PINK1 provided a major breakthrough linking two fundamental signalling pathways in cells; phosphorylation and ubiquitylation [13–15]. Ubiquitin can be phosphorylated on Ser, Thr and Tyr residues, which may further modulate its regulatory functions; however, the full physiological and indeed pathological significance of these additional modifications is unclear. In addition to activation of parkin, the consequences of Ser65 phosphorylation of ubiquitin on the structure, polyubiquitin chain assembly, recognition, hydrolysis and downstream mitochondrial homeostasis have been reported [16, 13–15, 17, 18]. Studies have suggested that phospho-Ser65-ubiquitin is the parkin receptor on damaged mitochondria [19], but the pathophysiological relevance of this post-translational modification in the PINK1-parkin mitophagy pathway in neurons and brain tissue is not fully understood. While phosphorylation of the modifier protein, ubiquitin, further increases complexity, it also provides more selectivity and specificity for an apparently universal ubiquitylation process [20, 21]. Although the function of Lys63-linked phospho-Ser65-(poly)ubiquitin in parkin activation and directing damaged mitochondria to the autophagy pathway has been established, the broader molecular consequences of ubiquitin phosphorylation, including targets of phosphoubiquitin, have not been fully explored [20, 14, 22, 23, 18, 24].

Further, comparatively little is known about the regulation of unanchored polyubiquitin chains. In the same way that conjugated polyubiquitin chains can be considered to represent an 'ubiquitin code', distinct sub-populations of unanchored polyubiquitin chains are likely to underlie different biological activities [25]. An unanchored polyubiquitin chain is a free ubiquitin chain that is not conjugated to a substrate protein. Knowledge of the range of fundamental processes that may be regulated by unanchored polyubiquitin and unanchored phosphorylated-polyubiquitin chains, as well as the precise molecular composition of these ubiquitin polymers (i.e., presence of different isopeptide linkages, etc.) is also lacking. Whether, like conjugated polyubiquitin, such substrate-free ubiquitin assemblies are also regulated by modifications such as phosphorylation, is unclear, but previously established robust purification protocols mean that such questions can now be addressed [26].

Significance Statement

Eukaryotes have an extensive repertoire of PTMs such as phosphorylation and ubiquitylation. Phosphorylation of Serine residues of ubiquitin modifies its structure, polyubiquitin chain assembly and deubiquitylase-dependent chain hydrolysis, providing an additional layer of regulation in ubiquitin signal functions. Site-specific ubiquitin phosphorylation generating pSer65-ubiquitin in the PINK1-parkin pathway is relevant in diseases associated with mitochondrial dysfunction including Parkinson's disease, cancer, cardiovascular and metabolic disorders.

In the same way that conjugated polyubiquitin chains can be considered to represent an 'ubiquitin code', distinct subpopulations of unanchored (substrate-free) polyubiquitin chains are likely to exert different biological activities. Knowledge of the range of fundamental processes that may be regulated by unanchored polyubiquitin as well as the precise molecular composition of these ubiquitin polymers is also lacking.

This project used proteomic approaches to identify and catalogue mammalian target proteins modified by covalent attachment of pSer65-(poly) ubiquitin from cultured human embryonic kidney cells and identified SUMO2 as a candidate, and indicated that unanchored polyubiquitin chains may also be subject to phosphorylation.

We hypothesised that there are many other protein targets beyond mitochondrial outer membrane proteins [18] that are covalently modified by phosphorylated ubiquitin. This investigation therefore attempted to identify novel mammalian proteins that are modified by covalent modification with phospho-Ser65-(poly)ubiquitin in HEK293T cells.

2 | MATERIALS AND METHODS

2.1 | Expression of His FLAG-tagged ubiquitin and phosphomimetic Ser65Asp mutant

Human ubiquitin wild-type (UbWT) and Ser65Asp (UbS65D) phosphomimetic mutant genes were amplified by PCR from a Ubpkk223-3 construct (human ubiquitin cloned into a pKK223-3 vector, GenBank: M77749) and S65D_Ubpkk223-3 mutant constructs to engineer a *Not*I cleavage site directly upstream of the *atg* ubiquitin start codon in the forward primer (CACACGCGGCCGCATGCAGATCTTCGTCAAGACGTTA) and an *Apa*I recognition site downstream of the ubiquitin stop codon (*tga*) in the reverse primer (CACACGGGCCCTCAACCACCTCTTAGTCTTAAGAC) (SIGMA-ALDRICH Company Ltd, Gillingham, UK). Following gel purification, cDNAs obtained were digested with *Apa*I (1 U/50 μL) at 25°C. After 3 h of incubation, *Apa*I was

heat-inactivated at 65°C on an Incublock Microtube Incubator (Labnet International *Inc*., NJ, USA) for 20 min before *Not*I-HF (1 U/50 μL) was added at 37°C for another 3 h. cDNA digests were purified by gel extraction and ligated into the *Not*I-HF/*Apa*I restriction sites of the pre-cut *N*-terminal His FLAG-tagged pcDNA3.1Zeo+ vector (i.e., pcDNA3.1Zeo+, addgene Catalog Number V86020, into which a His_6-FLAG oligo was ligated to generate the His-FLAG tag) following post-restriction alkaline phosphatase treatment, to allow expression in mammalian cells. Construct DNAs were amplified by transforming into competent *Escherichia coli* XL10-Gold strains and extracted by the QIAprep Spin Maxiprep protocol. Sequences were confirmed by DNA sequencing using CMV promoter 5'/Bovine growth hormone poly (A) 3' primer pair.

Cultured HEK293T cells at ~80% confluence were transiently transfected with the purified His FLAG-tagged pcDNA3.1Zeo+ empty vector, UbWT or UbS65D mutant construct (HF-EV, HF-UbWT, HF-UbS65D) by the polyethylenimine (PEI) transfection protocol [27, 28]. 10 μg of each DNA was diluted in 250 μL of PBS while 30 μL of 1 mg/mL PEI solution was diluted in 250 μL of PBS per 10 cm dish. The PEI/DNA complex was prepared by adding PEI dropwise to each DNA solution, mixing gently and allowing to sit at room temperature for 15 –30 min. Each complex was then added dropwise to the appropriate dish of cells in media containing 2% FBS (DMEM high glucose, 2% FBS, 1% Penicillin-Streptomycin). Cells were returned to the tissue culture incubator at 5% CO_2, 100% humidity for 18 h after which media containing 2% FBS was replaced with complete media (DMEM high glucose, 10% FBS, 1% Penicillin-Streptomycin) and further incubated for 48 h in the tissue culture incubator.

