Erschienen in: FEBS Journal ; 281 (2014), 7. - S. 1848-1859
https://dx.doi.org/10.1111/febs.12745

Investigations into the auto-FAT10ylation of the bispecific E2 conjugating enzyme UBA6-specific E2 enzyme 1

Annette Aichem[1], Nicola Catone[1] and Marcus Groettrup[1,2]

1 Biotechnology Institute Thurgau at the University of Konstanz, Kreuzlingen, Switzerland
2 Division of Immunology, Department of Biology, University of Konstanz, Germany

The cytokine-inducible ubiquitin-like modifier HLA-F adjacent transcript 10 (FAT10) targets its substrates for degradation by the proteasome. FAT10 is conjugated to its substrates via the bispecific, ubiquitin-activating and FAT10-activating enzyme UBA6, the likewise bispecific conjugating enzyme UBA6-specific E2 enzyme 1 (USE1), and possibly E3 ligases. By MS analysis, we found that USE1 undergoes self-FAT10ylation *in cis*, mainly at Lys323. Mutation of Lys323 to an arginine did not abolish auto-FAT10ylation of USE1, but every other lysine could instead be modified with FAT10. Similarly to bulk FAT10 substrates, FAT10ylation of USE1 accelerated its proteasomal degradation. Interestingly, the USE1–FAT10 conjugate continued to be an active E2 enzyme, because both FAT10 and ubiquitin could still be thioester-linked to the USE1–FAT10 conjugate. We therefore suggest that the major function of USE1 auto-FAT10ylation is to serve as a negative feedback mechanism to limit the conjugation of FAT10 upon its cytokine-mediated induction by reducing the amount of USE1 through proteasomal degradation of the USE1–FAT10 conjugate.

Introduction

Proteins that are modified with ubiquitin chains are mostly targeted for degradation by the 26S proteasome [1]. Apart from ubiquitin, several ubiquitin-like modifiers (ULMs), such as HLA-F adjacent transcript 10 (FAT10), NEDD8, small-ubiquitin-like modifier (SUMO)-1/2/3, ISG15; ATG8, ATG12, URM-1, and UFM-1, have been identified [2]. The ubiquitin-like modifier FAT10 was described as a transferable tag for proteasomal degradation that directly targets proteins for degradation by the proteasome [3–5]. FAT10 is an interferon (IFN)-γ- and tumour necrosis factor (TNF)-α-inducible protein that is encoded in the major histocompatibility complex class I locus [6,7]. FAT10 is constitutively expressed in mature dendritic cells, and the basal level of expression is highest in organs of the immune system [8–10]. FAT10 seems to play a role in several different cellular processes, such as MHC class I restricted antigen presentation [11,12], nuclear factor-κB-mediated signal transduction [13], cell cycle control, and chromosomal segregation [14]. Recently, we have shown that endogenous FAT10 becomes attached to hundreds of proteins with functions in numerous cell biological pathways [15].

Abbreviations

CHX, cycloheximide; ECL, enhanced chemiluminescence; FAT10, HLA-F adjacent transcript 10; HA, haemagglutinin; HRP, horseradish peroxidase; IFN, interferon; SUMO, small-ubiquitin-like modifier; TNF, tumour necrosis factor; ULM, ubiquitin-like modifier; USE1, UBA6-specific E2 enzyme 1.

The activation and conjugation of FAT10 is conducted by an enzyme cascade, as is known for other ULMs. In general, each subfamily of modifiers uses its private set of E1, E2 and E3 enzymes to ensure the specificity of the conjugation pathway [16,17]. In the case of FAT10, this concept seems to be violated, because FAT10 is activated by the E1 enzyme UBA6, which was first identified as the second E1 enzyme for ubiquitin before it was shown that it also activates FAT10 [18–21]. Interestingly, UBA6 binds FAT10 with higher affinity than ubiquitin, but the adenylation and transthiolation reaction is slower for FAT10 than for ubiquitin [22]. Similarly, the FAT10 and ubiquitin bispecific conjugating enzyme UBA6-specific E2 enzyme 1 (USE1) was first reported to accept activated ubiquitin exclusively from UBA6, and not from UBE1 [19,23]. USE1 was subsequently shown to interact with FAT10, and silencing of USE1 greatly reduced the formation of bulk FAT10 conjugates. Interestingly, we showed that USE1 undergoes self-FAT10ylation *in cis*, which means that the same USE1 transfers the thioester-bound FAT10 from its active site cysteine onto a lysine of itself and not of another USE1. Once the isopeptide-linked USE1–FAT10 conjugate has been formed, it resists degradation by 10% 2-mercaptoethanol or 4 M urea [21]. The consequences of USE1 auto-FAT10ylation have not been investigated so far. We show in this study that auto-FAT10ylation of USE1 leads to its accelerated degradation by the proteasome. Interestingly, auto-FAT10ylation of USE1 does not produce an inactive E2 enzyme, because we show that both ubiquitin and FAT10 can still be thioester-linked to the USE1–FAT10 conjugate. Auto-FAT10ylation of USE1 therefore seems not to have an influence on the ability of USE1 to discriminate between FAT10 and ubiquitin, but represents a negative feedback regulation of the FAT10 conjugation pathway by reducing the amount of USE1 by FAT10-mediated proteasomal degradation.

Results

The bulk of endogenously FAT10ylated protein is rapidly degraded by the proteasome

Covalent modification of proteins with the ubiquitin-like modifier FAT10 strongly shortened the half-life of artificial substrates such as FAT10–green fluorescent protein and FAT10–dihydrofolate reductase [3]. To investigate the proteasomal degradation of endogenous FAT10-modified proteins, HEK293 cells were treated for 24 h with the proinflammatory cytokines IFN-γ and TNF-α to induce FAT10 expression, and endogenous FAT10 and FAT10 conjugates were immunoprecipitated. To monitor the degradation of FAT10ylated proteins under endogenous conditions, cells were treated for 1, 3 or 5 h with the translation inhibitor cycloheximide (CHX). As shown in Fig. 1A,B, both monomeric FAT10 and the conjugates were rapidly degraded within 3 h. Degradation of monomeric FAT10 and FAT10 conjugates was inhibited by the proteasome inhibitor MG132, confirming that FAT10ylation is also a general signal for proteasomal degradation [3,24,25] under endogenous conditions.

USE1–FAT10 is guided to proteasomal degradation

As FAT10ylation of p62/SQSTM1 and UBE1 leads to their degradation by the proteasome [15, 28], we investigated whether auto-FAT10ylation of USE1 promotes its degradation by the proteasome. Treatment of HEK293 cells for up to 6 h with the proteasome inhibitor MG132 led to the accumulation of the endogenous USE1–FAT10 conjugate (Fig. S1). In CHX chase experiments, monomeric FAT10 was degraded rapidly, whereas the USE1–FAT10 conjugate was degraded more slowly, but was also rescued by MG132 (Fig. 2A), strongly suggesting that FAT10ylation of USE1 leads to its proteasomal degradation. Approximately 30–40% of the USE1–FAT10 conjugate was degraded during 5 h of CHX chase (Fig. 2C, left graph). In contrast, the degradation of unconjugated USE1 in the presence (Fig. 2C, middle graph) or absence (Fig. 2B, and right graph in Fig. 2C) of FAT10 induction was negligible. Even when cells were stimulated with IFN-γ and TNF-α for up to 72 h to strongly upregulate FAT10 expression and formation of USE1–FAT10 conjugates (Fig. S2), or when the cells were treated with MG132 (Fig. S1), changes in the steady-state quantities of unconjugated USE1 were not observed, most probably because of the relatively small amount of the USE1–FAT10 conjugate as compared with the large amount of unmodified endogenous USE1 in the cells.

