# Regulation of target proteins by small ubiquitin-like modifiers
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Cover Page

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# Chapter 5

# UFMylation: an emerging post-translational modification regulating protein synthesis

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## Summary

UFMylation is a reversible post-translational modification implicated in a variety of biological processes including ER homeostasis upon unfolded protein response (UPR), erythroid differentiation, and more recently coordinating interaction of mRNA and other proteins to the ribosome. We studied global UFMylation in a site-specific manner in both wildtype and UFSP2 knockout cells. We identified seven proteins (RPL26, RPL26L1, TUBA1B, MCM5, SLC26A7, SCYL2, WDR63) for which we could map the modified lysines. Interestingly, we found UFMylation of RPL26 (L24), a 60S ribosomal protein, that we demonstrate to specifically interact with the signal recognition particle receptor (SRPR). Together, these results shed a new light on the biological role of UFM1 and open new avenues of research to clarify the physiological role of the UFM1-system.

# 1 Introduction

Post-translational modifications (PTMs) tightly regulate protein functions either through the addition of chemical moieties like methylation, acetylation and phosphorylation or small protein modifiers that belong to the ubiquitin-like (Ubl) family to specific residues of their target proteins[1,2]. Besides ubiquitin, other proteins such as Small Ubiquitin-like Modifiers (SUMOs), Atg8, Atg12, FUBI, FAT10, HUB1, ISG15, Nedd8, URM1 and UFM belong to the Ubl family[3,4]. As a result of these PTMs, functional study on proteomics become extremely complex[5,6] .

Phosphorylation and ubiquitination are the most common and well-studied post-translational modification mechanisms in eukaryotes[7-9]. Small ubiquitin-like modifiers (SUMOs), have also been extensively studied in the past two decades. Thousands of SUMO substrates as well as acceptor lysines were discovered by mass spectrometry (MS) analyses[10-14], which provided a wealth of resources for further functional study of these modifications. However, only limited information is available about other Ubl conjugation systems such as UFM1.

UFM1, a recently identified ubiquitin-like protein, resembles ubiquitin, covalently modifies lysine residues of its substrates through a three components enzymatic cascade[15-19]. UFM1 is translated as an inactive precursor form (pro-UFM1) which has one or two additional amino acids beyond the conserved single glycine and are cleaved by UFSP2 to expose the UFM1- single active C-terminal glycine. Mature UFM1 is activated as an adenylate by UBA5 forming a high-energy thioester bond with the active-site cysteine of UBA5. This allows the transfer onto the catalytic site of the E2 enzyme-UFC1, where again a thioester bond is formed. With the action of the E3-like enzyme, UFL1, activated UFM1 is transferred onto the lysine residues of the protein substrates, forming an isopeptide bond. Similar to ubiquitin, UFMylation is reversed by cleavage of UFM1 specific protease, UFSP2. UFM1 has six lysines which are amenable to forming poly-UFMylation[20,21].

The main conserved function of the ribosome is translation of the genomic code into proteins[22,23]. The eukaryotic ribosome consists of 80 ribosomal proteins and a ribosomal RNA shell[24,25]. Secretory proteins are delivered through the secretory pathway. In this pathway proteins possessing signal sequences are recognized at the ribosome by the signal recognition particle (SRP) while they are still undergoing synthesis. With the help of SRP and its membrane bound receptor (SRPR or SR), co-translational secretory and membrane proteins are directly translocated into the translocation channel and finally into the ER[26].

UFMylation has been connected to biological processes including ER homeostasis, vesicle trafficking, autophagy and more recently it has been demonstrated to modify ribosomal proteins[27, 29,30]. A failure to conjugate UFM1 to target proteins results in the promotion of tumor formation[20]. Loss of function of UFMylation in mice leads to apoptosis in fetal liver cells and pancreatic beta cells[21]. Using an improved immunoprecipitation approach, Simsiek et al. reported UFMylation of a ribosomal protein (eL36) and its interaction with the UFM1 E3 ligase UFL1[27]. More recently, Pirone et al reported their

identification of 494 UFMylated proteins, of which 82 survived stringent filtering[28]. However, none of them been independently validated and no biological function studies have been well conducted to elucidate the role of protein UFMylation. Despite these interesting findings, the biological consequences of UFMylation remain obscure.

Given the lack of understanding of biological processes involving UFMylation, as well as the transient nature of UFM1 modification, substrate identification has posed major technical challenges and urgently calls for the development of proteomics methods. To address this problem, we adapted the mass spec technology developed by Hendriks et al[10,13]. by combining it with CRISPR/Cas9-mediated gene knockout of the only known UFM1 specific protease-UFSP2 that is active in mammalian cells, to enrich UFMylated substrates and to identify UFM1 specific acceptor lysines. Seven proteins with UFM1 acceptor lysines have been identified in our screen. One of the major UFM1 modifed proteins was the ribosomal protein RPL26. We found that UFMylated RPL26 can efficiently interact with signal recognition particle receptor (SRPR). Our findings provide new insight in UFM1 signal transduction.