2.2 | IMAC purification of ubiquitin-modified proteins

Cells from each transfection were scraped in 6 mL pre-chilled PBS after which 10% of each cell suspension was pelleted at 3000 × *g* for 5 min at 4°C. After discarding the PBS by carefully pipetting, each pellet was re-suspended in 50 μL of 1 × Laemmli sample loading buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) containing 200 mM imidazole in order to subsequently elute His-tagged protein from the Ni^{2+} resin. The remaining 90% of cell suspensions were in turn pelleted at 3000 *g* for 5 min and re-suspended in 4 mL Denaturing Lysis Buffer, pH 8.0 (DLB; 6 M Guanidine.HCl (GnHCl), 20 mM Na_2HPO_4/NaH_2PO_4, pH 8.0, 500 mM NaCl, 20 mM imidazole). Proteins that had covalently attached to HF-UbWT or HF-S65D mutant proteins in cell sample lysate were purified by small-scale denaturing IMAC. Briefly, 30 μL of His-Select Nickel Affinity Gel resin for purification of His-tagged proteins per 10 mL QIAGEN disposable affinity column were prepared by washing each resin 1 × 15 min with 10 mL of DLB rotating gently at room temperature after which DLB was allowed to flow through columns by gravity. To shear released genomic DNA and make the extracted solution less viscous to pipette, cells were sonicated 3 × 30 s on ice and rotated gently on an end–over-end rotator mixer for 10 min at 4°C to ensure complete cell lysis. Cell fragments were pelleted at 13,000 rpm in a benchtop centrifuge for 10 min. Supernatants containing protein extracts were transferred to previously prepared IMAC resin in affinity chromatography columns and rotated gently for 4 h at room temperature. Following protein binding, the supernatant was allowed to flow through by gravity after which beads were successively washed 1 × 15 min with 10 mL of wash buffer-1 (WB1; 8 M Urea, 20 mM Na_2HPO_4/NaH_2PO_4, pH 8.0, 500 mM NaCl, 20 mM imidazole), 2 (WB2; 8 M Urea, 20 mM Na_2HPO_4/NaH_2PO_4, pH 6.0, 500 mM NaCl, 20 mM imidazole), 3 (WB3; WB2 + 0.2% v/v Triton X-100), 4 (WB4; WB3 + 0.1% v/v Triton X-100) and lastly with PBS. 25 μL of beads with associated proteins were dried at 2000 *g* for 1 min and stored at -20°C for LC-MS/MS identification of bound proteins. Proteins on remaining 5 μL of beads were eluted with Laemmli sample loading buffer containing 200 mM imidazole. SDS-PAGE was performed on eluted samples and analysed by immunoblotting resolved proteins with anti-His-tag (AbD Serotec, Kidlington, UK) or anti-(VU-1) ubiquitin (Life Sensors, PA, USA) antibodies and visualised by ECL. Following ECL, blots were stripped and reprobed with anti-ß-actin antibodies (SIGMA-ALDRICH, Gillingham, UK).

2.3 | Identification of proteins modified by covalent attachment of phosphoubiquitin by LC-MS/MS

The shotgun proteomic approach was employed to identify proteins in IMAC-purified mixtures as detailed in literature [29–31]. Protein on ~25 μL of beads were initially reduced by addition of 50 mM DTT, alkylated by treatment with 100 mM chloroacetamide and subjected to tryptic digestion using 0.02 mg/mL sequencing-grade trypsin. Resulting peptide mixtures obtained, were separated by tandem mass spectrometry (MS/MS) on an LTQ-Orbitrap-Velos spectrometer (Thermo Scientific, Wilmington, USA) with nano-flow liquid chromatography (Dionex Ltd, Camberley, UK). Proteome Discoverer (version 1.4.0.288, Thermo Scientific, Wilmington, USA) was used to process the raw data file obtained from the LC-MS/MS acquisition. The file was then searched using Mascot (version 2.2.04, Matrix Science Ltd, Marylebone, London, UK) against the UniProt Human_2018_03 database. Peptides were identified in data-dependent mode combining Mascot and X! Tandem search engines to validate MS/MS assigned spectra from Proteome Discoverer. The peptide and MS/MS tolerance were set to 10 ppm and 0.02 Da, respectively. Fixed modifications were set as alkylation of cysteine, and variable modifications set as deamidation of Asn and Gln, and oxidation of Met and Pro residues. Peptides were identified at greater than 95.0% probability, with a minimum of two peptides required for protein identification. IMAC and LC-MS/MS was performed on an initial small-scale pilot screen, and a subsequent more preparative scaled-up repeat, with proteins of interest found to be overlapping in the analyses triaged for biochemical confirmation. In each case, LC-MS/MS was conducted in six replicates.

2.4 | Confirmation of the covalent modification of transfected SUMO2 by ubiquitin *in vivo*

SUMO2 and SUMO2ΔGG (a *C*-terminal—GG deleted SUMO2 variant incapable of substrate conjugation but able to accept post-translational modifications) cDNAs engineered in pEGFP-C1 vector to generate GFP-tagged SUMO2-GG and GFP-tagged SUMO2ΔGG constructs were co-transfected with equal amounts of HF-UbWT construct created earlier into HEK293T cells as follows; GFP-SUMO2-GG and HF-UbWT, and GFP-SUMO2ΔGG and HF-UbWT. Following harvesting, cells were lysed in DLB and proteins were purified by IMAC as described previously. After washes, 25 μL of 1 × Laemmli loading buffer containing 200 mM imidazole was added to elute bound proteins. Eluted proteins were separated by SDS-PAGE and analysed by western blotting with anti-(VU-1) ubiquitin and rabbit anti-GFP (Abcam *plc*., Cambridge, UK) (1:2000) antibodies.