USE1 is mainly FAT10ylated at Lys323

The amino acid sequence of USE1 contains 11 lysines. To identify the lysine or lysines that become modified by USE1 auto-FAT10ylation, His–3xFLAG–USE1–hemagglutinin (HA)–FAT10 conjugate was immunoprecipitated from transiently transfected HEK293 cells and analysed by MS. As a tryptic digest of FAT10 results in peptides modified with a 13-residue C-terminal FAT10 fragment that are difficult to assign with

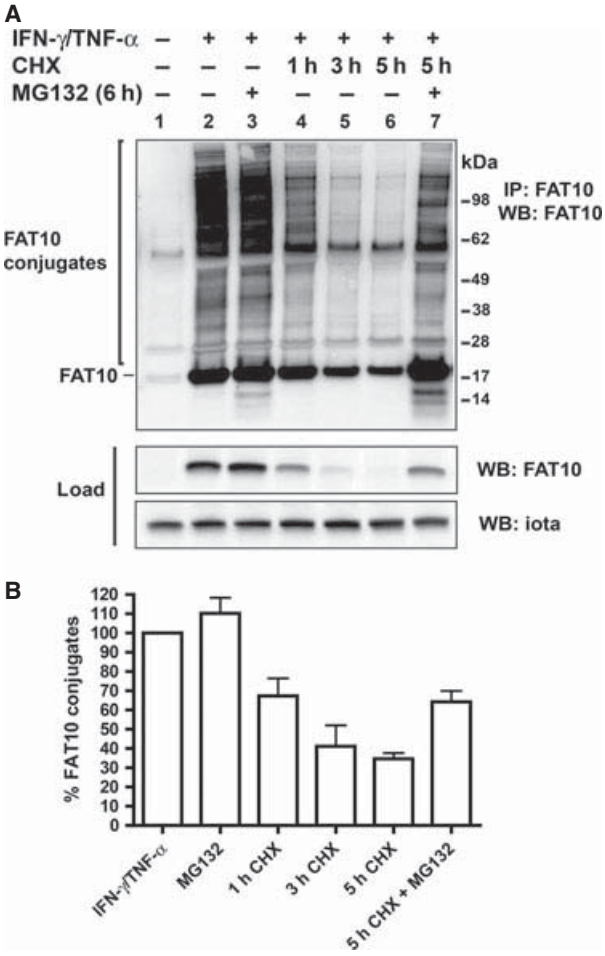

Fig. 1. Proteasomal degradation of endogenous FAT10 conjugates. (A) Total cell extracts of untreated or IFN-γ/TNF-α-stimulated HEK293 cells were used to immunoprecipitate endogenous FAT10 and its conjugates with a mAb against FAT10 (4F1), and this was followed by western blot (WB) analysis with a polyclonal antibody against FAT10. Cells were additionally treated for the indicated times with CHX and/or the proteasome inhibitor MG132. Proteins were separated under reducing conditions (10% 2-mercaptoethanol) on 4–12% Bis/Tris NuPAGE gels. The upper western blot panel shows the immunoprecipitated FAT10 conjugates; the lower panels show the expression level of endogenous FAT10 in the cell lysates. Iota was used as a loading control. One representative experiment of three experiments with similar outcomes is shown. (B) Quantification of endogenous FAT10 conjugates. The amount of conjugates in IFN-γ-treated and TNF-α-treated cells (lane 2) was set to unity. ECL signals between the bracket in (A) delineating the formed FAT10 conjugates were calculated and normalized to the respective iota loading control. The graph shows the mean of three independent experiments with similar outcomes.

conventional algorithms, the raw data were analysed with a program called CHOPNSPICE, which was originally developed for the identification of peptides modified with SUMOs [26]. This analysis revealed that Lys323 is the main FAT10ylated lysine within USE1. To validate this result, Lys323 was mutated to arginine, and USE1-K323R was tested for stable conjugate formation with FLAG–FAT10 in HEK293 cells. In transfected HEK293 cells expressing HA–USE1-K323R and FLAG–FAT10, the HA–USE1-K323R–FLAG–FAT10 conjugate was still detectable, but the amount was much smaller than that of the wild-type HA–USE1–FLAG–FAT10 conjugate (Fig. 3A, lanes 1 and 3). Inhibition of the proteasome resulted in accumulation of both FAT10 conjugates of wild-type and mutant USE1 (Fig. 3A, lanes 2 and 4). This result indicates that Lys323 is the major lysine that becomes FAT10ylated, but that, upon substitution of this by an arginine, USE1 auto-FAT10ylation can switch at least partially to another lysine within USE1. To further investigate this issue, each single lysine of USE1 was mutated to arginine, and the resulting HA-tagged USE1 variants were transiently expressed together with FLAG–FAT10. As shown in Fig. 3B, each mutant was still able to form a stable conjugate with FAT10. To compare the amounts of the mutant USE1–FAT10 conjugates, the enhanced chemiluminescence (ECL) signals of the USE1–FAT10 conjugates were quantified and normalized to the respective USE1 variants in the lysate (load) (Fig. 3B,C). Statistical analysis revealed significantly lower amounts of USE1-K323R–FAT10 ($P = 0.0156$), whereas all other mutant USE1–FAT10 conjugates did not significantly differ in quantity from wild-type USE1–FAT10. Taken together, our mutagenesis experiments confirmed the results from MS that USE1 is mainly FAT10ylated on Lys323.

Auto-FAT10ylation does not inactivate USE1

Modification of enzymes with ULMs can result in changes in their function. In the case of the bispecific enzyme USE1, we investigated, whether auto-FAT10ylation has an influence on the general activity of USE1 with respect to its preference for one of the two modifiers. Therefore, we investigated whether the free active site cysteine of the USE1–FAT10 conjugate can still be loaded with activated ubiquitin or FAT10. To this end, we performed *in vitro* experiments with purified HA–USE1–FLAG–FAT10 conjugate, recombinant FAT10, ubiquitin, and FLAG–UBA6. To obtain sufficient quantities of the HA–USE1–FLAG–FAT10 conjugate, HEK293 cells were transiently transfected with expression plasmids for HA–USE1 with or without a His–3xFLAG–FAT10 plasmid (FLAG–FAT10). Unconjugated HA–USE1 was immunopurified with antibody against HA coupled to

