The proteomic analysis of endogenous FAT10 substrates identifies p62/SQSTM1 as a substrate of FAT10ylation

Annette Aichem¹, Birte Kalveram², Valentina Spinnenhirn², Kathrin Kluge², Nicola Catone¹, Terje Johansen³ and Marcus Groettrup¹,²,*

¹Biotechnology Institute Thurgau at the University of Constance, CH-8280 Kreuzlingen, Switzerland
²Division of Immunology, Department of Biology, University of Constance, 78457 Konstanz, Germany
³Molecular Cancer Research Group, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway

*Author for correspondence (marcus.groettrup@uni-konstanz.de)

Summary

FAT10 is a ubiquitin-like modifier proposed to function in apoptosis induction, cell cycle control and NF-κB activation. Upon induction by pro-inflammatory cytokines, hundreds of endogenous substrates become covalently conjugated to FAT10 leading to their proteasomal degradation. Nevertheless, only three substrates have been identified so far to which FAT10 becomes covalently attached through a non-reducible isopeptide bond, and these are the FAT10-conjugating enzyme USE1 which auto-FAT10ylates itself in cis, the tumor suppressor p53 and the ubiquitin-activating enzyme UBE1 (UBA1). To identify additional FAT10 substrates and interaction partners, we used a new monoclonal FAT10-specific antibody to immunopurify endogenous FAT10 conjugates from interferon (IFN)γ-stimulated cells for identification by mass spectrometry. In addition to two already known FAT10-interacting proteins, histone deacetylase 6 and UBA6, we identified 569 novel FAT10-interacting proteins involved in different functional pathways such as autophagy, cell cycle regulation, apoptosis and cancer. Thirty-one percent of all identified proteins were categorized as putative covalently linked substrates. One of the identified proteins, the autophagosomal receptor p62/SQSTM1, was further investigated. p62 becomes covalently mono-FAT10ylated at several lysines, and FAT10 colocalizes with p62 in p62 bodies. Strikingly, FAT10ylation of p62 leads to its proteasomal degradation, and prolonged induction of endogenous FAT10 expression by pro-inflammatory cytokines leads to a decrease of endogenous p62. The elucidation of the FAT10 degradome should enable a better understanding of why FAT10 has evolved as an additional transferable tag for proteasomal degradation.

Key words: Ubiquitin, Proteasome, FAT10, Proteomics, p62

Introduction

Covalently tagging proteins with ubiquitin or ubiquitin-like modifiers (ULM) is a widely used posttranslational modification to change the localization, function or fate of specific target proteins. Similar to the modification with poly-ubiquitin chains (Xu et al., 2009), the covalent modification with the ubiquitin-like modifier FAT10 (HLA-F adjacent transcript 10; Ubiquitin D) via its C-terminal diglycine motif was shown to target proteins in a ubiquitin-independent manner for proteasomal degradation (Hipp et al., 2005; Raasi et al., 2001; Schmidtke et al., 2009). FAT10 consists of two ubiquitin-like (UBLs) domains that are connected by a short linker (Fan et al., 1996). It is mainly expressed in mature dendritic cells and the basal level of expression is the highest in organs of the immune system such as thymus, fetal liver, lymph nodes and spleen (Canaan et al., 2006; Lee et al., 2003; Lukasiak et al., 2008). In addition, expression of FAT10 can be synergistically induced in many tissues by IFNγ and TNFα (Liu et al., 1999; Raasi et al., 1999). As other ULMs, FAT10 becomes covalently attached to its substrates via an E1-E2-E3 enzyme cascade, where UBA6 (UBE1L2, E1-L2 or MOP-4) serves as an E1 activating enzyme (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007) in combination with the recently identified E2 conjugating enzyme USE1 (UBA6-specific E2 enzyme) (Aichem et al., 2010; Jin et al., 2007) and probably E3 ligases which await their identification. Upregulation of FAT10-induced apoptosis in mouse fibroblasts (Raasi et al., 2001), human renal epithelial cells (Ross et al., 2006), and HeLa cells (Liu et al., 1999). FAT10 was shown to be upregulated in gastrointestinal and gynecological cancers (Lee et al., 2003; Lukasiak et al., 2008) and seems to play a role in spindle check point control during mitosis (Liu et al., 1999; Ren et al., 2006; Ren et al., 2011) as well as NF-κB activation (Gong et al., 2010).

Upon proteasomal inhibition, FAT10 is shuttled via histone deacetylase (HDAC)6 to aggresomes by traveling on dynein motors along microtubules towards the microtubule organizing center (Kalveram et al., 2008).

