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Making FAT10 with a Reactive C-terminus for E3 Ligase Screening

Jinjing Cao and Gunter Schmidtke*

Abstract

Background: FAT10, a ubiquitin-like modifier, targets proteins to the 26S proteasome for degradation, similar and in addition to ubiquitin. However, FAT10 utilizes its own enzyme cascade, including E1 (UBA6), E2 (USE1), and various E3 ligases. To date, Parkin is the only identified E3 ligase for FAT10, but about 800 different E3-enzymes were reported for ubiquitin. In this study, we aimed to generate a branched FAT10-USE1 conjugate with a reactive C-terminus for the purpose of screening potential E3 ligases. Our method is simple, cost effective and does not require expensive lab equipment. We were able to avoid organic solvents and extreme pH or salt conditions.

Methods: The proteins were expressed at a low temperature for 7–8 hours and purified using affinity resin. Conjugation was achieved through a radical thiol–yne coupling reaction. The resulting products were analyzed by Coomassie Brilliant Blue staining and Western blotting.

Results: FAT10-SH was successfully purified using a Strep-Tactin column and chitin resin, while USE1-GFP was purified via Ni-affinity chromatography. Notably, FAT10-alkene-USE1-GFP conjugates were successfully generated using IA-alkyne, but not STP-alkyne.

Conclusion: In this study, we developed novel FAT10-based probes suitable for E3 ligase screening, which may facilitate future investigations into FAT10 biology. The method can be easily adapted to other ubiquitin like modifiers.

Keywords: FAT10; USE1-GFP; Ni-affinity chromatography; Strep-Tactin; thiol–yne coupling reaction; IA-alkyne; E3 ligase screening

Abbreviation: FAT10- Human leukocyte antigen-F adjacent transcript 10; TNF- tumor necrosis factor; IFN-γ- interferon-gamma; ABPs- activity-based probes; TYC- thiol-yne coupling

Introduction

Human leukocyte antigen-F adjacent transcript 10 (FAT10) is a member of the ubiquitin-like modifier family and is encoded within the MHC class I locus [1]. It contains two ubiquitin-like domains—an N-terminal domain and a C-terminal domain—jointed by a short linker (KPSDE) [2]. FAT10 is predominantly expressed in the immune system and is strongly upregulated by tumor necrosis factor (TNF) and interferon-gamma (IFN-γ) [3,4]. FAT10 was reported to play a role in certain carcinomas and proposed as a tumor marker [5-10]. FAT10 utilizes its own enzyme cascade to directly target proteins, including itself, for degradation by the 26S proteasome [11]—a process known as FAT10ylation. Similar to the ubiquitination pathway, FAT10ylation involves three key enzymes: an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase. UBA6 has been identified as the E1

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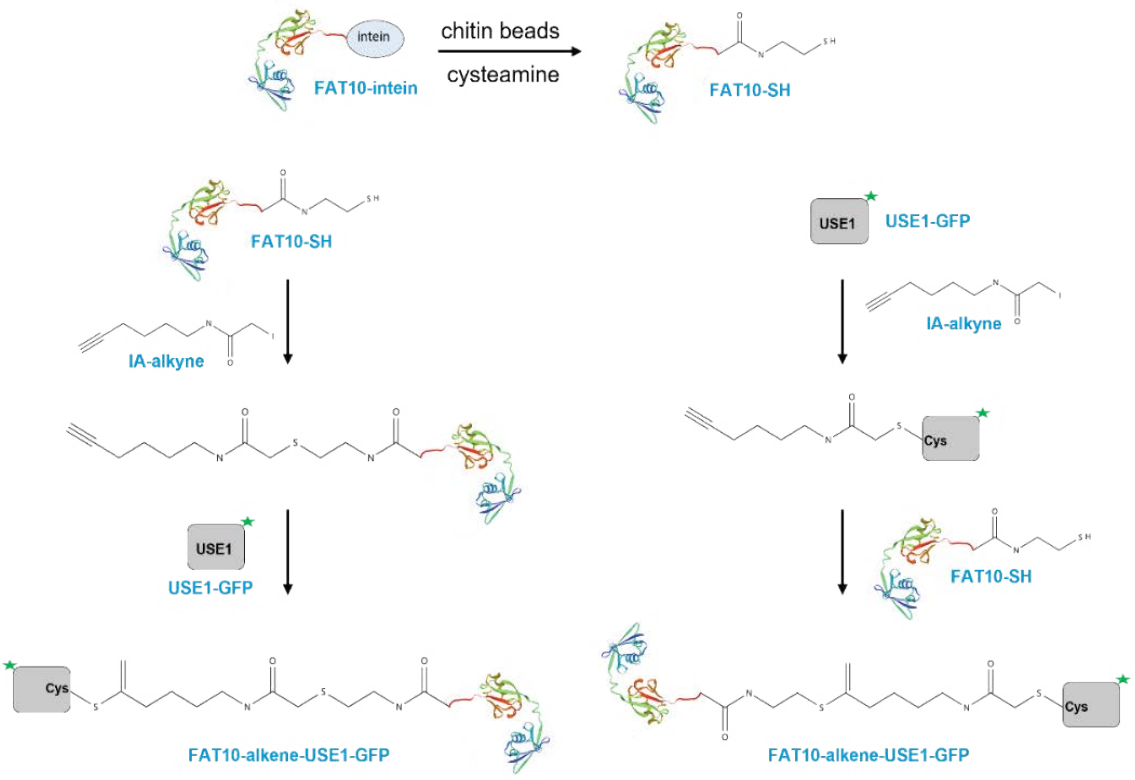

