The ubiquitin-like modifier FAT10 – much more than a proteasome-targeting signal

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ABSTRACT

Human leukocyte antigen (HLA)-F adjacent transcript 10 (FAT10) also called ubiquitin D (UBD) is a member of the ubiquitin-like modifier (ULM) family. The *FAT10* gene is localized in the MHC class I locus and FAT10 protein expression is mainly restricted to cells and organs of the immune system. In all other cell types and tissues, FAT10 expression is highly inducible by the pro-inflammatory cytokines interferon (IFN)-γ and tumor necrosis factor (TNF). Besides ubiquitin, FAT10 is the only ULM which directly targets its substrates for degradation by the 26S proteasome. This poses the question as to why two ULMs sharing the proteasome-targeting function have evolved and how they differ from each other. This Review summarizes the current knowledge of the special structure of FAT10 and highlights its differences from ubiquitin. We discuss how these differences might result in differential outcomes concerning proteasomal degradation mechanisms and non-covalent target interactions. Moreover, recent insights about the structural and functional impact of FAT10 interacting with specific non-covalent interaction partners are reviewed.

KEY WORDS: FAT10, Ubiquitin, Ubiquitin-like modifier, Proteasome, VCP, Autophagy, Proteostasis

Introduction

The post-translational modification of proteins with ubiquitin or ubiquitin-like modifiers (ULMs) is a central mechanism to regulate the stability, cellular localization or function of such modified proteins, and also to regulate protein–protein interactions. In case of ubiquitin, the outcome for the modified protein is strongly dependent on the way how ubiquitin is conjugated to the substrate. Ubiquitin molecules linked via their N-terminal methionine α-amino groups form linear chains (M1-chains) and play an important role in the regulation of NF-κB signaling (Ikeda et al., 2011; Iwai, 2011; Rittinger and Ikeda, 2017). While it is still under debate whether modification of proteins with one single ubiquitin is sufficient to guide them to proteasomal degradation, the predominant proteasome-targeting signals are ubiquitin chains (Ding et al., 2019; Lu et al., 2015; Shabek et al., 2012; Thrower et al., 2000). Depending on which of the seven lysine residues within ubiquitin (at positions 6, 11, 27, 29, 33, 48, and 63) are used for chain elongation, different polyubiquitin-linkage types with different functions arise. With the exception of Lys-63 chains, all other ubiquitin chain types guide substrates to degradation by the 26S proteasome (Xu et al., 2009), a 2.5 MDa barrel-shaped multi-subunit complex consisting of a 20S cylindrical core particle and one or two 19S regulatory particles

(see Box 1) (Groll et al., 1997; Huber et al., 2012). Besides ubiquitin, several ULMs have been described, such as human leukocyte antigen (HLA)-F adjacent transcript 10 (FAT10; also known as UBD), small-ubiquitin-like modifier (SUMO) 1, SUMO2 and SUMO3, interferon-stimulated gene 15 (ISG15), neural precursor cell expressed developmentally downregulated 8 (NEDD8), ubiquitin-fold modifier protein 1 (UFM1) and ubiquitin-related modifier 1 (URM1), as well as the autophagy related modifiers ATG8 (LC3 family in mammals) and ATG12 (Albert et al., 2018; Cappadocia and Lima, 2018; Dzimianski et al., 2019; Flotho and Melchior, 2013; Mizushima, 2019; Pichler et al., 2017; van der Veen and Ploegh, 2012). For most modifiers, the function of these modifications is not direct proteasomal degradation, and instead, for example, in the case of SUMO proteins, results in changes in the activity or localization of the target proteins (Flotho and Melchior, 2013). While FAT10 expression is induced by the type II interferon IFNγ in combination with TNF, the structurally very similar UBL modifier ISG15 is inducible with type I interferons IFNα and IFNβ and plays a role in the anti-viral immune response (Durfee et al., 2010; Lenschow et al., 2007). As for FAT10, ISG15 is composed of two UBL domains, connected by a short linker (Haas et al., 1987; Loeb and Haas, 1992; Narasimhan et al., 2005). Although structurally both modifiers appear to be related this notion is not fully supported from an evolutionary point of view. FAT10 is expressed in mammals only, whereas ISG15 is found in earlier vertebrates (e.g. fish). The nearest relative of ubiquitin is NEDD8 (also known as Rub1), followed by FAT10 and ISG15, which are most closely related to each other (Cajee et al., 2012). However, they largely differ in their surface-charge distribution (Aichem et al., 2018; Narasimhan et al., 2005). In addition, the N- and C-terminal UBL domains of both modifiers are not found in the same branch of an evolutionary tree, suggesting that they probably have evolved independently from each other (Cajee et al., 2012). These differences are also reflected by their very different function in that FAT10 does serve as a proteasomal degradation signal whereas ISG15 does not.

Common to all ULMs is the conserved three-dimensional (3D) structure of ubiquitin, the so called β-grasp fold (Vijay-Kumar et al., 1987). Similarly, the C-terminal diglycine motif is common to most UBLs, except for ATG8, ATG12 and UFM1, which possess a single C-terminal glycine (Ichimura et al., 2000; Komatsu et al., 2004; Mizushima et al., 1998). The diglycine motif in ubiquitin and FAT10 are necessary for activation and covalent attachment to substrates by enzymatic cascades involving E1, E2 and E3 enzymes (Ciechanover et al., 1982; Handley et al., 1991; Hochstrasser, 2009; Huibregtse et al., 1995; Jin et al., 2007; Lorick et al., 1999; Pelzer et al., 2007; Pickart and Vella, 1988).

The *FAT10* gene was identified in 1996 by genomic sequencing of the human major histocompatibility complex (MHC) class I locus (Fan et al., 1996). The 18.3 kDa FAT10 protein has a special structure because it is composed of two UBL domains, which are connected by a short, flexible linker of five amino acids (Fig. 1A).