Despite all these functional implications, only three substrates of FAT10ylation have been identified so far. These are its own E2 conjugating enzyme USE1, which undergoes self-FAT10ylation in cis (Aichem et al., 2010), the tumor suppressor p53 (Li et al., 2011), and the ubiquitin-specific E1-activating enzyme UBE1 (UBA1) (Rani et al., 2012). To gain more information about the function of FAT10, the identification of additional FAT10-interacting proteins and substrates is pertinent. Owing to the
The proteome of FAT10ylation

Fig. 1. Detection and isolation of endogenous FAT10 conjugates from cytokine-stimulated HEK293 cells. (A) Total cell extracts of untreated or IFNγ/TNFα-stimulated HEK293 cells were used to immunoprecipitate endogenous FAT10 and its interacting partners with the monoclonal FAT10 antibody 4F1 followed by western blot analysis using a polyclonal FAT10 antibody. The upper western blot panel shows the immunoprecipitated FAT10 conjugates, the lower panels show the expression level of endogenous FAT10 in the cell lysates. β-actin was used as a loading control. (B) Detection of FAT10 conjugates on Coomassie- and silver-stained gels for subsequent mass spectrometry. All experiments were performed under reducing conditions (10% β-mercaptoethanol). Asterisks indicate the heavy and light chains of the FAT10-reactive antibody used for the immunoprecipitation. Arrows indicate the gel slices that were cut out and sent for mass spectrometry. One representative experiment out of four experiments with similar outcome is shown.

Results
Identification of FAT10-interacting proteins and substrates by immunoprecipitation and mass spectrometry

In order to identify novel putative FAT10 targets we performed immunoprecipitation of endogenous FAT10 from IFNγ and TNFα-stimulated HEK293 cells. The immunoprecipitation was performed under non-denaturing conditions in order not to isolate only substrates but also additional proteins non-covalently interacting with FAT10. After immunoprecipitation of endogenous FAT10 with the monoclonal FAT10-reactive antibody 4F1 (Aichem et al., 2010) and subsequent western blot analysis using a polyclonal FAT10-specific antibody (Hipp et al., 2005), we could detect large amounts of endogenous monomeric FAT10 as well as numerous FAT10-associated proteins of higher molecular mass in cytokine-treated cells that were absent in untreated control cells (Fig. 1A). The same conditions were used to analyze the samples on an SDS-PAGE stained with Coomassie Blue or silver (Fig. 1B). Thirteen gel slices corresponding to molecular masses between 17 kDa and approximately 150 kDa (Fig. 1B, arrows), that were absent in the untreated control lane, were cut out and sent together with their corresponding control bands from the untreated cells for mass spectrometric analysis with an LTQ Orbitrap Hybrid mass spectrometer. Proteins which were identified in gel slices from both, unstimulated control and IFNγ/TNFα-stimulated samples, as well as all identified chaperones and heat shock proteins were excluded from further analysis to eliminate false positives. The published or predicted molecular masses of the proteins were further compared to the apparent size assigned to the respective gel slice to determine if the protein could be a putative substrate or a protein non-covalently interacting with FAT10. Proteins were categorized as putative covalently FAT10ylated substrates when the apparent molecular mass assigned to the respective gel slice was at least 18 kDa (i.e. the molecular mass of FAT10) higher than the predicted molecular mass. In total we identified with this method 571 putative FAT10-interacting proteins with 31% of the proteins (176 proteins) assigned to be putative FAT10 substrates and the remaining 69% (395 proteins) assigned to be putative FAT10-interacting proteins (Fig. 2). Among these we identified two already published FAT10-interacting proteins: the ubiquitin and FAT10-specific E1 enzyme UBA6 (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007) and HDAC6, which we have recently described as a FAT10 binding protein (Kalveram et al., 2008). As shown in Fig. 2, proteins of known function were subdivided into functionally defined categories. Interestingly, the putative FAT10 interactors do not primarily fall into a specific category but cover a broad spectrum of diverse cellular functions. The biggest group comprises proteins involved in binding DNA or RNA with many transcription factors as well as DNA or RNA polymerases followed by the group of cancer-related proteins and the group of putative E3 ligases. Among the 20 identified E3 ligases, we found 10 belonging to the RING (really interesting new gene)-finger family, two HECT domain, two F-box-containing and two SUMO-specific ligases, one anaphase-...
promoting complex E3 ligase, and two zinc-finger and two cullin E3 ligases. A complete list of all identified proteins of both screens is available online as supplementary material Table S1.

