USP5 enhances SGTA mediated protein quality control.

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Abstract

Mislocalised membrane proteins (MLPs) present a risk to the cell due to exposed hydrophobic amino acids which cause MLPs to aggregate. Previous studies identified SGTA as a key component of the machinery that regulates the quality control of MLPs. Overexpression of SGTA promotes deubiquitination of MLPs resulting in their accumulation in cytosolic inclusions, suggesting SGTA acts in collaboration with deubiquitinating enzymes (DUBs) to exert these effects. However, the DUBs that play a role in this process have not been identified. In this study we have identified the ubiquitin specific peptidase 5 (USP5) as a DUB important in regulating the quality control of MLPs. We show that USP5 is in complex with SGTA, and this association is increased in the presence of an MLP. Overexpression of SGTA results in an increase in steady-state levels of MLPs suggesting a delay in proteasomal degradation of substrates. However, our results show that this effect is strongly dependent on the presence of USP5. We find that in the absence of USP5, the ability of SGTA to increase the steady state levels of MLPs is compromised. Moreover, knockdown of USP5 results in a reduction in the steady state levels of MLPs, while overexpression of USP5 increases the steady state levels. Our findings suggest that the interaction of SGTA with USP5 enables specific MLPs to escape proteasomal degradation allowing selective modulation of MLP quality control. These findings progress our understanding of aggregate formation, a hallmark in a range of neurodegenerative diseases and type II diabetes, as well as physiological processes of aggregate clearance.

Keywords: deubiquitination, mislocalised membrane proteins, protein quality control, aggregation.

Introduction

Newly synthesised membrane proteins are triaged between biosynthetic pathways targeting them to subcellular compartments such as the mitochondria and the endoplasmic reticulum (ER) and degradative pathways operating in the cytosol [1]. Timely degradation of membrane proteins that mislocalise to the cytosol is necessary to prevent their accumulation and aggregation which is linked to a range of pathologies [2, 3]. Therefore, a variety of intricate protein quality control mechanisms are dedicated to eliminating these mislocalised membrane proteins (MLPs) and maintaining protein homeostasis in mammalian cells [4]. Another layer of protein quality control for membrane proteins occurs at the ER membrane. Misfolded membrane proteins that fail to integrate into the ER membrane are targeted for ubiquitination and subsequent degradation via the endoplasmic-reticulum-associated protein degradation pathway (ERAD) [5, 6]. These quality control mechanisms collectively act to ensure that mislocalised or misfolded membrane proteins are degraded at any stage during their targeting and integration into the ER membrane [7].

The fate of MLPs that expose hydrophobic amino acids in the cytosol is decided via a delicate collaboration between factors that promote ubiquitination and those that promote deubiquitination of the MLPs [8, 9]. Ubiquitination occurs via the action of an array of enzymes; an E1 ubiquitin activating enzyme, an E2 ubiquitin- conjugating enzyme and an E3 ubiquitin ligase, acting in sequence to polyubiquitinate the substrate for subsequent proteasomal degradation [10]. Ubiquitination is a highly dynamic and reversible process due to the action of deubiquitinating enzymes (DUBs) which act as ubiquitin proteases, thereby antagonising the action of the E3 ligases and preventing the proteasomal degradation of substrates [11], [12].

73 It has long been known that DUBs are important for generating a pool of free ubiquitin in the
 74 cell [13],[14]. However, recent evidence suggests a role for DUBs in the regulation of many
 75 cellular processes by fine-tuning or modulating substrate ubiquitination and degradation
 76 [14]. A typical example is the DUB Ataxin-3, which acts to limit the length of ubiquitin chains
 77 built on substrates by CHIP (C-terminus of Hsc70 Interacting Protein) a mammalian E3
 78 ligase involved in cytosolic protein quality control [15]. In addition, some cytosolic localised
 79 DUBs are important in kinetic proofreading and discrimination of membrane proteins that
 80 should be degraded by the proteasome [16]. While DUBs localised on the proteasome, such
 81 as UCHL5 and USP14 can delay the degradation of proteasomal substrates [17]. Moreover,
 82 DUBs are now known to have an important role in ubiquitin chain editing by promoting either
 83 K48 ubiquitin conjugates or K63 linked ubiquitin chains on substrates to regulate
 84 degradation by the proteasome or via autophagy, respectively [18, 19].

85
 86 Recent studies suggest a role for USP13 in ERAD [20, 21], and USP20/33 in
 87 deubiquitinating tail anchored membrane proteins prior to their insertion into the ER
 88 membrane [22]. Although such details about the role of specific DUBs in membrane protein
 89 quality control is beginning to emerge, this knowledge is still in its infancy. This is in stark
 90 contrast to the tremendous progress that has been made towards our understanding of the
 91 E3 ligases that play a role in membrane protein quality control. In yeast the cytosolic
 92 ubiquitin ligases Ubr1p [23], San1P [24] and the ER resident Doa10p and Hrd1p ubiquitinate
 93 substrates for proteasomal degradation [25, 26]. The mammalian orthologues of Hrd1p
 94 (HRD1) [27], Hrd1p homologue gp78 [28] and Doa10p (MARCH6) [29] are known to
 95 promote ERAD. Moreover, the ligases MARCH6 and TRC8 were also shown to promote the
 96 ubiquitination of tail anchored proteins at the cytosolic face of the ER [30], while RNF126
 97 was shown to promote ubiquitination of MLPs [31].

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99 Several studies have also demonstrated a role for the ubiquitin proteasome system in the
 100 degradation of MLPs [4, 9, 31-35]. Among the factors known to be involved in regulating
 101 this degradation is the BCL2-associated athanogene 6 (BAG6) [1, 36, 37], the cochaperone
 102 SGTA (small glutamine-rich tetratricopeptide repeat protein alpha) [1, 9, 32] and a host of
 103 their diverse interacting partners. SGTA, is a homodimer, with an N-terminal domain which
 104 interacts with UBL4A (the ubiquitin-like protein 4A), and BAG6 [38, 39], a central TPR
 105 domain which interacts with Hsp70 and Hsp90 chaperones [40, 41], and a C-terminal
 106 domain capable of binding hydrophobic substrates [42].

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108 A BAG6/SGTA quality control cycle dictating the fate of MLPs is proposed to be operating
 109 at the proteasome [43]. In this cycle, BAG6 recruits RNF126, an E3 ubiquitin ligase which
 110 catalyses the selective ubiquitination of MLPs for subsequent degradation by the
 111 proteasome [31, 44]. On the other hand, SGTA promotes deubiquitination thereby giving
 112 substrates a chance to escape proteasomal degradation [9, 32]. Therefore, in the context of
 113 the BAG6/SGTA cycle, it is plausible that an interaction of SGTA with deubiquitinating
 114 enzymes could provide SGTA-bound MLPs access to the enzyme(s) which mediate
 115 deubiquitination, thereby delaying the clearance of the MLPs and perhaps favour their
 116 targeting to the ER and subsequent membrane integration [43]. However, the
 117 deubiquitinating enzyme(s) that play a role in this process have not been defined to date. In
 118 this study we employed pull downs and mass spectrometry to identify DUBs that interact
 119 with SGTA. We show that USP5 is in complex with SGTA using co-immunoprecipitation,
 120 and that this interaction is necessary for the ability of SGTA to increase the steady state
 121 levels of MLPs. The implications of this study to our understanding of mislocalised
 122 membrane protein quality control is discussed.