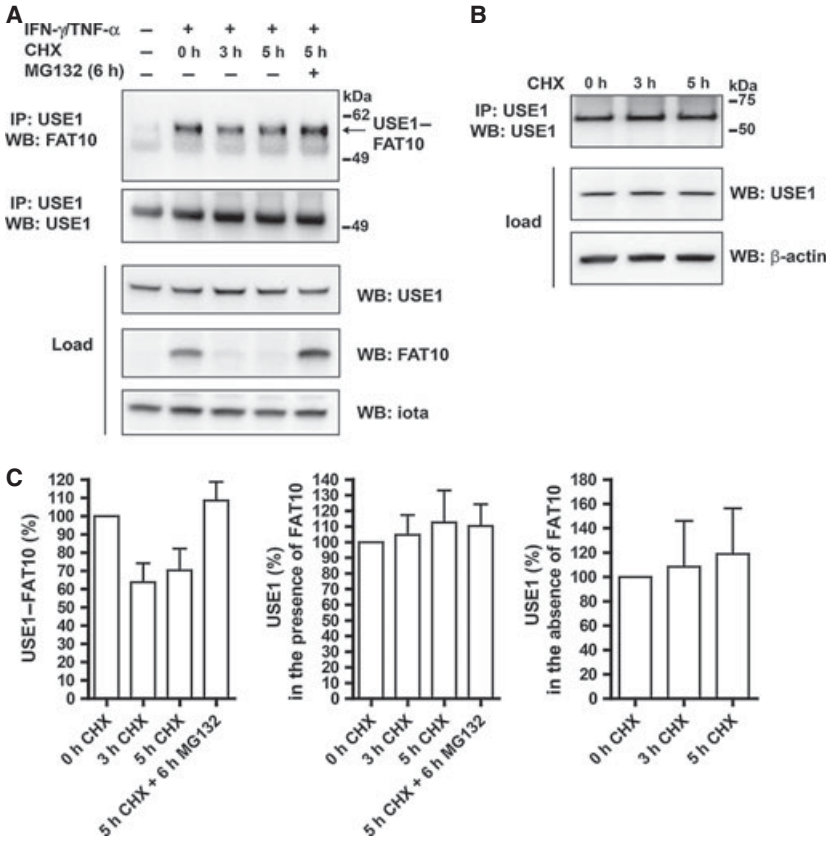

Fig. 2. Proteasomal degradation of the endogenous USE1–FAT10 conjugate. (A) HEK293 cells were treated for 24 h with IFN-γ and TNF-α to induce endogenous FAT10 expression. Prior to harvesting, cells were treated for the given time periods with CHX and the proteasome inhibitor MG132, as indicated. Total cell extracts were used to immunoprecipitate endogenous USE1–FAT10 conjugate with a rabbit mAb against USE1 (31-10). Proteins were separated under reducing conditions (10% 2-mercaptoethanol) on 4–12% Bis/Tris NuPAGE gels, and visualized with either a mAb against FAT10 (4F1) or a polyclonal antibody against USE1. The proteasome subunit iota (α1) was used as a loading control. (B) HEK293 cells were treated for 0, 3 or 5 h with CHX, and lysates were subjected to immunoprecipitation (IP) with a mAb against USE1, as described in (A). Western blot (WB) analysis was performed with a polyclonal antibody against USE1 and an antibody against β-actin as a loading control. One of five independent experiments with similar outcomes is shown. (C) ECL signals of the immunoprecipitated USE1–FAT10 conjugate (left graph), of unconjugated USE1 (middle graph), as shown in (A), or of USE1 in untreated cells (right graph), as shown in (B). ECL signals of five independent experiments were quantified and normalized to the loading control, and the amount of the respective protein in IFN-γ-treated and TNF-α-treated cells in the absence of CHX treatment (0 h CHX) was set to unity.

sepharose, whereas the HA–USE1–FLAG–FAT10 conjugate was purified with anti-FLAG affinity matrix. After immunoprecipitation, beads were washed, and the proteins bound on the beads were incubated with the indicated recombinant proteins (Fig. 4). Western blot analysis was performed under nonreducing and reducing (10% 2-mercaptoethanol) conditions, to monitor thioester bond formation. As a positive control, transfer of recombinant FAT10 or ubiquitin onto HA–USE1 was performed (Fig. 4A,B, lanes 1–5). Recombinant FAT10 was loaded in a UBA6-dependent manner onto HA–USE1 (Fig. 4A, lanes 2 and 3; Fig. S3A, lanes 2 and 6), and formed a stable, nonreducible conjugate with HA–USE1, although with low efficiency. Ubiquitin could also be transferred from UBA6 onto HA–USE1 (Fig. 4A,B, lanes 4 and 5), but, in this case, a thioester bond was formed, as it was reducible, confirming previous results [21]. As can be seen (Fig. 4A,B, lanes 1–5), HA–USE1 was already loaded with a slight amount of endogenous ubiquitin upon purification from HEK293 cells, but the amount of ubiquitin-loaded HA–USE1 was strongly enhanced in the presence of recombinant ubiquitin and FLAG–UBA6 (Fig. 4A, top panel, lane 4), showing that endogenous ubiquitin did not impair the *in vitro* reaction. Interestingly, USE1 auto-FAT10ylation did not inactivate USE1 regarding ubiquitin loading, because ubiquitin was also transferred in a UBA6-dependent manner onto the HA–USE1–FLAG–FAT10 conjugate (Fig. 4A, lanes 9 and 10), and also, in this case,

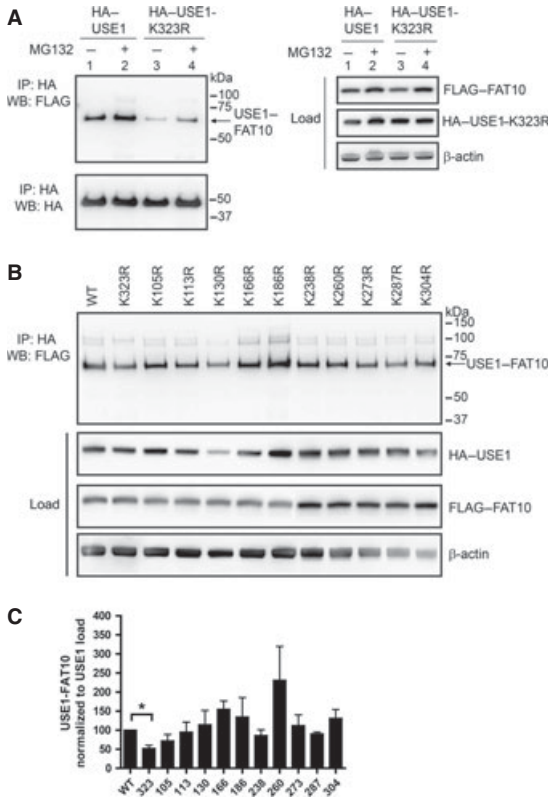

Fig. 3. Lys323 is the main FAT10ylation target in USE1. (A) HEK293 cells were transiently transfected with expression plasmids for His–3xFLAG–FAT10 (FLAG–FAT10) and pcDNA3.1–HA–USE1 (HA–USE1) or the lysine-to-arginine mutant pcDNA3.1–HA-USE1-K323R (HA–USE1-K323R). Before harvesting, cells were treated for 6 h with 10 μM MG132, where indicated. Lysates were subjected to immunoprecipitation (IP) with anti-HA–agarose. Proteins were separated under reducing conditions (10% 2-mercaptoethanol) on 4–12% Bis/Tris NuPAGe gels, and formation of the USE1–FAT10 conjugate was investigated by western blot (WB) analysis with peroxidase-labeled antibodies against HA and FLAG. The left panels show the immunoprecipitated proteins, and the right panels show the protein expression levels in the lysate (load). β-Actin was used as a loading control. One representative experiment of three with similar outcomes is shown. (B) HEK293 cells transiently expressing FLAG–FAT10 and HA–USE1 or a mutant form of HA–USE1 in which one of 11 lysines of USE1 was mutated to arginine (pcDNA3.1–HA–USE1-K*R). Immunoprecipitation and western blot analysis were performed as described in (A). WT, wild-type HA–USE1. (C) USE1–FAT10 conjugate amounts in three independent experiments as shown in (B), from cells expressing either wild-type HA–USE1 or one of the lysine-to-arginine mutants together with FLAG–FAT10. The ECL signals of three independent experiments were quantified and normalized to the respective USE1 amount in the load. The amount of wild-type USE1–FAT10 conjugate was set to unity, and the amounts of the mutant USE1–FAT10 conjugates were calculated according to this value. The mean of three independent experiments was calculated ± standard deviation. Statistical analysis was performed on raw data before normalization to the USE1 load. The asterisk indicates a significant *P*-value of 0.0156.

ubiquitin formed a reducible thioester bond with the HA–USE1–FLAG–FAT10 conjugate (Fig. 4A, lowest panel, reducing conditions, lane 9). In addition, a linear diubiquitin was also transferable onto both proteins, HA–USE1 and HA–USE1–FLAG–FAT10 (Fig. S3A,B, lanes 4), further supporting the finding that auto-FAT10ylation did not inactivate USE1 with respect to ubiquitin transfer.