activating enzyme [12,13] and USE1 as the E2 conjugating enzyme [14]. Both are bispecific, functioning in both the ubiquitin and FAT10 pathways. First, FAT10 is activated by an E1 activating enzyme, forming a thioester bond between the C-terminal glycine of FAT10 and the catalytic cysteine in the adenylation domain of E1 via a covalent interaction. Subsequently, FAT10 is transferred to an E2 conjugating enzyme, forming a thioester bond between its C-terminal domain and the active-site cysteine of E2. Finally, with the assistance of an E3 ligase, an isopeptide bond is formed between the C-terminal glycine of FAT10 and a lysine residue on the substrate protein [2]. The E3 ligase, which plays an essential and non-redundant role in substrate recognition and transfer, remains less well characterized in the context of FAT10. Thus far, Parkin has been identified as an E3 ligase for FAT10 [15]. For ubiquitin, for instance, about 800 different E3 enzymes were identified [16,17]. Therefore, it is likely that other E3 ligases for FAT10 exist, the identification of which would help to understand the function of FAT10 in cancer.

To date, numerous studies have reported the mechanisms of ubiquitination[18-23]. Additionally, activity-based probes (ABPs) have been developed to investigate the enzymatic activity of E3 [24-26] or DUB [27]. Several approaches were used to identify components of the ubiquitin conjugation and de-conjugation pathway. A method using intein chemistry for such analysis was reported by Wilkinson et al. [28]. Several such reports were published which modified the C-terminus of ubiquitin to enable reactions with the active sites of cascade enzymes. However the methods described sometimes required HPLC purification of intermediates or final products [24,28], used low pH [29], used organic solvents [30], or used cupper click reactions [31], all of those conditions will lead to denaturation of FAT10 and loss of function. Some of the published reactions required the synthesis of components [31-33] or used expensive methods for analysis [30,32-34]. We would like to avoid all of the mentioned conditions and components. Some reports made advantage of the thiol–yne coupling reaction [32-35] as the enzymes involved have a thiol in the active center. Because the product of the thiol–yne coupling reaction can react with thiols to form a stable bisthioether bond [31,32]. This is a promising approach if we link an alkyne to FAT10 C-terminus and let it react with the active site cysteine of USE1. If the active site cysteine of an E3 comes close, it can form a stable bisthioether bond. Such an approach was used in [31], but with help of a cupper click reaction. We also employed a reverse strategy by attaching a linker to the active-site cysteine of USE1, enabling the alkyne to react with a thiol at the FAT10 C-terminus, which was generated using intein technology. So we generated two branched FAT10-USE1 conjugates, with the thiol-reactive group in a different position to maximize chances to react with an E3. We also tried to link an alkyne to any of the lysines of USE1 in order to make the branch mimicking an isopeptide bond and trap an isopeptidase, but failed to link FAT10 to it.

Cao J and Schmidtke G., Arch Clin Biomed Res 2025
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Material and Methods

Plasmid

The plasmids for expression of Strep-FAT10-intein and pET28-USE1-GFP have been previously described [14,36].