The autophagic receptor p62/SQSTM1 is a novel substrate of FAT10

One protein that was identified in several gel slices was the autophagic receptor p62/SQSTM1 (p62) which we decided to investigate further to initiate the validation of our mass spectrometry results. To recapitulate the conditions used for the mass spectrometric analysis we first performed an immunoprecipitation of endogenous FAT10 from IFNγ- and TNFα-treated HEK293 cells with the monoclonal FAT10-reactive antibody 4F1 followed by a western blot analysis using a p62-reactive antibody. As shown in Fig. 3A,B, FAT10 was strongly expressed after 24 hours of cytokine treatment, and cytokine treatment also slightly increased the amount of endogenous p62 in the lysate, confirming previous results from Mostowy et al. who also showed an p62 increase upon TNFα treatment (Mostowy et al., 2011). A strong non-covalent interaction of p62 and FAT10 could be detected after immunoprecipitation of endogenous FAT10 and western blot analysis using a p62-reactive antibody (marked by an arrow in Fig. 3A, lanes 2 and 4). In addition, four non-reducible stable p62-FAT10 conjugates were detected (marked by arrowheads in Fig. 3A, lanes 2 and 4) which were absent in untreated cells thus confirming our results that p62 was identified in gel slices corresponding to unconjugated p62, and p62 conjugated to several FAT10 molecules at the same time. The non-covalent interaction between p62 and FAT10 as well as the smallest p62-FAT10 conjugate could further be confirmed by performing the immunoprecipitation in the other direction by using a p62-reactive antibody for immunoprecipitation and p62 or FAT10-reactive antibodies for detection (data not shown). Treatment of the cells for five hours with cycloheximide led to a complete degradation of the p62-FAT10 conjugates while unconjugated p62 in the lysate was degraded only slightly. The degradation of the p62-FAT10 conjugates could be completely rescued by treatment of the cells with the proteasome inhibitor MG132 (Fig. 3A, lane 4) whereas at the same time MG132 treatment alone did not significantly increase the amount of endogenous, not FAT10-modified p62 (Fig. 3B) thus confirming that the accumulation of endogenous p62-FAT10 conjugates upon MG132 treatment was specific. Therefore it seems that FAT10ylation of p62 leads to its proteasomal degradation. Interestingly, cytokine treatment led to a clear increase in p62 (Fig. 3A,B) while at the same time it induced FAT10 expression which in turn caused degradation of p62. Therefore one would expect to see less p62 in cells treated with IFNγ and TNFα due to upregulation of FAT10 expression. To further investigate this issue, we performed time course experiments with IFNγ- and TNFα-treated HEK293 cells (Fig. 3C,D). Strikingly, the p62 mRNA as well as protein level increased much faster than the FAT10 mRNA and protein level (Fig. 3C) and p62 accumulated before detectable amounts of FAT10 were visible after 24 hours of IFNγ/TNFα treatment. To investigate later time points up to 72 hours of IFNγ/TNFα treatment, a second series of time course experiments was performed. As shown in Fig. 3D, the amount of endogenous p62 increased up to 24 hours of cytokine stimulation but then declined as soon as high amounts of FAT10 were expressed. These results show that sustained expression of FAT10 diminishes the level of endogenous p62 in HEK293 cells.

To demonstrate that the degradation of p62 is dependent on FAT10 and that it is not a cytokine-dependent effect unrelated to FAT10 expression, we overexpressed HA-FAT10 or, as a control, its diglycine mutant HA-FAT10ΔGG in HEK293 cells instead of inducing FAT10 expression with cytokines. As shown in Fig. 3E, overexpression of HA-FAT10 led to a significant decrease of endogenous p62 (as quantified and normalized to the signals of HA-FAT10 or HA-FAT10ΔGG) whereas overexpression of the diglycine mutant HA-FAT10ΔGG did not de-stabilize endogenous p62. This further confirms that FAT10ylation of p62 leads to its proteasomal degradation.

To test if FAT10 conjugation to p62 relied on the involved E1 and E2 enzymes, we downregulated the FAT10-specific E1 enzyme UBA6 (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007) or the FAT10-specific E2-conjugating enzyme USE1 (Aichem et al., 2010; Jin et al., 2007) by specific siRNA treatment (Fig. 3F, lanes 3 and 4, respectively) before induction of endogenous FAT10 expression by IFNγ and TNFα. Downregulation of each of the two proteins clearly inhibited the formation of the p62-FAT10 conjugates which was not the case, when a negative control siRNA was used. The amount of non-covalent interacting p62 was slightly decreased in the cells treated with UBA6 or USE1-specific siRNA because the level of endogenous FAT10 or p62 was slightly reduced in these samples, respectively (Fig. 3F, lanes 3, load FAT10, and 4, load p62). Taken together, these results show, that p62 is not only a non-covalent interaction partner of FAT10 but also a novel substrate of FAT10 conjugation and that this modification leads to proteasomal degradation of p62.

The p62-FAT10 interaction is independent of the p62-UBA domain but dependent on the C-terminal diglycine motif of FAT10

To further characterize the p62-FAT10 conjugate formation we expressed tagged FAT10 and tagged p62 in HEK293T cells. In a first approach, cells were transfected with expression constructs for either His-3xFLAG-FAT10 (FLAG-FAT10) or its mutant form where the C-terminal diglycine motif (GG), necessary for conjugate formation, was mutated (FLAG-FAT10ΔGG) together with HA-tagged p62 (HA-p62). In contrast to the conjugate formation under endogenous conditions, only one single HA-p62~FLAG-FAT10 conjugate of about 130 kDa could be detected (Fig. 4A, lane 2). The formation of the conjugate was dependent on the C-terminal diglycine motif of FAT10 since it was absent when the diglycine mutant FLAG-FAT10ΔGG was expressed together with HA-p62 (Fig. 4A, lane 3). To investigate if FAT10 interacts with the p62-UBA domain which is described as binding domain for polyubiquitylated proteins (Long et al., 2008; Tan et al., 2008; Wooten et al., 2008), we co-expressed FLAG-FAT10 or FLAG-FAT10ΔGG together with a mutant form of p62 where the UBA domain was deleted (HA-p62ΔUBA). Independent of the missing UBA domain, a HA-p62ΔUBA-FLAG-FAT10 conjugate was formed (Fig. 4A, lane 5). The conjugate size was reduced by the size of the deleted UBA domain, further confirming the conjugate formation of p62 and FAT10.