## 2 Results

UFM1 is a 9.1-kDa protein with a similar tertiary structure to ubiquitin and with a similar three component enzymatic cascade. Mature UFM1 can be attached to target proteins. Deconjugation of UFM1 is mediated by the two UFM1-specific proteases, UFSP1 and UFSP2 **(Figure 1 A and B).** Previously Ishimura et al. has shown that knockdown of UFSP2 leads to an increase in UFMylation, thereby enabling the enrichment of UFM1 modified proteins[31]. We employed CRISPR-Cas9 mediated genome editing to knock out UFSP2 in Hela cells **(Figure 2A).**

A
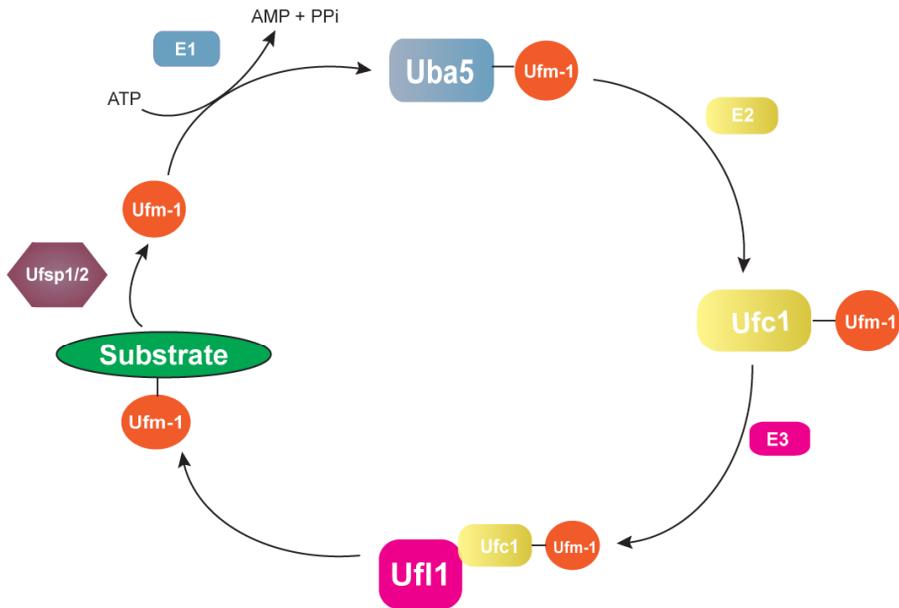


B     **Alignment of Ufm1 and Ub**



**Figure 1. UFM-1 cascade and UFM-1. A)** Ufm-1 conjugation machinery **B)** UFM-1 sequence alignment with Ubiquitin (Ub) shows that the C-terminal di-glycine motif characteristic of Ub is replaced by a valine- glycine motif in UFM-1.

## 2.1 Proteomics approach to study global UFMylation in UFSP2 knockout cells

Given that the knockout of UFSP2 in HeLa cells caused a significant increase in overall UFMylation and slightly decreased cell proliferation, we were interested in identifying UFM1 target proteins as well as acceptor lysines in UFSP2 knock out cells using a proteomics approach. Due to the nature of UFM1, one major challenge in addressing this is that analogous technologies used for identification of ubiquitinated substrates (e.g. di-Gly proteomics) are unavailable for UFM1, as antibodies targeting VG-sites are still not available. Furthermore, the abundance of UFMylated proteins is magnitudes lower than that of ubiquitination or SUMOylation, posing a major challenge for proteomics[32].

To address this, we have adapted the biochemical purification of SUMO target proteins and SUMO sites developed by Hendriks et al[10]. We generated a HeLa cell line and a HeLa cell line with UFSP2 knockout stably expressing a His10-tagged lysine-deficient UFM1 (His10-UFM1-K0) to enable UFM1 acceptor lysine mapping （**Figure 2B**）. To generate a stable cell line, we used puromycin as marker