Induction of protein

Induction of protein was performed as previously described [36]. The bacterial culture was shaken at 180 rpm at 37°C. When the OD600 of the bacteria reached 0.5, 1 mM IPTG (ROTH, 2316.4) was added to induce protein expression. Meanwhile, the bacteria were shaken at 180 rpm at 37°C for 7 to 8 hours. Lastly, the bacteria were harvested by centrifugation at 5000 rpm for 10 minutes and then stored at -20°C.

Extraction of protein

Protein extraction followed the protocol described in [36]. The bacteria were resuspended in 10 mL of buffer. Then, 1 mg/mL lysozyme (Fluka, 62971), 0.1 mg/mL DNase I (Roche, 3724778103), and 1x EDTA-free Protease Inhibitor Cocktail (Roche, 4693132001) were added to the mixture and incubated on ice for 1 hour. Next, the bacteria were sonicated, followed by centrifugation at 18,000 rpm at 4°C for 1 hour. The supernatant was collected and sonicated again. Lastly, the supernatant was filtered using a 0.22 µm filter (TPP, 99722).

Purification of FAT10-SH

Purification of FAT10 was performed as described by Brockmann et al. [36]. The lysate was applied to the Strep-Tactin column (iba, 2-5998-000), following the manufacturer's instructions. Subsequently, the appropriate amount of chitin resin was added to the desalted FAT10-intein and incubated at 4°C with rocking for 5 hours. The supernatant was then discarded. Then, a suitable volume of 50 mM cysteamine (Sigma-Aldrich, 30070) was added to the resin and incubated at 4°C overnight with rocking. Lastly, the supernatant was desalted using a Nap-10 column with PBS, yielding the desired FAT10-SH protein.

Purification of USE1-GFP

The lysate was applied to Ni-NTA agarose (Macherey-Nagel, 745400), following the company manual. The purified USE1-GFP was concentrated using a 30 kDa centrifugal filter and desalted with a Nap-10 (cytiva, 17085401) column, yielding the desired USE1-GFP.

The reaction of compounds

The purified protein (50 µL, 2 mg/mL in PBS) was treated with 1 µM iodoacetamide alkyne (IA-alkyne, MCE, HY-136205) or 1 µM pentynoic acid STP ester (STP-alkyne, lumiprobe, 40720) and incubated at room temperature for 1 hour. The reaction was quenched by desalting via a NAP-5 column according to instruction by the manufacterer. Next, FAT10-alkyne or USE1-GFP-alkyne was reacted with USE1-GFP or FAT10 via a thiol-yne coupling (TYC) reaction under 365 nm UV light irradiation in the presence of 1,5 mM reduced glutathione (GSH, Sigma-Aldrich, G4251) and 5 mM VA-044 (FUJIFILM, 225-02111) for 4 hours. The resulting product was first purified using 50µl Ni-IDA resin (Macherey-Nagel, 745210), followed by further purification using a 100 µl Strep-Tactin column. The eluted fraction were collected and analyzed by Western blotting. The information about the materials can be found in Table S1.

Coomassie Brilliant Blue staining and silver staining

The SDS-PAGE gels were soaked in Coomassie Brilliant Blue (Abcam, ab119211, UK) for 1 to 2 hours. Afterwards, the SDS-PAGE gels were submerged in ultrapure water for at least 1 hour or overnight. We used the silver staining kit (Thermo Scientific, 24612) according to the manufacturer's instructions.

Western blotting

The sample was boiled in SDS sample buffer. SDS-PAGE gel electrophoresis was then performed, followed by transferring the SDS-PAGE gel onto a nitrocellulose membrane using the Trans-Blot Turbo Transfer System at 25 V for 10 minutes. The membrane was blocked with Intercept (TBS) Blocking Buffer (LICOR, 927-60001) at room temperature for 1 hour, then incubated with the primary antibody at 4 °C overnight. Afterward, the membrane was washed with TBS-T for 5 minutes, repeating this step three times. Next, the membrane was incubated with the secondary antibody at room temperature for 1 hour, followed by another round of washing with TBS-T for 5 minutes, repeated three times. Finally, signal detection was performed using an ODYSSEY XF processor (LICOR, model 2800).