2.5 | Modification of transfected SUMO2 by endogenous phospho-Ser65-ubiquitin

HEK293T cells seeded at 1.5 × 10^6 cells per 10 cm dish were transfected at ∼80% confluence with SUMO2 cloned into pcDNA3.1Zeo+ to express a His-FLAG-tagged SUMO2 (HF-SUMO2) by the PEI protocol outlined above. 40 h after incubation in fresh complete culture media, the media was replaced with complete culture media containing 10 μM CCCP, an inhibitor of oxidative phosphorylation and returned to the tissue culture incubator (5% CO_2, 100% humidity) for 8 h prior to harvesting in PBS. CCCP is known to increase total or conjugated phospho-Ser65-(poly)ubiquitin and has been used to demonstrate the subsequent recovery of cells from oxidative stress and in mitophagy [16, 13]. The cells were then lysed in DLB and proteins bound to HF-SUMO2 were purified by IMAC as previously described. Bound proteins were eluted in 1 × Laemmli loading buffer containing 200 mM imidazole, resolved by SDS-PAGE and immunoblotted with anti-(VU-1) ubiquitin and phospho-Ser65-ubiquitin (Merck KGaA, Darmstadt, Germany) antibodies. Following western blot analysis, the ubiquitin blot was stripped and reprobed with anti-His-tag antibody while the phospho-Ser65-ubiquitin blot was reprobed with monoclonal anti-FLAG M2 antibody (Millipore, Watford, UK) (1:2500). Throughout this experiment Empty His-tagged vector (HF-EV) was added as an expression and SUMO2 modification control.

2.6 | Detection of Ser65 phosphorylation in purified unanchored (poly)ubiquitin chains in HEK293T cells

Cultured HEK293T cells at ∼80% confluence were treated with 10 μM CCCP by replacing complete media with fresh media containing 10 μM CCCP in DMSO or diluent DMSO only and placed in a tissue culture incubator at 5% CO_2, 100% humidity for 8 h. Cell debris was pelleted following cell lysis and sonication at 3 × 30 s on ice. The ZnF-UBP domain affinity chromatography protocol [26] was used to purify unanchored (poly)ubiquitin from 6.0 mg protein of CCCP-treated HEK293T cells in NETN buffer (200 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.1% NP-40, pH 8.0) containing phosphatase inhibitor cocktail (1:1000) and mammalian protease inhibitors (1:1000). Protein quantitation was achieved by BCA assay using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, USA). Subsequently, 50 μL of ZnF-UBP-Sepharose bait captures were eluted into 50 μL of sample loading buffer. Eluted unanchored polyubiquitin chains obtained were resolved on 5–20% SDS-PAGE gradient gels, transferred onto nitrocellulose membrane and western blotted. After probing with both rabbit anti-phospho-Ser65-ubiquitin primary (1:1000) and rabbit polyclonal secondary (Dako Denmark, Glostrup, Denmark, 1:2000) antibodies, the blot was visualised on X-ray film after ECL treatment.

3 | RESULTS AND DISCUSSION

3.1 | IMAC-purification of ubiquitin-modified proteins from HEK293T cells

We performed a pilot screen of cellular targets of (phosphomimetic)ubiquitin by initially transfecting HEK293T cells with HF-UbWT or phosphomimetic HF-UbS65D constructs, and purified covalently modified endogenous proteins by denaturing IMAC. Ubiquitin immunoreactivity, a combination of endogenous and transfected, was detected in all input samples as expected (Figure 1) with high molecular weight smears indicating incorporation of transfected ubiquitin (anti-His) into protein conjugates. As an indication of successful IMAC purification, no ubiquitin immunoreactive smears were observed in the IMAC-purified eluates of HF-EV (empty vector) control. Anti-His blotting of inputs and purified samples from HF-UbWT and HF-UbS65D transfections revealed (transfected ubiquitin) reactivity with apparently higher abundance for the phosphomimetic, with anti-β-actin immunoblotting confirming approximately equal loads in the input samples. Overall, this is suggestive of increased conjugation of endogenous proteins with HF-UbS65D compared to HF-UbWT in HEK293T cells, and/or that the phosphomimetic is more resistant to deubiquitylation, consistent with previous observations in yeast cells [23, 18].

3.2 | Identification of ubiquitin-modified proteins from HEK293T cells by mass spectrometry

Denaturing IMAC-purified samples were subjected to LC-MS/MS analysis in order to catalogue endogenous proteins modified by transfected ubiquitin. The initial analysis identified a total of 32 proteins in purified fractions from transfections with HF-UbWT, HF-UbS65D, or both (but not HF-EV controls), from a minimum of two peptide sequences, including 19 associated with HF-UbS65D only (Table S1). Data presented on Table 1 represent data from which controls

