The Ubiquitin-like Modifier FAT10
in Dendritic Cell Aggresome-like Induced Structures

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Abstract

The ubiquitin-like modifier ‘HLA-F adjacent transcript 10’ (FAT10) is encoded in the MHC class I locus and is predominantly expressed in tissues and cells of the immune system, such as spleen, thymus, and maturing dendritic cells. However, FAT10 can be induced by pro-inflammatory stimuli, such as the cytokines tumour necrosis factor and interferon gamma, in virtually all cells. FAT10 can be conjugated to substrate proteins via its C-terminal diglycine motif and thereby serves as a degradation signal for the proteasome similar to, yet independent of ubiquitin. By means of proteasome degradation FAT10 can promote antigen presentation. Upon inhibition of the proteasome, however, FAT10 and FAT10ylated substrates are re-localised to aggresomes in a largely HDAC6-dependent manner.

The first part of this study showed co-localisation of FAT10 to dendritic cell aggresome-like induced structures (DALIS) in murine and human dendritic cells (DCs). DALIS are similar to aggresomes with specific distinctions, such as their transient formation as well as their random distribution throughout the cytosol without accumulation at the microtubule organising centre, and among others form during maturation of dendritic cells. It has been proposed that DALIS serve as storage sites for polyubiquitylated antigens and mainly consist of defective ribosomal products, which are a source of antigens for MHC class I-restricted antigen presentation. Investigation into the formation of DALIS in FAT10-deficient and -proficient dendritic cells showed FAT10-mediated clearance of DALIS but no involvement of FAT10 in the formation of DALIS. Moreover, it could be shown that DALIS formed independent of HDAC6. Thus, this study found further evidence for a role of FAT10 in antigen processing and presentation by degradation of antigens that accumulated in DALIS during DC maturation.

Furthermore, the CCR7-mediated migration of DCs in dependence of FAT10 was examined since recent reports assigned a migration-promoting function to FAT10. Contrary to these reports, FAT10 reduced CCR7-dependent migration of human DCs only marginally and had no influence on the CCR7-mediated migration of murine DCs. Nonetheless, this provides first insights into the role of FAT10 in the migration of DCs and maybe immune cells in general.

Additionally, the expression profile of FAT10 in the major human and murine immune cell subsets was determined because there was extensive knowledge about tissue-specific expression of FAT10 that, however, was incomplete at the cellular level. The expression profile revealed cell-type specific expression of FAT10 in the different human and murine leukocyte
populations. This suggests cell type-specific functions of FAT10 and will help to prevent that new functions of FAT10 are overlooked.

Finally, the epitope peptide of the anti-FAT10 antibody 4F1 was identified and characterised. The epitope peptide was successfully applied to the elution of FAT10 from the FAT10-reactive antibody 4F1 demonstrating its usefulness. The epitope peptide represents a new tool for the investigation into FAT10 and will potentially enable the identification of novel low abundant interacting partners and substrates as well as allow for purification of the otherwise unstable FAT10 at optimal physiological conditions.

**Zusammenfassung**


Zuletzt wurde das Epitop-Peptid des anti-FAT10 Antikörpers 4F1 bestimmt und charakterisiert. Das Epitop-Peptid wurde erfolgreich für die Elution von FAT10 vom FAT10-reactiven Antikörper 4F1 verwendet und damit seine Nützlichkeit demonstriert. Das Epitop-Peptid stellt ein neues Werkzeug für die Untersuchung von FAT10 dar und wird die Identifizierung von neuen Interaktionspartner und Substratproteinen, welche in nur geringer Konzentration in der Zelle vorkommen, sowie die Aufreinigung des sonst unstabilen FAT10 unter optimalen physiologischen Bedingungen ermöglichen.
Preface

Data presented in chapter 3.3 and figure 10 has been published in Schregle R, Mah MM, Mueller S, Aichem A, Basler M, Groettrup M (2018) The expression profile of the ubiquitin-like modifier FAT10 in immune cells suggests cell type-specific functions. Immunogenetics. doi: 10.1007/s00251-018-1055-5. The data in subchapter 3.3.2, including table 14 and figure 10B, and in subchapter 3.3.3 has been provided by Mei Min Mah (University of Konstanz) and Annette Aichem (Biotechnology Institute Thurgau, Switzerland), respectively, and were included in this thesis with permission for the sake of completeness. Mei Min Mah and I wrote the manuscript with some editing by Marcus Groettrup and Michael Basler.