Results

Expression of FAT10-intein and USE1-GFP proteins

In this study, we aim to generate the FAT10-USE1 conjugate. First, the expression of FAT10 and USE1-GFP proteins was carried out. The FAT10 and USE1-GFP plasmids were separately transferred into B834 competent cells. After 7–8 hours of induction with IPTG, the expression of FAT10 (Figure 1A) and USE1-GFP (Figure 1B) was analyzed using Coomassie Brilliant Blue staining and Western blotting.

Purification of FAT10-SH protein

The lysate of FAT10-intein-chitin binding was applied to a 1 mL Strep-tag column, following the manufacturer's manual. The protein was eluted with buffer BXT. The results showed that FAT10-intein was successfully purified. Afterward, chitin resin was added to the purified FAT10-

Cao J and Schmidtke G., Arch Clin Biomed Res 2025
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intein protein. FAT10-SH was removed from the chitin resin using 50 mM cysteamine. The results showed that FAT10-SH was successfully separated from the chitin resin (Figure 2).

Purification of USE1-GFP protein

The lysate of USE1-GFP was applied to Ni-NTA agarose, following the manufacturer's manual. Additionally, we purified USE1. As shown in Figure 3, the purification of USE1-GFP was more efficient than that of USE1 alone. GFP not only serves as a reporter gene but also facilitates protein purification.

The generation of FAT10 and USE1 conjugates

Conjugation was carried out after all proteins had been prepared. Initially, FAT10 or USE1-GFP were incubated with IA-alkyne or STP-alkyne at room temperature for 1 hour, after which the reaction was quenched by by passing the proteins through a NAP-5 column. Subsequently, a

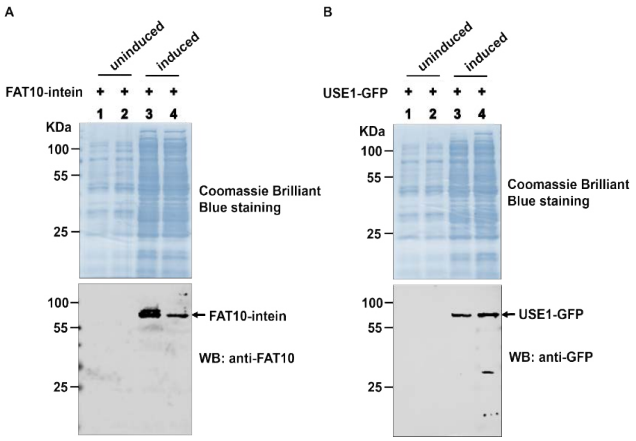
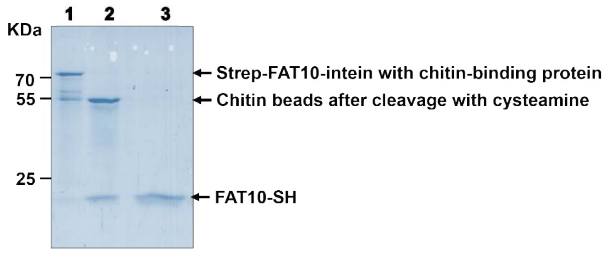


Figure 1: Expression of FAT10-intein and USE1-GFP proteins. (**A**) FAT10-intein expression was induced with 1 mM IPTG for 7–8 hours and analyzed by Western blotting. (**B**) USE1-GFP expression was induced with 1 mM IPTG for 7–8 hours and analyzed by Western blotting.



1. Strep-FAT10-intein chitin-binding protein bound to chitin beads before the addition of cysteamine
2. Chitin beads after cleavage of the supernatant
3. Supernatant cleavage with cysteamine removed from chitin beads, containing FAT10-SH

Figure 2: Purification of FAT10-SH protein. FAT10-SH was eluted from chitin beads with 50 mM cysteamine and analyzed by Coomassie Brilliant Blue staining.