for selection. Immunoblot analysis confirmed the slightly increased expression of His10-UFM1-K0 compared with endogenous levels of UFM1 in HeLa cells and the efficient purification of UFM1 conjugates by the His10-UFM1-K0 method **(Figure 2 C and D).**
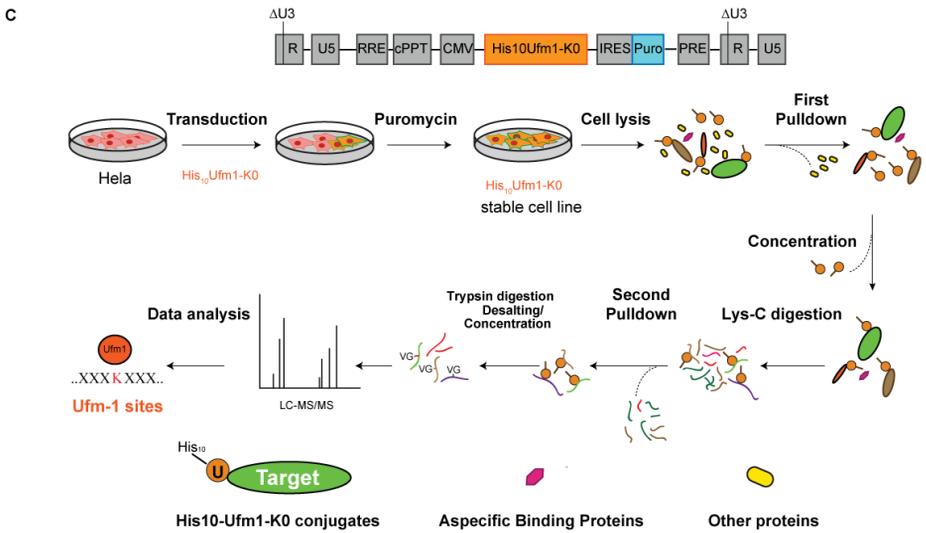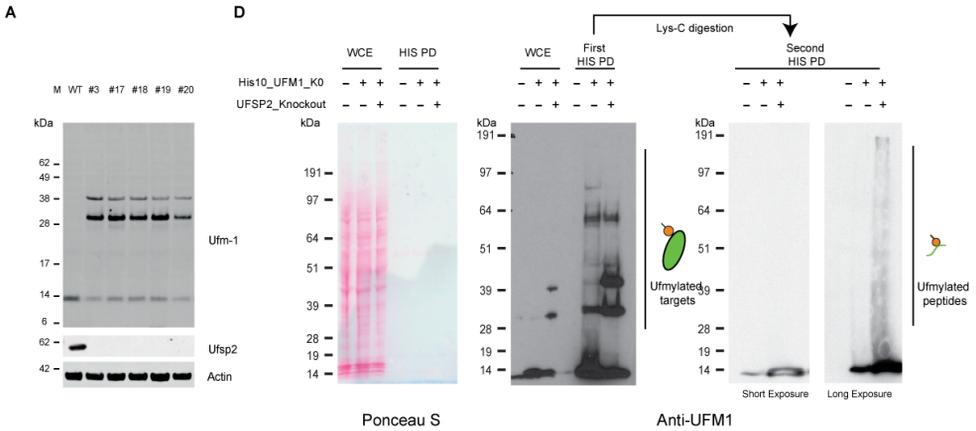
**Figure 2. Generation and validation of Hela cells stably expressing His10-UFM1-K0 with or without UFSP2. A)** UFSP2 depletion increases UFMylation. UFSP2 was knocked out using CRISPR-CAS9 gene editing technology. The efficiency of UFSP2 knock out was assessed by immunoblotting using an antibody against UFSP2. Depletion of UFSP2 caused an increase of UFMylation as shown by immunoblotting using an antibody against UFM1. **B)** Sequence of His10-UFM1-K0 protein used to generate stable cell lines in either HeLa or Hela UFSP2 knockout cells. **C)** Cartoon depicting the strategy to identify UFM1 targets as well as acceptor lysines. **D)** Purification of His10-UFM1-K0 conjugates via NTA purification was confirmed by immunoblotting. Whole cell extracts and UFM1 purified proteins of cells were run on 4-12% Bis-Tris polyacrylamide gels and levels of His10-UFM1 conjugates were compared by immunoblotting using anti-UFM1 antibody. Ponceau-S staining was performed to confirm the purity of the final fraction.

We used this cell line to enrich and purify UFMylation peptides from HeLa cells (His10-UFM1-K0) or HeLa cells(His10-UFM1-K0) without UFSP2. Wild type Hela cells were used as the negative control. Cells were cultured using a lower puromycin concentration to ensure stable expression. To enrich the His10-UFM1 modified proteins, a two-step purification strategy was employed as described in Figure 2 C[10]. For proteomic analysis, three biological replicates with two technical repeats for all cell lines were analyzed in this study. In total, 13 sites were identified in seven proteins, which are RPL26, RPL26L1, TUBA1B, MCM5, SLC26A7, SCYL2, WDR63 **(Figure 3A).**

## 2.2 RPL26 is extensively UFMylated

The ribosome production is a fundamental process in cell biology. RPL26 overexpression inhibits cell proliferation and induces cell cycle arrest through activating p53[33] and later more studies indicated that RPL26 controls p53 protein levels through binding to the 5' untranslated region of the p53 mRNA[34]. However, little is known about its post translational modifications and its role in protein synthesis. We selected RPL26 for further validation to study its regulation by UFMylation because it is the top UFM1 target identified in our screen.

We found four UFM1 acceptor lysines in the C-terminus of RPL26 (K132, K134, K136 and K142) **(Figure 3 A and B).** Interestingly, alignment of orthologous genes showed that these four acceptor lysines are highly conserved across species probably due to their functional importance **(Figure 3C).** Immunoblotting analysis of endogenous RPL26 and Flag-RPL26 in His10-UFM1-K0 HeLa cells and UFSP2 knockout HeLa cells revealed that UFMylation of RPL26 strongly increased in the absence of UFSP2 (**Figure 3 D and E).** Increased RPL26-UFMylation thus can be explained by depletion of UFSP2.