The lentiviral vectors generated from the plasmid pCDH-EF1α-hFAT10-IRES-copGFP were published in Schmidtke G, Schregle R, Alvarez G, Huber EM, Groettrup M (2017) The 20S immunoproteasome and constitutive proteasome bind with the same affinity to PA28αβ and equally degrade FAT10. Mol Immunol. 2017 Dec 2. pii: S0161-5890(17)30593-X. doi: 10.1016/j.molimm.2017.11.030. I cloned the plasmid vector and provided the lentiviruses generated from it, which were used in the study, as well as wrote a part of the methods section and designed the graphical abstract for the publication.

Record of Contribution

The data presented in the subchapter 3.3.2 including table 14 and figure 10B was generated and provided by Mei Min Mah (University of Konstanz) and data shown in subchapter 3.3.3 has been generated and provided by Annette Aichem (Biotechnology Institute Thurgau, Switzerland). Parts of the data presented in the subchapter 3.3.1, in figure 12, and the supplementary figure 1 and 2 and the plasmid vector pCDH-EF1α-UbΔGG-IRES-copGFP have been generated by Stefanie Müller under my supervision (University of Konstanz) in the context of her bachelor thesis.
1 Introduction

1.1 The Ubiquitin-Proteasome System

Due to the dynamic nature of the proteome present in all living cells, a complex network has evolved enabling proper protein homeostasis. This protein homeostasis network fulfils the challenging task to maintain the proteome in response to the ever-changing influences of the environment. In doing so, it controls protein synthesis and proper folding, post-translational modifications (PTM) and the consequences thereof, and the ultimate destruction and recycling of proteins in the densely packed milieu of the cell. Degradation and recycling of cellular components is achieved by several pathways. These include the ubiquitin-proteasome system (UPS), which degrades proteins in a selective manner, and the lysosomal targeting pathways, which involve bulk protein degradation and are less selective (Powers et al., 2009). Central to the degradation of proteins by the UPS is the proteasome. Proteasomes degrade proteins dependent on PTMs such as ubiquitin or FAT10, but degradation independent of these PTMs also occurs mediated through unstructured or highly flexible regions within proteins and through proximity to proteasomes as observed for the antizyme-mediated degradation of ornithine decarboxylase. After degradation, peptides are further processed by cytosolic proteases to produce amino acids, which are used for the neosynthesis of proteins, and peptides, which can be presented by major histocompatibility complex (MHC) class I molecules (Cohen-Kaplan et al., 2016).

1.1.1 The Proteasome

Proteasomes are large cylindrical and multi-enzymatic complexes of approximately 2,000 kDa that are necessary for the turnover of short-lived, misfolded, and damaged proteins within cells in an ATP-dependent manner (Coux et al., 1996). Proteasomes are evolutionary highly conserved and, besides eukaryotes, homologous structures can be found in archaea (Maupin-Furlow, 2013) and in certain classes of bacteria (Becker & Darwin, 2017).