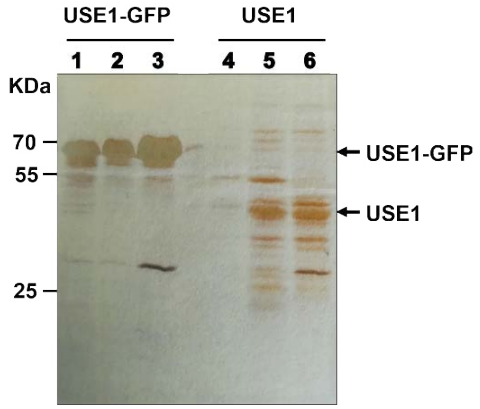


Figure 3: Purification of USE1-GFP and USE1 protein. Shown are three subsequent elution fractions from Ni-NTA agarose, each 1.5 times the volume of the batch. USE1-GFP and USE1 was analyzed by silver staining.

radical TYC reaction was performed by irradiating a mixture of either FAT10-alkyne or USE1-GFP-alkyne with USE1-GFP or FAT10, VA-044, and GSH under 365 nm UV light for 4 hours. The product was first purified using Ni-IDA resin, removing all smal components and proteins without Hexa-His tag, e.g. unreacted FAT10. Final purification was achieved using a Strep-Tactin column. All componets without the strep tag, e.g. USE1-GFP which did not react with FAT10 were removed.

As all proteins bound on the column must have a Strep tag and a Hexa-His tag, which is only the case for Strep-FAT10-Hexa-His-USE1-GFP conjugates. Flow-through and eluted fractions from the Strep-Tactin column were collected and analyzed by western blotting. As shown in Figure 4B, the GFP Western blot revealed a difference of approximately 15 kDa in molecular mass between the flow-through of the Strep-Tactin column and the eluate, which is consistent with the expected size increase resulting from the addition of FAT10 to USE1-GFP. The USE1-GFP is, expectetly, in the low through, whereas the FAT10-USE1-GFP is in the eluate. The higher molecular band in lane 2 and 4 reacted positive with an anti-FAT10 antibody, no reaction was observed in the other lanes. Therefore, FAT10-alkene-USE1-GFP conjugates were detected in reactions involving either FAT10-IA-alkyne + USE1-GFP or USE1-GFP-IA-alkyne + FAT10-SH, but not in the USE1-GFP-STP-alkyne + FAT10-SH condition. These findings indicate that FAT10 binds to the catalytic center of USE1-GFP with considerable affinity, in a manner consistent with its native degradation pathway. Additionally, when any lysine of USE1-GFP is modified with an alkyne group, it does not react with FAT10-SH. This is probably because the affinity of FAT10 to the active center of USE1 is high, whereas FAT10 has no affinity to the surface of USE1 to react with exposed lysines, at least not under the consentration applied and the condition tested (Figure 4).

Cao J and Schmidtke G., Arch Clin Biomed Res 2025
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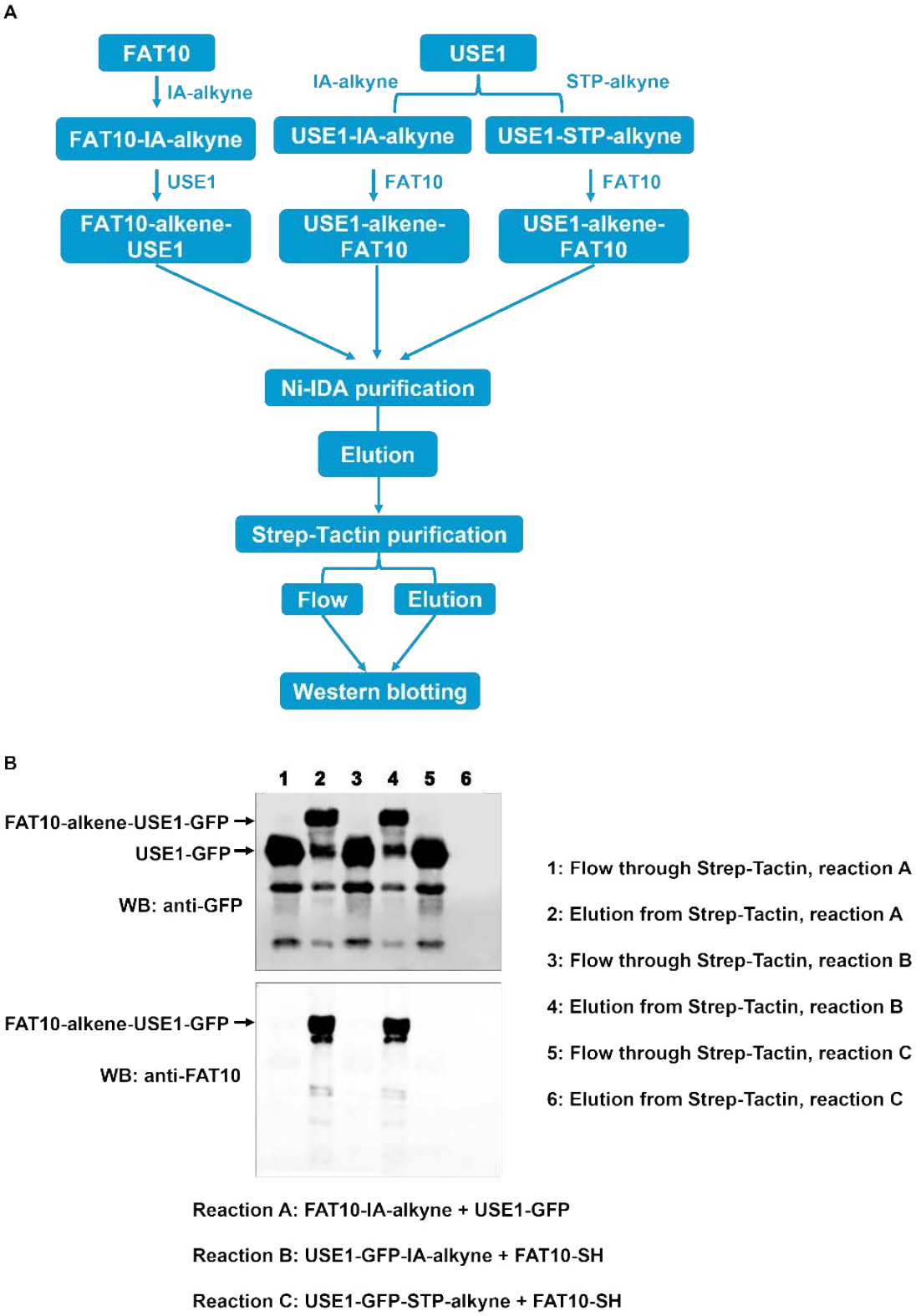