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Materials and methods

All cell culture and standard reagents were purchased from Fisher Scientific (Loughborough, UK). Antibodies to opsin and V5 were previously described [32]. Commercially available antibodies against the following targets were purchased from the indicated suppliers, tubulin and GFP were purchased from Abcam (Cambridge, UK), USP9X (#A301-350A), USP5 (#A301-542A), USP14 (#A300-920A) and USP10 (#A300-900A) antibodies were purchased from Bethyl Laboratories Inc. (Montgomery, USA). Bortezomib was purchased from Selleckchem (Munich, Germany), Leupeptin and Pepstatin A were purchased from BIOMOL (Hamburg, Germany).

Plasmids

Plasmids for the overexpression of SGTA-V5 and Pex19-V5 were previously described [43]. Ub-M-GFP (#11938) and Ub-R-GFP (#11939) plasmids described in [45] were purchased from Addgene. HA tagged USP5 (#22590) was purchased from Addgene and subcloned into pcDNA5 mammalian expression vector. The 3x NNP/AAA (NNP positions at 226-228, 239-241 and 255-257) variant of SGTA-V5 was previously described [46]. The pET28-TxA-SGTA_wild type plasmid for the expression and purification of WT SGTA was previously described [46]. This plasmid was generated by cloning WT SGTA into BamHI/XhoI restriction sites of a pET28c vector modified to encode an N-terminal thioredoxin A (TxA) fusion protein followed by a hexa-histidine tag. Plasmids for the expression and purification of SGTA_TPR double mutant (SGTA^{K160E/R164E}) and SGTA_UBL double mutant (SGTA^{D27R/E30R}) mutant were created by site directed mutagenesis using pET28-TxA-SGTA wild type plasmid as template and primers carrying the mutated codons. DNA sequencing was used to verify the mutants prior to use.

Cell culture

The inducible stable HeLa Flp-In T-REx cell lines expressing OP91 or opsin degran (OpD) were previously described in [32] and [47], respectively. The HeLa cells were maintained in DMEM containing 10% fetal bovine serum and 2 mM L-glutamine at 37°C under 5% CO₂. At alternate passages, DMEM was supplemented with 100 µg/ml hygromycin B and 4 µg/ml blasticidin S. Transfections of plasmid DNA were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. 6 hours post-transfection, OP91 or OpD expression was induced by treating the cells with DMEM containing 1 µg/ml tetracycline followed by growth for an additional 16-18 hours. Where indicated, 10 nM bortezomib or 100 µM leupeptin and 1 µg/ml pepstatin were added to cells 20 hours prior to analysis.

Knockdown experiments

For siRNA experiments, 20 nM of siRNAi duplexes were transfected into cells using INTERFERin (Polyplus, Illkirch, France) according to the manufacturer's instructions. 48 hours post-transfection the cells were transiently transfected with the appropriate DNA constructs (where necessary) and harvested after a further 24-hour incubation. Non-targeting control duplex [Cat# D-001810-01-20] and synthetic siRNA duplexes, USP5 siRNA1; [Cat#: D-006095-02-0002] and USP5 siRNA2; [Cat# D-006095-03-0002] targeting the USP5 gene [13], and the USP9x ON-TARGETplus siRNA [Cat# J-006099-06-0002] were purchased from Dharmacon (Chicago, IL, USA). SiRNA target sequences for SGTA and USP14 were 5'-ACAAGAAGCGCCUGGCCTATT-3' [48] and 5'-AGAAATGCCTTGTATATCATT-3' [49], respectively. SiRNA duplexes for these were obtained from Dharmacon (Chicago, IL, USA).

Real-time RT-PCR

To quantify OP91 expression levels, total cellular RNAs were isolated using TRIzol™ Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) and used for first-strand cDNA using the SensiFAST™ cDNA Synthesis (Bioline, Memphis, TN, USA). Quantitation of the OP91 transcript was done by quantitative PCR using the SensiFAST SYBR® Hi-ROX Kit (Bioline, Memphis, TN, USA) and the StepOnePlus™ Real-Time PCR detection system (Applied biosystems by Thermo Fisher Scientific). The expression of GAPDH was used as the internal control. For the primers 5 pmol of OP91 AGGGCCCAAACCTTCTACGTG (forward) and AGCGTGAGGAAGTTGATGGG (reverse) and GAPDH, GAAGGTGAAGGTCGGAGTC (forward) and GAAGATGGTGATGGGATTTC (reverse) were used. PCR conditions were 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt curve analysis was performed to verify the specificity of the reaction and cycle threshold values (Ct values) were used to calculate the -fold differences using the comparative Ct ($\Delta\Delta C_t$) method.

Protein purification

Plasmids for protein expression were transfected into *E. coli* BL21 (DE3) strains and protein expression was induced by using 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD600 ≈ 0.8, followed by overnight incubation at 22 °C. Cells were harvested and resuspended in lysis buffer (20 mM potassium phosphate, pH 8.0, 300 mM NaCl, 10 mM Imidazole, 50 mM Tris-HCl, 10% glycerol, supplemented with protease inhibitors -EDTA (Roche, Burgess Hill, UK), and 1 mM PMSF and lysed by sonication. Cell debris was removed by centrifugation, and the soluble fractions were purified using nickel affinity chromatography (Qiagen, Manchester, UK) and eluted with lysis buffer containing 300 mM imidazole, followed by dialysis into phosphate buffered saline pH 7.4. Gel filtration was carried out using a HiLoad 16/60 Superdex 200 column (GE Healthcare, Amersham, UK),

209 previously equilibrated in buffer containing 10 mM potassium phosphate pH 6.0, 100 mM
210 NaCl and 50 mM Tris-HCl. Proteins were concentrated using Vivaspin concentrators
211 (Sartorius Stedin) and the sample purity and homogeneity was assessed by SDS-PAGE.

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213 **Coupling of purified recombinant protein to resin**

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215 UltraLink Biosupport resin (Thermo Scientific, Pierce) were incubated with coupling buffer
216 (0.6M Sodium Citrate, 0.1M MOPS, pH 7.5) and equimolar amounts of recombinant protein
217 WT SGTA, SGTA mutants (SGTA^{K160E/R164E} and SGTA^{D27R/E30R}) or Thioredoxin, overnight
218 at 4°C, to allow covalent coupling via primary amines. 3M ethanolamine pH 9.0 was used to
219 quench the reaction after coupling. Beads with coupled recombinant proteins were washed
220 for 15 minutes with PBS and 1M NaCl in turn. Beads were further washed 3 times in PBS
221 and re-suspended in PBS for storage at 4 °C.

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223 **Pull-down assays**

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225 Beads coupled to recombinant WT SGTA, SGTA mutants (SGTA^{K160E/R164E} and
226 SGTA^{D27R/E30R}) or Thioredoxin were incubated with rabbit reticulocyte lysate or HeLa lysate
227 overnight. Resins were washed three times in buffer containing 40mM HEPES-KOH, pH
228 7.5, 40mM potassium acetate, 5mM MgCl₂ followed by incubation in Laemmli sample buffer
229 at 70 °C for 10 minutes. Samples were analysed by SDS-PAGE and western blotting.