**A**

| Protein names | VG (UFM1) site within proteins | VG (UFM1) site positions | Score |
|---|---|---|---|
| RPL26 / RPL26L1 | 132 | KILERKAKSRQVGKEKGKYKEETIEKMQE__ | 210.35/140.14 |
| RPL26 / RPL26L1 | 134 | LERKAKSRQVGKEKGKYKEETIEKMQE____ | 100.22/62.16 |
| RPL26 / RPL26L1 | 136 | RKAKSRQVGKEKGKYKEETIEKMQE_____ | 69.03/60.16 |
| RPL26 | 142 | QVGKEKGKYKEETIEKMQE_____ | 64.84 |
| TUBA1B | 370 | INYQPPTVVPGGDLAKVQRAVCMLSNTTAIA | 92.93 |
| MCM5 | 582 | VRQLEAIVRIAEALSKMKLQPFATEADVEEA | 61.16 |
| SLC26A7 | 120 | SIVVLVLVKELNEQFKRKIKVVLPVDLVLII | 60.91 |
| SCYL2 | 73 | KQEVAVFVFDKKLIDKYQKFEKDQIIDSLKR | 59.91 |
| SCYL2 | 76 | VAVFVFDKKLIDKYQKFEKDQIIDSLKRGVQ | 59.91 |
| WDR63 | 564 | DHLLCKTQDKMLAQSKTEKAEEMNPYHNLES | 45.28 |

**B**



**C**



**D**



Anti-RPL26

**E**



Anti-Flag

**Figure 3. Label Free Quantification Results. A)** VG-sites identified for UFM1 modified proteins by mass spectrometry. **B)** Domain structure of RPL26 highlighting the C-terminal UFM1 modification. **C)** Sequence Alignment of RPL26 reveals that the C-terminal lysine residues of this ribosomal protein are conserved across species except for yeast. **D)** Hela cells with His10-UFM1-K0 and with or without UFSP2 were lysed and proteins were subjected to NTA purification. Whole cell extracts and UFM1 purified samples were analysed by immunoblotting using an antibody against RPL26. **E)** Flag-RPL26 was overexpressed in His10-UFM-K0 cell lines with or without UFSP2. Cells were lysed and proteins subjected to NTA purification. Whole cell extracts and UFM1 purified samples were analysed by immunoblotting using antibody against Flag.

## 2.3 UFMylated RPL26 can directly interact with SRPR

Next, we asked whether UFMylation of RPL26 affected ribosome associated protein interaction. To investigate such protein interactions, we performed immunoprecipitation of ribosomes with modified or unmodified RPL26 coupled with a label free quantitative proteomics approach to identify proteins preferentially binding to UFMylated RPL26. In this case, Flag-RPL26 was transiently overexpressed in both HeLa cells and HeLa cells UFSP2 knockout cells stably expressing His10-UFM1-K0 and immunoprecipitated using an anti-Flag antibody. Proteins were digested on beads with trypsin and four independent experiments were performed **(Figure 4A)**. Proteins were identified by mass spectrometry and silver staining were performed to confirm the purity of the final fraction **(Figure 4B).** Volcano plots shown in Figure 4C indicate the proteins preferentially binding to UFMylated RPL26.

**A**



Flag-IP

LC-MS/MS

His10

UFM1  UFM1

RPL26 — Flag

RPL26 — Flag

Interactors          Other proteins

**B**

Immunoprecipitation
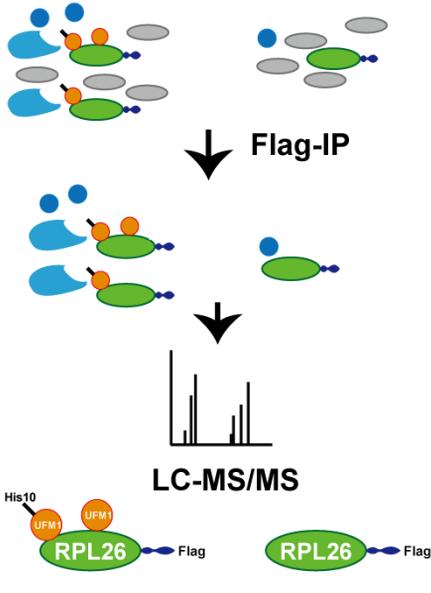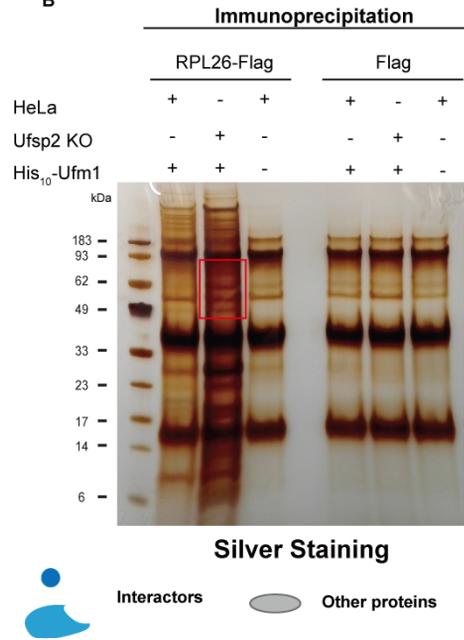