As the *in vitro* transfer of recombinant FAT10 onto HA–USE1 was not as efficient as transfer of ubiquitin (Fig. 4A, lanes 2 and 4) or even diubiquitin (Fig. S3A, B, lanes 2 and 4), and as the amount of purified HA–USE1–FLAG–FAT10 conjugate was lower than that of HA–USE1, we looked in more detail at longer exposure times to determine whether FAT10 could also be loaded onto the HA–USE1–FLAG–FAT10 conjugate (Fig. 4A, nonreducing conditions, middle panels). By looking at increasing exposure times of the same western blot under nonreducing conditions, we could indeed identify, at a size of ~ 80 kDa, a conjugate representing the HA–USE1–FLAG–FAT10 conjugate loaded with one additional FAT10 molecule (Fig. 4A, asterisk, lane 7). To validate this finding, we performed an additional experiment with transiently transfected HEK293 cells, expressing 6His–USE1 and His–3xFLAG–FAT10 (FLAG–FAT10) in combination with either HA–FAT10, its diglycine mutant HA–FAT10ΔGG, which cannot be conjugated to a lysine, or HA–ubiquitin (Fig. 5). The 6His–USE1–FLAG–FAT10 conjugate was immunoprecipitated with anti-FLAG–sepharose, and the additional HA–FAT10 molecule bound to the 6His–USE1–FLAG–FAT10 conjugate was detected under nonreducing and reducing (10% 2-mercaptoethanol) conditions by western blot analysis with an HA-specific antibody (Fig. 5, top panels). Under nonreducing conditions, both the 6His–USE1–FLAG–FAT10–HA–FAT10 conjugate and the 6His–USE1–FLAG–FAT10–HA–ubiquitin conjugate were clearly detectable (Fig. 5, left panel, lanes 3 and 5, respectively), and both conjugates were almost completely reducible (Fig. 5, right panel, lanes 3 and 5), suggesting that both HA–FAT10 and HA–ubiquitin were thioester-bound to the 6His–USE1–FLAG–FAT10 conjugate. No signal was detectable when the FAT10 diglycine mutant HA–FAT10ΔGG was coexpressed (Fig. 5, lane 4).

Taking all results together, we suggest that the main function of USE1 auto-FAT10ylation is to guide USE1 to proteasomal degradation, although with a slow degradation rate. Therefore, auto-FAT10ylation seems not to influence the ability of USE1 to distinguish between the two modifiers FAT10 and ubiquitin, and USE1 auto-FAT10ylation seems not to produce

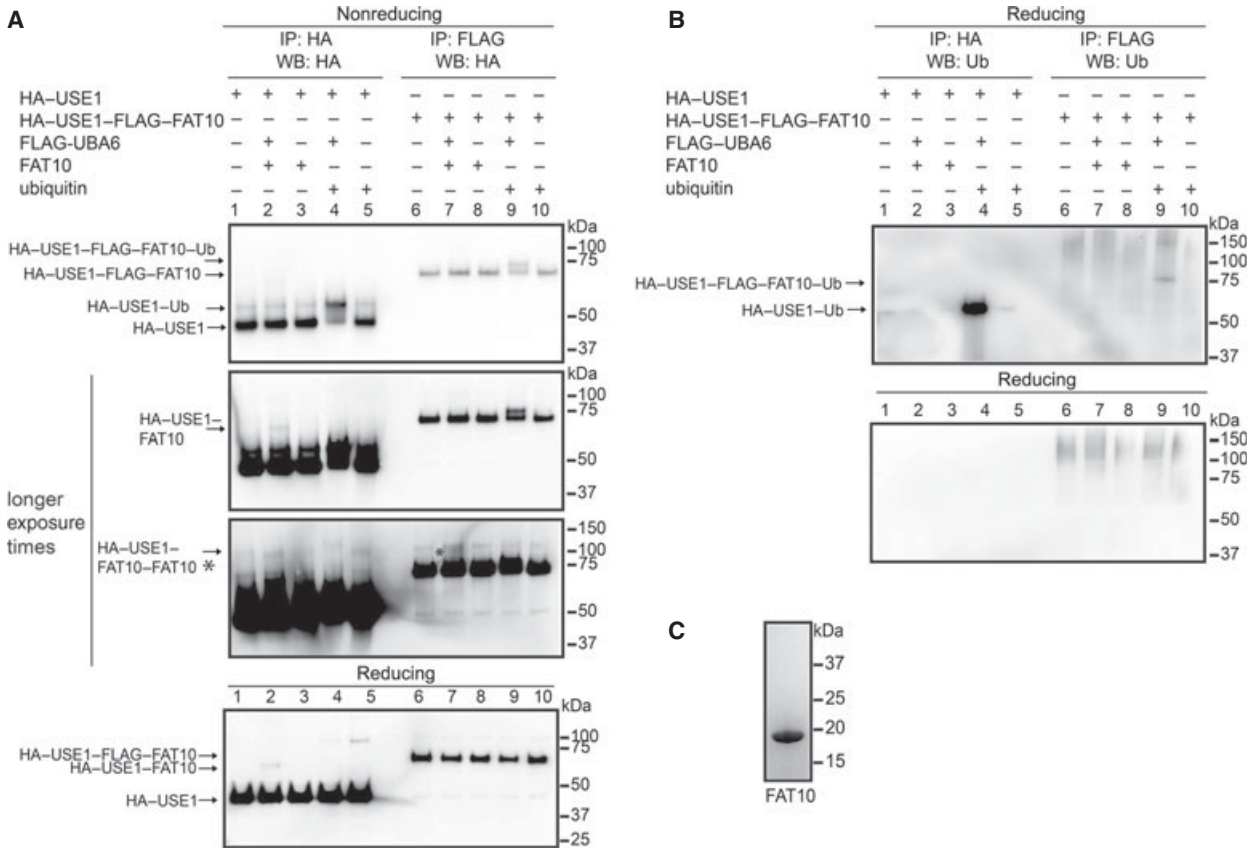

Fig. 4. The USE1–FAT10 conjugate accepts both, activated ubiquitin (Ub) and activated FAT10 from UBA6 *in vitro*. HA–USE1 or HA–USE1–FLAG–FAT10 conjugate was purified from transiently transfected HEK293 cells, and used for *in vitro* transfer experiments with recombinant FLAG–UBA6, FAT10, or ubiquitin. HA–USE1 and HA–USE1–FLAG–FAT10 were left bound to HA-beads and FLAG-beads used for immunoprecipitation (IP), respectively, and incubated for 45 min at 30 °C in reaction buffer containing ATP and the indicated recombinant proteins. Proteins were separated under nonreducing or reducing (10% 2-mercaptoethanol) conditions, and subjected to western blot (WB) analysis with peroxidase-labeled antibody against HA (A) or antibody against ubiquitin (B). Different exposure times of the same blot under nonreducing conditions are shown in (A) to visualize the HA–USE1–FLAG–FAT10–FAT10 conjugate formation indicated by the asterisk in lane 7. (C) Coomassie-stained SDS/PAGE gel showing 100% input of purified recombinant FAT10 (10 μg). One representative experiment of three with similar outcomes is shown.

an inactive E2 enzyme, as has been described for enzymatic pathways of other modifiers. Whether USE1 auto-FAT10ylation has an influence on its ability to interact with specific E3 ligases remains to be investigated.