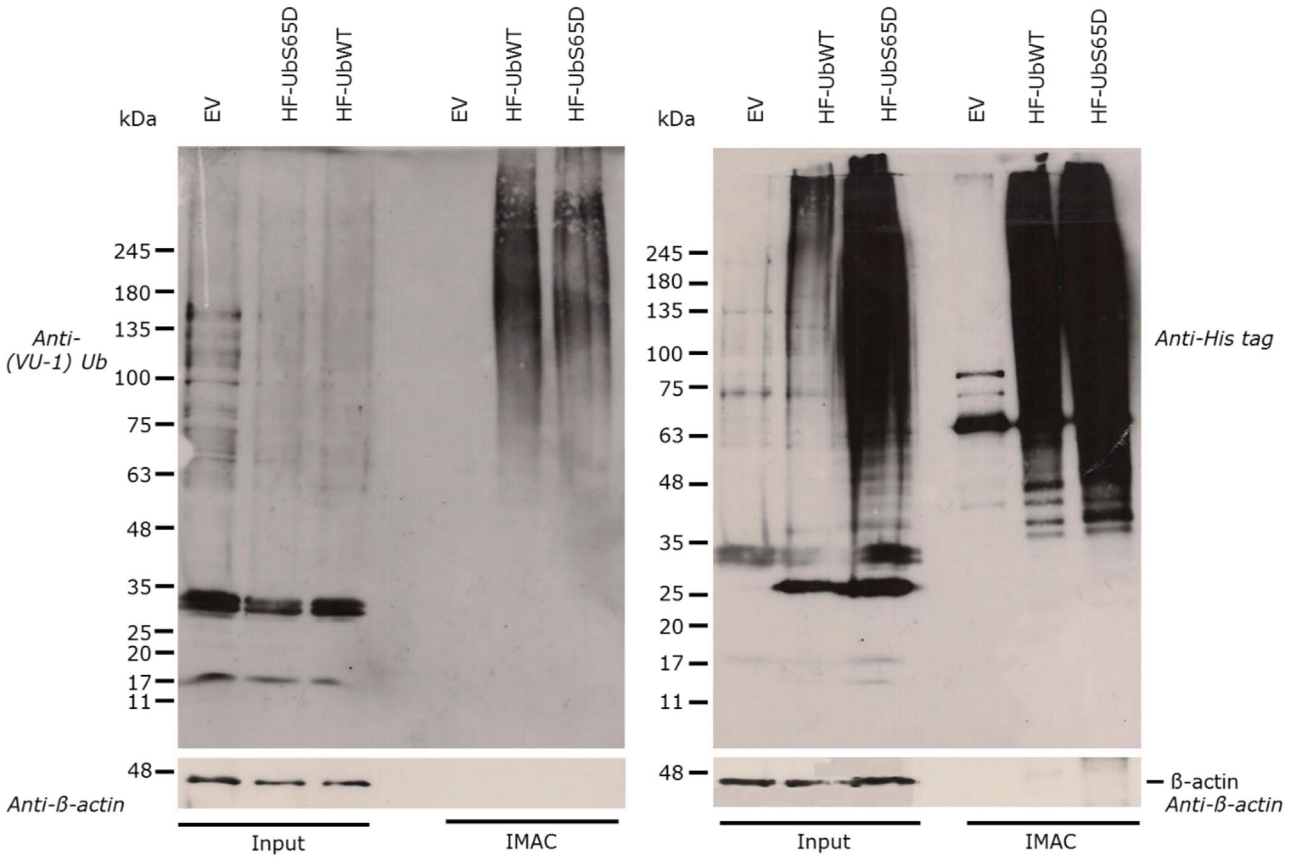

FIGURE 1 Immunoblot of purified protein targets of ubiquitylation by recombinant wild-type and Ser65Asp-ubiquitin in HEK293T cells. His FLAG-tagged UbWT and UbS65D mutant proteins were expressed in HEK293T cells. Following cell lysis in DLB buffer and sonication at 3 x 30 s on ice, cell fragments were pelleted at 13,000 *g* for 10 min. Proteins bound to UbWT and UbS65D were purified by IMAC, resolved by SDS-PAGE and immunoblotted against VU-1 ubiquitin (left panel) or anti-His-tag antibodies (right panel). Blots were stripped and reprobed with anti-*β*-actin antibodies (lower panels)

(proteins identified in transfects with HF-EV controls) had been manually excluded. One of these HF-UbS65D-modified proteins was SUMO2 (Figure 2). A subsequent more preparative scaled-up repeat using four times the amount (by protein) of transfected cell lysates following the same protocol described above, identified a total of 316 different proteins in corresponding IMAC-purified samples were detected, again including SUMO2 (Table S1) in the HF-UbWT purifications, but with increased 'total spectrum counts' (semi-quantitative indicator of protein abundance in Scaffold) in the HF-UbS65D purification. Although different scales do not permit detailed comparison of the proteomic screens, together eight overlapping proteins; ALDOA, CD9, CD47, ESYT1, HNRNPC, HSPA8, SLC6A15 and SUMO2 were identified in both experiments (~25% of proteins in the small-scale purification found to be modified in the more preparative analysis regardless of whether they were modified by UbWT or UbS65D (Table 1 and Table S1). Across both experiments, a total of 22 out of 93 residues of the endogenous SUMO2 sequence were detected in the purified samples from HF-UbS65D-transfected cells, representing sequence coverage of 24% (Figure 2). These analyses therefore suggested (preferential) modification of SUMO2 by transfected phosphomimetic HF-UbS65D compared to HF-UbWT in HEK293T cells.

3.3 | Validation of SUMO2 as a target of ubiquitin in HEK293T cells

SUMO2 has not previously been described as target of phospho-Ser65-ubiquitin, although mixed/hybrid chains with ubiquitin have been reported [32, 33], and ubiquitylation prediction software programmes (i.e., UbiSite, RUBI and MuBiSiDa) analyses conducted in this project indicate the presence of ubiquitylation sites. SUMO2 was therefore considered a potentially novel covalent target of phospho-Ser65-ubiquitin in HEK293T cells. As SUMO2 itself is an ubiquitin-like modifier, we first determined whether it was indeed undergoing modification with ubiquitin, or itself was the modifier of ubiquitin, in subsequent experiments. To this end, we co-transfected HEK293T cells with HF-UbWT and GFP-SUMO2 with or without its critical C-terminal diglycine residues that are required to mediate conjugation to targets, whilst not affecting the ability to 'accept' conjugation (i.e., GFP-SUMO2ΔGG). After denaturing IMAC-purifications, anti-ubiquitin immunoblotting indicated that deletion of the diglycine residues of transfected SUMO2 had no effect on its ability to be modified by HF-UbWT, with high molecular weight ubiquitin conjugates present in purified fractions (Figure 3). GFP-tagged protein reactive bands

TABLE 1 LC-MS/MS analyses of HEK293T proteins captured on denaturing IMAC after expression of HF-EV control, UbWT and UbS65D