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231 **Mass spectrometry analysis**

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233 Mass spectrometry was performed at the Sanford-Burnham Proteomics Facility, Sanford-
234 Burnham Medical Research Institute, (La Jolla) using the method previously described [50].
235 Briefly, beads from pull down assays were resuspended in a buffer containing 8M urea and
236 50mM ammonium bicarbonate followed by reduction with Tris(2-carboxyethyl) phosphine,

(TCEP), alkylation with iodoacetamide and digestion with mass spectrometry grade trypsin (Promega). Samples were washed with 50mM ammonium bicarbonate and formic acid was added, followed by desalting. Peptides were analysed by high-resolution and high-accuracy LC-MS/MS. The LC-MS/MS raw data of three technical replicates were combined and submitted to Sorcerer Enterprise v.3.5 release (Sage-N Research Inc.) with SEQUEST algorithm as the search program for peptide/protein identification. The LC-MS/MS raw data were also submitted to Integrated Proteomics Pipelines (IP2) Version IP2 1.01 (Integrated Proteomics Applications, Inc.) with ProLucid algorithm as the search program for peptide/protein identification. ProLucid and SEQUEST were both set up to also search the target-decoy ipi. Human.v3.73 protein database. The search results were viewed, sorted, filtered, and statically analysed by using comprehensive proteomics data analysis software, Peptide/Protein prophet v.4.02 (ISB) and DTA Select for proteins. The differential spectral count analysis was done by QTools, an open-source tool for automated differential peptide/protein spectral count analysis and Gene Ontology. The reviewed human UniProt database was used for identifying SGTA interacting partners.

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253 **Preparation of HeLa lysate**

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255 HeLa M cells were plated in 10-cm dishes at 100% confluency and washed twice with PBS
256 at 4°C. Following washes, cells were lysed in a buffer containing 20mM HEPES pH 7.4,
257 5mM MgCl₂, 0.1M NaCl, 0.5% Triton x100, protease inhibitor and 1mM PMSF. Cells were
258 harvested from the dish using a cell scraper followed by incubation on ice for 10-15
259 minutes. The cell suspension was then centrifuged to remove cell debris at 13000 rpm at 4
260 °C for 20 min and the supernatant used for analysis.

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Western blotting

Western blot experiments were performed according to standard procedures previously described [32]. Briefly, cells were lysed directly into Laemmli sample buffer and subjected to SDS-PAGE followed by infrared immunoblotting as described previously. Fluorescent bands for specific proteins were quantified using Image Studio-Lite (Li-Cor Biosciences) employing data from at least three independent experiments expressed as the mean $\pm$ s.e.m. The statistical significance of the results was assessed by applying a student's t-test using Prism 7 (GraphPad).

Co-immunoprecipitation

Stable HeLa Flp-In T-REx cell lines expressing OP91 or OpD were grown as detailed. Half of the samples were induced while the other half remained as uninduced samples. Cell lysates were prepared by harvesting cells in solubilisation buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% n-dodecyl β -D-maltopyranoside (DDM) and 1 mM EDTA supplemented with protease inhibitor tablets (Roche) and 1 mM PMSF for 1 hour at 4°C. The lysates were centrifuged at 16,000 g for 20 minutes at 4°C to remove insoluble material and 10% of the supernatant was retained as the input sample. Approximately 500 μ L of the clarified lysate was incubated with IgG control or SGTA antibodies (1–2 μ g) for 12 h at 4 °C with constant rotation. Protein A Sepharose (GenScript Biotech, Piscataway USA) was used to precipitate complexes by adding a 50% slurry and incubating for further 2 h. Beads were then washed five times in lysis buffer followed by elution of immune complexes by resuspending the beads in 2 $\times$ Laemmli sample buffer followed by immunoblotting with appropriate antibodies.

Cycloheximide chase

Transfected HeLa Flp-In T-REx cells were treated with non-targeting siRNAi duplexes or siRNAi targeting USP5, USP9X, USP14, and SGTA as described above. Prior to harvesting transfected cells were treated with 100 µg/ml cycloheximide (Sigma, Aldrich) to inhibit protein synthesis. Cells were lysed directly into SDS-PAGE sample buffer at specific time-points followed by Western blotting analysis.

Results

Previous studies suggest that SGTA regulates the fate of membrane proteins that mislocalise to the cytosol [9, 32]. Overexpression of exogenous SGTA led to an increase in the steady-state levels of MLPs. One such model MLP is OP91, an N-terminal fragment of opsin that is inefficiently targeted to the endoplasmic reticulum [32]. SGTA also promoted the deubiquitination of MLPs [9, 32]. Since, SGTA itself has no domains typical for DUBs, it was proposed that SGTA recruits one or more DUBs to exert these effects [9, 32, 43]. In this study we aimed to identify the DUBs that interact with SGTA in mammalian cells and dissect their role in MLP quality control.

Identification of SGTA Interacting DUBs.

To identify SGTA interacting DUBs, we took a proteomic based approach by isolating protein complexes that interact with SGTA using a pull-down assay. We made use of SGTA mutants that are defective in interacting with known SGTA binding partners to develop and validate a robust assay for the identification of specific SGTA interactors. The SGTA^{K160E/R164E} mutant which does not interact with Hsp70 [48] and the (SGTA^{D27R/E30R}) which does not interact with BAG6 and Ubl4A [20], were utilised in the pull-downs. WT SGTA, and the two SGTA mutants

(SGTA^{D27R/E30R} and SGTA^{K160E/R164E}) were purified as thioredoxin (TXN) fusion proteins and immobilised to UltraLink Biosupport beads. Protein complexes interacting with these immobilised proteins were isolated from rabbit reticulocyte lysate under native conditions, with recombinant TXN immobilised to beads as negative control. The pull-downs were validated by Western blotting analysis, probing with BAG6, Ubl4A and Hsp70 specific antibodies. As expected, WT SGTA pulled-down high levels of BAG6, Ubl4A and Hsp70, while the TXN control did not pull-down any significant amounts of these three proteins (Fig. 1A, cf. lanes 2 and 3). Consistent with previous publications [20], very low levels of BAG6 and Ubl4A were detected in the SGTA^{D27R/E30R} mutant and Hsp70 levels were low in the SGTA^{K160E/R164E} mutant (Fig. 1A, cf. lanes 2, 3 and 4) [48].

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328 **Fig 1. Identification of SGTA Interacting DUBs**

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330 **A)** Beads coupled to recombinant Thioredoxin-SGTA fusion proteins as indicated or Thioredoxin only (Control) were incubated with rabbit reticulocyte lysate for 16 hours at 4 °C. The beads were washed as detailed in Materials and Methods. Twice the bead volume of SDS sample buffer was added, and samples were processed for SDS PAGE and Western blotting and probed with antibodies against Bag6, UBL4A and Hsp70 proteins using a fluorescence- based Odyssey ® Fc Imaging System (LICOR). **B)** Beads described in A, were incubated with HeLa lysate for 16 hours, washed and processed for Western blotting as detailed in A. Blots were probed with antibodies against USP5, USP14, USP9X and USP10 proteins using fluorescence- based detection on an Odyssey ® Fc Imaging System (LICOR).

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341 **Table 1. SGTA interacting partners identified by mass spectrometry.**

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343 Pull-down experiments were performed as described in Materials and Methods. The beads were then analysed for bound proteins by mass spectrometry as detailed in the materials and methods followed by interrogating the reviewed human UniProt database for SGTA interacting partners.

345

Identified proteins known to interact with SGTA are indicated together with the spectral counts. SGTA peptides detected reflect comparative efficiency for the coupling of SGTA or the mutants to beads.