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Box 1. The 26S proteasome
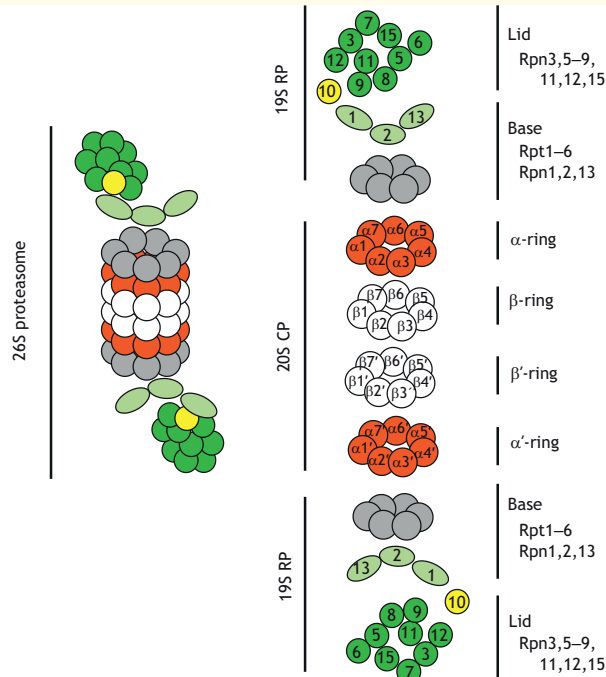
The 26S proteasome is the major protease in eukaryotic cells and is responsible for regulated ATP-dependent degradation of cytosolic, as well as nuclear, proteins in order to maintain cellular homeostasis. It has a size of 2.5 MDa and consists of a barrel-shaped 20S core particle (20S CP), and one or two regulatory particles (19S RP, PA700), which are required for binding and unfolding of ubiquitylated proteins, thus regulating the entry of substrates (Groll et al., 1997; Huber et al., 2012) (see figure). The proteolytic activity of the holoenzyme lies within the 20S CP and is accessible only for unfolded proteins through a narrow axial pore (Bard et al., 2018; Groll et al., 2000). The CP is built of four heptameric rings, two outer rings, each with seven α-subunits and two inner rings, each with seven β-subunits (see figure). The inner rings include the three catalytically active subunits characterized by N-terminal threonine residues acting as active-site nucleophiles with different cleavage specificities; β1 (PSMB6, Y, δ) with caspase-like activity for cleaving after acidic amino acids, β2 (PSMB7, Z, MC14) with a trypsin-like activity for cleavage after basic amino acids, and β5 (PSMB5, X, MB1) with a chymotrypsin-like activity for cleavage after hydrophobic residues (Groll et al., 1997; Huber et al., 2012). Under inflammatory conditions in the presence of IFNγ and TNF, these three subunits are exchanged to low-molecular-mass peptide 2 (LMP2, β1i), multicatalytic endopeptidase complex like-1 (MECL-1, β2i) and LMP7 (β5i), respectively, giving rise to the 20S immunoproteasome (Griffin et al., 1998); it has a lower caspase-like activity and a higher chymotrypsin-like activity, resulting in the generation of different subsets of MHC I ligands (Gaczynska et al., 1994). The 19S regulatory particle consists of a base and a lid. The base is composed of six ATPases (Rpt1–Rpt6) and the three non-ATPases Rpn1 (named S2 in humans), Rpn2 (S1) and Rpn13 (Adrm1), of which Rpn1 and Rpn13 function as binding sites for ubiquitin and ubiquitin-like proteins. The lid is composed of nine structurally diverse subunits (Glickman et al., 1998; He et al., 2012; Husnjak et al., 2008; Shi et al., 2016) (see figure). Rpn10 (S5a) lies at the interface of lid and base and binds ubiquitin and FAT10 (Deveraux et al., 1994; Glickman et al., 1998; Rani et al., 2012; van Nocker et al., 1996). In addition, several soluble ubiquitin receptors, which only interact transiently with the proteasomal subunits Rpn1, Rpn2 or Rpn10, are known, such as Rad23, Dsk2 and Ddi1 (Elsasser et al., 2004; Funakoshi et al., 2002; Saeki et al., 2002; Schauber et al., 1998; Verma et al., 2004). Nub1L, a soluble proteasome receptor for FAT10 interacts with both, Rpn10 and Rpn1 and facilitates enhanced degradation of FAT10 and FAT10ylated proteins (Hipp et al., 2004; Rani et al., 2012).

The N- and C-terminal UBL domains share an amino acid sequence identity of 29% and 36% with ubiquitin, respectively (Aichem et al., 2018; Fan et al., 1996; Theng et al., 2014). In contrast to ubiquitin, which is ubiquitously expressed, and in line with the location of the *FAT10* gene within the MHC, a basal constitutive FAT10 protein expression is restricted to cells and organs of the immune system, such as thymus, spleen or lymph nodes (Lukasiak et al., 2008) where it displays immune-system-specific functions (see Box 2). Other cell types and tissues do not constitutively express FAT10; however, upon exposure to the pro-inflammatory cytokines interferon (IFN)-γ and tumor necrosis factor (TNF), a strong synergistic upregulation of FAT10 mRNA and protein is observed (Choi et al., 2014; Liu et al., 1999; Mah et al., 2019; Raasi et al., 1999). This is in line with earlier findings of TNF and IFNγ-inducible genes encoded within the MHC class I locus, all of which play a role in antigen processing (Raval et al., 1998; Wallach et al., 1982). In HEK293 cells, the FAT10 mRNA expression is detectable after 4–5 h, whereas FAT10 protein expression can be detected by western blot analysis after 24 h of cytokine treatment (Aichem et al., 2012). In addition, FAT10 expression is highly upregulated in more than a dozen different cancer types (Lee et al., 2003; Lukasiak et al., 2008), and is further enhanced by the inflammatory microenvironment of the tumors (Lukasiak et al., 2008). In cancer cells, FAT10 expression is described to have a positive impact on cell proliferation and migration and to promote invasion and metastasis formation (Gao et al., 2015, 2014; Lee et al., 2003; Li et al., 2018; Lim et al., 2006; Liu et al., 2014, 1999; Lukasiak et al., 2008; Theng et al., 2014; Yuan et al., 2014; Zhao et al., 2015; Zou et al., 2018).