In a second approach, FLAG-FAT10 was co-expressed with either HA-tagged p62 (HA-p62) or a lysine-less p62 mutant [HA-p62(K0)] in which all lysines were mutated to arginines. As compared to the co-expression of HA-p62 and FLAG-FAT10 where a clear conjugate was detected (Fig. 4B, lane 6), no stable conjugate was formed between the lysine-less mutant
HA-p62(K0) and FLAG-FAT10 (Fig. 4B, lane 7). As a further control, HA-p62 was again co-expressed together with the diglycine mutant FLAG-FAT10ΔGG (Fig. 4B, lane 8) where after immunoprecipitation no conjugate was detectable as already shown in the total cell lysate used in Fig. 4A.

**p62 becomes mono-FAT10ylated at several lysines**

Our mass spectrometric analysis identified p62 in several gel slices containing proteins with increasing molecular masses and immunoprecipitation of endogenous p62-FAT10 conjugates detected four conjugates of different size. Therefore we were interested to investigate if p62 becomes modified with a FAT10 chain or if it becomes mono-FAT10ylated at several different lysines. To this aim, HEK293T cells were transfected with expression plasmids for p62-6His in combination with either HA-tagged FAT10 or a HA-FAT10(K0) mutant protein, in which all lysines were mutated to arginines. As shown in Fig. 4C, independent of the FAT10 protein co-expressed along with p62-6His, only one single p62-6His~HA-FAT10/FAT10(K0) conjugate of approximately 130 kDa was detectable (Fig. 4C, A).

**Fig. 3.** See next page for legend.
lanes 4 and 5), strongly suggesting that no FAT10 chains were formed but that p62 becomes mono-FAT10ylated at three or four different lysine residues.

**p62 and FAT10 colocalize in p62 bodies**

To further confirm the obtained results under endogenous in vivo conditions, HeLa cells were stimulated with IFNγ and TNFα to induce FAT10 or were left unstimulated and stained with the FAT10-specific monoclonal antibody 4F1 and the p62-specific polyclonal antibody H290. As previously reported, p62 localized both in cytokine-treated and untreated HeLa cells in numerous small dots designated p62 bodies or ALIS (Bjørkøy et al., 2005; Pankiv et al., 2007). Interestingly, FAT10 completely colocalized with p62 in these bodies upon induction with IFNγ and TNFα (Fig. 5) thus supporting further the finding of their interaction shown by immunoprecipitation under overexpression and endogenous conditions.

Inspired by these colocalization data we investigated if non-covalent interaction of FAT10 with p62 might have some functional consequences for p62 and its role in autophagy. To this aim, we expressed the tandem fluorescence tagged proteins mCherry-EGFP-p62, mCherry-EGFP-FAT10 or, as a positive control, mCherry-EGFP-4xUbiquitin in HeLa cells. By using this protein tag, it is possible to monitor the autophagic sequestration of these fusion proteins with the red, acid-insensitive mCherry and the acid-sensitive green fluorescent proteins (Pankiv et al., 2007). Although we could show the non-covalent interaction of mCherry-EGFP-FAT10 and HA-p62 by co-immunoprecipitation (supplementary material Fig. S1) as well as a specific targeting of mCherry-EGFP-p62 or mCherry-EGFP-4xUbiquitin to autophagolysosomal degradation, no significant increase in red fluorescent bodies of mCherry-EGFP-FAT10 compared to the mCherry-EGFP fluorescence tag alone was detectable (supplementary material Fig. S2), neither in the absence nor in the presence of the proteasome inhibitor MG132 (supplementary material Fig. S3). Therefore we failed to obtain evidence that FAT10 serves as a signal for autophagosomal degradation.

To investigate if conversely p62 might have an influence on the turnover of FAT10 and its conjugates we downregulated p62 in HEK293 cells with siRNA treatment and subsequently overexpressed His-3xFLAG-tagged FAT10. Interestingly, knockdown of p62 led only to a significant accumulation of monomeric FAT10 but not of FAT10 conjugates (supplementary material Fig. S4), pointing to a minor effect of p62 on the turnover of FAT10. Taken all results together, our data indicate that a function of FAT10ylation of p62 is to target it for proteasomal degradation.