Figure 4: Formation of the FAT10-alkene-USE1-GFP conjugate. The conjugate was purified using Ni-IDA resin, followed by purification with a Strep-Tactin column. (**A**) Workflow for the generation of the conjugates. (**B**) Lanes 1, 3, and 5 correspond to the flow-through collection after loading of the Strep-Tactin column, while lanes 2, 4, and 6 represent the eluted fractions containing the purified protein. FAT10-alkene-USE1-GFP was analyzed by Western blotting.

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Cao J and Schmidtke G., Arch Clin Biomed Res 2025
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Discussion

FAT10, unlike ubiquitin—which is ubiquitously expressed—is restricted to the immune system [, and is upregulated in various cancers [5-9,36]. FAT10ylation is a unique protein degradation pathway—FAT10 employs its own enzymatic cascade [12-14]. In this process, FAT10 is covalently attached to substrate proteins and co-degraded with them by the 26S proteasome [11]. To date, Parkin is the only known E3 ligase mediating FAT10ylation [15]. In this study, we aimed to generate FAT10-USE1-GFP conjugates, as this is more promising as compared to just a C-terminal reactive group on FAT10 only, to facilitate the screening of potential E3 ligases involved in FAT10ylation. Just a modified C-terminus on FAT10 is more likely to react with an E1 and E2, forming stable conjugates with these, and will not be able to react with an E3. This was also the rationale in [31]. The advantages of our conjugates are the simple preparation on the bench top without expensive equipment. The avoidance of any hard condition, the use of cost effective commercially available components, and the easy and fast final purification via two consecutive affinity chromatographies, which leave only the conjugate of FAT10 and USE1-GFP in the eluate, as it is the only one having both affinity tags. We used two strategies for the generation of the conjugates which link the C-terminus of FAT10 to USE1-GFP active site cysteine. We assume it is the active site cysteine without formal confirmation, because it is the most reactive one. Other publication, which did in depth analysis via mass spectrometry [30,31,34,35] always found reaction with the active site cysteine only. The affinity of the UBL-modifier to the active center is much higher than to any other part of the protein, which keeps the reactive groups long enough together to react. One report notes that the length of the linker has an influence on the reaction efficacy [35], whereas another report did not see such a dependency [34]. The low concentration and affinity are probably the reason why we did not see any reaction between the alkyne labeled lysine of USE1-GFP and FAT10-SH, at least not with our conditions, because there is no binding of the surface of USE1-GFP and the C-terminus of FAT10-SH. Rather the active center of USE1-GFP might bind the FAT10-SH C-terminus, preventing it from reacting with the alkyne. We generated two different FAT10-USE1-GFP conjugates, with the difference being the position of the reactive group. It is possible that the position will have an influence on the reaction with a potential E3, so it is better to use different probes. As there is only one known E3 for FAT10, but several hundred for ubiquitin, we are confident that our tool will enable the identification of several E3 enzymes for FAT10, should suitable patient material available. Our method of generation of branched FAT10-E2 conjugates with two different positions of a thiol-reactive group can as well be easily adapted to any other ubiquitin like modifier, and any other enzyme of the conjugation cascade, as well as to isopeptidases, many of them have a cysteine in the active center. This is of great advantage if the budget is limited an access to expensive equipment is impossible.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Cao J and Schmidtke G., Arch Clin Biomed Res 2025
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SUPPLEMENTARY FILES