Spectral counts				
SGTA interacting proteins	Thrx	SGTA	SGTA ^{D27R/E30R}	SGTA ^{K160E/R164E}
SGTA	28.8794	1813.40948	1799.50341	1561.96154
BAG6	70.6523	188.85411	26.81825	192.46266
HSPA2	96.34687	37.44345	34.27598	0
UBL4A	10.9873	15.07829	2.7107	17.22824
Number of SGTA peptides detected	28	1813	1753	1799

Having validated that the pull-down approach was working as expected, pull-downs were performed as detailed above and the samples were analysed by mass spectrometry followed by interrogating the reviewed human UniProt database to identify SGTA interactors. Consistent with the Western blotting results (Fig. 1 A), beads containing WT SGTA were able to pull-down high levels of Bag6, Ubl4A and Hsp70 (Table 1) as judged from the spectral counts. The SGTA^{D27R/E30R} mutant had very low spectral counts for Bag6 and Ubl4A, while the SGTA^{K160E/R164E} mutant had very low levels of some isoforms of Hsp70, notably HspA2 known to specifically interact with SGTA [51]. Based on these results we gained confidence that this proteomic based approach was reliable and likely to reflect *bona fide* and novel SGTA interacting partners.

We then looked at all the DUBs that had been identified by mass spectrometry (Table 2). Based on spectra counts, USP5, USP9X, USP14, and USP7 were identified as candidates for follow-up studies (Table 2). Since our goal was to focus on the DUBs that play a role in the quality control of membrane proteins mislocalised to the cytosol, we looked in published literature for the subcellular localisation of the DUBs that we had identified. Based on this

criteria USP7 was not included in further analysis as it is reported to localise mainly to the nucleus, while USP5, USP9X and USP14 localise to both the nucleus and cytosol [52].

Table 2. Putative SGTA interacting DUBs identified by mass spectrometry.

Pull-down experiments were performed as described in Materials and Methods. The beads were then analysed for bound proteins by mass spectrometry as detailed in the materials and methods. All DUBs identified from the mass spectrometry are shown together with the spectral counts. The DUBs highlighted were prioritised for further studies based on high spectral counts and low background in the negative control.

Name of enzyme	Spectral			
	Thr _x	SGTA WT	SGTA ^{D27R/E30R}	SGTA ^{K160E/R164E}
Ubiquitin carboxyl-terminal hydrolase 10 (USP10)	0	0	0	0.36738
Ubiquitin carboxyl-terminal hydrolase 19 (USP19)	0	0	0.68318	0
Ubiquitin carboxyl-terminal hydrolase 5 (USP5)	2.11094	9.27228	10.51442	8.43043
Ubiquitin carboxyl-terminal hydrolase 14 (USP14)	0	8.48274	14.24989	7.69728
Ubiquitin carboxyl-terminal hydrolase 15 (USP15)	6.86995	7.72786	7.81252	6.96413
Ubiquitin carboxyl-terminal hydrolase 7 (USP7)	0	6.18345	5.75859	3.66414
Ubiquitin carboxyl-terminal hydrolase 47 (USP47)	0	1.5502	1.35755	0
Ubiquitin carboxyl-terminal hydrolase 32 (USP32)	0	1.53864	0	0.36577
Ubiquitin carboxyl-terminal hydrolase 4 (USP4)	0	1.1612	1.02477	0
Ubiquitin carboxyl-terminal hydrolase 24 (USP24)	0	0.77221	1.69914	0
Probable ubiquitin carboxyl-terminal hydrolase FAF-X (USP9X)	0	5.40546	6.45057	4.39891
Ubiquitin carboxyl-terminal hydrolase isozyme L5 (UCHL5)	0	1.93919	1.69474	0.36738
Ubiquitin thioesterase (OTUB1)	0	1.93341	1.35755	0

Since the initial pull-downs which identified, these DUBs were performed using rabbit reticulocyte lysate we validated these results by repeating the pulldowns using lysate from HeLa cells. Pull-downs were performed as described above followed by Western blotting for the identified DUBs and USP10 which had very low spectral counts from the mass spectrometry results (Table 2). Consistent with the mass spectrometry data, WT SGTA and both mutants were able to pull down comparable amounts of USP5, USP9X, and USP14

when compared to TXN control, while USP10 was barely detectable, consistent with the very low spectral counts obtained for this DUB (Fig. 1B, cf. lanes 2, 3, 4 and 5). From these results we decided to further investigate the role of USP5, USP9X, and USP14 in SGTA mediated quality control of MLPs.

Knocking down USP5 reduces the steady state of a model MLP.

To investigate whether the identified DUBs play a role in MLP quality control, we reduced the level of endogenous DUBs using an siRNA approach and studied the effect on steady-state levels of the model MLP, OP91. We reasoned that if a DUB is responsible for the deubiquitination and stabilisation of OP91, its knock down would enhance MLP degradation through favouring the ubiquitinated form which is subject to proteasomal clearance. Knocking down USP9X did not reduce the steady state levels of OP91 when compared to the non-targeting siRNAi (Fig. 2A, lane 4), instead it led to a slight increase in the steady state levels of OP91 when signals were quantified and normalised to tubulin loading control (Figure 2B). Knocking down USP5 on the contrary led to a marked decrease in the steady state levels of non-glycosylated form of OP9, indicating a role on MLPs (Fig. 2A, cf. lane 1 and 2). The significant changes in the OP91 signal were equivalent to ~50% reduction when compared to the non -targeting control (Figure 2B). Knockdown of USP14 also led to a decrease in the steady state levels of OP91, although the effect was less marked than that of USP5 (Fig. 2A, cf. lane 1, 2 and 3), possibly because the knockdown of USP14 was less efficient. In these studies, knocking down any of these DUBs had no effect on SGTA levels (Fig 2A), suggesting these results are directly related to changes in levels of the DUBs. The effect of these DUBs was specific for the non-glycosylated form of OP91, indicating that they act on MLPs.

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Fig 2. Knocking down USP5 reduces the steady state of a model MLP.

A) HeLa TRex Flp-In cells stably expressing OP91 after induction, were seeded at 50 % confluence and transfected with 1 nM of siRNAi targeting the indicated DUBs. OP91 expression was induced 48 h later and cells grown for 20 hrs post induction. Total cell lysates were prepared, and products analysed by Western blotting with antibodies against OP91, SGTA, USP5, USP9X, USP14 and tubulin (loading control) using fluorescence- based detection (LICOR). The % knockdown efficiency is indicated for each DUB in lane 2, 3 and 4. **B)** OP91 signals were quantified using Odyssey 2.1 software and normalised to the tubulin loading control, values show standard errors for n = 3. Pairwise comparisons relative to the NT control, were performed using GraphPad Prism. Student t-test: $P > 0.05 = \text{ns}$, $P \leq 0.05 = *$, $P \leq 0.01 = **$, $P \leq 0.001 = ***$ **C)** HeLa TRex Flp-In cells stably expressing OP91 were transfected with non-targeting siRNAi or two independent siRNAi duplexes targeting USP5. Cells were treated as described in A, followed by blotting for OP91, USP5 and tubulin. **D)** OP91 signals were quantified using Odyssey ® Fc Imaging system and normalised to the tubulin loading control, values show standard errors for n = 3. Pairwise comparisons relative to the NT control, were performed using GraphPad Prism. Student t-test: $P > 0.05 = \text{ns}$, $P \leq 0.05 = *$, $P \leq 0.01 = **$, $P \leq 0.001 = ***$ and $P \leq 0.0001 = ****$.