Discussion

Previously, it was assumed that each ULM uses its private set of E1, E2 and E3 enzymes, which are necessary to accomplish the modification of their specific substrate pools [17]. However, it has recently emerged that different modifiers can share enzymes, as it is the case for FAT10 and ubiquitin, which are both activated by UBA6 [18–20] and conjugated by USE1 [19,21]. It is therefore pertinent to elucidate how the use of the same enzymes by different modifiers is regulated to achieve specificity. We recently identified USE1 as the E2 conjugating enzyme for FAT10, and showed that it undergoes self-FAT10ylation *in cis* but not *in trans*, and forms an isopeptide-linked USE1–FAT10 conjugate [21]. In the current study, we provide evidence that auto-FAT10ylation of USE1 leads to moderately accelerated proteasomal degradation of the USE1–FAT10 conjugate. We show that auto-FAT10ylation has no influence on the activity of the remaining USE1–FAT10 conjugate, and that USE1 – although covalently modified by one FAT10 molecule – can still be loaded with an additional FAT10, ubiquitin or even diubiquitin molecule (Figs 4, 5 and S3). As these conjugates were almost completely reducible, and as the diglycine mutant of

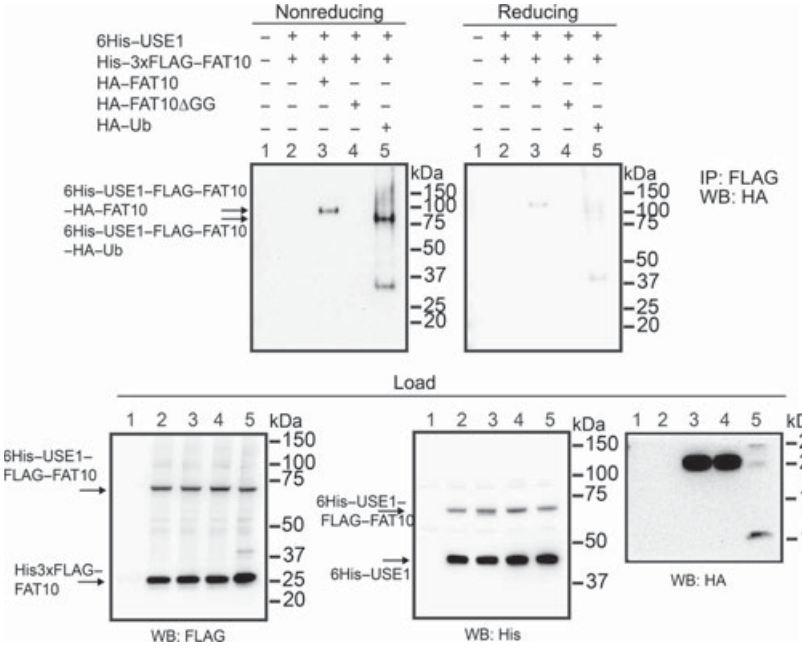

Fig. 5. Loading of HA–FAT10 or HA–ubiquitin onto the 6His–USE1–FLAG–FAT10 conjugate. HEK293 cells were transiently transfected with expression plasmids for 6His–USE1, His–3xFLAG–FAT10 (FLAG–FAT10), HA–FAT10, HA–FAT10ΔGG, or HA–ubiquitin. After 24 h, cells were harvested, and lysates were subjected to immunoprecipitation (IP) with anti-FLAG–sepharose to isolate the HA–USE1–FLAG–FAT10 conjugate. Additionally bound HA-tagged proteins were visualized by western blot (WB) analysis under nonreducing and reducing (10% 2-mercaptoethanol) conditions, using a peroxidase-labeled antibody against HA (upper panels). Lower panels show the expression of the different proteins in the cell lysate (load). One representative experiment of three with similar outcomes is shown.

FAT10 (HA–FAT10ΔGG) did not form a further conjugate with the USE1–FAT10 conjugate, we suggest that the additional modifier was bound by a thioester bond to the active site cysteine of USE1. It was recently reported that UBA6 binds FAT10 with higher affinity than ubiquitin, but that the adenylation and transthiolation reaction is slower for FAT10 than for ubiquitin [22]. Our *in vitro* experiments confirm these findings, and we show that this is also true for the transfer of ubiquitin, diubiquitin or FAT10 onto the USE1–FAT10 conjugate. This difference seems not to arise from the size of FAT10, which is double that of ubiquitin, because the loading of a linear diubiquitin molecule, which is approximately the same size as FAT10, was also much more efficient than FAT10 loading onto USE1. FAT10 consists of two ubiquitin-like domains that are separated by a short, and possibly flexible, linker [6]. So far, no crystal structure of FAT10 has been reported, which may be attributable to the high tendency of FAT10 to precipitate. Nevertheless, differences in the extent of interdomain motions of diubiquitin and FAT10 may account for their different abilities to access the active site cysteine of USE1. The successful loading of an additional modifier onto the USE1–FAT10 conjugate further shows that USE1 auto-FAT10ylation does not inhibit the interaction of the E2 enzyme USE1 with the E1 enzyme UBA6, as has been described for the E2 enzyme E2-25K and the E1 enzyme UBE1 [27]. E2-25K becomes sumoylated on the N-terminal helix, and this, in turn, inhibits its interaction with UBE1 by masking the E1 interaction site, resulting in impaired ubiquitin–E2-25K thioester formation. Therefore, USE1 auto-FAT10ylation seems to have no influence on the activity of USE1 or on the ability of USE1 to discriminate between a ubiquitin or a FAT10 molecule.

By combined MS analysis and site-directed mutagenesis of each single lysine of USE1, we were able to identify Lys323 as the major lysine that becomes FAT10ylated. Replacement of this lysine with an arginine still resulted in the formation of a stable USE1–FAT10 conjugate, but the amount was significantly diminished as compared with the formation of wild-type USE1–FAT10 (Fig. 3). In line with this finding, mutation of the FAT10ylated lysine of the recently identified FAT10 substrate LRRFIP2 also did not abrogate conjugate formation [13], suggesting that the function of FAT10ylation is not necessarily dependent on the position of the covalently attached FAT10

within the substrate. Our systematic comparison of USE1 mutants in which each of the 11 lysines was individually replaced by arginine revealed that each of these USE1 variants formed a stable conjugate with FAT10, but, as compared with wild-type USE1–FAT10, USE1-K323R–FAT10 was the only mutant forming significantly less conjugate with FAT10 (Fig. 3C), in accordance with our MS analysis.

With CHX chase experiments, we showed that the USE1–FAT10 conjugate is degraded by the proteasome (Fig. 2), and this finding is consistent with earlier results showing that FAT10 is a ubiquitin-independent signal for proteasomal degradation [3–5,28]. The FAT10ylation of two newly identified FAT10 substrates, the autophagosomal receptor p62/SQSTM1 [15] and the ubiquitin-activating enzyme UBE1 [28], also led to their degradation by the proteasome. These data from individual FAT10ylation substrates fully agree with the rapid degradation of the bulk of endogenous FAT10 conjugates in cytokine-stimulated cells (Fig. 1) reported here, and together emphasize the role of FAT10 as a transferable tag for proteasomal degradation.