1.1.1.1 The Constitutive 20S Proteasome

All proteasomes harbour a 20S catalytic core particle (CP) that can bind to different regulatory particles (Tanaka, 2009). This core particle is composed of four stacked rings consisting of two
identical α- and β-rings arranged in an α-β-β-α series. In eukaryotes, each α- and β-ring is made up of seven different subunits α1-α7 and β1-β7. The outer α-rings form a gate-like structure which represents a physical barrier to the inner β-rings that form the catalytic chamber (Figure 1A) (Groll et al., 1997). In the absence of proteasome activators (PAs) the N-terminal peptides of the α-subunits protrude into the opening of the α-ring gate and physically restrict access to the catalytic chamber, where the N-terminal peptide of the α3-subunit very likely plays a superior role in stabilising the closed gate (Groll et al., 2000; Groll & Huber, 2003). Thus, the 20S proteasome is almost completely latent in cells, although there is evidence for specific catalytic activity of the 20S proteasome (Kisselev et al., 2002; Liu et al., 2003; Jung et al., 2009; Osmulski et al., 2009). Within the catalytic chamber three of the seven β-subunits in each β-ring show proteolytic activity, which differs between the subunits. The β1-subunit is associated with a caspase-like or peptidyl-glutamyl-peptide-hydrolysing activity, the β2-subunit reflects a trypsin-like activity, and the β5-subunit exhibits a chymotrypsin-like activity. The individual activities of these subunits result in the preferential cleavage after acidic (β1), basic (β2), and hydrophobic (β5) amino acids (Figure 1C, left proteasome) (Orlowski, 1990; Jung et al., 2009). Inside the catalytic chamber, proteins are cleaved completely in a processive manner giving short peptides of 3-25 amino acids. The processive manner of the proteolysis by the three active subunits ensures complete degradation and prevents toxic effects of otherwise partially degraded proteins (Kisselev et al., 1999). Yet, some transcriptions factors, such as NF-κB and NRF1, are activated upon partial degradation by the proteasome in a process termed ‘regulated ubiquitin–proteasome-dependent processing’ or RUP (Hoppe, 2014).

1.1.1.2 Proteasome Regulators

To open the α-ring gate and activate the 20S CP, proteasome activators must bind. In case the 20S CP binds one or two 19S regulatory particles (RP; PA700) this results in the 26S and 30S proteasome, respectively (Figure 1B, top panel) (Tanaka, 2009). The 19S RP is composed of two sub-complexes called the lid and the base (Glickman et al., 1998). The base consists of a ring-shaped heteromeric complex formed by six regulatory particle triple-A ATPases (RPT1-6) and three regulatory particle non-ATPases (RPN1, RPN2, & RPN13) (Förster et al., 2013; Schweitzer et al., 2016). When the hetero-hexamer formed by RPT1-6 binds to the α-ring of the 20S CP, it prompts the α-ring to open, and mediates unfolding and translocation of proteins into the catalytic chamber in an ATP-dependent manner (Smith et al., 2007; Lasker et al., 2012). RPN1 and RPN2 bind to the RPT1-6 complex and serve as adaptors for shuttling ubiquitin receptors or ubiquitin-like (UBL)-ubiquitin-associated (UBA) domain proteins, such as
HR23A/B, ubiquilin-2, and NUB1L, as well as the ubiquitin receptor RPN13 and the associated de-ubiquitylating enzymes (DUBs), such as USP14 and UCH5L (Förster et al., 2013). Although initially thought to be part of the base, the ubiquitin receptor RPN10 now is believed to be part of the lid complex that is formed additionally by nine other non-ATPase subunits (RPN3, RPN5-9, RPN11, RPN12, RPN15) (Lander et al., 2012; Schweitzer et al., 2016). RPN11 is probably the most important component of the lid since it is necessary for complete de-ubiquitylation of proteins prior to their unfolding and translocation by the triple-A ATPase-ring into the CP (Förster et al., 2013).