Mammalian USP14 and the yeast homologue, Ubp6 both associate with the proteasome [53], [54] and catalyse cleavage of ubiquitin subunits from substrates thereby delaying their proteasomal degradation [55]. OP91 was previously shown to be degraded by the proteasome [9], [32]. Hence, the destabilisation of OP91 upon knocking down USP14 is consistent with the effect of USP14 on proteasomal degradation of substrates. The destabilisation of MLPs upon USP5 knockdown is a novel finding that has not been previously reported. Hence, we focussed our attention on the role of USP5 in MLP quality control.

439 To validate the effects of USP5 on reducing the steady state levels of OP91, we made use
440 of another siRNA oligonucleotide to independently target USP5. This siRNA oligonucleotide
441 also led to a marked decrease in the level of OP91 (Fig. 2C). The effect of reducing OP91
442 levels was reproducible as both siRNA oligonucleotides targeting USP5 led to ~ 50%
443 reduction in OP91 levels (Fig. 2D).

444

445 **Loss of USP5 does not affect other substrates.**

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447 Next, we wanted to confirm that the destabilisation effect observed after knocking down
448 USP5 was specific for MLPs and not due to changes in ubiquitin homeostasis. To this effect,
449 we made use of ubiquitin–methionine–GFP (Ub-M-GFP) which upon cleavage of ubiquitin
450 does not have a degradation signal and is therefore stable [45]. USP9X and USP14 were
451 included in this experiment for comparison. Knocking down any of the DUBs did not affect
452 the steady state levels of Ub-M-GFP (Fig. 3A and 3B). We also made use of ubiquitin–
453 arginine–GFP (Ub-R-GFP) [45]), a proteasomal N-end rule substrate whose degradation
454 was previously shown to be independent of SGTA [43]. Knocking down all the DUBs led to
455 comparable steady-state levels of Ub-R-GFP (Fig. 3A and 3B), suggesting the effects
456 observed with USP5 are specific for OP91.

457

458 **Fig 3. Loss of USP5 does not affect other substrates.**

459

460 **A)** HeLa M cells were seeded at 50 % confluence and transfected with 1 nM of siRNAi targeting the
461 indicated DUBs. Ub-MGFP and Ub-RGFP were transfected 48 h later and cells grown for 20 hrs
462 post-transfection. Total cell lysates were prepared and analysed by Western blotting using GFP
463 antibodies and tubulin (loading control). **B)** Ub-RGFP signals were quantified using the Odyssey ®
464 Fc imaging system and normalised to the tubulin loading control, values show standard errors for n
465 = 3. Pairwise comparisons relative to the NT control, were performed using GraphPad Prism.
466 Student t-test: P > 0.05 = ns, P ≤ 0.05= *, P ≤ 0.01= **, P ≤ 0.001= ***. **C)** HeLa TRex Flp-In cells

467 stably expressing OpD after induction were seeded at 50 % confluence and transfected with 1 nM
 468 of siRNAi targeting the indicated DUBs. OpD expression was induced 48 h later, and cells grown for
 469 20 hrs post induction. Total cell lysates were prepared and analysed by Western blotting using opsin
 470 antibodies to detect OpD, and tubulin (loading control). **D)** OP91 signals were quantified using
 471 Odyssey ® Fc imaging system and normalised to the tubulin loading control, values show standard
 472 errors for n = 3. Pairwise comparisons relative to the NT control, were performed using GraphPad
 473 Prism. Student t-test: $P > 0.05 = \text{ns}$, $P \leq 0.05 = *$, $P \leq 0.01 = **$, $P \leq 0.001 = ***$ and $P \leq 0.0001 = ****$.

474

475 To further explore the substrate specificity of USP5, we looked at the effects of knocking
 476 down USP5 on the stability of the opsin degron (OpD), an ERAD substrate derived from
 477 wild-type opsin by the introduction of a degron motif into the first transmembrane domain
 478 [56]. The SGTA/ Bag6 complex was previously shown to play an important role in ERAD of
 479 OpD [20], , [47]. Knocking down USP5 did not change the steady state levels of OpD when
 480 compared to the non-targeting control (Fig. 3C, cf. lane 1 and 2). On the other hand,
 481 knocking down USP14 and USP9X resulted in an accumulation of OpD (Fig. 3C, cf. lane 1,
 482 3 and 4). These effects mirrored what was observed when the proteasome is inhibited by
 483 Bortezomib (Fig. 3C, lane 5). This observation is consistent with a perturbation of ERAD
 484 upon knocking down USP14 or USP9X but not USP5. Based on these results, we
 485 concluded that USP5 is a DUB that specifically plays a role in the cytosolic quality control of
 486 MLPs.

487

488 **USP5 protects OP91 against proteasomal degradation.**

489

490 Having established that knocking down USP5 reduces the steady state levels of OP91. We
 491 next investigated whether knocking down USP5 changes the OP91 mRNA levels. HeLa T-
 492 REx cells were transfected with oligonucleotides to knockdown USP5 or a non-targeting
 493 control. After 72 hours, cells were harvested and total RNA was extracted, followed by

analysis of OP91 mRNA levels using quantitative RT-PCR. In contrast to the observed decrease in steady state levels of the OP91 protein, mRNA levels were comparable between the non-targeting control and a USP5 knockdown (Fig. 4A), suggesting that the reduction in OP91 levels in a USP5 knockdown occurs post-translationally.

498

499 **Fig 4. USP5 protects OP91 against proteasomal degradation.**

500

501 **A)** HeLa T-REx Flp-In cells stably expressing OP91 were seeded at 50% confluence followed by
502 transfection of oligos for knockdown of USP5 or a non-targeting oligo. Cells were grown for 48 hrs
503 followed by induction of OP91 expression and growth for a further 20 hrs post. Cells were harvested
504 and total RNA extracted and used in a qPCR reaction following standard qPCR protocol. **B)** Cells
505 grown as described in A, inhibitors of the proteasome and autophagy were added 24 hours before
506 cells were harvested. Samples were analysed by SDS-PAGE and Western blotting for OP91 and
507 Tubulin loading control. **C)** OP91 signals were quantified using Odyssey® Fc Imaging System and
508 normalised to the tubulin loading control, values show standard errors for n = 3. Pairwise
509 comparisons were performed using GraphPad Prism. Student t-test: $P > 0.05 = \text{ns}$, $P \leq 0.05 = *$, $P \leq$
510 $0.01 = **$, $P \leq 0.001 = ***$ and $P \leq 0.0001 = ****$.

511

512 OP91 was previously shown to be degraded by the proteasome [32]. Therefore, we
513 investigated whether OP91 was stabilised by proteasome inhibition when USP5 levels are
514 reduced. Upon addition of Bortezomib, a proteasome inhibitor, there was an increase in
515 steady state levels of OP91 in both the nontargeting control and in a USP5 knockdown (Fig.
516 4B cf. lanes 2 and 5). Although, Leupeptin/Pepstatin which inhibits autophagy did not
517 increase the steady state levels of OP91 in the non- targeting control (Fig. 4B cf. lanes 3),
518 this treatment led to stabilisation of a small proportion of OP91 in a USP5 knockdown (Fig.
519 5B cf. lane 6). This observation suggests that in the absence of USP5 a small pool of OP91
520 is degraded by autophagy, consistent with the cross talk observed between the proteasomal
521 and autophagic pathways of degradation [57].