Experiment 1							
No	GN	Identified proteins	Accession number	Mwt (kDa)	HF-EV	UbWT	UbS65D
1	ALDOA	Fructose-bisphosphate aldolase A (Fragment) OS = *Homo sapiens* PE = 1 SV = 1	H3BMQ8 (+6)	15	0	2	0
2	CASP14	Caspase-14 OS = *Homo sapiens* PE = 1 SV = 2	P31944	28	0	4	0
3	CD47	Leukocyte surface antigen CD47 OS = *Homo sapiens* PE = 1 SV = 1	Q08722	35	0	0	2
4	CD9	Tetraspanin OS = *Homo sapiens* PE = 1 SV = 1	A6NNI4 (+2)	18	0	0	2
5	DCD	Dermcidin OS = *Homo sapiens* PE = 1 SV = 2	P81605	11	0	2	0
6	DERL1	Derlin OS = *Homo sapiens* PE = 1 SV = 1	B4E1G1 (+1)	17	0	0	2
7	DSG1	Desmoglein-1 OS = *Homo sapiens* PE = 1 SV = 2	Q02413	114	0	0	4
8	EIF3CL	Eukaryotic translation initiation factor 3 subunit C-like protein OS = *Homo sapiens* PE = 3 SV = 1	B5ME19 (+1)	105	0	0	2
9	ESYT1	Extended synaptotagmin-1 OS = *Homo sapiens* PE = 1 SV = 1	Q9BSJ8	123	0	0	3
10	FAF2	FAS-associated factor 2 OS = *Homo sapiens* PE = 1 SV = 2	Q96CS3	53	0	2	2
11	FLG	Filaggrin OS = *Homo sapiens* PE = 1 SV = 3	P20930	435	0	0	2
12	FNDC3A	Fibronectin type III domain containing 3A, isoform CRA_f OS = *Homo sapiens* PE = 1 SV = 1	G5E9 × 3 (+1)	75	0	0	2
13	HIST2H3PS2	Histone H3 OS = *Homo sapiens* PE = 1 SV = 1	Q5TEC6	15	0	4	0
14	HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2 OS = *Homo sapiens* PE = 1 SV = 1	B2R5W2 (+6)	32	0	3	0
15	HSPA8	Heat shock cognate 71 kDa protein OS = *Homo sapiens* PE = 1 SV = 1	E9PKE3 (+2)	69	0	0	3
16	JUP	Junction plakoglobin OS = *Homo sapiens* PE = 1 SV = 3	P14923	82	0	0	9
17	KDELR1	ER lumen protein-retaining receptor 1 OS = *Homo sapiens* PE = 1 SV = 1	P24390	25	0	0	3
18	PARP1	Poly [ADP-ribose] polymerase 1 OS = *Homo sapiens* PE = 1 SV = 4	P09874	113	0	2	0
19	POC1B	Polypeptide N-acetyl galactosaminyl transferase OS = *Homo sapiens* -GALNT4 PE = 3 SV = 1	F8VUJ3 (+1)	66	0	2	0
20	PTK7	Inactive tyrosine-protein kinase 7 OS = *Homo sapiens* PE = 1 SV = 2	Q13308	118	0	0	2
21	S100A8	Protein S100-A8 OS = *Homo sapiens* PE = 1 SV = 1	P05109	11	0	3	0
22	S100A9	Protein S100-A9 OS = *Homo sapiens* PE = 1 SV = 1	P06702	13	0	2	0
23	SBSN	Suprabasin OS = *Homo sapiens* PE = 1 SV = 2	Q6UWP8	61	0	0	2
24	SLC39A7	Zinc transporter SLC39A7 OS = *Homo sapiens* PE = 1 SV = 2	Q92504	50	0	3	0
25	SLC6A15	Sodium-dependent neutral amino acid transporter B(0)AT2 OS = *Homo sapiens* PE = 1 SV = 1	Q9H2J7	82	0	0	2
26	SRRM2	Serine/arginine repetitive matrix protein 2 OS = Homo sapiens PE = 1 SV = 2	Q9UQ35	300	0	2	0
27	STX4	Syntaxin-4 OS = *Homo sapiens* PE = 1 SV = 2	Q12846	34	0	0	2
28	SUMO2	Small ubiquitin-related modifier 2 OS = *Homo sapiens* PE = 1 SV = 3	P61956	11	0	0	3
29	TMEM38B	Transmembrane protein 38B, isoform CRA_b OS = *Homo sapiens* PE = 1 SV = 1	A0A0A0MRS4 (+1)	26	0	0	2

(Continues)

TABLE 1 (Continued)

Experiment 1							
No	GN	Identified proteins	Accession number	Mwt (kDa)	HF-EV	UbWT	UbS65D
30	TMEM65	Transmembrane protein 65 OS = *Homo sapiens* PE = 1 SV = 2	Q6PI78	25	0	2	0
31	TTYH3	Protein tweety homolog 3 OS = *Homo sapiens* PE = 1 SV = 3	Q9C0H2	58	0	0	2
32	YIF1A	Protein YIF1A OS = *Homo sapiens* PE = 1 SV = 1	A6NGW1 (+1)	26	0	0	2

Numerical values in final three columns indicate *total spectrum counts*.
Minimum # peptides = 2, Number of replicates = 6.
Abbreviations: HF-EV, His FLAG-tagged empty vector; IMAC, immobilised metal affinity chromatography; UbWT, ubiquitin wild-type; UbS65D, Ser65Asp-ubiquitin; SUMO2, small ubiquitin-related modifier 2

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MADEKPKEGVKTENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMRQIRF 60

RFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG                              93
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FIGURE 2 LC-MS/MS analyses of endogenous SUMO2 captured by denaturing IMAC after expression of His FLAG-tagged Ser65Asp-ubiquitin (HF-UbS65D) in HEK293T cells. Across two experiments, a total of 22 out of 93 residues of the endogenous SUMO2 primary sequence (highlighted) were detected, representing sequence coverage of 24%