Table S1. Regents and materials

Reagents or kit	Company	Cat No.
Cysteamine	Sigma-Aldrich	30070
Kanamycin	ROTH	T832.2
Ampicillin sodium salt	ROTH	K029.1
Tryptone/Peptone ex casein	ROTH	8952.2
TWEEN® 20	Sigma-Aldrich	P7949
Triton™ X-100	Sigma-Aldrich	X100
Trizma® base	Sigma-Aldrich	T1503
Glycine	ROTH	3908.2
Sodium chloride	ROTH	3957.2
Yeast Extract	ROTH	2363.4
InstantBlue® Coomassie Protein Stain (ISB1L)	Abcam	ab119211
β-mercaptoethanol	ROTH	4227.3
Acrylamide/Bis Solution, 37.5:1	SERVA	10688.01
Strep-Tactin®XT 4Flow® high capacity resin	iba	2-5030-002
Strep-Tactin®XT 4Flow® Starter Kit	iba	2-5998-000
Pentinsäure-STP-Ester	lumiprobe	40720
IA-Alkyne	MCE	HY-136205
Chitin Resin	NEB	S6651S
Anti-GFP	Roche	11814460001
Anti-His Tag Antibody	Sigma-Aldrich	05-531
IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody	LICORbio	926-32210
IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody	LICORbio	926-68070
Amicon® Ultra Centrifugal Filter, 3 kDa MWCO	Millipore	UFC500308
Amicon® Ultra Centrifugal Filter, 30 kDa MWCO	Millipore	UFC903024
Amicon® Ultra Centrifugal Filter, 30 kDa MWCO	Millipore	UFC803024
Pierce™ BCA Protein Assay Kits	Thermo Scientific™	23225
1,4-Dithioerythritol	Sigma-Aldrich	D8255
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche	4693132001
Intercept® (TBS) Blocking Buffer	LICOR	927-60001
IPTG	ROTH	2316.4
Lysozyme	Fluka	62971
DNase I	Roche	3724778103
syringe filter 22	TPP	99722
PETRI DISH, PS, 94/16 MM, WITHOUT VENTS	greiner	632180
Screw cap tube, 15 ml, (LxØ): 120 x 17 mm, PP, with print	SARSTEDT	62.554.502
Screw cap tube, 50 ml, (LxØ): 114 x 28 mm, PP, with print	SARSTEDT	62.547.254
Agar	Millipore	1016141000
NAP-5 Columns Sephadex G-25 DNA Grade	cytiva	17085302
NAP-10 Columns Sephadex G-25 DNA Grade	cytiva	17085401
2-Propanol ≥99.7%, AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical reagent	VWR Chemicals	20842.312
Trans-Blot Turbo 5x Transfer Buffer	BIO-RAD	10026938
Magnesium chloride hexahydrate	ROTH	2189.1
Ethylenediamine tetraacetic acid disodium salt dihydrate	ROTH	8043.2
Ethanol	ROTH	9065.3
VA-044	FUJIFILM	225-02111

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DOI:10.26502/acbr.50170459

L-Glutathione reduced (GSH)	Sigma-Aldrich	G4251
Protino Ni-IDA Resin für His-Tag-Proteinreinigung	Macherey-Nagel	745210
Protino Ni-NTA Agarose für His-Tag-Proteinreinigung	Macherey-Nagel	745400
Pierce™ Silver Stain Kit	Thermo Scientific	24612
All applications are based on the manufacturing processes of the company.		