**Discussion**

The ubiquitin-like modifier FAT10 has been proposed to function in several different cell biological pathways. Numerous traits of FAT10 are consistent with a function in the immune system for instance the localization of the FAT10 gene in the MHC locus (Fan et al., 1996), the synergistic inducibility by IFNγ and TNFα (Liu et al., 1999; Raasi et al., 1999), the high expression in lymphoid organs (Canaan et al., 2006; Lee et al., 2003; Lukasiak et al., 2008) and the upregulation in dendritic cells upon their maturation (Lukasiak et al., 2008). Further studies implied FAT10 in playing a role in TNFα/IFNγ-induced apoptosis (Raasi et al., 2001; Ross et al., 2006), in the spindle assembly checkpoint during mitosis (Liu et al., 1999; Ren et al., 2006; Ren et al., 2011), in carcinogenesis (Lee et al., 2003) and recently also in NF-kB activation (Gong et al., 2010). This multitude of options for FAT10 function urged us to investigate a pivotal question with respect to this search for function, namely to which proteins does FAT10 become covalently linked.

Here, we identified proteins which become endogenously modified with FAT10 in human cells after the stimulation with IFNγ and TNFα. This approach has become possible through the recent generation of the high affinity monoclonal antibody 4F1 which specifically immunoprecipitates monomeric FAT10 as well as FAT10-conjugated substrates (Aichem et al., 2010). We confirmed, what has been suggested earlier in overexpression studies, that a large variety of different substrate proteins become covalently linked to FAT10 (Aichem et al., 2010; Chiu et al., 2007). Of the 571 proteins which we identified in the FAT10 immunoprecipitates of cytokine-induced but not of untreated cells, 176 migrated in the SDS-PAGE at a position which was at least 18 kDa higher than their respective predicted molecular mass. These proteins we classified as ‘putative conjugates’ being
aware that their non-covalent linkage to FAT10 in cytokine-induced cells awaits their confirmation by combined immunoprecipitation and western blot analysis. The residual 395 proteins we classified as putative non-covalently bound interaction partners of FAT10 given that they were found in FAT10 immunoprecipitates in spite of stringent lysis and washing conditions applied during immunoprecipitation. While quite a few of these proteins may not directly interact with FAT10 but may be linked to FAT10 via other proteins, our data collection is nevertheless a precious database for future work on the function of FAT10 especially because many of the proteins in the list were identified in both rounds of immunoprecipitation.

Fig. 5. Endogenous p62 colocalizes with FAT10. HeLa cells were grown on coverslips and stimulated with IFNγ and TNFα to induce FAT10 expression for 20 hours, or left unstimulated. Cells were fixed, permeabilized and stained as indicated with the FAT10-specific mAb 4F1 and the p62-specific pAb H290 and subsequently with the respective Alexa Fluor®-labeled secondary antibody (Fab)(2) fragments. One representative experiment out of three with similar outcome is shown.

Fig. 4. p62 becomes oligo-mono-FAT10ylated, dependent on the diglycine motif of FAT10 and independent of the p62-UBA domain. (A) HEK293T cells were transiently transfected with expression constructs for His-3xFLAG-FAT10 (FLAG-FAT10), His-3xFLAG-FAT10ΔGG (FLAG-FAT10ΔGG), HA-p62 or HA-p62ΔUBA as indicated above the panel to detect p62-FAT10 conjugate formation. After 24 hours, lysates were subjected to western blot analysis using monoclonal anti-FLAG-HRP (M2) or monoclonal anti-HA-HRP (HA-7) antibody as indicated. (B) Immunoprecipitation of His-3xFLAG-FAT10 (FLAG-FAT10) and HA-p62 or its lysine-less mutant HA-p62(K0) from lysates of transiently transfected HEK293T cells using an EZview Red ANTI-FLAG M2 affinity gel. Samples were subsequently subjected to western blot analysis using monoclonal anti-HA-HRP (HA-7) antibody or the monoclonal anti-FLAG (M2) antibody. The lower panels show the expression level of HA-p62, HA-p62(K0) or FLAG-FAT10 in the lysates (load). (C) Total cell extracts of transiently transfected HEK293T cells were used to detect p62-6His conjugate formation with either HA-FAT10 or its lysine-less mutant form HA-FAT10(K0) on a western blot incubated with monoclonal anti-HA-HRP (HA-7) antibody (top). The lower panel shows the p62-6His expression level using monoclonal anti-6His-HRP antibody. One representative experiment out of three with similar outcome is shown.
which we performed (see supplementary material Table S1). In contrast to earlier approaches to identify substrates of ubiquitylation (Peng et al., 2003), sumoylation (Vertegaal et al., 2004; Zhao et al., 2004), and ISGylation (Zhao et al., 2005) our endogenous approach does not rely on overexpression of a tagged modifier which bears the risk of yielding false positives.