Our results imply negative feedback regulation of the FAT10 conjugation pathway through USE1 auto-FAT10ylation, which limits the FAT10ylation pathway by subsequent degradation of its principle E2 enzyme USE1. Such an automodification is reminiscent of certain ubiquitin E3 ligases such as Mdm2, Itch, BCA2, and TRIM22, which all autoubiquitylate themselves as a mechanism of autoregulation. Automodification of E2 enzymes seems to be a much rarer event. It has been described for the SUMO-specific conjugating enzyme Ubc9, which undergoes autosumoylation *in vitro* and *in vivo* [29,30]. As is the case for auto-FAT10ylation of USE1, autosumoylation also does not impair UBC9–SUMO thioester formation with an additional SUMO molecule, and it does also not inhibit E2 activity as such, but serves as a modulator for target recognition. Whereas sumoylation of some targets is not affected by UBC9 autosumoylation, SUMO modification of RanGAP1 is strongly impaired [27]. On the other hand, UBC9 autosumoylation strongly increases SUMO modification of Sp100. This is achieved by increasing the interaction of UBC9 with the SUMO-interacting motif of Sp100. In the case of auto-FAT10ylated USE1, our data imply that the function seems to be selective inactivation of FAT10ylation rather than a change in substrate specificity. However, we cannot exclude the possibility that it might have an effect on the FAT10ylation of specific single substrates, as has been described in the case of UBC9 auto-SUMOylation, or that USE1 auto-FAT10ylation might be a prerequisite for the choice between ubiquitin-specific and FAT10-specific E3 ligases, which still await identification. Moreover, specific interaction domains for FAT10, such as the three UBA domains of NUB1L [31] or the CAT1 and BUZ domain of histone deacetylase 6 [32], have been described, and we cannot rule out the possibility that, in cells, the function of the USE1–FAT10 conjugate is influenced by a so far unidentified FAT10-binding protein.

Our data provide evidence that, at the level of the E2 conjugating enzyme USE1, a regulation step takes place, which seems to be a negative feedback loop to dampen USE1 activity. This negative feedback regulation could be a means to limit or to fine-tune FAT10ylation activity after the synergistic induction of FAT10 by IFN-γ and TNF-α [7,33]. Overexpression or induction of FAT10 in several cell types induces apoptosis [24,33,34] and chromosomal missegregation [14,35], which might be kept in check by avoiding excessive FAT10ylation through USE1 self-FAT10ylation. It will be very interesting to investigate whether an additional mode of regulation exists at the level of putative FAT10-specific E3 ligases. So far, only one single ubiquitin-specific (but not FAT10-specific) E3 ligase of the UBA6–USE1 pathway has been identified, which is UBR2 [36]. During a proteomic analysis of FAT10-interacting proteins, we have identified 20 E3 ligases that remain to be scrutinized for their activity as FAT10-specific E3 ligases [15]. Further work will be necessary to fully understand the regulation of the partially overlapping conjugation pathways of ubiquitin and FAT10.

Experimental procedures

Primers and constructs

The plasmids used for transient transfection of HEK293 cells were pcDNA3.1–HA–FAT10 for expression of HA-tagged FAT10 [37], and pcDNA3.1–HA–FAT10ΔGG [21], pcDNA3.1–HA–ubiquitin (M. Basler, unpublished) or pcDNA3.1–His–3xFLAG–FAT10 [20] for the expression of His–3xFLAG–FAT10 (FLAG–FAT10). pcDNA3.1-HisA-Xpress-USE1 was used to express His-Xpress-tagged USE1 (6His–USE1), and pcDNA3.1–His–3xFLAG–USE1 was used to express His–3xFLAG–USE1, as previously described [21]. pcDNA3.1–HA–USE1 was used to express HA–USE1, as previously described [21]. For the generation of single lysine-to-arginine mutants of HA–USE1, the QuikChange II Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with pcDNA3.1–HA–USE1 as template and the following

primers for the exchange of lysine to arginine were used: K105R, 5′-GCCGCAGTGTCTACTCCGGATCAGGCGG GATATCATGTCCATTTATAAGG-3′; K113R, 5′-CAAG CGGGATATCATGTCCATTTATAGGGAGCCTCCTCC AGGAATGTTCGTTG-3′; K130R, 5-′CCTGATACTGTT GACATGACTAGGATTCATGCATTGATCACAGG-3′; K166R, 5′-TCCCATCCACCCACCTCGGGTCAGACTG ATGACAACGGGCAATAAC-3′; K186R, 5-′CCAACT TCTACCGCAATGGGAGAGTCTGCTTGAGTATTCTA GGTACATGG-3′; K238R, 5′-GGAGAGACATCCAGGA GACAGCAGAAACTATAATGAATGTATCCGGCACG-3′; K260R, 5′-CTGTGACATGATGGAAGGAAGGTGTC CCTGTCCTGAACCC-3′; K273R, 5′-CCTACGAGGGG TGATGGAGAGGTCCTTTCTGGAGTATTACG-3′; K28 7R, 5′-CGACTTCTACGAGGTGGCCTGCAGAGATCG CCTGCACCTTCAAGGCC-3′; K304R, 5′-GGACCCTTT TGGAGAGAGGCGGGGCCACTTTGACTACCAGTC C-3′; and K323R, 5′-GCGCCTGGGACTGATACGTCAG AGAGTGCTGGAGAGGCTCCATAATG-3′.

Induction of endogenous FAT10 expression, CHX chase experiments, and immunoprecipitation

Induction of endogenous FAT10 expression by the proinflammatory cytokines IFN-γ (200 $U \cdot mL^{-1}$) and TNF-α (400 $U \cdot mL^{-1}$) (both from Peprotech GmbH, Hamburg, Germany) was performed as recently described [15]. Before harvesting, 10 μM of the proteasome inhibitor MG132 (Enzo Lifesciences, Lausen, Switzerland), was added and the cells were incubated for an additional 6 h. Where indicated, cells were additionally treated with 50 $\mu g \cdot mL^{-1}$ CHX (Sigma-Aldrich, Buchs, Switzerland) for 5 h in parallel with MG132 treatment. Cells were harvested and lysed for 30 min on ice in lysis buffer containing 20 mM Tris/HCl (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, and 1% NP-40, supplemented with 1× protease inhibitor mix (complete, mini, EDTA-free protease-inhibitor cocktail; Roche, Rotkreuz, Switzerland). Cleared lysates were subjected to immunoprecipitation with either a mouse mAb against FAT10 (4F1) [21] or a rabbit mAb against USE1 (clone 31-10; kindly provided by B. Amidon, Millennium Pharmaceuticals) bound to protein A sepharose, as recently described [21]. Proteins were separated on 4–12% gradient Bis/Tris NuPAGE SDS gels (Invitrogen, Lucerne, Switzerland), and subjected to western blot analysis with either mAb 4F1, rabbit polyclonal antibody against huFAT10, which is crossreactive with moFAT10 [3], or rabbit polyclonal antibody against USE1 [21]. Antibody against iota or antibody against β-actin (Abcam, Cambridge, UK) was used as a loading control. Immunoprecipitation of overexpressed HA-tagged or FLAG-tagged proteins was performed with anti-HA–agarose conjugate HA-7 or EZview Red Anti-FLAG-M2 Affinity Gel, respectively (both from Sigma). Proteins were detected with directly labeled peroxidase-conjugated mAb against HA-7 or horseradish peroxidase (HRP)-conjugated mAb against FLAG M2 antibody (both from Sigma).