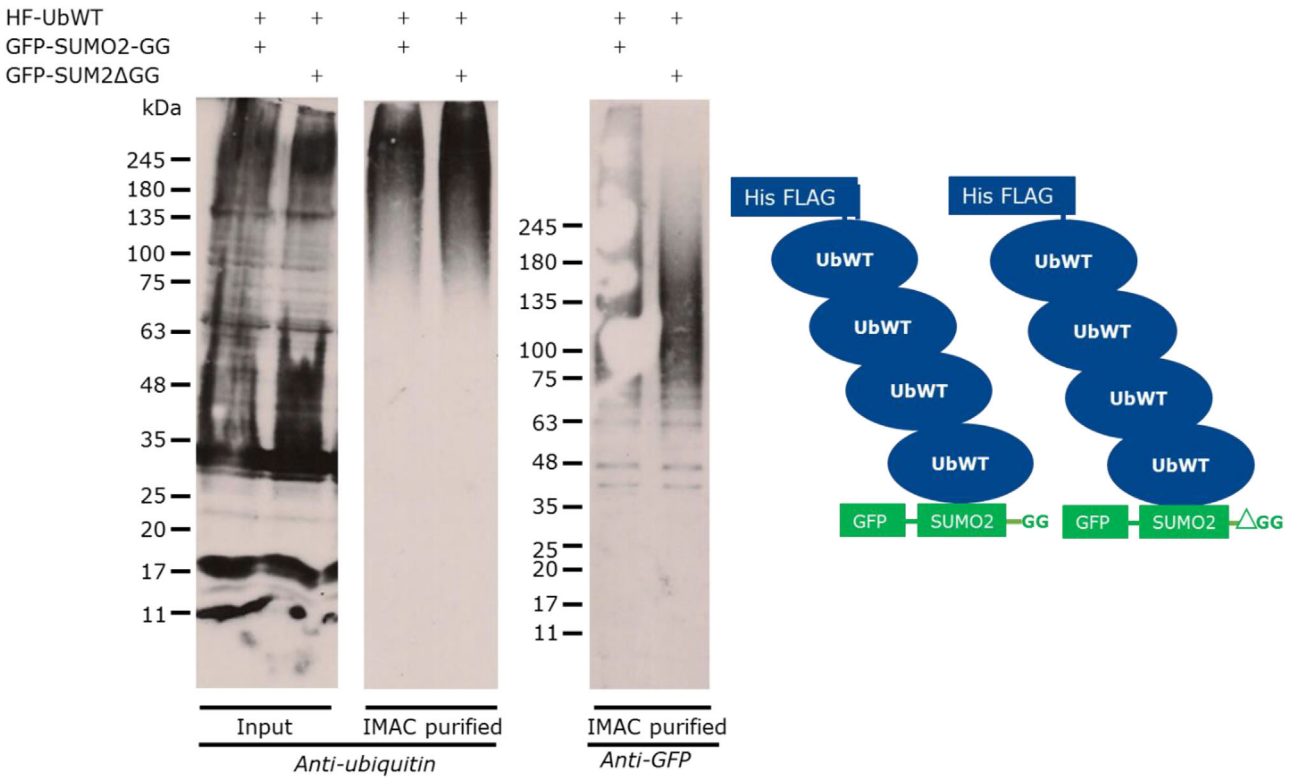

FIGURE 3 Validation of the covalent modification of proteins by phospho-Ser65-ubiquitin in HEK293T cells. His FLAG-tagged wild-type ubiquitin (HF-UbWT) was co-transfected with GFP-tagged SUMO2 or GFP-tagged SUMO2ΔGG (a *C*-terminal –GG deleted SUMO2 variant incapable of substrate conjugation but able to accept post-translational modifications) in HEK293T cells. Cells were lysed in DLB and sonicated 3x 30 s on ice. Cell protein extracts were purified by the IMAC protocol. Proteins bound to resin were eluted with 1x Laemmli loading buffer containing 200 mM imidazole after washes and further resolved by SDS-PAGE prior to western blot analysis against anti-(VU-1) ubiquitin and anti-GFP antibodies. GFP-tagged protein reactive bands were observed at the expected weight of ~48.4 kDa which suggests that IMAC-purified proteins retained GFP reactivity in the absence of *C*-terminal diglycine residues of SUMO2 (SUMO2ΔGG) indicative of modification of SUMO2 sequences by ubiquitin in HEK293T cells

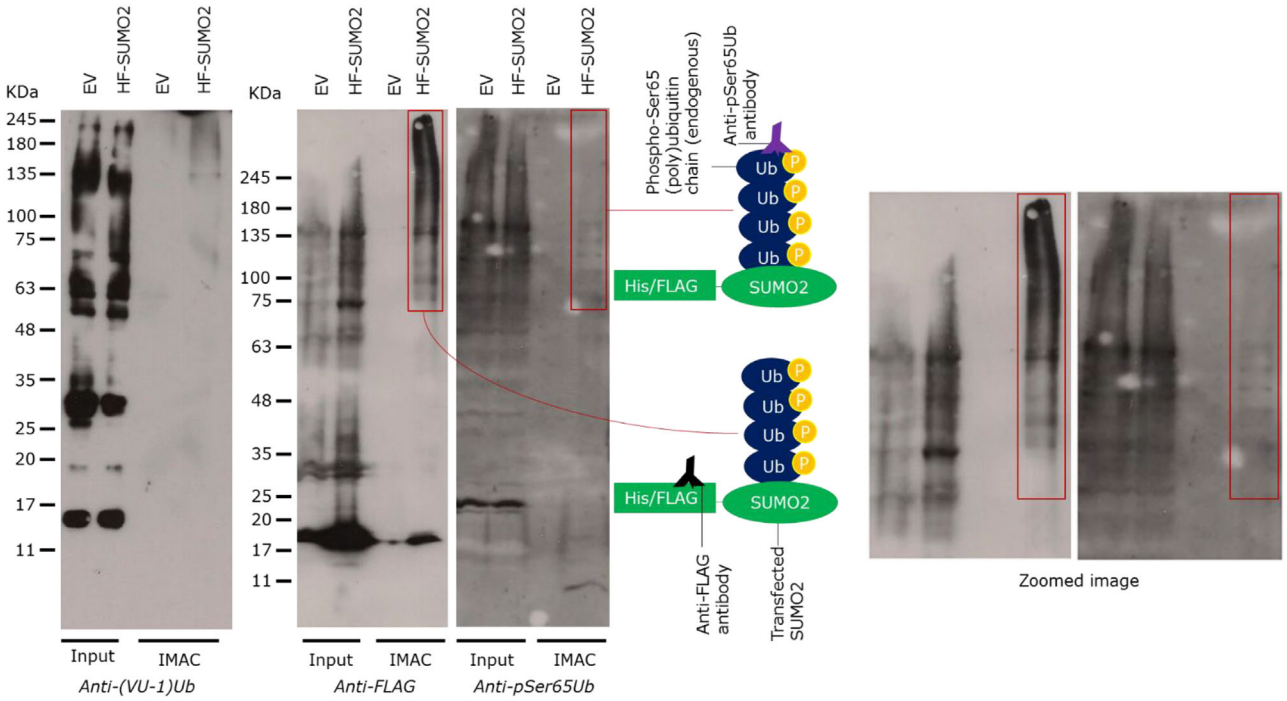

FIGURE 4 Biochemical validation of SUMO2 as covalent target of endogenous phospho-Ser65-ubiquitin. His FLAG-tagged SUMO2 was overexpressed in HEK293T cells. Forty hours after transfection, cells were treated with 10 μM CCCP which is known to increase total or conjugated phospho-Ser65-(poly)ubiquitin, for extra 8 h prior to harvesting in PBS. Cells were lysed in DLB and proteins bound to His FLAG-tagged SUMO2 were purified by IMAC. Proteins were eluted from the resin with 1X Laemmli loading buffer containing 200 mM imidazole following washes. Eluted proteins were then resolved by SDS-PAGE and immunoblotted with anti-(VU-1) ubiquitin and anti-pSer65-ubiquitin antibodies. After ECL, blots were stripped and respectively reprobed with anti-His-tag and anti-FLAG-tag antibodies