When we set out to determine the FAT10 proteome, we were hoping that protein substrates may be revealed which point at one or a few cell biological pathways in which FAT10 is involved. However, as previously found for ubiquitin, SUMO-1,2,3, or ISG15, which all modify hundreds of proteins, we identified also for FAT10 putative conjugation substrates with functions in numerous cell biological pathways (Fig. 2). Merely by inspection of the nature of the putative FAT10 conjugates it is impossible to substantiate or refute one of the pathways in which FAT10 was suggested to be functionally involved. The number of putative FAT10 conjugates and interaction partners found in the cytokine-stimulated human embryonic kidney cell line HEK293 probably understimates the true complexity which can be expected to emerge when cells of other tissue origin would be investigated in the same manner. Apart from analyzing the consequences of ubiquitylation, sumoylation or ISGylation for every single substrate, which is by far not yet accomplished, it has been attempted to extract unifying themes for each modifier. Polyubiquitylation most often leads to proteasomal degradation, sumoylation is often involved in changing the intracellular localization of substrate proteins and ISGylation has recently been suggested to be involved in the functional inactivation of newly synthesized proteins (Durfee et al., 2010). The fact that FAT10 very efficiently targets proteins for proteasomal degradation (Hipp et al., 2005; Schmidtke et al., 2009) opens up the possibility that it may be generally involved in targeting proteins for antigen processing by the proteasome for the production of peptides presented by MHC class I molecules. However, since previously we had only been able to detect one single prominent FAT10 conjugate of 35 kDa in a murine fibroblast line after induction of FAT10 expression (Raasi et al., 2001) we have not pursued this hypothesis for some time. Now, that we have identified a multitude of putative FAT10 conjugates this hypothesis needs to be reinvestigated.

While we have been unable to simultaneously work on many of the putative FAT10 conjugates, we have at first focused our efforts to validate and further investigate p62/Sequestosome-1 as a very interesting substrate of FAT10ylation which appeared in several of the excised gel slices submitted to mass spectrometric analysis (Fig. 1B) which we could assign to derive from non-covalent as well as covalent interactions of FAT10 and p62 (Fig. 3A). We could show that the covalent conjugation of FAT10 leads to proteasomal degradation of endogenous p62-FAT10 conjugates (Fig. 3A) and that overexpression of HA-tagged FAT10 significantly decreased the amount of endogenous FAT10 whereas overexpression of its diglycine mutant HA-FAT10AGG had no significant influence on the amount of endogenous p62 (Fig. 3E). Therefore we suggest, that the covalent interaction of p62 and FAT10 has the function to target p62 to proteasomal degradation, which is a very interesting finding since up to now it was not clear, how p62 turnover is regulated other than by its degradation via autophagy. Since treatment of the cells with the proteasome inhibitor MG132 alone did not increase the amount of endogenous p62 (Fig. 3B) we find it rather unlikely, that degradation of p62 by the proteasome is due to ubiquitylation of p62. We have recently shown that the proteasome subunit Rpn10/S5a is a receptor not only for ubiquitylated proteins but also for FAT10 and that a knockdown of Rpn10/S5a leads to a strong inhibition of FAT10 degradation (Rani et al., 2012). Therefore, we suggest that also FAT10ylated p62 is degraded by the proteasome via binding to Rpn10/S5a.

Mostowy et al. showed that the endogenous p62 protein level increases upon treatment of cells with TNFα and IFNγ, a finding that we could confirm in our experiments (Mostowy et al., 2011). Interestingly, p62 protein levels in HEK293 cells treated for 24 hours with IFNγ/TNFα increased while at the same time FAT10 expression was induced (Fig. 3A,B). Since we show here that a covalent modification of p62 with FAT10 leads to degradation of p62 one would expect to detect a lower p62 level in cytokine-treated cells. To explain this apparent contradictory finding, we performed time course experiments and stimulated HEK293 cells for 0–72 hours with IFNγ and TNFα (Fig. 3C,D). While FAT10 protein in cell lysates was detectable not before 24 hours of cytokine treatment, the p62 protein level increased much earlier. At later time points (32–72 hours) with prolonged and increased FAT10 expression, the p62 protein level decreased again to almost basal level comparable to untreated cells thereby explaining the initial increase of p62 after 24 hours of cytokine treatment. This finding is very interesting since it could mean that early in an innate immune response p62 becomes upregulated to clear pathogens and viruses from cells and later on, FAT10 becomes upregulated to remove the excess of p62 again. Further investigations will be needed to test this hypothesis in more detail.