MS

His–3xFLAG–USE1–HA–FAT10 conjugate was immunoprecipitated from HEK293 cells transiently transfected with pcDNA3.1–His–3xFLAG–USE1 and pcDNA3.1–HA–FAT10. Approximately 2×10^7 cells were lysed in 4 mL of lysis buffer [20 mM Tris/HCl, pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 1% NP-40, 1× protease inhibitor mix (complete, mini, EDTA-free protease-inhibitor cocktail; Roche)] for 30 min on ice. Lysates were centrifuged for 30 min at 20 000 ***g***, and subjected to immunoprecipitation with anti-HA–agarose (Sigma). Immunoprecipitates were washed twice with NET-TN buffer (50 mM Tris/HCl, pH 8.0, 650 mM NaCl, 5 mM EDTA, 0.5% Triton X-100) and twice with NET-T buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100) as previously described [15], separated on NuPAGE Bis/Tris 4–12% gradient gels (Invitrogen) with 1× Mes running buffer under reducing conditions [10% β-mercaptoethanol), and directly stained with a standard Coomassie Blue solution. Protein bands containing His–3xFLAG–USE1–HA–FAT10 conjugate were cut out and sent for analysis on an LTQ OrbiTrap Hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) at the Proteomics Facility of the University of Konstanz (Germany). Raw data were analyzed with the CHOPNSPICE algorithm [26].

In vitro conjugation experiments

HA–USE1–FLAG–FAT10 conjugate was purified from HEK293 cells, transiently transfected with pcDNA3.1–HA–USE1 and pcDNA3.1–His–3xFLAG–FAT10. For purification of HA–USE1 or HA–USE1–FLAG–FAT10, 2×10^6 or 1.5×10^8 HEK293 cells, respectively, were transiently transfected with the respective plasmids, and, after 24 h harvested and lysed for 30 min on ice in lysis buffer containing 20 mM Tris/HCl (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, and 1% NP-40, supplemented with 1× protease inhibitor mix (complete, mini, EDTA-free protease-inhibitor cocktail; Roche). Cleared lysates were equally divided into 10 immunoprecipitation samples for either HA–USE1 or HA–USE1–FLAG–FAT10, and supplemented with 20 μL of anti-HA–agarose conjugate HA-7 or 20 μL of EZview Red Anti-FLAG-M2 Affinity Gel (both from Sigma) per reaction, respectively. After 2 h of incubation at 4 °C with rolling, beads were washed with NET-T/dithiothreitol buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5 mM dithiothreitol) to remove copurified endogenous ubiquitin, twice with wash buffer NET-T (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100), and twice with reaction

buffer containing 20 mM Tris/HCl (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, 4 mM ATP, 0.1 mM dithiothreitol, 5 units·mL^{-1} inorganic pyrophosphatase, 20 mM creatine phosphate, and 4 μg·mL^{-1} creatine phosphokinase (all from Sigma), supplemented with 1× protease inhibitor mix. Immunoprecipitated HA–USE1 or HA–USE1–FLAG–FAT10 was left bound to the beads, and, where indicated, recombinant proteins were added to a final volume of 120 μL of reaction buffer in the following amounts: FLAG–UBA6, 1 μg; FAT10, 10 μg; ubiquitin, 25 μg; and linear diubiquitin, 10 μg. The reaction mixture was incubated at 30 °C for 45 min with shaking, and subsequently washed three times with wash buffer containing 20 mM Tris/HCl (pH 7.6), 50 mM NaCl, and 10 mM $MgCl_2$. Proteins were separated on 4–12% gradient Bis/Tris NuPAGE SDS gels (Invitrogen), and subjected to western blot analysis with peroxidase-conjugated mAb against HA-7, HRP-conjugated mAb against FLAG M2 (both from Sigma), and a mAb against ubiquitin (clone VU-1; Lifesensors, Malvern, PA, USA) in combination with a secondary antibody (peroxidase-conjugated goat anti-mouse) (Jackson Immuno Research, Newmarket, Suffolk, UK).

Quantification of ECL signals and statistical analysis

For quantification of detected protein amounts, ECL signals were quantified with QUANTITY ONE (BioRad, Cressier, Switzerland). Signals for bulk FAT10 conjugates from three independent experiments were calculated (excluding monomeric FAT10 in the immunoprecipitation) and normalized to the ECL signals of the loading control. Signals for the endogenous USE1–FAT10 conjugate or the overexpressed HA–USE1-K*R–FLAG–FAT10 conjugates from five or three independent experiments, respectively, were normalized to the loading control or the USE1 protein level in the lysate (load), respectively. Values in the figures are given as means ± standard errors of the mean. Statistical analysis of the amounts of HA–USE1-K*R–FLAG–FAT10 conjugate was performed with raw data before normalization to the USE1 load by the use of GRAPHPAD INSTAT (Instat Statistics; GraphPad Software, San Diego, CA, USA). For all data, paired non-parametric *t*-tests were performed. A *P*-value of < 0.05 was considered to indicate statistical significance.

Acknowledgements

We thank A. Marquardt for MS, and H. Urlaub and H.-H. Hsiao for MS data analysis with CHOPNSPICE. We acknowledge B. Amidon for the gift of antibody against USE1 (31-10), and J. Wade Harper, Z. J. Chen and E. Deuerling for the contribution of plasmids. This work was supported by the German Research Foundation (DFG) Collaborative Research Centre SFB969 (Project C01), the DFG Priority Programme 'SPP1365 Ubiquitin Family Network' (grant GR1517/10-1), the Thurgauische Stiftung für Wissenschaft und Forschung, and the Swiss State Secretariat for Education and Research.

References

1 Finley D (2009) Recognition and processing of ubiquitin–protein conjugates by the proteasome. *Annu Rev Biochem* **78**, 477–513.
2 van der Veen AG & Ploegh HL (2012) Ubiquitin-like proteins. *Annu Rev Biochem* **81**, 323–357.
3 Hipp MS, Kalveram B, Raasi S, Groettrup M & Schmidtke G (2005) FAT10, a ubiquitin-independent signal for proteasomal degradation. *Mol Cell Biol* **25**, 3483–3491.
4 Schmidtke G, Kalveram B & Groettrup M (2009) Degradation of FAT10 by the 26S proteasome is independent of ubiquitylation but relies on NUB1L. *FEBS Lett* **583**, 591–594.
5 Schmidtke G, Aichem A & Groettrup M (2013) FAT10ylation as a signal for proteasomal degradation. *Biochim Biophys Acta* **1843**, 97–102.
6 Fan W, Cai W, Parimoo S, Lennon GG & Weissman SM (1996) Identification of seven new human MHC class I region genes around the HLA-F locus. *Immunogenetics* **44**, 97–103.
7 Raasi S, Schmidtke G, Giuli RD & Groettrup M (1999) A ubiquitin-like protein which is synergistically inducible by interferon-g and tumor necrosis factor-a. *Eur J Immunol* **29**, 4030–4036.
8 Canaan A, Yu XF, Booth CJ, Lian J, Lazar I, Gamfi SL, Castille K, Kohya N, Nakayama Y, Liu YC *et al.* (2006) FAT10/diubiquitin-like protein-deficient mice exhibit minimal phenotypic differences. *Mol Cell Biol* **26**, 5180–5189.
9 Lee CG, Ren J, Cheong IS, Ban KH, Ooi LL, Yong Tan S, Kan A, Nuchprayoon I, Jin R, Lee KH *et al.* (2003) Expression of the FAT10 gene is highly upregulated in hepatocellular carcinoma and other gastrointestinal and gynecological cancers. *Oncogene* **22**, 2592–2603.
10 Lukasiak S, Schiller C, Oehlschlaeger P, Schmidtke G, Krause P, Legler DF, Autschbach F, Schirmacher P, Breuhahn K & Groettrup M (2008) Proinflammatory cytokines cause FAT10 upregulation in cancers of liver and colon. *Oncogene* **27**, 6068–6074.
11 Schliehe C, Bitzer A, van den Broek M & Groettrup M (2012) Stable antigen is most effective for eliciting CD8+ T-cell responses after DNA vaccination and infection with recombinant vaccinia virus *in vivo*. *J Virol* **86**, 9782–9793.