Silver Staining

**C**
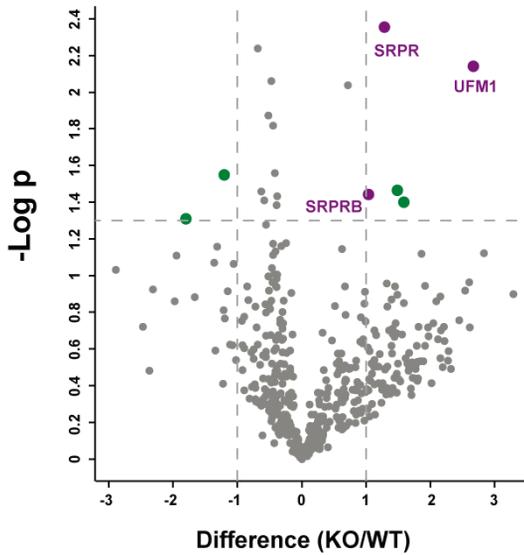
**Figure 4. RPL26-UFMylation alters the interaction of ribosome associated proteins. A)** Cartoon depicting the strategy to identify interactors of UFMylated RPL26. **B)** Silver staining of mass spec samples. Co-Immunoprecipitation of Flag-RPL26 from either the wild type or the UFSP2 knockout HeLa cells stably expressing His10-UFM-K0. The silver stained gels reveal that there are indeed differences between the modified and the unmodified RPL26 containing ribosomes. **C)** Volcano plots showing proteins preferentially binding to UFMylated RPL26.

Most notably, the signal recognition particle alpha (SRPR) interacts with UFM1 modified ribosomes in the UFSP2 KO His10-UFM1-K0 cells, but less when UFM1 is removed by the UFSP2 protease. This interaction could be confirmed by co-immunoprecipitation, which clearly showed that the UFM1 modified ribosome preferentially interacts with SRPR **(Figure 5 A and B).**

# 3 Discussion

It has been found that UFMylation is important to promote interactions between proteins[15]. Only little is known about the target proteins regulated by UFM1[20,27,28,35]. To identify UFM1 target proteins as well as acceptor lysines, we have adopted a pioneering and well-developed method that has been proven to enable identification of SUMO acceptor lysines[13]. As a result, we successfully identified 8 novel UFM1 target proteins with 23 sites including RPL26 as a major UFM1 target in the absence of UFSP2. We confirmed RPL26 as a key UFM1 target and further confirmed that the UFMylated form of RPL26 can efficiently interact with SRPR. Functional analysis of RPL26 such as RNA profiling of UFSP2 knockout HeLa cells revealed a role for UFM1-modified RPL26 in promoting translation elongation (data not shown).

## 3.1 PTM of ribosomal proteins

The mammalian ribosomes are composed of two subunits: 40S (small) and 60S (large) subunits. The 40S subunit is composed of 33 proteins and 18S rRNA, whereas the 60S subunit contains 46 proteins and three rRNAs (5S, 5.8S and 28S). While the 40S subunit is involved in the decoding of mRNA, the 60S subunit is responsible for peptide bone formation and export of nascent peptides[22-24,36-41]. The rim of the exit point is composed of RNA, and a ring of four ubiquitously conserved ribosomal proteins (L22, L23, L24 and L29). L24 is RPL26[42,43]. **(Supplementary Table 1)**

Among all ribosomal proteins, RPS6 is the first reported and best characterized phosphorylated ribosomal protein which is involved in PI3K, mTOR and Ras signalling pathways[30,44,45]. Later, mouse models linked phosphorylation of RPS6 to the initiation of pancreatic cancer, however the physiological roles are still elusive[46]. Another PTM modifying ribosomal protein is ubiquitin, which forms K63-linked chains on the RPL28/uL15 protein in yeast[47]. Proteomic analysis of cells with chemically induced unfolded protein stress (UPR), revealed that mono-ubiquitination of small ribosomal proteins Rps2/uS5 and Rps3/uS3 was altered dynamically, most likely affecting protein-protein interaction[48]. Using an improved immunoprecipitation approach, Simsiek et al. first report UFMylation of a

ribosomal protein (eL36) and its interaction with the UFM1 E3 ligase UFL1. However, they did not further investigate its biological impact on protein translation or protein-protein interaction[27].

## 3.2 UFMylation of RPL26 stimulates its interaction with SRPR.

Synthesis of new proteins is a very complex process. During translation of mRNA transcripts, various factors including enzymes and chaperones are involved in the processing, folding and targeting of nascent peptide chains to translocation pores at membranes[49]. The ribosome plays a key role in these different tasks and serves as a platform for the regulated enzymes, targeting factors and chaperones that work on the newly translated polypeptides emerging from the exit tunnel. Secretory proteins are translated with an N-terminal signal sequence that can be recognized by the signal recognition particle (SRP). In order to target the ribosome-nascent chain complex to the endoplasmic reticulum membrane, SRP contacts the SRP receptor (SRPR) and leads the nascent chain transformation from the SRP into the lumen of the ER.