Specific downregulation of the FAT10-specific E1 and E2 enzymes, UBA6 and USE1, led to a strong reduction in the amount of endogenous p62-FAT10 conjugates but also to a decrease of non-covalently interacting p62 (Fig. 3F). The decrease in the amount of non-covalently interacting p62 in UBA6 or USE1 siRNA-treated cells can be explained by a slightly reduced expression of endogenous FAT10 and p62 in these samples, respectively (Fig. 3F, load). Treatment of the cells with an unspecific control siRNA always considerably increased the amount of FAT10ylated p62 (Fig. 3F, IP), whereas the endogenous protein levels of p62, UBA6, USE1 and FAT10 remained stable at the same time. One explanation could be that control siRNA treatment downregulates a not yet identified de-FAT10ylation enzyme, resulting in higher amounts of FAT10ylated p62. Another possibility would be that control siRNA treatment somehow upregulates or induces the activity of a FAT10-specific E3 ligase, which in turn also would result in a higher amount of FAT10ylated p62. Since both proteins, a de-FAT10ylation enzyme as well as a FAT10-specific E3 ligase responsible for the FAT10ylation of p62, await their identification, this notion will have to be investigated in the future.

Since a large amount of p62 interacted also non-covalently with FAT10, we were interested in investigating the function of this interaction further. FAT10 is functionally linked to HDAC6, a previously investigated interaction partner of FAT10 (Kalveram et al., 2008). In this former project we found striking parallels between ubiquitin and FAT10 in that ubiquitin and FAT10 both were shown to interact with HDAC6 when proteasome activity was inhibited, and both ubiquitin- and FAT10-linked proteins are shuttled via HDAC6 and dynein motors along microtubules
towards the microtubule organizing center where aggresomes are formed (Kawaguchi et al., 2003). For poly-ubiquitylated proteins, it has been shown that they bind to p62 via the C-terminal UBA domain (Seibenhener et al., 2004) and that p62 links them to nascent autophagosomes by binding to LC3/ATG8 via its LIR domain (Pankiv et al., 2007). The identification of p62 as an endogenously expressed conjugate of FAT10 modification lets us suggest that another parallel between ubiquitin and FAT10 may exist along this pathway. Indeed, we could show that p62 and FAT10 colocalize in p62 bodies in HeLa cells (Fig. 5). Therefore, we went one step further and expressed tandem tagged mCherry-EGFP-FAT10 or, as positive controls, mCherry-EGFP-p62 or mCherry-EGFP-4xUbiquitin in HeLa cells to investigate, if FAT10 localizes to acidifying autophagosomal vesicles. Whereas both, mCherry-EGFP-p62 as well as mCherry-EGFP-4xUbiquitin relocated to lysosomes, this was not the case for mCherry-EGFP-FAT10 (supplementary material Fig. S2). As it has been hypothesized by Myeku et al. that only aggregates can be cleared by autophagy but diffuse ubiquitylated proteins cannot, we tested whether the expression of mCherry-EGFP-FAT10 under proteasome impairment would result in autophagosomal targeting (Myeku and Figueiredo-Pereira, 2011). Although we could observe an increase of green bodies suggesting aggregate formation, no significant enhancement of autophagosomal targeting of any of the tested constructs was detected (supplementary material Fig. S3).

On the other hand, p62 might contribute to targeting FAT10 and FAT10-conjugates to proteasomal degradation as it was shown that p62 directly interacts with the proteasome (Seibenhener et al., 2004) and shuttles polyubiquitylated tau to the proteasome (Babu et al., 2005). To investigate this hypothesis we downregulated p62 with specific siRNAs in HEK293 cells and determined the amount of monomeric and conjugated FAT10 by western blot analysis. Interestingly, knockdown of p62 led to a significant accumulation of monomeric FAT10, but not of FAT10 conjugates (supplementary material Fig. S4). However, the overall effect of knocking down p62 on monomeric and conjugated FAT10 was only minor.

Since p62 is, apart from the cognate E2 enzyme USE1 (Aichem et al., 2010), p53 (Li et al., 2011) and UBE1 (Rani et al., 2012), a new confirmed substrate shown to become covalently conjugated FAT10 was only minor.

Plasmids used for transient FAT10 expression were pcDNA3.1-His-3xFLAG-FAT10 (Chiu et al., 2007), or its mutated form pcDNA3.1-His-3xFLAG-FAT10(K0) (Hipp et al., 2005). For expression of HA-tagged p62, pDESTHA-p62 (Lamarck et al., 2003), pDESTHA-p62AUBA (Bjorkoy et al., 2005) or pDESTHA-p62(K0) were used. Expression of 6His-tagged p62 was performed with plasmid pcDNA6-6His-2. For their generation, p62 was PCR amplified and the cDNA was inserted into the EcoRI and BstII sites of pcDNA6/myc-His-A (Invitrogen, Karlsruhe, Germany). pDESTHA-p62(K0) was obtained by mutation of all lysines to arginines using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands). The constructs pmCherry-EGFP-p62 (Pankiv et al., 2007) and pmCherry-EGFP-4xUbiquitin were kindly provided by the group of T. Johansen. For generation of pcDNA3.1mCherry-EGFP, mCherry-EGFP was PCR amplified from pDest-mCherry-EGFP-p62 and the PCR product was inserted into the Nhel and HindIII sites of pcDNA3.1-3xFlag-FAT10. For the generation of pcDNA3.1mCherry-EGFP-Flag-FAT10 the stop codon of pcDNA3.1mCherry-EGFP-3xFlag-FAT10 was eliminated by the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands). All constructs were verified by sequencing (GATC Biotech, Konstanz, Germany).