Compared to other UBL modifiers, the proteome of FAT10-conjugation substrates has the smallest overlap with that of ubiquitin (Merbl et al., 2013), and dozens of covalent conjugation substrates as well as non-covalent interaction partners have been identified by independent mass spectrometry analyses performed under non-denaturing conditions (Aichem et al., 2012; Leng et al., 2014). However, only a few covalent FAT10-modified substrates have been fully confirmed until now, including the autophagy receptor p62 (also known as SQSTM1) (Aichem et al., 2012), proliferating cell nuclear antigen (PCNA), which plays an important role in DNA damage repair (Chen et al., 2018), the deubiquitylating enzyme otubain 1 (OTUB1) (Bialas et al., 2019) and the transcription factor JunB (Aichem et al., 2019). The E1 activating and E2 conjugating enzymes, which activate and finally link FAT10 via an isopeptide linkage to an internal lysine residue of the substrate (referred to as 'FAT10ylation'), are the ubiquitin- and FAT10-specific enzymes UBA6 (also known as UBE1L2 or MOP4) (Chiu et al., 2007; Pelzer et al., 2007) and USE1 (also known as UBE2z) (Aichem et al., 2010; Jin et al., 2007), respectively, whereas putative E3 ligases still await identification (Fig. 1B). A prevalent question in the field is why there is the need for a second transferable tag for proteasomal degradation in addition to ubiquitin, and what distinguishes these two modifiers in this respect. The unique expression profile of FAT10 in cells of the immune system and its upregulation under inflammatory conditions already suggests that FAT10 fulfills specific functions within the immune system. Besides this notion, the recent resolution of the 3D structure of FAT10 (Aichem et al., 2018) has provided interesting structural insights that allow us to answer several open questions. This Review will outline the most important differences to ubiquitin-mediated proteasomal degradation, as well as the non-covalent interactions FAT10 undergoes with its specific binding partners. Moreover, the functional outcome of these non-covalent interactions will be highlighted.
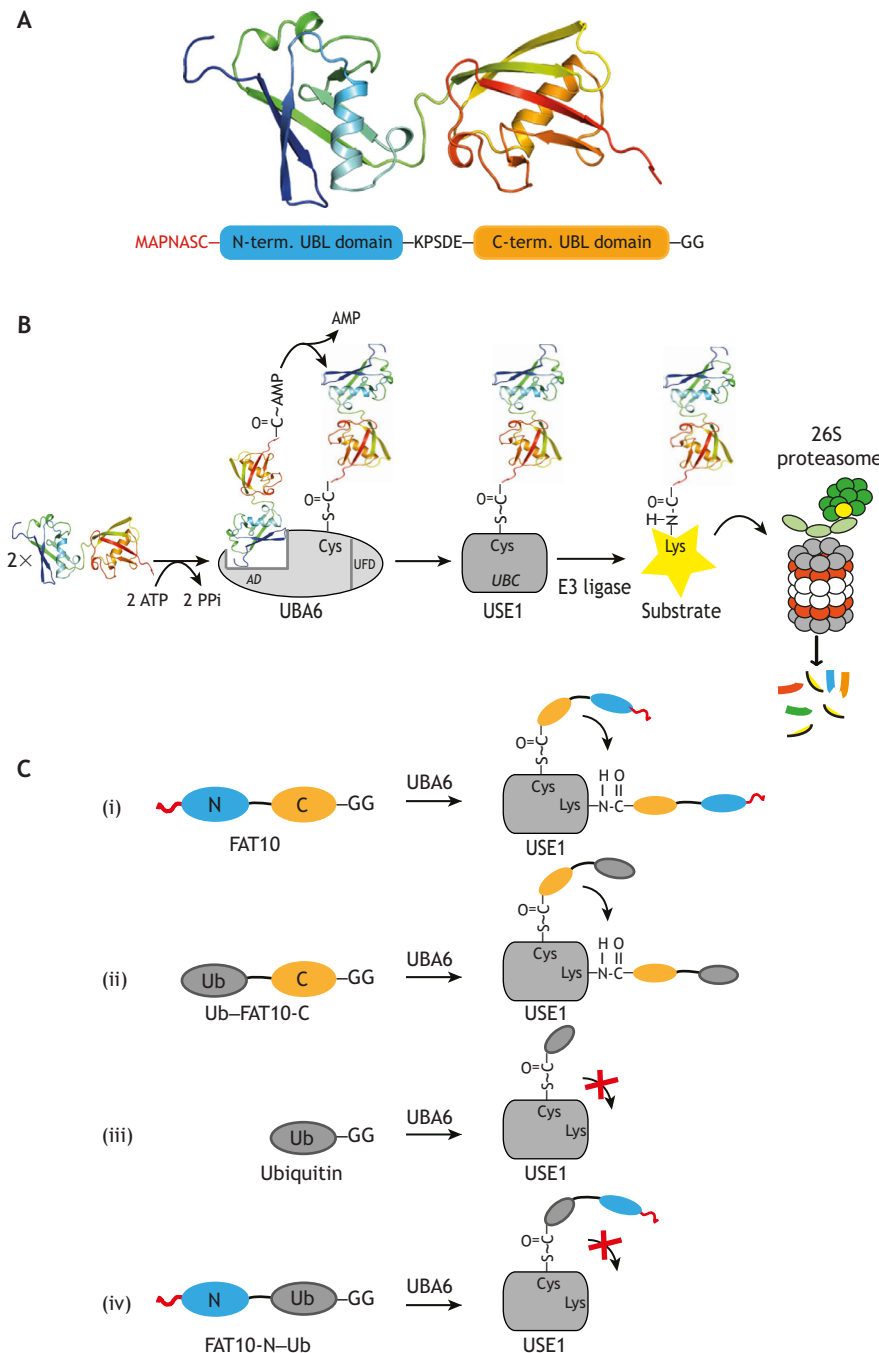
Fig. 1. The structure and conjugation cascade of FAT10. (A) Ribbon representation of the 3D structure of human FAT10 [kindly provided by our cooperation partner Silke Wiesner, University of Regensburg, Germany; PDB codes 6GF1 and 6GF2 (Aichem et al., 2018)]. The unstructured N-terminal extension (MAPNASC), the linker sequence (KPSDE) as well as the C-terminal diglycine motif (-GG) are highlighted. The N-terminal ubiquitin-like (UBL) domain is shown in blue, the C-terminal UBL domain in orange. (B) FAT10ylation is an ATP-consuming process that involves the likely action of three enzymes, namely the E1 activating enzyme UBA6, the E2 conjugating enzyme USE1 and yet unidentified E3 ligase(s), which work sequentially in a cascade. Briefly, FAT10 is activated by non-covalently interacting with its C-terminal UBL domain with the adenylation domain (AD) of UBA6, which uses ATP to form a high energy FAT10-AMP intermediate. In a next step, a thioester linkage between the carboxyl group of the C-terminal glycine of FAT10 and the active-site cysteine of UBA6 is formed upon the nucleophilic attack of the active-site cysteine on the FAT10-AMP intermediate, with the release of AMP. In a subsequent transthiolation reaction, FAT10 is transferred onto the active-site cysteine of USE1, where again a thioester is formed. As final step, probable E3 ligases catalyze the covalent isopeptide linkage of FAT10 to an ε-amino group of an internal lysine residue of a substrate. Covalently FAT10-modified proteins are targeted together with FAT10 for degradation by the 26S proteasome. AD, adenylation domain; UFD, ubiquitin-fold domain; UBC, ubiquitin conjugating domain; PPi, free pyrophosphate. (C) Covalent auto-modification of USE1 is performed by formation of an isopeptide linkage between the C-terminal glycine of UBA6-activated FAT10 (i), or a ubiquitin–FAT10-C fusion protein (Ub–FAT10-C) (ii) and an internal lysine residue of USE1. No USE1 auto-modification is observed for ubiquitin (Ub) (iii), or a FAT10-N–Ub fusion protein (iv).

What the structure of FAT10 tells us – it is not just a 'di-ubiquitin'

In contrast to ubiquitin, FAT10 has a relatively short half-life in cells of ∼1 h (Aichem et al., 2014; Hipp et al., 2005). This is primarily based on the fact that ubiquitin is recycled at the proteasome by the action of deubiquitylating enzymes (DUBs), which enzymatically remove ubiquitin prior to the degradation of the substrate (Reyes-Turcu et al., 2009). In case of FAT10, no such deconjugating enzymes have been identified to date, and FAT10 appears to be degraded along with its substrates by the proteasome (Aichem et al., 2014; Hipp et al., 2005; Schmidtke et al., 2006). Moreover, the interaction of FAT10 with its non-covalent interaction partner the long isoform of NEDD8-ultimate buster 1 (Nub1L) accelerates the degradation rate of FAT10 by about eight-fold (Hipp et al., 2004;

Schmidtke et al., 2009). However, the differences in the stability of the two modifiers also rely on their structure. As noted above, FAT10 is composed of two tethered UBL domains, which both possess the typical ubiquitin-like β-grasp fold (Fig. 1A) (Aichem et al., 2018; Fan et al., 1996; Theng et al., 2014). Previous attempts to purify FAT10 in high concentrations in order to determine its 3D structure were not very successful because of the poor solubility of FAT10, which most probably can be attributed to intramolecular and intermolecular disulfide bridge formations between the four cysteine residues of FAT10 (Aichem et al., 2018, 2014). However, by using a mutated form of FAT10, in which all cysteine residues except Cys-134, were mutated to serine residues, we were able to determine the 3D structure of FAT10 (Aichem et al., 2018). By further mutating Cys-134 to a leucine [FAT10(C0)-C134L], the

Box 2. Roles of FAT10 in the immune system

FAT10 is part of the immune system and functions in both adaptive and innate immune responses. For instance in MHC-class I peptide presentation, linkage of FAT10 to antigens affects the MHC class I processing pathway by several means: (1) the presence of FAT10 leads to an altered peptide presentation on MHC class I molecules in medullary thymic epithelial cells (mTECs), which have an important role in thymic T-cell selection (Buerger et al., 2015); (2) MHC class I presentation of the human cytomegalovirus (HCMV)-derived antigen pp65 is enhanced by about two-fold in HeLa cells when FAT10 is N-terminally fused to pp65 (Ebstein et al., 2012); (3) the degradation rate, as well as the presentation, of specific lymphocyte choriomeningitis virus (LCMV) epitopes on MHC class I molecules is enhanced when FAT10 is fused to the N-terminus of the LCMV nucleoprotein (Schliehe et al., 2012). However, a selective cooperation of FAT10 with the similarly MHC-encoded and antigen-processing-related immunoproteasome has not been observed (Schmidtke et al., 2019). In addition, FAT10 may function in autophagy as it interacts covalently and non-covalently with the autophagosomal receptor p62, and localizes together with p62 in so-called p62 bodies (Aichem et al., 2012). An involvement of FAT10 in MHC class II presentation to CD4$^+$ T cells has also been suggested (Buerger et al., 2015). While most of the MHC class II non-self-peptides are delivered by endocytosis, a substantial portion of class II peptides originates from cytosolic or nuclear antigens, and these are degraded by autophagy (Münz, 2012). The high FAT10 expression in thymic medullary epithelial cells supports this idea as in the thymus, autophagy is essential in mediating tolerance of CD4$^+$ T cells (Buerger et al., 2015).