We immunoprecipitated ribosomes with modified or unmodified RPL26 coupled with a label free quantitative proteomics approach in order to identify associated factors. We identified the signal recognition particle receptor alpha (SRPR), to be significantly enriched in UFSP2 KO cells, suggesting that this protein might be preferentially regulated by UFMylated RPL26. As expected, immunoblot analysis revealed that SRPR coimmunoprecipitated more efficiently with UFMylated RPL26 than with the non-UFMylated RPL26. These results suggest that the ribosome contacts the SRPR directly in the presence of UFM1 modification of RPL26 and does not require the signal recognition particle (SRP) to mediate the interaction. Upon examination of literature[50], the scenario seems to be the case, during the "handover" state of the ribosome, that is when the ribosome has been properly positioned over the SEC translocon and the SRP is being displaced. Taking our results into consideration, we hypothesize that UFM1 seems to mediate direct contact of the tunnel exit of the ribosome with the SRPR during the last step of direct peptides to the Sec61 translocon (**Figure 5C**). Our data indicate that RPL26 is one of the UFM1 target proteins serving as a platform for ribosome associated protein interaction.

The observation that SRPR can preferentially interact with UFMylated RPL26 in our experiments further raises the question how UFMylation specifically regulates translation. Given the novelty of this discovery, more experiments addressing the functional consequences on translation need to be performed to fully understand the role of UFM1 in this context. To answer this question, more studies are being performed (i.e. ribosome profiling, data not shown).

**Figure 5. Validation of Interaction between SRPR and RPL26-UFM1. A, B)** Co-immunoprecipitation of SRPR and RPL26 modified with UFM1. Hela cells, Hela cells without UFSP2 were lysed and proteins were incubated with RPL26 antibody (A) or SRPR antibody (B). Whole cell extracts and IP samples were analysed by immunoblotting using antibodies against UFM1 or SRPR as indicated in the figures. **C)** Model illustrating our current hypothesis of how UFM1 interacts with SRPR in during the "handover" state of the ribosome.

# 4 Conclusion

In this study, we identified seven proteins (RPL26, RPL26L1, TUBA1B, MCM5, SLC26A7, SCYL2, WDR63) that are covalently modified by UFM1. We describe for the first time a novel mechanism by which UFMylation might regulate proteins during the last step of the translation (elongation), after (or shortly before) the SRP leaves the ribosome and the ribosome correctly binds to the Sec61 translocon. The identification of the ribosomal protein RPL26 being modified by UFM1 might help to understand how UFM1 regulates protein synthesis by coordinating its targets.

# 5 Experimental procedures

## Antibodies

The primary antibodies used for this study are: anti-UFSP2 (Abcam ab185965), anti-Actin (Sigma Aldrich A5941, Clone AC-15, ascites fluid), anti-UFM1 (Abcam ab109305), anti-RPL26 (Thermo Fisher Scientific, PA5-17093), anti-Flag (Sigma Aldrich, M2), anti-SRPR (Abnova H00006734-B02P). Secondary antibodies used for this study are: anti-mouse-800 (1: 10000 dilution; LiCOR, 926-3210) and anti-rabbit-800 (1: 10000 dilution; LiCOR, 926-3211). Fluorescent second antibody were used for visualization of labeled proteins on LICOR Odyssey system v3.0.

For immunofluorescence, goat anti-rabbit and goat anti-mouse AlexaFluor-488/568 conjugates (Thermo Fisher Scientific) were used.

## Cloning, cell culture and cell line generation

The His10-UFM1-K0 we described and used in this manuscript has the following amino acid sequence:

MHHHHHHHHHHGGSMSRVSFRITLTSDPRLPYRVLSVPESTPFTAVLRFAAEEFRVPAA TSAIITNDGIGINPAQTAGNVFLRHGSELRIIPRDRVG.

The corresponding nucleotide sequence was cloned in plasmid pLV-CMV-IRES-puro[51]. HeLa cell lines originated from ATCC, they were cultured under standard conditions (DMEM (Gibco) supplemented with 10% FCS (Sigma-Aldrich) at 37 °C with 5% CO2). Hela cells at 60% confluency were infected using a bicistronic lentivirus at an MOI of 3 encoding His10_UFM1_K0_IRES_puro. Following infection, cells were cultured under standard conditions as described before for two weeks under puromycin selection (2.5 µM). All cell lines used in this study were tested for mycoplasma contamination routinely with consistently negative outcome.

## CRISPR-Cas mediated gene editing

UFSP2 guide RNA (gRNA) was designed using the CRSIPR Design tool (http://crispr.mit.edu/), subcloned into a pX260 vector (Addgene).