Immunoprecipitation, detection and mass spectrometry of FAT10 conjugates

Experiments were performed as described recently (Aichem and Grotepurp, 2012). Briefly, for induction of endogenous FAT10 expression, HEK293 cells were cultivated for 6 hours in 200 U/ml IFNγ and 400 U/ml TNFα (both from Peprotech, London, UK). Before harvesting, 10 μM of the proteasome inhibitor MG132 (Calbiochem, Darmstadt, Germany) was added and cells were grown for additional 6 hours. Approximately 8×10⁶ cells were lysed in 8 ml lysis buffer (20 mM Tris-HCl pH 8.0, 650 mM NaCl, 5 mM EDTA, 0.5% Triton X-100) on ice. Lysates were centrifuged for 30 minutes at 3000 rpm to remove insoluble material and clarified supernatants were used for immunoprecipitation using the anti-huFAT10 rabbit polyclonal antibody, cross reactive with the proteasome inhibitor (eBioscience, San Diego, CA). Immunoprecipitation was performed using Protein-A–Sepharose 4B beads at a 1:10 ratio. After 3 hours at 4°C, beads were washed twice with 1 ml NET-TON buffer (50 mM Tris-HCl pH 8.0, 600 mM NaCl, 5 mM EDTA, 0.5% Triton X-100) and twice with 1 ml NET-T buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100) and subsequently separated on NuPAGE® Bis-Tris 4-12% gradient gels (Invitrogen) with 1× MRS running buffer under reducing conditions (10% f-mercaptoethanol). FAT10 conjugates were analyzed by western blotting using an anti-huFAT10 rabbit polyclonal antibody, cross reactive with mFAT10 (Hipp et al., 2005) or directly stained with a standard Coomassie Blue solution or stained with Pierce® silver stain kit (Pierce, Bonn, Germany) as described by the manufacturer. FAT10 conjugate bands were cut out and sent for analysis on a LTQ Orbitrap Hybrid mass spectrometer (Thermo Life Sciences, Erlbsbach, Germany) at the Proteomics Facility of the University of Constance (Germany).

Target verification experiments

HEK293 cells were transfected using FuGene6 (Roche) as described by the manufacturer. After 24 hours, cells were collected and lysed in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100. Immunoprecipitation was performed using 40 μl of EZview® Red Anti-FLAG M2 Affinity Gel (Sigma). Proteins were separated on 12% polyacrylamid SDS gels and subjected to western blot analysis using directly labeled anti-HA-HPR (HA-7) or anti-FLAG-HPR (M2) antibodies.
Confocal microscopy

HeLa cells were grown on coverslips and stimulated with 200 μM IFNγ and 400 μM TNFa for 20 hours. Cells were fixed for 20 min with 4% paraformaldehyde and subsequently permeabilized with 0.2% Triton X-100 for 10 min at room temperature and stained with anti-FAT10 4F1 monoclonal antibody (dilution 1:100) (Aichem et al., 2010) or anti-p62 H290 (dilution 1:100, Santa Cruz, Heidelberg, Germany). Cells were first labeled with the primary antibodies, followed by washing and incubation with the respective Alexa Fluor® labeled secondary antibodies [F(ab)2; dilution 1:400, Invitrogen]. All antibodies were diluted in 0.2% gelatine. All incubations were carried out for 1 h at room temperature. Images were acquired and analyzed with a LSM 510 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany) using a 63× plan-apochromat, oil-immersion objective (NA=1.4).

Spinning disc microscopy

HeLa cells were grown in CELL view™ glass bottom dishes (Greiner bio one, Kremsmünster, Austria) with IMDM glutamax containing 25 mM HEPES. After 42 h of transient transfection (FuGene HD, Roche) cells were incubated with proteasome inhibitor (MG132, 5 μM) for additional 6 hours or left untreated. Live cell microscopy was performed with a Zeiss CellObserver HS microscope equipped with a Zeiss spinning Dics Scanning Unit and a Zeiss Axiocam MRm detector using a 63× objective with temperature control was used for stable live cell conditions. Images were processed using Imaged software. Statistical analysis was performed with GraphPad Prism software (GraphPad, San Diego, CA).

p62 knockdown

siRNA transfection was performed as recently described (Aichem et al., 2010). Briefly, 1.6×10⁶ HEK293 cells were transfected with a pool of four different human p62/SQSTM1-specific siRNAs (HS_SQSTM1_2, HS_SQSTM1_5, HS_SQSTM1_6, HS_SQSTM1_7, Qiagen) or with a control siRNA (dilution 1:100). An incubation chamber with temperature control was used for stable live cell conditions. Images were processed using Imaged software. Statistical analysis was performed with GraphPad Prism software (GraphPad, San Diego, CA).

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