were observed at the expected weight of ~48 kDa which suggests that IMAC-purified proteins retained GFP reactivity in the absence of SUMO2 *C*-terminal diglycine residues (SUMO2ΔGG) indicative of modification of SUMO2 sequences by ubiquitin. These observations suggest that ubiquitin covalently modified SUMO2 (Figure 3, right panel) with SUMO2 representing the 'proximal' moiety in the 'hybrid' chain in HEK293T cell line.

3.4 | Confirmation of SUMO2 as a covalent target of endogenous phospho-Ser65-ubiquitin

In order to induce the formation of endogenous phospho-Ser65-ubiquitin since steady-state levels in cells are relatively low [34], HEK293T cells transfected with His FLAG-tagged SUMO2 (HF-SUMO2) or empty vector (HF-EV) control were treated with 10 μM CCCP (an inhibitor of oxidative phosphorylation which is also known to increase phosphorylation of endogenous phospho-Ser65-ubiquitin) for 8 h after which proteins covalently linked to HF-SUMO2 were purified by denaturing IMAC and analysed by western blotting (Figure 4). Ubiquitin-reactive smears were observed in eluates from IMAC-purified samples only when HF-SUMO2 was transfected, again with SUMO2 modification with endogenous ubiquitin evident (Figure 4, left panel). Accordingly, IMAC-purified samples were also positive for FLAG reactivity only when HF-SUMO2 was transfected. The anti-phospho-Ser65-ubiquitin blot of purified samples revealed distinct reactive bands indicative of the presence of endogenous phospho-Ser65-(poly)ubiquitin which had been purified by denaturing IMAC along with HF-SUMO2. Phospho-Ser65-(poly)ubiquitin immunoreactive bands were absent in controls (Figure 4, right panel). The pattern of phospho-Ser65-(poly)ubiquitin observed also corresponded to that on the anti-FLAG reactive smear, indicating covalent interaction of phosphoubiquitin with HF-SUMO2. The biochemical detection of the interaction between endogenous phospho-Ser65-(poly)ubiquitin and SUMO2 is illustrated by annotation in Figure 4 (extreme right).

Classically, protein SUMOylation is predominantly confined to the nucleus and unlike ubiquitylation, SUMOylated proteins are not targeted for degradation. SUMOylation of target proteins have several functions such as protein stability, nuclear-cytosolic transport, transcriptional regulation, cell cycle regulation in invertebrates and signal transduction. Results obtained therefore suggests that modification of SUMO2 with phospho-Ser65-ubiquitin might have several consequences in SUMO2 function by altering signals for SUMO2 degradation, DUB-mediated deubiquitylation or induction of unknown phospho-ubiquitin-SUMO2 hybrid chain effects in HEK293T cells. Nevertheless, replicating these experiments in different cell lines using HEK293T as vehicle will enable conclusive evidence to be drawn.