UFSP2 guide RNA was subcloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene #42230, Cambridge, MA, USA), a human codon-optimized SpCas9 and chimeric guide RNA expression plasmid. CRISPR-mediated UFSP2 depletion in Hela cells was performed by co-transfecting confluent HeLa with a vector harbouring the gRNA and the Cas9 and a construct conferring blasticidin resistance. After blasticidin selection and clonal expansion, UFSP2 depletion was verified by immunoblotting against using anti-UFSP2 antibody (1:1000 dilution, Abcam ab185965).

## Electrophoresis and immunoblotting

Whole cell extracts or purified protein samples were separated on Novex Bolt 4-12% Bis-Tris Plus gradient gels (Life Technologies) using MOPS buffer or standard SDS-PAGE using a Tris-glycine

buffer and transferred onto Hybond-C nitrocellulose membranes (GE Healthcare Life Sciences) using a submarine system (Life Technologies). Membranes were stained with Ponceau S (Sigma) to visualize total protein amounts and subsequently blocked with 8% milk in PBS containing 0.05% Tween-20 before incubating with the primary antibodies as indicated.

## Silver staining

For silver staining, gels were stained as described before[52]. Gels were incubated in Fixer (30% ethanol, 10% acetic acid) for 30 minutes and then washed two time in 20% ethanol and two times in water, 10 minutes each. Gels were sensitized in 0.8mM sodium thiosulfate for one minute and then rinsed in water twice for one minutes. Gels were then impregnated with 12 mM silver nitrate for 20 minutes and transferred to stop solution (40g of Tris and 20 ml of acetic acid per liter) for 30 minutes. Gels were then washed twice in water for 30 minutes.

## Co-immunoprecipitation

For co-immunoprecipitation of RPL26-FLAG with endogenous interacting proteins, both HeLa cell lines stably expressing His10-UFM1 were transiently transfected with RPL26-FLAG or FLAG only as a background control. Cells were scraped on ice into ice cold co-immunoprecipitation buffer (50mM tris-HCl, pH 7.5, 1.5mM MgCl2, 0.5% NP-40 and 5% Glycerol supplemented with 1mM DTT and a Complete Protease Inhibitor Tablet (Roche)). Lysates were clarified by centrifugation (4000rpm, 4°C, 20 min) and immunoprecipitated for 1h at 4°C with 3 µg Flag antibody followed by a 3h incubation with pre-equilibrated Protein-G Sepharose beads (GE Healthcare) at 4°C while rotating. After immunoprecipitation, samples were washed four times with ice-cold co-immunoprecipitation buffer and processed further for mass spectrometric analysis.

## Purification of His10-UFM1 conjugates

Hela cells expressing His10-UFM1-K0 were washed, and scraped into ice-cold PBS. For total lysates, a small aliquot of cells was kept separately and lysed in 2% SDS, 1% N-P40, 50 mM TRIS pH 7.5, 150 mM NaCl. The remaining parts of the cell pellets were lysed in 6 M guanidine-HCl pH 8.0 (6 M guanidine-HCl, 0.1 M Na2HPO4/NaH2PO4, 10 mM TRIS, pH 8.0). The samples were snap frozen using liquid nitrogen, and stored at -80°C.

For His10-UFM1-K0 purification, the cell lysates were first thawed at room temperature and sonicated for 5 sec using a sonicator (Misonix Sonicator 3000) at 30 Watts to homogenize the lysate. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific) and lysate concentrations were equalized. Subsequently, imidazole as well as β-mercaptoethanol were added to a final concentration of 50 mM and 5 mM, respectively. His10-UFM1-K0 conjugates were enriched on nickel-nitrilotriacetic acid-agarose beads (Ni-NTA) (Qiagen), followed by washes with the following buffers A-D: Wash buffer A: 6 M guanidine-HCl, 0.1 M

Na2HPO4/NaH2PO4 pH 8.0, 0.01 M Tris-HCl pH 8.0, 10 mM imidazole pH 8.0, 5 mM β-mercaptoethanol, 0.1% Triton X-100. Wash buffer B: 8 M urea, 0.1 M Na2HPO4/NaH2PO4 pH 8.0, 0.01 M Tris-HCl pH 8.0, 10 mM imidazole pH 8.0, 5 mM β-mercaptoethanol, 0.1% Triton X-100. Wash buffer C: 8 M urea, 0.1 M Na2HPO4/NaH2PO4 pH 6.3, 0.01 M Tris-HCl pH 6.3, 10 mM imidazole pH 7.0, 5 mM β-mercaptoethanol, no Triton X-100. Wash buffer D: 8 M urea, 0.1 M Na2HPO4/NaH2PO4 pH 6.3, 0.01 M Tris-HCl, pH 6.3, 5 mM β-mercaptoethanol. For samples subjected to immunoblotting, wash buffers containing 0.2% Triton X-100, were used. Samples were eluted in 7 M urea, 0.1 M NaH2PO4/Na2HPO4, 0.01 M Tris/HCl, pH 7.0, 500 mM imidazole pH 7.0. For site-specific purification, we used the strategy developed previously[10,13].