Besides its role in adaptive immunity, FAT10 also impacts innate immune responses. For example, (1) FAT10 is conjugated to autophagy-targeted cytoplasmic *Salmonella Typhimurium* bacteria in mice and promotes resistance to *Salmonella* (Spinnenhirn et al., 2014); (2) FAT10 downregulates type-I interferon expression during an anti-viral immune response (Nguyen et al., 2016; Wang et al., 2019); (3) FAT10 is necessary for a normal type II interferon secretion by activated CD8$^+$ T cells in LCMV-infected mice by fine-tuning the balance between type I and type II interferons produced during an LCMV infection. Here, FAT10 lowers the production of interferon-α and -β while it enhances the production of interferon-γ (Mah et al., 2019). The FAT10-mediated attenuation of type I interferon production has been shown to lead to enhanced replication of influenza A virus *in vitro* (Zhang et al., 2016).

C-terminal UBL domain could finally be stabilized, as this domain was still heavily unstructured in the FAT10(C0) mutant (Aichem et al., 2018). The obtained nuclear magnetic resonance (NMR) and crystal structure data now show that the FAT10 N- and C-terminal UBL domains are folded in a very similar manner to ubiquitin; however, they are much less stable than ubiquitin and move independently of each other. Furthermore, determination of the melting temperature revealed that wild-type FAT10 and FAT10(C0)-C134L melt at 41°C and 47°C, respectively, while ubiquitin is very stable and melts at 83°C (Aichem et al., 2018), providing another rationale for the poor solubility of FAT10 (Aichem et al., 2018).

Nevertheless, it is not fully understood why FAT10 has evolved as a di-ubiquitin-like protein containing a linker. The determination of the 3D structure confined the position of the flexible linker to amino acids 82–86 (KPSDE) (Fig. 1A), and our functional studies of the linker revealed that it is indispensable for FAT10 activation and conjugation to its substrates (Fig. 1B) (Aichem et al., 2018). In fact, while UBA6, which is specific to both ubiquitin and FAT10, is able to activate a recombinant linear di-ubiquitin under *in vitro* conditions (Aichem et al., 2014), this does not occur for a FAT10 variant that lacks the entire linker, thus resembling a linear di-ubiquitin. This points to a specific function of the linker region during the activation

process mediated by UBA6. Moreover, in case of all FAT10 linker mutants we have tested (i.e. KPSDE linker sequence mutated to either five proline, five glycine or five alanine residue), no auto-FAT10ylation of USE1, the cognate E2-conjugating enzyme for FAT10, was observed (Aichem et al., 2018, 2014, 2010). Auto-modification of USE1 normally takes place upon UBA6-mediated activation and loading of FAT10 onto Cys-188 in the active-site of USE1, but not upon activation and transfer of ubiquitin (Fig. 1C) (Aichem et al., 2010). Experiments with FAT10 C-terminal mutants (Schelpe et al., 2016), as well as artificial FAT10-N-domain–ubiquitin or ubiquitin–FAT10-C-domain fusion proteins (Fig. 1C) (Aichem et al., 2018) have shown that the very C-terminal part of FAT10 is indispensable for USE1 to distinguish between the two modifiers. In detail, when ubiquitin was N-terminally fused to the C-terminal FAT10 UBL domain (ubiquitin–FAT10-C-domain fusion; see Fig. 1C), the fusion protein behaved like wild-type FAT10, resulting in USE1 auto-modification. However, when ubiquitin was fused to the N-terminal UBL domain of FAT10 (FAT10-N-domain–ubiquitin fusion), thus creating a FAT10–ubiquitin hybrid protein with a ubiquitin C-terminus, USE1 was not auto-modified (Aichem et al., 2018), as earlier described for wild-type ubiquitin (see Fig. 1C) (Aichem et al., 2010). Moreover, mutations of the FAT10 C-terminal sequence (CYCIGG) that generate a C-terminus similar to ubiquitin (LRLRGG) also result in a FAT10 variant that behaves like ubiquitin, as this mutant, but not wild-type FAT10, can be activated by the ubiquitin E1 UBE1 (also named UBA1) (Schelpe et al., 2016). Thus, the very C-terminus of FAT10 and ubiquitin appears to be essential for being recognized and distinguished by the E1 and E2 conjugating enzymes UBE1, UBA6 and USE1. These data (Schelpe et al., 2016) also confirm previous work showing that UBA6 has a higher affinity for FAT10, but shows a faster transthiolation reaction for ubiquitin, meaning that the transfer of ubiquitin from the active-site cysteine residue of UBA6 onto the active-site cysteine residue of USE1 is faster for ubiquitin than for FAT10 (Fig. 1B) (Gavin et al., 2012), with the latter relying on the FAT10 C-terminal sequence CYCIGG (Schelpe et al., 2016).
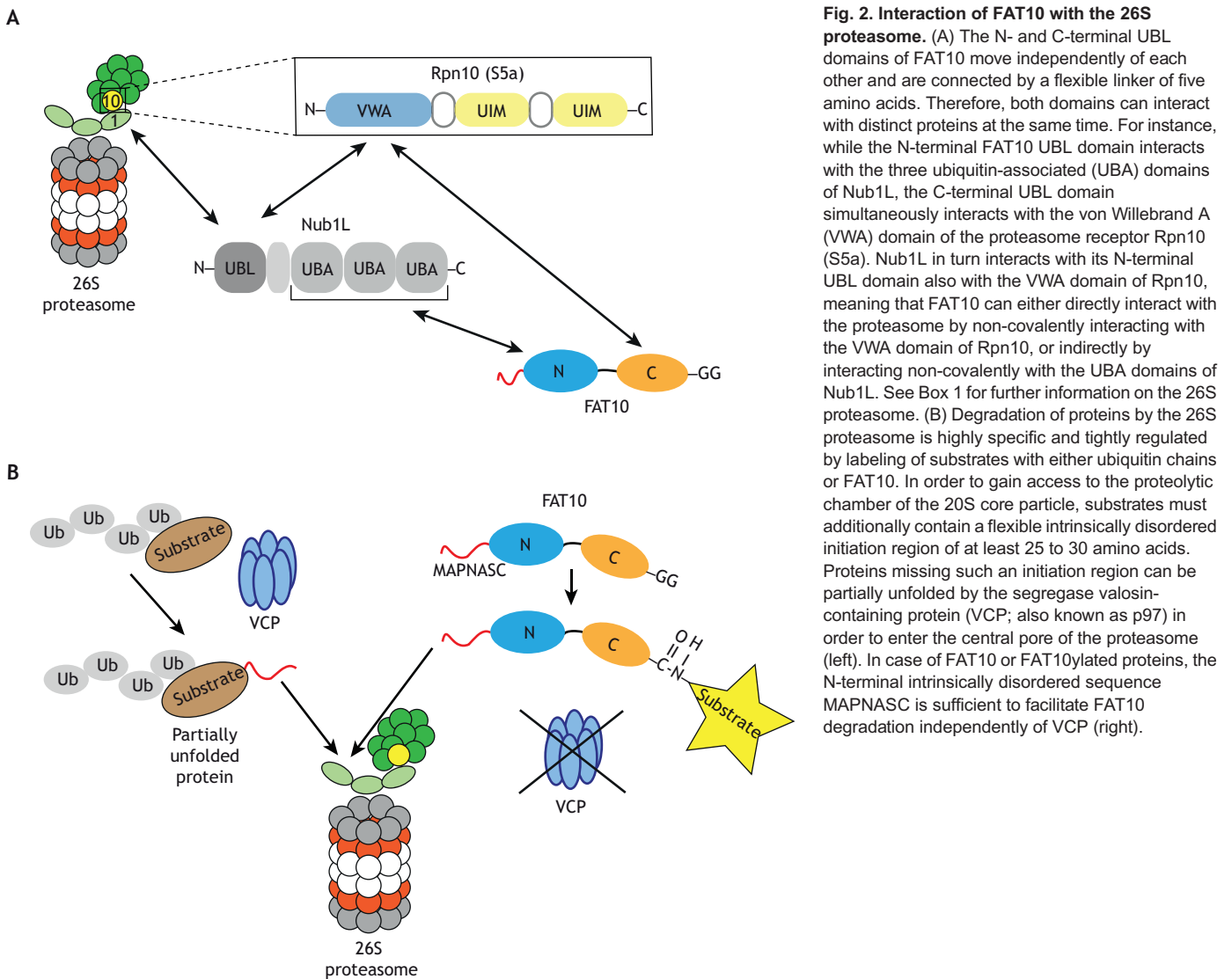
In summary, although FAT10 and ubiquitin can be activated and transferred by the same E1 and E2 enzymes (UBA6 and USE1), there are striking differences in the affinity of the two modifiers for the E1 and E2 enzymes, their activation kinetics and their transfer rates, and this is most probably based on differences in their structures and surface charges.