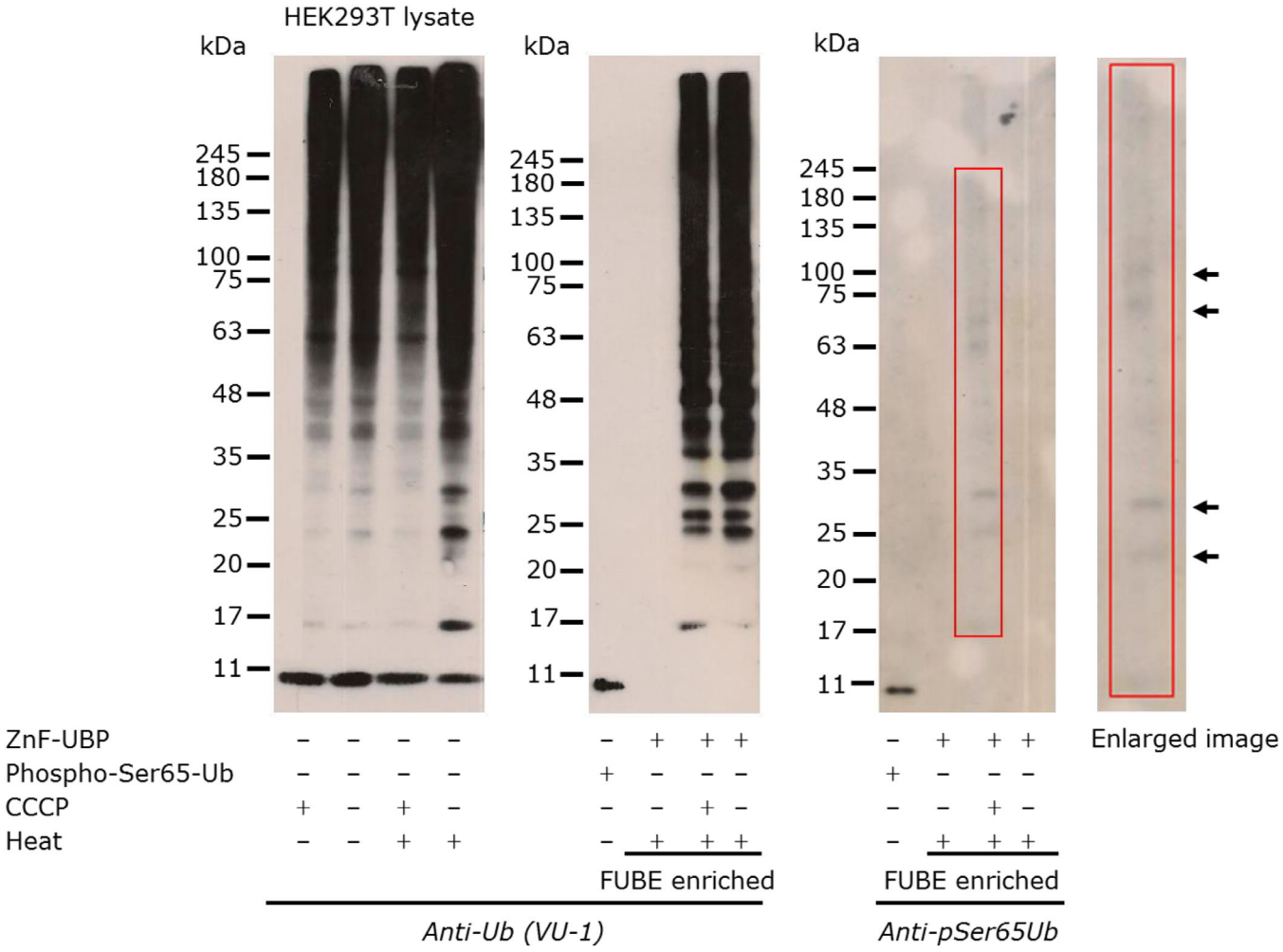

FIGURE 5 Detection of Ser65 phosphorylation in endogenous unanchored (poly)ubiquitin in HEK293T cells. 6.0 mg of HEK293T protein lysate in NETN buffer previously treated for 8 h with 10 μM CCCP (+CCCP) or diluent DMSO (-CCCP) were purified by ZnF-UBP domain affinity chromatography using 100 μL of 10 mg/mL ZnF-UBP protein on beads. Captured proteins were resolved by SDS-PAGE and immunoblotted with VU-1 ubiquitin or phospho-Ser65-ubiquitin antibodies. Commercial phospho-Ser65-ubiquitin was used as a positive control of Ser65 phosphorylation of unanchored (poly)ubiquitin chains. Traces of weak phospho-Ser65-ubiquitin reactivity is evident in purified fractions of unanchored polyubiquitin, consistent with the presence of endogenous phospho-Ser65-ubiquitin

3.5 | Detection of phospho-Ser65-ubiquitin in affinity purified unanchored (poly)ubiquitin following CCCP treatment of HEK293T cells

Unanchored polyubiquitin chains are free ubiquitin chains that are not conjugated to a substrate protein. Given our observations that 'hybrid' chains consisting of phospho-Ser65-ubiquitin and SUMO2 may be physiologically relevant, we finally investigated if unanchored polyubiquitin chains, that is, covalent assemblies of multiple ubiquitins in a substrate-free form that regulate a range of different biological pathways, also represent overlooked substrates of modification by phosphorylation [35–39, 6, 40]. Such chains (as well as monoubiquitin) can be affinity purified using an ubiquitin-binding domain (UBD) with high affinity exclusively for the free *C*-terminus of ubiquitin (but not SUMO2), the ZnF-UBP domain denoted as FUBE: that is, free ubiquitin-binding entity [25]. Heating of cell lysates prior to purification is a critical step in order to resolve unanchored polyubiquitin chains from heat-sensitive ubiquitin-protein conjugates (Figure 5, right panel) [41]. Unanchored polyubiquitin chains were successfully purified by ZnF-UBP-Sepharose affinity chromatography [26] both from cells with (+CCCP) and without CCCP (+DMSO) treatment, and not with control ZnF-UBP-Sepharose (no protein) (Figure 5). To investigate whether unanchored polyubiquitin chains (like substrate/SUMO2 conjugated forms) can be modified by phosphorylation, captured endogenous unanchored (poly)ubiquitin chains from +CCCP-treated HEK293T cells were resolved and immunoblotted with anti-phospho-Ser65-ubiquitin and VU-1 (ubiquitin) antibodies. At the 8 h +CCCP time point there was evidence of weak phospho-ubiquitin reactivity in the purified unanchored polyubiquitin fraction of treated HEK293T cells consistent with low-level Ser65 phosphorylation of unanchored polyubiquitin chains (Figure 5, third panel) [16].

3.6 | Concluding remarks

His FLAG-tagged human recombinant UbWT and UbS65D mutant were created and employed to purify protein targets modified by covalent attachment of (poly)ubiquitin or phospho-Ser65-(poly)ubiquitin in HEK293T cells by the denaturing IMAC protocol. Proteomic analysis suggested differential modification of various potentially novel target proteins by ubiquitin compared to UbS65D including endogenous SUMO2, with S65D phosphomimetic mutant modifying proteins more efficiently. By transfecting GFP-SUMO2 and its *C*-terminal–GG deletion mutant, along with ubiquitin, we confirm that ubiquitin modifies SUMO2 rather than vice versa. Further, we found that transfected HF-SUMO2 is modified by endogenous phospho-Ser65-ubiquitin in HEK293T cells. SUMO2 therefore represents a novel target of Ser65-phosphorylated ubiquitin in HEK293T cells. Endogenous unanchored (substrate-free) polyubiquitin chains from HEK293T cells were successfully purified by ZnF-UBP domain affinity chromatography. Additionally, we established that unanchored polyubiquitin chains may be regulated by phosphorylation on Ser65 and illustrated that CCCP treatment augments phosphorylation of unanchored polyubiquitin in HEK293T cells. Further studies will investigate the biological importance of (i) ubiquitin modification of SUMO2, (ii) phospho-ubiquitin modification of SUMO2, (iii) the role of phospho-ubiquitin in unanchored polyubiquitin chains in HEK293T cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Robert Layfield and Simon P. Dawson conceived the idea and supervised the project. Julius T. Dongdem contributed to development of the research hypotheses. Experiments were designed and performed by Julius T. Dongdem with inputs from Robert Layfield and Simon P. Dawson. Julius T. Dongdem analysed all data with inputs from Robert Layfield and Simon P. Dawson. Julius T. Dongdem drafted the manuscript with suggestions from Robert Layfield. Simon P. Dawson and Robert Layfield contributed to reviewing the manuscript.

DATA AVAILABILITY STATEMENT

Protein identification datasets generated and analysed during the current study are available in Zenodo open data repository (CERN) (Zenodo. http://doi.org/10.5281/zenodo.4776429) as an integral part of the supporting data.

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