12 Ebstein F, Lehmann A & Kloetzel PM (2012) The FAT10- and ubiquitin-dependent degradation machineries exhibit common and distinct requirements for MHC class I antigen presentation. *Cell Mol Life Sci* **69**, 2443–2454.
13 Buchsbaum S, Bercovich B, Ziv T & Ciechanover A (2012) Modification of the inflammatory mediator LRRFIP2 by the ubiquitin-like protein FAT10 inhibits its activity during cellular response to LPS. *Biochem Biophys Res Commun* **428**, 11–16.
14 Ren JW, Wang Y, Gao Y, Mehta SBK & Lee CGL (2011) FAT10 mediates the effect of TNF-alpha in inducing chromosomal instability. *J Cell Sci* **124**, 3665–3675.
15 Aichem A, Kalveram B, Spinnenhirn V, Kluge K, Catone N, Johansen T & Groettrup M (2012) The proteomic analysis of endogenous FAT10 substrates identifies p62/SQSTM1 as a substrate of FAT10ylation. *J Cell Sci* **125**, 4576–4585.
16 Kerscher O, Felberbaum R & Hochstrasser M (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* **22**, 159–180.
17 Groettrup M, Pelzer C, Schmidtke G & Hofmann K (2008) Activating the ubiquitin family: UBA6 challenges the field. *Trends Biochem Sci* **33**, 230–237.
18 Pelzer C, Kassner I, Matentzoglu K, Singh RK, Wollscheid HP, Scheffner M, Schmidtke G & Groettrup M (2007) UBE1L2, a novel E1 enzyme specific for ubiquitin. *J Biol Chem* **282**, 23010–23014.
19 Jin JP, Li X, Gygi SP & Harper JW (2007) Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging. *Nature* **447**, 1135–1137.
20 Chiu YH, Sun Q & Chen ZJ (2007) E1-L2 activates both ubiquitin and FAT10. *Mol Cell* **27**, 1014–1023.
21 Aichem A, Pelzer C, Lukasiak S, Kalveram B, Sheppard PW, Rani N, Schmidtke G & Groettrup M (2010) USE1 is a bispecific conjugating enzyme for ubiquitin and FAT10, which FAT10ylates itself in cis. *Nat Commun* **1**, 13, doi:10.1038/ncomms1012.
22 Gavin JM, Chen JJ, Liao H, Rollins N, Yang XF, Xu Q, Ma JY, Loke HK, Lingaraj T, Brownell JE *et al.* (2012) Mechanistic studies on activation of ubiquitin and di-ubiquitin-like protein, FAT10, by ubiquitin-like modifier activating enzyme 6, Uba6. *J Biol Chem* **287**, 15512–15522.
23 Gu X, Zhao F, Zheng M, Fei X, Chen X, Huang S, Xie Y & Mao Y (2007) Cloning and characterization of a gene encoding the human putative ubiquitin conjugating enzyme E2Z (UBE2Z). *Mol Biol Rep* **34**, 183–188.
24 Raasi S, Schmidtke G & Groettrup M (2001) The ubiquitin-like protein FAT10 forms covalent conjugates and induces apoptosis. *J Biol Chem* **276**, 35334–35343.
25 Buchsbaum S, Bercovich B & Ciechanover A (2012) FAT10 is a proteasomal degradation signal that is itself regulated by ubiquitination. *Mol Biol Cell* **23**, 225–232.
26 Hsiao HH, Meulmeester E, Frank BTC, Melchior F & Urlaub H (2009) 'ChopNSpice', a mass spectrometric approach that allows identification of endogenous small ubiquitin-like modifier-conjugated peptides. *Mol Cell Proteomics* **8**, 2664–2675.
27 Pichler A, Knipscheer P, Oberhofer E, vanDijk WJ, Korner R, Olsen JV, Jentsch S, Melchior F & Sixma TK (2005) SUMO modification of the ubiquitin-conjugating enzyme E2-25K. *Nat Struct Mol Biol* **12**, 264–269.
28 Rani N, Aichem A, Schmidtke G, Kreft SG & Groettrup M (2012) FAT10 and NUB1L bind to the VWA domain of Rpn10 and Rpn1 to enable proteasome-mediated proteolysis. *Nat Commun* **3**, 749.
29 Bencsath KP, Podgorski MS, Pagala VR, Slaughter CA & Schulman BA (2002) Identification of a multifunctional binding site on Ubc9p required for Smt3p conjugation. *J Biol Chem* **277**, 47938–47945.
30 Knipscheer P, Flotho A, Klug H, Olsen JV, van Dijk WJ, Fish A, Johnson ES, Mann M, Sixma TK & Pichler A (2008) Ubc9 sumoylation regulates SUMO target discrimination. *Mol Cell* **31**, 371–382.
31 Schmidtke G, Kalveram B, Weber E, Bochtler P, Lukasiak S, Hipp MS & Groettrup M (2006) The UBA domains of NUB1L are required for binding but not for accelerated degradation of the ubiquitin-like modifier FAT10. *J Biol Chem* **281**, 20045–20054.
32 Kalveram B, Schmidtke G & Groettrup M (2008) The ubiquitin-like modifier FAT10 interacts with HDAC6 and localizes to aggresomes under proteasome inhibition. *J Cell Sci* **121**, 4079–4088.
33 Liu Y, Pan J, Zhang C, Fan W, Collinge M, Bender JR & Weissman SM (1999) A MHC-encoded ubiquitin-like protein (FAT10) binds noncovalently to the spindle assembly checkpoint protein MAD2. *Proc Natl Acad Sci USA* **96**, 4313–4318.
34 Ross MJ, Wosnitzer MS, Ross MD, Granelli B, Gusella GL, Husain M, Kaufman L, Vasievich M, D'Agati VD, Wilson PD *et al.* (2006) Role of ubiquitin-like protein FAT10 in epithelial apoptosis in renal disease. *J Am Soc Nephrol* **17**, 996–1004.
35 Ren JW, Kan A, Leong SH, Ooi LLPJ, Jeang KT, Chong SS, Kon OL & Lee CGL (2006) FAT10 plays a role in the regulation of chromosomal stability. *J Biol Chem* **281**, 11413–11421.
36 Lee PC, Sowa ME, Gygi SP & Harper JW (2011) Alternative ubiquitin activation/conjugation cascades interact with N-end rule ubiquitin ligases to control degradation of RGS proteins. *Mol Cell* **43**, 392–405.
37 Hipp MS, Raasi S, Groettrup M & Schmidtke G (2004) NEDD8 ultimate buster-1L interacts with the ubiquitin-like protein FAT10 and accelerates its degradation. *J Biol Chem* **279**, 16503–16510.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Accumulation of the endogenous USE1–FAT10 conjugate upon proteasomal inhibition.

Fig. S2. Unaffected USE1 protein level upon prolonged expression of endogenous FAT10.

Fig. S3. Linear diubiquitin loading onto USE1–FAT10.

Doc. S1. Recombinant proteins and purification of recombinant FAT10.

Doc. S2. Time course experiments.

Doc. S3. Supplementary references.