Proteasomal degradation of FAT10 and its independency of VCP/p97

The best-investigated function of FAT10 is that it targets proteins for degradation by the 26S proteasome, with FAT10 being degraded along with its substrates (Hipp et al., 2005). Interestingly, FAT10 itself does not need to become ubiquitylated as degradation of a lysine-less FAT10 mutant (FAT10-K0) is as fast as that of wild-type FAT10 (Schmidtke et al., 2014, 2009). In contrast to ubiquitin, in which a Lys-48 tetramer represents an optimal proteasomal-targeting signal (Ding et al., 2019; Thrower et al., 2000), thus far, there are no indications for FAT10 chain formation, except for the observation that some FAT10 substrates become at least multi-monoFAT10ylated, as for example the autophagy receptor p62 (Aichem et al., 2012). Thus, modification of a substrate with a single FAT10 moiety appears to be sufficient to target the substrate for proteasomal degradation. Also, in contrast to ubiquitin, no FAT10-specific de-conjugating enzymes, which might be able to recycle FAT10, have been identified to date, and the degradation rate of monomeric FAT10 is the same as that of the bulk of FAT10ylated substrates (Aichem et al., 2014; Hipp et al., 2005).

Arguing against the existence of FAT10-specific deconjugating enzymes is the fact that FAT10, in contrast to other UBL modifiers, is expressed as mature protein that contains a free diglycine at its C-terminus and therefore does not require proteolytic activation at the C-terminus (Fan et al., 1996). Nonetheless, as long as the opposite is not proven, we cannot exclude that FAT10-deconjugating enzymes exist, which might have specific roles in certain cellular processes to fine-tune the stability of specific proteins. Active site probes have been successfully used in the past to identify new conjugating as well as deconjugating enzymes. For this strategy, the UBL modifier is chemically linked at its C-terminus to a chemical group (designated a 'warhead'), such as dehydroalanine (Dha) for identification of active-site cysteine-containing enzymes of the conjugation cascade, or to vinyl sulfone (VS), bromoethylamine (BEA) or propargylamine (PA), for the identification of deconjugating enzymes (Borodovsky et al., 2001, 2002; Cravatt et al., 2008; Hewings et al., 2017; Mulder et al., 2016). Most deconjugating enzymes are cysteine proteases and are well suited for being identified by activity-based probes. Deconjugating enzymes attack the probes and become covalently linked to the modifier, allowing a subsequent immunoprecipitation and mass spectrometric analysis of the trapped enzymes. The generation of FAT10-based activity probes containing such chemical warheads might help to definitely answer this question.

There are two possibilities for how FAT10 can interact with the proteasome in order to be degraded. Either FAT10 directly binds, through its C-terminal UBL domain, to the von Willebrand A (VWA) domain of the Rpn10 (S5a) subunit of the proteasome (Box 1; Fig. 2A) (Rani et al., 2012), or it interacts through its N-terminal UBL domain with the ubiquitin-associated (UBA) domains of Nub1L (Fig. 2A), which acts as a soluble receptor and transports FAT10 and FAT10ylated proteins to the proteasome. There, Nub1L binds with its UBA domains to the VWA domain of Rpn10, or to Rpn1 (Box 1; Fig. 2A) (Rani et al., 2012). The interaction of FAT10 with Nub1L leads to an acceleration of the degradation rate of FAT10 and FAT10ylated proteins by about eight-fold as compared to the situation when Nub1L is not expressed (Schmidtke et al., 2006). Besides the VWA domain, Rpn10 contains two ubiquitin-interacting motifs (UIMs). Interestingly, while ubiquitin binds – as expected – to the Rpn10 UIMs, FAT10 interacts only with the VWA domain, but not the UIMs (Fig. 2A) (Rani et al., 2012). A closer look at the FAT10 structure now explains this discrepancy; the two modifiers possess distinct hydrophobic and electrostatic surface landscapes, which enable them to interact with different types of interaction motifs (Aichem et al., 2018). While ubiquitin binds most 'readers' through its hydrophobic patch (Leu-8, Ile-44 and Val-70), this patch is not

Fig. 2. Interaction of FAT10 with the 26S proteasome. (A) The N- and C-terminal UBL domains of FAT10 move independently of each other and are connected by a flexible linker of five amino acids. Therefore, both domains can interact with distinct proteins at the same time. For instance, while the N-terminal FAT10 UBL domain interacts with the three ubiquitin-associated (UBA) domains of Nub1L, the C-terminal UBL domain simultaneously interacts with the von Willebrand A (VWA) domain of the proteasome receptor Rpn10 (S5a). Nub1L in turn interacts with its N-terminal UBL domain also with the VWA domain of Rpn10, meaning that FAT10 can either directly interact with the proteasome by non-covalently interacting with the VWA domain of Rpn10, or indirectly by interacting non-covalently with the UBA domains of Nub1L. See Box 1 for further information on the 26S proteasome. (B) Degradation of proteins by the 26S proteasome is highly specific and tightly regulated by labeling of substrates with either ubiquitin chains or FAT10. In order to gain access to the proteolytic chamber of the 20S core particle, substrates must additionally contain a flexible intrinsically disordered initiation region of at least 25 to 30 amino acids. Proteins missing such an initiation region can be partially unfolded by the segregase valosin-containing protein (VCP; also known as p97) in order to enter the central pore of the proteasome (left). In case of FAT10 or FAT10ylated proteins, the N-terminal intrinsically disordered sequence MAPNASC is sufficient to facilitate FAT10 degradation independently of VCP (right).

conserved in neither the FAT10 N- (Glu-15, Leu-51 and Lys-79) nor the C-terminal (Ser-95, Thr-133 and Ala-159) UBL domain (Aichem et al., 2018). Moreover, since the two FAT10 UBLs are structurally independent of each other, FAT10 can simultaneously interact with different binding motifs present on two different interaction partners, for example the UBA domain of Nub1L and the VWA domain of Rpn10 at the proteasome (Fig. 2A) (Rani et al., 2012). In this context, the flexible linker joining the FAT10 UBL domains might provide the necessary flexibility for the formation of such trimeric complexes.

Ubiquitin-dependent substrate degradation by the 26S proteasome does not only rely on a covalently attached ubiquitin chain, but the substrate must additionally be partially unfolded in order to gain access to the ATPase ring of the proteasome (see Box 1). Such unfolding is performed by a discrete multimeric complex, the segregase valosing-containing protein (VCP; also known as p97), a member of the AAA-ATPase family, which partially loosens tightly folded substrates to facilitate their binding and entry into the proteasome (Fig. 2B) (Gödderz et al., 2015; Matyskiela et al., 2013; Olszewski et al., 2019; Schweitzer et al., 2016). An earlier study has shown that the dependency of a substrate on the unfolding activity of VCP can be bypassed when it contains an unstructured C-terminal extension of at least 20 amino acids (Gödderz et al., 2015). This has also been shown for a naturally occurring protein containing such a native degron, ornithine decarboxylase (Takeuchi et al., 2007), and appears also to be valid for FAT10. Ubiquitin adopts a highly compact fold, whereas FAT10 is much more loosely folded and contains an N-terminal unstructured extension of seven amino acids (amino acids MAPNASC) (Fig. 1A) (Aichem et al., 2018). Although the degradation of FAT10 and FAT10ylated proteins is unaffected by the presence of specific inhibitors of VCP, their degradation becomes completely dependent on the unfolding activity of VCP when the intrinsically disordered N-terminus of FAT10 that is localized outside the N-terminal UBL domain is missing (FAT10-ΔMAPNASC) (Aichem et al., 2018). Thus, proteasomal degradation mediated by covalent attachment of a single FAT10 molecule is entirely independent of ubiquitin and the segregase VCP and thus represents an alternative degradation mechanism in mammals (Fig. 2B).