522

523 To better understand the basis for the reduced steady-state levels of MLPs observed in a
 524 USP5 knockdown, we analysed OP91 levels over a 1-hour time course by blocking protein
 525 synthesis with cycloheximide and following degradation of OP91 in knockdowns of USP5,
 526 USP9X and in a nontargeting control. Previous studies showed that knocking down SGTA
 527 leads to a reduction in OP91 levels (S1. Fig cf. lane 2). We reasoned that if USP5 and SGTA
 528 act in the same pathway, their knockdown should affect the degradation of MLPs in a similar
 529 way. Therefore, an SGTA knockdown was included in the time-course experiments for
 530 comparison. Pulse chase experiments revealed that in the non-targeting and USP9X
 531 knockdowns, ~50 % of OP91 was degraded in the first 20 minutes with the rate of
 532 degradation slowing after this point (see supplementary material, S2A. Fig). While, in the
 533 SGTA and USP5 knockdowns, there was a less marked reduction as the steady state levels
 534 of OP91 were already low before the start of the chase experiment (S2A. Fig). Therefore, to
 535 better compare the degradation kinetics in these knockdowns, OP91 levels were expressed
 536 as a percentage of the total OP91 signal in the negative control (NT) at time zero (S2B. Fig).
 537 This revealed similar degradation kinetics for all conditions, notably however, these
 538 experiments also revealed that when USP5 and SGTA are reduced there is loss of a
 539 protected pool of OP91 which is rapidly degraded.

540

541 **Overexpression of USP5 stabilises OP91.**

542

543 To gain confidence of the role of USP5 in MLP quality control, we investigated the effect of
 544 overexpressing USP5 on the stability of OP91. To this end HA tagged USP5, and V5 tagged
 545 SGTA [43] were transfected into HeLa cells followed by induction of OP91 expression. We
 546 have previously shown that overexpression of a chaperone protein, Pex19 does not stabilise
 547 OP91 [43]. Therefore, this was used as a negative control to reveal the basal level of OP91.
 548 After growth, cells were harvested into Laemmli sample buffer and analysed by Western

549 blotting, probing for OP91, Tubulin, USP5, SGTA and PEX19. Consistent with previous
550 studies (Wunderley, Leznicki et al. 2014), overexpression of SGTA led to an increase in the
551 steady state levels of OP91 (Fig 5A cf. lane 2), above those seen in the presence of
552 exogenous PEX19 control (Fig. 5A cf. lane 1). Overexpression of USP5 led to 2-fold
553 increase in the steady state levels of OP91 when compared to the Pex19 control (Fig. 5A
554 cf. lane 3). These results demonstrated that modulating USP5 levels directly affect MLP
555 quality control.

556

557 **Fig 5. Overexpression of USP5 stabilises OP91.**

558

559 **A)** HeLa T-REx Flp-In cells stably expressing OP91 upon induction were transfected with V5-tagged
560 Pex19 and SGTA or HA -tagged USP5. OP91 expression was induced 6 hours post-transfection.
561 Cells were grown for a further 20 hours, post-induction. Total cell lysates were prepared and
562 analysed by western blotting using antibodies against OP91, V5 tagged SGTA and Pex19, HA
563 tagged USP5 and tubulin (loading control). **B)** OP91 signals were quantified using Odyssey ® Fc
564 Imaging System and normalised to the tubulin loading control, values show standard errors for n =
565 3. Pairwise comparisons relative to Pex19, were performed using GraphPad Prism. Student t-test:
566 $P > 0.05 = \text{ns}$, $P \leq 0.05 = *$, $P \leq 0.01 = **$, $P \leq 0.001 = ***$ and $P \leq 0.0001 = ****$.

567

568 **USP5 specifically enhances SGTA activity.**

569

570 It was previously shown that increasing exogenous SGTA levels leads to an increase in
571 steady state levels of OP91 [32],[43]. Having shown that USP5 is also required for the
572 stabilisation of OP91 in this study (Fig. 2A & B), we next asked whether the stabilising effect
573 of SGTA is dependent on USP5. To this end we performed a knockdown of USP5 and used
574 non-targeting siRNAi as control. After 48 hours, V5 tagged SGTA or Pex19 control were
575 transfected followed by induction of OP91 expression. After growth, cells were analysed by
576 Western blotting for OP91, tubulin, and V5 tagged SGTA or Pex19. Overexpression of

577 SGTA led to stabilisation of OP91 in the presence of the nontargeting siRNAi (Fig. 6A cf.
578 lane 2), when compared to the Pex19 control (Fig. 6A cf. lane 1). However, in the presence
579 of USP5 siRNAi, overexpression of SGTA did not stabilise OP91 to the same level as
580 observed in the presence of non-targeting siRNAi (Fig. 6A cf. lane 2 and 4), suggesting that
581 USP5 is required for the stabilisation of OP91 observed during SGTA overexpression.

582

583 **Fig 6. USP5 specifically enhances SGTA activity.**

584

585 **A)** HeLa T-REx Flp-In cells stably expressing OP91 after induction were seeded at 50 % confluence
586 and transfected with 1 nM of siRNAi targeting USP5 or a nontargeting control and incubated for 48
587 hours. After this, plasmids expressing V5 tagged SGTA or PEX19 were transfected followed by
588 incubation for 6 hours before the medium was replaced with medium containing 1 mg/ml of
589 tetracycline to induce OP91 expression. Cells were grown for 20 hrs post induction and total cell
590 lysate were prepared and products analysed by western blotting using antibodies for opsin (OP91),
591 V5 (SGTA and PEX19), and tubulin (loading control). **B)** OP91 signals were quantified using
592 Odyssey ® Fc Imaging System and normalised to the tubulin loading control, values show standard
593 errors for n = 3. Pairwise comparisons relative to Pex19, were performed using GraphPad Prism.
594 Student t-test: $P > 0.05 = \text{ns}$, $P \leq 0.05 = *$, $P \leq 0.01 = **$, $P \leq 0.001 = ***$ and $P \leq 0.0001 = ****$. **C)** HeLa
595 T-REx Flp-In cells were treated with siRNAi targeting USP5, USP9X, USP14 and a nontargeting
596 control and analysed as detailed in A. **D)** OP91 signals were quantified using Odyssey ® Fc Imaging
597 System and normalised to the tubulin loading control, values show standard errors for n = 3. Pairwise
598 comparisons relative to Pex19, were performed using GraphPad Prism. Student t-test: $P > 0.05 =$
599 ns , $P \leq 0.05 = *$, $P \leq 0.01 = **$, $P \leq 0.001 = ***$ and $P \leq 0.0001 = ****$.

600

601 We also revisited the effect we observed that upon knockdown of USP14 destabilisation of
602 OP91 occurred (Fig. 2A). To investigate whether USP14 is required for the stabilisation
603 effect observed with exogenous SGTA, we knocked down USP14 and overexpressed
604 SGTA. We also looked at the effects in a USP9X knockdown as this did not affect the steady

state levels of OP91 (Fig 2A & B). As expected, reducing USP9X did not affect the ability of SGTA to stabilise OP91 (Fig. 6C cf. lane 5 and 6). On the other hand, lack of USP5 led to a reduction in the ability of SGTA to stabilise OP91 (Fig. 6C cf. lane 3 and 4). However, the knockdown of USP14 did not affect the ability of exogenous SGTA to stabilise OP91 (Fig. 6C cf. lane 7 and 8), suggesting that USP14 is not directly required for the SGTA mediated stabilisation of OP91.