**Sample preparation and mass spectrometry**

UFM1-enriched samples were supplemented with 1 M Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) to a final concentration of 5 mM, and incubated for 20 minutes at room temperature. Iodoacetamide (IAA) was then added to the samples to a final concentration of 10 mM and samples were incubated in the dark for 15 minutes at room temperature. Lys-C and Trypsin digestions were performed according to the manufacturer's specifications. Lys-C was added in a 1:50 enzyme-to-protein ratio, samples were incubated at 37 °C for 4 hours, and subsequently 3 volumes of 100 mM Tris-HCl pH 8.5 were added to dilute urea to 2 M. Trypsin (V5111, Promega) was added in a 1:50 enzyme-to-protein ratio and samples were incubated overnight at 37 °C. For site-specific sample preparation, we used the strategy developed previously[10,13].

Then, digested samples were desalted, desalted samples were further concentrated on STAGE-tips as described previously[10,13]. Samples were eluted with 0.1% formic acid in 80% acetonitrile. Elution step has been vacuum dried using a SpeedVac RC10.10 (Jouan, France) and before online nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS), final fractions were dissolved in 10 μL 0.1% formic acid.

Experiments were performed on a Q-Exactive Orbitrap (Thermo Fisher Scientific, Germany) connected with an EASY-nLC 1000 system (Proxeon, Odense, Denmark) through a nano-electrospray ion source. Peptides were separated in a 15 cm analytical column with an inner-diameter of 75 μm, in-house packed with 1.9AQ - μm C18 beads (Reprospher-DE, Pur, Dr. Manish, Ammerbuch-Entringen, Germany).

The gradient length was 120 minutes from 2% to 95% acetonitrile in 0.1% formic acid at a flow rate of 200 nL/minute. The mass spectrometer was operated in a data-dependent acquisition mode with a top 10 method. Full-scan MS spectra were acquired at a target value of $3 \times 10^6$ and a resolution of 70,000, and the Higher-Collisional Dissociation (HCD) tandem mass spectra (MS/MS) were recorded at a target value of $1 \times 10^5$ and with a resolution of 17,500 with a normalized collision energy (NCE) of 25%. The maximum MS1 and MS2 injection times were 20 ms and 60 ms, respectively. The

precursor ion masses of scanned ions were dynamically excluded (DE) from MS/MS analysis for 60 sec. Ions with charge 1, and greater than 6 were excluded from triggering MS2 events.

For samples enriched for identification of UFM1 acceptor lysines, a 120-minute gradient was used for chromatography. Data dependent acquisition with a top 5 method was used. Maximum MS1 and MS2 injection times were 20 ms and 250 ms, respectively. Resolutions, normalized collision energy and automatic gain control target were set as mentioned previously. Dynamic exclusion was set to 20 sec.

## Mass spec data analysis

### Site level UFMylation data analysis

Site-specific purification was performed in three biological replicates, and all samples were measured in technical duplicates. All 18 RAW files were analyzed by MaxQuant (version 1.5.3.30). The first search was carried out with a mass accuracy of 20 ppm, while the main search used 64.5 ppm for precursor ions. Database searches were performed with Trypsin/P specificity, allowing three missed cleavages. Carbamidomethylation of cysteine residues was considered as a fixed modification. Mass tolerance of MS/MS spectra was set to 20 ppm to search against an in silico digested UniProt reference proteome for Homo sapiens (2017-30-01). Additionally, MS/MS data were searched against a list of 245 common mass spectrometry contaminants by Andromeda. Oxidation (M) and Acetyl (Protein N-term) were set as variable modifications. Additionally, VG modified lysine was introduced as as a variable modification with a composition of $C_7H_{12}N_2O_2$ and a monoisotopic mass of 156.090 Da. Match between runs was used with 0.7 min match time window and 20 min alignment time window.

### Interactors of Ufmylated RPL26

Protein lists generated by MaxQuant were further analyzed by Perseus (Version 1.5.5.3). Proteins identified only by site and as a common contaminant were filtered out, and then all the LFQ intensities were $\log_2$ transformed. Different experiments were annotated in to six group, Hela-Flag, Hela-Flag-RPL26, Hela-His10-Ufm1-K0-Flag, Hela-His10-Ufm1-K0-Flag-RPL26, Hela-His10-Ufm1-K0-Flag (without UFSP2), Hela-His10-Ufm1-K0-Flag-RPL26 (without UFSP2). Proteins identified in at least one treatment condition and found in at least three biological replicates were included for further analysis. For each experimental condition individually, missing values were imputed using Perseus software by normally distributed values with a 1.8 downshift ($\log_2$) and a randomized 0.3 width ($\log_2$). Final corrected P values were filtered to be less than 0.05. Then, average $\log_2$ ratios for Hela-His10-Ufm1-K0-Flag-RPL26 vs Hela-His10-Ufm1-K0-Flag were calculated and *P* values of each protein across all treatment conditions were calculated by T-tests. Proteins were selected as Ufmylated RPL26 interactors when their average $\log_2$ ratios are greater than 1 and corresponding p values were less than 0.05. Volcano plots to demonstrate identified proteins preferentially binding to UFMylated RPL26.

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