What remains to be answered is why there is the need for such a fast degradation of FAT10? As FAT10 is highly upregulated during inflammatory processes, one might speculate that the fast degradation of FAT10ylated substrates, as well as of FAT10 itself, might be a regulatory mechanism to prevent or to limit excessive inflammatory reactions, which might result in tissue damage or apoptosis. In fact, it has been reported that overexpression of FAT10 in HeLa and in renal tubular epithelial cells induces apoptosis (Raasi et al., 2001; Ross et al., 2006). Thus, the fast upregulation of FAT10 combined with the short half-live of FAT10 might provide a tool for a prompt but time-limited response of a cell to handle eventually harmful factors during inflammatory processes. One alternative explanation, namely that some specific proteins can only be conjugated and removed by FAT10 but not ubiquitin, has no evidence up to date because all confirmed FAT10 substrates, such as p53, JunB and p62 additionally become ubiquitylated (Aichem et al., 2012; 2019; Fang and Kerppola, 2004; Li et al., 2011; Peng et al., 2017; Scheffner et al., 1993).

Effects of FAT10 beyond proteasomal degradation

Ubiquitin and SUMO interact non-covalently with specific conserved motifs found on the surface of their respective interaction partners, such as UIMs (Hofmann and Falquet, 2001;

Young et al., 1998), or SUMO-interacting motifs (SIMs) (Minty et al., 2000). Although the structure of FAT10 has been resolved, no substrate-specific FAT10-interacting consensus motif that might mediate the non-covalent binding of FAT10 has been defined so far. This is quite astonishing considering that, although for several confirmed FAT10-conjugation substrates, such as UBE1, p62, translation elongation factor 1A1, JunB, OTUB1, PCNA and p53, the outcome is proteasomal degradation (Aichem et al., 2012; Aichem et al., 2019; Bialas et al., 2019; Bialas et al., 2015; Chen et al., 2018; Li et al., 2011; Rani et al., 2012; Yu et al., 2012), in most of these studies a non-covalent interaction of FAT10 with the substrate was also observed, but not always further investigated (see overview in Table 1). Even more remarkable is the fact that the proportion of a given substrate that becomes FAT10ylated in cells is always very low (5–10% as estimated from western blot data), leaving most of the substrate unmodified. However, out of this unmodified substrate pool, a considerable amount interacts non-covalently with FAT10 (Aichem et al., 2019; Bialas et al., 2015). This observation should not be overlooked because it suggests that additional functions of FAT10 prevail besides targeting of proteins to the 26S proteasome.

In fact, two decades ago, FAT10 had already been shown to interact non-covalently through its N-terminal UBL domain with mitotic arrest deficient 2 (MAD2; also known as MAD2L1) (Liu et al., 1999). MAD2 functionally serves as a mitotic spindle checkpoint protein that binds to the kinetochores of sister chromatids in the metaphase of the cell cycle and ensures the correct attachment of the chromosomes to the mitotic spindle along the metaphase plate before onset of anaphase (Li and Benezra, 1996). It was shown that FAT10 binds to free MAD2 and prevents it from localizing to kinetochores, resulting in incorrect separation of sister chromatids. As a result, multinucleated cells with abnormal nuclear morphology and long, incompletely condensed chromosomes are formed (Liu et al., 1999; Ren et al., 2006, 2011). These effects could be reversed when a FAT10 mutant deficient in MAD2 binding was expressed, further confirming the important role of this non-covalent interaction in the development of cancer-related chromosomal instability (Theng et al., 2014).

Furthermore, FAT10 has been described to stabilize several additional non-covalent interaction partners by preventing their ubiquitylation and subsequent degradation by the proteasome (Liu et al., 2016; Yi et al., 2019; Yu et al., 2012; Yuan et al., 2014; Zou et al., 2018). Of note, in none of the described cases, was a covalent FAT10 conjugate shown, suggesting that non-covalently interacting FAT10 mediates the observed inhibition of ubiquitylation. For example, the transcription factor β-catenin, which has a prominent role in the development of cancer and is involved in the regulation of proliferation, differentiation and cell survival, is stabilized by its interaction with FAT10 (Yuan et al., 2014). While non-phosphorylated β-catenin is found in the nucleus where it forms active dimers with other transcription factors, phosphorylation of β-catenin, mediated by serine/threonine glycogen synthase kinase 3β (GSK3β) and by casein kinase I, leads to its ubiquitylation by the E3 ligase β-TRCP and subsequent degradation by the proteasome (Amit et al., 2002; Polakis, 2000, 2012). Yuan and colleagues showed that the interaction with FAT10 prevents ubiquitylation of β-catenin, resulting in a higher amount of active β-catenin and thus an increase in cell survival and proliferation (Yuan et al., 2014). Consequently, they observed a decrease in the mRNA expression of Wnt-induced secreted protein-1 (WISP1; also known as CCN4), a downstream target of β-catenin, which plays a role in promoting hepatocellular carcinoma (HCC) proliferation, in the presence of

Table 1. List of all currently confirmed covalent and non-covalent interaction partners of FAT10

	Covalent conjugation partner (isopeptide linkage)	Non-covalent interaction partner	Influence on stability (proteasomal degradation)	Influence on activity	Reference
p53	Yes	–	–	Activated	Li et al., 2011
p62/SQSTM1	Yes	Yes	Degraded	–	Aichem et al., 2012
USE1	Yes	Yes	Degraded	No	Aichem et al., 2014, 2010
LRRFIP2	Yes	–	–	Inactivated (precipitates with FAT10 in insoluble cytosolic aggregates)	Buchsbaum et al., 2012
UBE1	Yes	Yes	Degraded	No	Bialas et al., 2015; Rani et al., 2012
JunB	Yes	Yes	Degraded	–	Aichem et al., 2019
PCNA	Yes	–	Degraded	–	Chen et al., 2018
eEF1A1	–	Yes	Stabilized	–	Liu et al., 2016
OTUB1	Yes	Yes	Degraded	Activated	Bialas et al., 2019
WISP1	Yes	–	Degraded	Increased WISP1 mRNA expression by stabilization of β-catenin	Yan et al., 2018
AIPL1	Yes	Yes	–	–	Bett et al., 2012
SUMO E1 AOS1/UBA2	Thioester	Yes	–	Inhibited	Aichem et al., 2019
β-catenin	–	Yes	Stabilized	–	Yuan et al., 2014
Caveolin-3	–	Yes	Stabilized	–	Zhou et al., 2018
YAP1	–	Yes	Stabilized	–	Yi et al., 2019
Survivin	–	Yes	Stabilized	–	Dong et al., 2016
RIG-I	–	Yes	–	Inactivated (precipitates with FAT10 in insoluble cytosolic aggregates)	Nguyen et al., 2016; Wang et al., 2019
MAD2	–	Yes	–	Inactivated	Liu et al., 1999; Ren et al., 2006, 2011; Theng et al., 2014
ZEB2	–	Yes	Stabilized	–	Zou et al., 2018
APOL1	–	Yes	Degraded	–	Zhang et al., 2018
HDAC6	–	Yes	Degradation via aggresomes	–	Kalveram et al., 2008
Nub1L	–	Yes	–	–	Schmidtke et al., 2009

'–' denotes that no data are available up to date.