611

612

613 **Association of SGTA and USP5 is enhanced in the presence of an MLP.**

614

615 Co-immunoprecipitation studies have shown that SGTA and OP91 are in a complex [32].
616 We next wanted to see whether USP5 was also part of this complex. To this effect, HeLa
617 cells were transfected with SGTA, followed by induction of OP91 expression in half of the
618 samples. After growth, cells were harvested into non-denaturing buffer and subjected to co-
619 immunoprecipitation using SGTA antibodies or IgG antibodies as negative control. SGTA
620 associated with USP5 in the absence of OP91 induction and this association was increased
621 upon OP91 induction (Fig. 7A cf. lane 5 and 6), suggesting that association of USP5 and
622 SGTA is substrate dependent. This association was more pronounced for the non-
623 glycosylated form [0-CHO] of OP91 which probably reflects the mislocalised form. Since the
624 IgG control did not show an increased association of USP5 with SGTA even upon OP91
625 induction (Fig. 7A cf. lane 3 and 4), these results suggested that this association was specific
626 for SGTA.

627

628 **Fig 7. Association of SGTA and USP5 is enhanced in the presence of an MLP.**

629

630 **A)** HeLa T-REx Flp-In cells stably expressing OP91 after induction were seeded at 50 % confluence
631 and transfected with SGTA, followed by induction of OP91 expression in half of the samples. After
632 growth, cells were harvested into non-denaturing buffer and subjected to co-immunoprecipitation

633 using SGTA antibodies or IgG antibodies as negative control. Products were analysed by western
634 blotting using antibodies against opsin (OP91), V5 (SGTA) and tubulin (loading control). **B)** HeLa
635 TRex Flp-In cells stably expressing OP91 after induction were transfected with V5 tagged WT SGTA
636 or SGTA-3xNNP/AAA C-terminal mutant (denoted here as SGTA C-term) followed by incubation for
637 6 hours before the medium was replaced with medium containing 1 mg/ml of tetracycline to induce
638 OP91 expression. Cells were grown for 20 hrs post induction and total cell lysate were prepared and
639 products analysed by western blotting using antibodies for opsin (OP91), V5 (SGTA and SGTA- C-
640 term), and tubulin (loading control). **C)** Cells transfected with WT SGTA or the SGTA C-terminal
641 mutant described in B were processed for immunoprecipitation using SGTA antibodies. Samples
642 were analysed by western blotting using antibodies against SGTA, OP91 and USP5. **D)** HeLa TRex
643 Flp-In cells stably expressing OpD upon induction were seeded at 50 % confluence and transfected
644 with SGTA, followed by induction of OpD expression in half of the samples. Cells were subjected to
645 immunoprecipitation as detailed in A.

646

647 SGTA contains a well conserved C-terminal NNP region characterized by three repeats of
648 the NNP motif. Previously, a mutant version of SGTA in which all three NNP motifs were
649 altered to alanine's (SGTA-3xNNP/AAA-V5), was shown to enhance the ability of SGTA to
650 stabilise OP91 five -fold [46], (Fig 7B, cf. lane 2 and 3). To further investigate the substrate
651 dependent association of SGTA and USP5 we made use of this SGTA mutant. We reasoned
652 that if the stabilisation of MLPs is linked to deubiquitination, this mutant will likely associate
653 with more USP5 than WT SGTA. We therefore asked whether this mutant associates with
654 more USP5 compared to the WT SGTA. To this end, HeLa cells were transfected with WT
655 SGTA or the SGTA mutant, followed by induction of OP91 expression in half of the samples.
656 Cells were harvested into non-denaturing buffer 24 hours post-induction and subjected to
657 co-immunoprecipitation using SGTA antibodies or IgG antibodies as a negative control. As
658 expected SGTA associated with USP5 in the absence of OP91 induction and this
659 association was increased upon OP91 induction (Fig. 7C cf. lane 3 and 4). Similarly, the

660 SGTA-3xNNP/AAA mutant associated with more USP5 in the absence of induction (Fig. 7C
661 cf. compare lane 1 and 3), consistent with an increase in stabilisation of OP91 seen in this
662 mutant before induction. Upon induction of OP91, there was an increase in association
663 compared to the WT (Fig. 7C cf. compare lane 2 and 4), concomitant with an increase in
664 OP91. Therefore, these results confirmed that this association is substrate dependent.

665

666 To validate these observations, we investigated the association of SGTA and USP5 in the
667 presence of the ERAD substrate, opsin degron (OpD) [20], [47]. Induction of OpD did not
668 result in an increase in the association of SGTA and USP5 when compared to the uninduced
669 sample (Fig. 7D, cf. lane 3 and 4). Based on these results, we concluded that association
670 of USP5 and SGTA is specifically enhanced by the presence of an MLP. Attempts to confirm
671 direct binding of SGTA to USP5 *in vitro* did not show direct binding, suggesting the
672 interaction may be via an adaptor protein.

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Discussion

A role for DUBs in SGTA mediated protein quality control was first apparent when SGTA was reported to promote deubiquitination of MLPs [9, 32]. However, until now the deubiquitinating enzymes that play a role in this process had not been identified. In this study, we identified DUBs associated with SGTA by using pull-down assays and mass spectrometry. We confirmed that knocking down USP5 specifically reduces the steady state levels of a model MLP (Fig. 2), while overexpression of USP5 leads to an increase (Fig. 5). Using co-immunoprecipitation, we show an association of USP5 and SGTA which is dependent on the presence of an MLP: association of USP5 with SGTA was increased upon induction of OP91 (Fig. 7). This association is specific for MLPs as we did not observe an increase in the association of USP5 with OpD, an ERAD substrate (Fig. 7). Moreover, a gain of function mutant of SGTA which leads to a 20-fold increase in the steady state levels of OP91 (Fig. 7) showed increased USP5 association when compared to WT SGTA (Fig. 7). The failure of SGTA to effectively stabilise OP91 in the absence of USP5 (Fig. 6) points to an important role of USP5 in SGTA mediated quality control of membrane proteins that mislocalise to the cytosol. This is supported by an increased association of USP5 with the non-glycosylated OP91 which is mislocalised but has not been ER translocated (Fig. 7). Overall, our results point to a role of USP5 in regulating the quality control of MLPs.

USP5 has a well characterised function regulating a stable pool of free ubiquitin in vivo by recycling unanchored polyubiquitin chains [13]. In this regard, USP5 hydrolyses K63-, K48-, K11-, K29- linked and linear polyubiquitin chains [58]. However, a role for USP5 in the stabilisation of substrates destined for degradation is not without precedence. Previously, overexpression of USP5 was shown to stabilise FoxM1, a protein with a key role in tumorigenesis and progression of pancreatic cancers [59]. In addition, USP5 was shown to associate with the linker III-IV of the Cav3.2 protein and its knockdown increased

714 ubiquitination of Cav3.2 protein and subsequent degradation, thus supporting the notion that
715 USP5 stabilises Cav3.2 [60].

716

717 USP5 shares 54.8% sequence identity with USP13, hence a partial functional overlap
718 between the two has been suggested. Indeed, USP5 and USP13 are both recruited to
719 stress granules containing K48 and K63 linked ubiquitin chains. In this case, depletion of
720 USP5 and USP13 led to an increase in ubiquitinated proteins in heat induced stress
721 granules, suggesting these DUBs play a role in ubiquitin editing of substrates to facilitate
722 disassembly of stress granules [61]. Despite these functional overlaps, there is evidence
723 supporting the idea that these closely related DUBs also have some distinct roles and
724 classes of substrates. For instance, USP13 was previously shown to promote a stable
725 interaction between BAG6 and SGTA during ERAD and its knockdown reduced this
726 interaction [20]. On the other hand, USP5 did not have a direct effect on the interaction of
727 SGTA and BAG6 [20]. Consistent with these observations, in this study we did not observe
728 an effect of USP5 on the stability of the opsin degron, an ERAD substrate. Moreover, in our
729 pulldown assays, we did not detect an association of SGTA with USP13, suggesting USP13
730 might have a limited or no role in MLP quality control.