FAT10 (Yan et al., 2018). Here, an additional covalent FAT10ylation of WISP1 leads to its subsequent proteasomal degradation and further decreases the protein levels of WISP1, thereby further promoting HCC proliferation (Yan et al., 2018). Similarly, other factors have also been shown to be stabilized by non-covalent interaction with FAT10, preventing their ubiquitylation and subsequent proteasomal degradation, including survivin (Dong et al., 2016), a member of the inhibitor of apoptosis (IAP) protein family, eukaryotic translation elongation factor 1A1 (eEF1A1) (Liu et al., 2016; Yu et al., 2012), caveolin-3, which plays a role in cardiovascular diseases (Zhou et al., 2018) and YAP1 (Yi et al., 2019).

However, FAT10 can also negatively influence its interaction partners. For instance, the non-covalent interaction of FAT10 with retinoic acid inducible gene 1 (RIG-I; also known as DDX58) interferes with RIG-I-mediated antiviral signaling that is necessary for the production of type I interferons upon viral infection (Nguyen et al., 2016; Wang et al., 2019). There are two possible means of how this might be achieved. One group proposed that FAT10 sequesters RIG-I into insoluble aggregates, leading to inactivation of RIG-I (Nguyen et al., 2016). Another study showed that non-covalently interacting FAT10 inhibits ubiquitylation of the RIG-I CARD domains, a post-translational modification that is normally necessary for activation of RIG-I (Wang et al., 2019). Both of these mechanisms would result in the inactivation of RIG-I and thus interfere with the type-I-dependent antiviral immune response.

Moreover, we recently reported a clear effect of non-covalently interacting FAT10 on the activity of the deubiquitylating enzyme OTUB1 (Bialas et al., 2019), a member of the OTU (ovarian tumor) superfamily of cysteine proteases (Komander et al., 2009). OTUB1 inhibits ubiquitylation of substrates in two different ways. Firstly, it

directly acts as a deubiquitylating enzyme (DUB) and removes Lys-48 polyubiquitin chains from substrates such as p53 (Sun et al., 2012). Secondly, it inhibits polyubiquitylation in a non-catalytic manner by binding to and thereby inhibiting its cognate E2 conjugating enzymes UbcH5b and Ubc13 (also known as UBE2D2 and UBE2N, respectively) (Edelmann et al., 2010; Sato et al., 2008). Although covalent FAT10ylation of OTUB1 targets it for degradation by the 26S proteasome, the non-covalent interaction with FAT10 has the opposite effect and instead stabilizes OTUB1, thereby resulting in an increase in its catalytic as well as non-catalytic activity (Bialas et al., 2019).

Taken together, FAT10 not only functions as a tool that mediates proteasomal degradation, but also is a factor that can positively or negatively influence the stability or activity of target proteins. Since the structure of FAT10 is now solved, the exact mechanism of how FAT10 is able to perform these functions might become more clear by further structural investigations in the years to come.

Interplay between FAT10 and other ULM pathways

For a long time it was expected that each ULM uses a dedicated set of E1 activating, E2 conjugating enzymes and E3 ligases to become conjugated to the respective target proteins. This assumption was proven to be incorrect upon the identification of the E1 activating enzyme UBA6 (Chiu et al., 2007; Pelzer et al., 2007) and the E2 conjugating enzyme USE1 (Aichem et al., 2010; Jin et al., 2007), which are bispecific for both ubiquitin and FAT10. Therefore, some crosstalk between the two modifiers must exist, and the activation of FAT10 or ubiquitin must be tightly regulated. In fact, it has been shown that UBA6 has a higher affinity for FAT10 upon its cytokine-mediated upregulation, although the transthiolation reaction is faster for ubiquitin (Gavin et al., 2012). This points to a preference of

UBA6 for FAT10 when both modifiers are expressed. It also suggests that, upon induction of FAT10 under inflammatory conditions, FAT10 can be conjugated very efficiently by an otherwise bispecific enzyme, because ubiquitin might still be activated by the second ubiquitin-activating enzyme UBE1, which only activates ubiquitin but not FAT10 (Bialas et al., 2015; Chiu et al., 2007). Interestingly, we identified UBE1 as a covalent FAT10 conjugation substrate that is subjected to proteasomal degradation (Bialas et al., 2015). However, the amount of the degraded UBE1– FAT10 conjugate was only very low and thus did not affect the amount of bulk ubiquitin conjugates. Moreover, the additional non-covalent interaction of FAT10 with UBE1 had no obvious impact on the ubiquitin-activation capacity of UBE1; therefore, a direct effect

of FAT10 on ubiquitin conjugation owing to inactivation and degradation of the E1 enzyme could not be demonstrated (Bialas et al., 2015).

In contrast, more recently, we were able to demonstrate a strong effect of non-covalent FAT10 interaction on the SUMO conjugation pathway by showing that FAT10 directly and very efficiently inhibits SUMO conjugation (Fig. 3A) (Aichem et al., 2019). The underlying mechanism involves a direct interaction between FAT10 and the SUMO E1 heterodimeric enzyme AOS1–UBA2 (also known as SAE1–SAE2). Here, FAT10 interacts non-covalently with the SUMO-adenylation domain, which spans over both E1 subunits, and so hinders access of SUMO to its cognate E1 enzyme. In addition, FAT10 is also transferred in an ATP-dependent manner
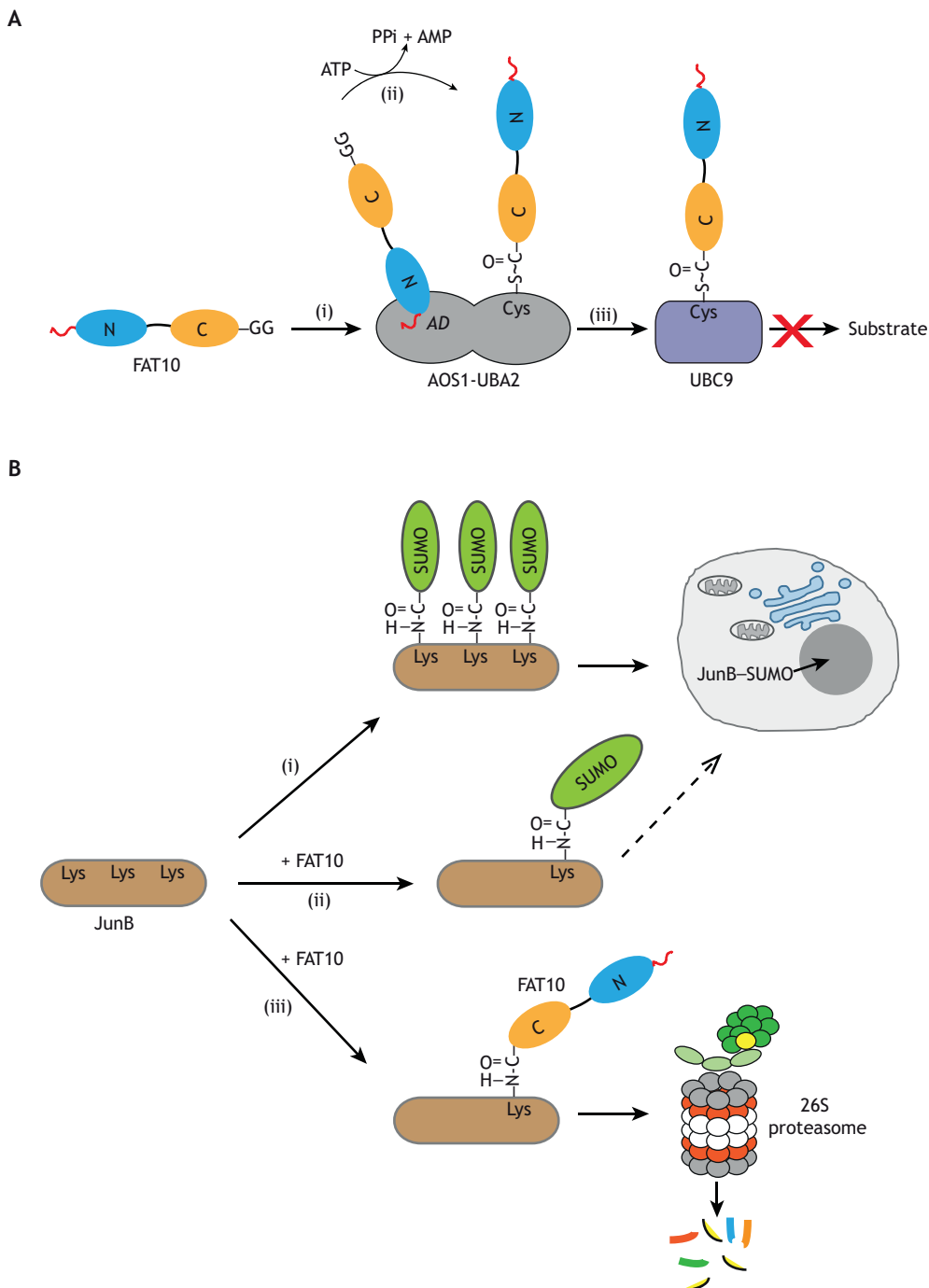

Fig. 3. Crosstalk between FAT10 and SUMO. (A) FAT10 non-covalently interacts with and occupies the adenylation domain (AD) of the SUMO E1 activating enzyme AOS1–UBA2 and it can eventually be activated in an ATP-dependent manner (i). Activated FAT10 is further transferred onto the active-site cysteine residue located on UBA2 where it forms a thioester with the active-site cysteine (ii) and handed over by a transthiolation reaction to the active-site cysteine of the SUMO-specific E2 conjugating enzyme UBC9 (iii). However, this does not lead to FAT10ylation of substrates but results in interference with SUMO activation and conjugation by blocking the access of SUMO to its own conjugation cascade. (B) (i) SUMOylation of the transcription factor JunB is necessary for its translocation into the nucleus and, subsequently, enhanced transcription if its target genes, such as IL-2 or IL-4. (ii) In the presence of FAT10, SUMOylation of JunB is heavily diminished due to FAT10-mediated inhibition of SUMO conjugation. (iii) Moreover, JunB becomes covalently modified by FAT10 at a SUMOylation-consensus site, leading to the degradation of the JunB–FAT10 conjugate by the 26S proteasome. Hence, in the presence of FAT10, the amount of active JunB is heavily reduced by two means, downregulation of its activation and degradation by the 26S proteasome.

onto the active-site cysteine residue of the E1 located on UBA2, and further onto the active-site cysteine of the SUMO E2 conjugating enzyme UBC9 (also known as UBE2I), suggesting, that FAT10 can be activated by AOS1–UBA2 (Fig. 3A). However, since the final transfer of FAT10 onto substrates was not observed, FAT10 activation by the SUMO E1 appears to be unproductive and instead results in a blockage of the SUMO-conjugation pathway (Aichem et al., 2019). Interestingly, SUMO activation is not impaired at all in the presence of other recombinant ULMs, such as ISG15, linear di-ubiquitin or ubiquitin itself, pointing to a unique function of FAT10 (Aichem et al., 2019). The specificity of this inhibition might again be explained by the unique surface properties of the different modifiers. Although all these ULMs possess the same conserved 3D structure of the ubiquitin β-grasp fold, their surface charges, as well as the ubiquitin-specific hydrophobic patches, are not very well conserved (Aichem et al., 2018; Bayer et al., 1998; Huang et al., 2004; Narasimhan et al., 2005; Vijay-Kumar et al., 1987). As compared to ubiquitin, which bears a rather positively charged surface (Vijay-Kumar et al., 1987), or ISG15, which contains a large apolar surface area in its N-terminal UBL domain (Narasimhan et al., 2005), both SUMO1 and FAT10 are rather negatively charged (Aichem et al., 2018; Bayer et al., 1998).

Of note, SUMO proteins play a role in numerous cellular processes, and therefore the question of the functional consequences of such a potent inhibition of the SUMO-conjugation pathway arises. In fact, in our study we identified JunB, a transcription factor of the AP-1 family, as a new FAT10 substrate (Fig. 3B) (Aichem et al., 2019). Modification of JunB with a SUMO protein is necessary for its nuclear translocation and increased transcriptional activity towards the interleukins IL-2 and IL-4, and thus regulates Th2 helper cell differentiation (Garaude et al., 2008). However, in the presence of FAT10, JunB SUMOylation was strongly diminished due to FAT10-mediated inhibition of the SUMO conjugation pathway. Moreover, the JunB–FAT10 conjugate was degraded by the proteasome upon covalent modification with FAT10, suggesting a strong decrease in the amount of active JunB in the presence of FAT10 (Aichem et al., 2019) (Fig. 3B). Interestingly, this might account for the observed elevation of Th2-derived cytokines such as IL-10 in the muscle and plasma of FAT10$^{-/-}$ mice (Canaan et al., 2006, 2014).

Promyelocytic leukemia (PML) bodies are important regulators of several cellular processes, such as DNA repair and genome maintenance, and SUMO modification is necessary for their function (Duprez et al., 1999; Kamitani et al., 1998a,b; Koidl et al., 2016; Muller et al., 1998; Sternsdorf et al., 1997). SUMO-dependent formation and the size of PML bodies are significantly diminished in the presence of FAT10 (Aichem et al., 2019; Li et al., 2011). Hence, a FAT10-mediated reduction in the amount of active PML bodies might promote the transforming capacities of FAT10 in the development of cancer and provide an explanation for the function of FAT10 as a proto-oncogene (Gao et al., 2015, 2014; Lee et al., 2003; Li et al., 2018; Lim et al., 2006; Liu et al., 2014, 1999; Lukasiak et al., 2008; Theng et al., 2014; Yuan et al., 2014; Zhao et al., 2015; Zou et al., 2018).

In summary, the outcome of a non-covalent interaction of FAT10 with one of its specific interaction partners appears to be entirely separate from proteasomal degradation and encompasses inhibition or activation of the respective proteins. Furthermore, dozens of proteins that have been identified in independent proteomic approaches (Aichem et al., 2012; Leng et al., 2014) still await their detailed characterization, which will further contribute to a better understanding of the function of FAT10 other than proteasomal degradation.

Conclusion and perspectives

The *FAT10* gene was identified by genomic sequencing of the human MHC class I locus in 1996 (Fan et al., 1996). During the last 24 years, major efforts have been invested to understand the biology and function of this unusual modifier, and only more recently has the 3D structure of FAT10 finally been determined (Aichem et al., 2018; Theng et al., 2014). Since the best-investigated function of FAT10 is the covalent modification of proteins collectively resulting in proteasomal degradation, the necessity of a second transferable tag – besides ubiquitin – for proteasomal degradation has bewildered researchers. The elucidation of the FAT10 structure has revealed that the two modifiers strongly differ from each other (Aichem et al., 2018). These findings now provide explanations as to why ubiquitin and FAT10 non-covalently interact with different interaction motifs on the surface of their respective binding partners. Moreover, in contrast to ubiquitin, FAT10-mediated degradation is independent of VCP activity and results in an irreversible, fast and very efficient degradation of the target substrates by the 26S proteasome. This feature might become important under inflammatory conditions or during an infection, where a fast cellular reaction is needed to fight against incoming pathogens that does not leave room for interference by such microorganisms.

Although until now no FAT10-specific consensus interaction motifs, such as the described SIM or UIM domains, have been identified, the outcomes of non-covalent binding to FAT10 are beginning to become the focus of ongoing research. Co-crystallization studies of FAT10 with its interaction partners, such as the SUMO E1 activating enzyme (Aichem et al., 2019) or OTUB1 (Bialas et al., 2019), or other non-covalent interaction partners will provide important information to further clarify the exact mechanism of how FAT10 exerts these functions. Such research might also open new avenues for the development of unprecedented therapeutic strategies for infectious diseases or cancer, where FAT10 has been shown to play a role.

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