731

732 Our results clearly indicate that USP5 enhances SGTA mediated MLP quality control: the
733 ability of SGTA to stabilise MLP was significantly reduced in the absence of USP5 (Fig. 6).
734 However, it is not clear how USP5 exerts these effects. This is complicated by the fact that
735 DUBs such as USP5 have many cellular targets. Therefore, the effects of USP5 may be
736 indirect as was reported for USP13 [21]. It was shown that, USP13 prevents promiscuous
737 gp78-mediated ubiquitination of Ubl4A, a BAG6 cofactor which promotes ERAD.
738 Ubiquitination of Ubl4A was accompanied by irreversible cleavage of BAG6 and attenuation
739 of ERAD [21]. Hence, the deubiquitination action of USP13 eliminates ubiquitin conjugates

740 from Ubl4A to maintain the function of BAB6, thereby ensuring efficient disposal of ERAD
741 substrates. Recently, it was also shown that USP13 and gp78 also regulate the caspase
742 activity of CASP3, which cleaves BAG6 and switches its function from an ERAD regulator
743 to an autophagy tuner [19]. It was shown that the LIR1 motif of BAG6 holds the
744 autophagosome structural membrane proteins LC3B-I and the unprocessed form of LC3B
745 to suppress autophagy. This inhibition is relieved by the ubiquitination of thioredoxin which
746 results in CASP3 activation and cleavage of BAG6 [19]. Hence, it may be that USP5 might
747 exerts its' effects by modulating the ubiquitination status of a key player in the degradation
748 pathway of MLPs rather than the MLP itself. Reconstitution of the DUB reaction *in vitro* is
749 necessary in order to fully defined the role of USP5 in MLP quality control.

750

751 Functional pairing of E3 ligases with DUBs, as discussed for gp78 and USP13, is an
752 important regulatory step in protein quality control [15]. In MLP quality control, BAG6 binds
753 hydrophobic substrates and recruits RNF126, an E3 ubiquitin ligase which catalyses the
754 selective ubiquitination of MLPs [31, 44]. On the other hand, DUBs such as USP5 promote
755 substrate deubiquitination thereby giving substrates a chance to avoid proteasomal
756 degradation [9, 32, 43]. Hence, future work should study the interplay of USP5 and RNF126
757 in regulating MLP quality control. Another possibility is that USP5 might play a key role in
758 ubiquitin chain editing, by promoting K-63 linked assembly of substrates which would favour
759 their accumulation in inclusion bodies. This would be the reverse of what is seen with the
760 DUBs, Ubp2 and Ubp3 which prevent assemble of K-63 chains on substrates thereby
761 promoting K-48 mediated proteasomal degradation [18]. To understand the role of USP5 in
762 MLP quality control, it is necessary to study the type of ubiquitin linkages of MLPs and how
763 these change upon rapid depletion of USP5 by using approaches such as the auxin-
764 inducible degron (AID) technology [62].

765

766 In this study we did not detect a direct interaction of USP5 and SGTA, suggesting the
 767 interaction might be via an adaptor protein. A key role of the adaptor protein UBXN1 in
 768 regulating the interaction of VCP with BAG6 clients occurring prior to ER insertion was
 769 recently demonstrated [63]. It was shown that BAG6 clients, once ubiquitylated, were not
 770 directly degraded by the proteasome, but were recognised by the VCP-UBXN1 complex
 771 prior to degradation. Failure of ubiquitinated substrates to engage the UBXN1 adaptor led
 772 to their accumulation as aggregates [63], underscoring the importance of the adaptor
 773 proteins in protein quality control. In the case of USP5, the dendritic cell-derived ubiquitin
 774 (Ub)-like protein (DC-UbP) functions as an adaptor protein reconciling the cellular
 775 ubiquitination and deubiquitination processes by regulating the function of USP5 and UbE1
 776 [64]. The C-terminal UbL domain of DC-UbP interacts with the UBA domains of USP5 and
 777 with the Ub-fold domain (UFD) of the Ub-activating enzyme UbE1, on the distinct surfaces.
 778 Overexpression of DC-UbP enhanced the association of these two enzymes and prompted
 779 cellular ubiquitination, whereas knockdown of the protein reduced the cellular ubiquitination
 780 level [64]. In the case of MLP quality control, SGTA may function in a similar way to DC-
 781 UbP as it has a ubiquitin (Ub)-like domain on the N-terminus where BAG6 binds and promote
 782 RNF126 recruitment [31]. It may be that this (Ub)-like domain also promotes recruitment of
 783 DUBs such as USP5, via another protein. Future work should investigate the nature of
 784 interaction between USP5 and SGTA and identify any adaptor proteins involved in this
 785 process.

786
 787 The number of DUBs that play important roles in the cytosolic protein quality control
 788 continues to increase, and this study adds USP5 to this list. This study suggests that USP5
 789 impacts on the disposal of MLPs and possibly other aggregation prone proteins when its
 790 levels are modulated. Therefore, future work should extend these studies to other
 791 membrane proteins and to substrates implicated in pathologies such as neurodegeneration
 792 and cancer in order to understand how USP5 regulates their fates. These studies will shed
 793 light on how USP5 can be modulated as a therapeutic target for prevention of terminal

794 aggregates which are linked to neurodegenerative diseases, cancer, and type II diabetes II.
 795 The apparent specificity of USP5 for cytosolic substrates, makes effective therapies more
 796 likely as there may be a limited crosstalk to other DUBs and pathways.
 797

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799
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Supporting information

Fig_S1. Knockdown of SGTA destabilises OP91.

HeLa T-REx Flp-In cells stably expressing OP91 after induction were seeded at 50 % confluence and transfected with 1 nM of siRNAi targeting SGTA or a nontargeting control and incubated for 48 hours. After this, OP91 expression was induced by replacing with medium containing 1 mg/ml of tetracycline. Cells were grown for 20 hrs post induction and total cell lysate were prepared and analysed by western blotting using antibodies for opsin (OP91), SGTA, and tubulin (loading control) and detected using an Odyssey ® Fc imaging system.

Fig_S2. USP5 and SGTA protect OP91 from degradation.

A. HeLa T-REx Flp-In cells stably expressing OP91 were seeded at 50 % confluence and transfected with 1 nM of siRNAi targeting USP5, USP9X, SGTA or a nontargeting control and incubated for 48 hours. After this OP91 expression was induced by addition of medium containing 1 µg/ml of tetracycline and grown for 20 hrs post induction. Prior to harvesting transfected cells were treated with 100 µg/ml cycloheximide (Sigma, Aldrich) to inhibit protein synthesis. Cells were lysed directly into SDS-PAGE sample buffer at specific time-points followed by Western blotting with antibodies against opsin (OP91) and tubulin (loading control) using fluorescence- based detection (LICOR). **B)** OP91 signals were quantified using Odyssey ® Fc imaging system and normalised to the tubulin loading control, values show standard errors for n = 3.

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