Apart from the 19S RP, there are other PAs, such as PA28 or 11S regulator and PA200 or bleomycin resistance protein 10 (BLM10) (Figure 1B). All PAs can bind to the 20S CP and open the α-ring gate, although by different mechanisms. This can lead to the formation of a range of differently capped so-called hybrid-proteasomes (Jung et al., 2009; Tanaka, 2009). PA28 is formed either as the hetero-heptamer PA28αβ composed of four α- and three β-subunits, or as the homo-heptamer PA28γ from γ-subunits (Chu-Ping et al., 1992b; Dubiel et al., 1992; Huber & Groll, 2017). PA28αβ and PA28γ localise to different subcellular compartments, where PA28αβ can be found in the cytoplasm as well as the nucleoli and PA28γ is present almost exclusively in the nucleus (Wójcik et al., 1998; Zannini et al., 2008). Interestingly, the α- and β-subunit are inducible by cytokines whereas the γ-subunit is not (Ahn et al., 1995; Tanahashi et al., 1997). Furthermore, both PA28 complexes seem to fulfil different functions. PA28αβ alters the generation of peptides and is required for processing of specific viral, bacterial, and tumour-derived antigens (Groettrup et al., 1995, 1996b; Dick et al., 1996; Schwarz et al., 2000; van Hall et al., 2000; Murata et al., 2001; Cascio et al., 2002; Yamano et al., 2002; de Graaf et al., 2011). The PA28γ complex on the other hand mediates nuclear proteolysis and seems to be involved in various different functions (Moriishi et al., 2003; Li et al., 2006, 2007; Zannini et al., 2008; Zhang & Zhang, 2008).

The regulator PA200 (PSME4) is, like PA28γ, preferentially localised in the nucleus and is a monomeric protein of approximately 200 kDa, hence the name (Hoffman et al., 1992; Ustrell et al., 2002). Functionally, PA200 is involved in spermatogenesis (Khor et al., 2006) and the maturation of nuclear 20S CP (Fehlker et al., 2003; Marques et al., 2007). Initially, Ustrell and colleagues suggested that PA200 is also involved in DNA repair (Ustrell et al., 2002), but this has been questioned since knockout (KO) and overexpression of PA200 in yeast only showed a mild phenotype (Iwanczyk et al., 2006). Moreover, PSME4-deficient mice are viable and develop normally and PSME4-deficient embryonic stem cells are not more sensitive to induced DNA damage (Khor et al., 2006).
Figure 1: The 20S proteasome with its subunits, its regulatory particles, and the different types. (A): The constitutive 20S core particle (CP) or proteasome consists of four stacked rings made of either seven α- or β-subunits. The outer α-rings (beige) form the gates to control access to the catalytic chamber, which is made of the two identical, inner β-rings (grey and blue). The β-subunits β1, β2, and β5 in each β-ring bear different, catalytic activities (blue). (B): The 20S CPs can interact with several proteasome regulators that increase the degradative capacity by opening the α-ring gates (19S RP, PA28αβ, PA28γ, & PA200). Furthermore, negative regulators, such as PI31, can bind to 20S CPs and negatively influence proteasome function or assembly. (C): Besides the constitutive or standard proteasome, other types of 20S CPs exist that are the thymoproteasome and intermediate or mixed forms of the constitutive and the immunoproteasome. In these types of 20S CP the constitutive subunits β1, β2, and β5 (blue) are exchanged partially or completely with the inducible subunits β1i, β2i, and β5i (red) or the tissue-specific subunit β5t (purple). Image is modified from (Vigneron et al., 2017).
There are not only regulatory particles that activate the proteasome, but also inhibitory proteins that negatively influence proteasome function, such as PR39 (Gaczynska et al., 2003) and PI31 (Figure 1B) (Chu-Ping et al., 1992a), but their functions and mechanisms are poorly understood. The proteasome inhibitor of 31 kDa, PI31, was initially thought to decrease proteasome activity in vitro by preventing its interaction with the 19S RP and PA28αβ (Zaiss et al., 1999; McCutchen-Maloney et al., 2000), but this could not be shown in cells (Zaiss et al., 2002; Li et al., 2014). Instead, it has been suggested that PI31 impairs maturation of immunoproteasomes and antigen processing (Zaiss et al., 2002) and positively regulates assembly of the 26S proteasome (Cho-Park & Steller, 2013), which again could not be confirmed in another study (Li et al., 2014). PR39 is a proline/arginine-rich, 39 amino acid-long peptide and interacts reversibly with the α7-subunit of the 20S CP. This interaction causes conformational changes within the entire 20S CP leading to decreased activity and impaired binding of the 19S RP (Gaczynska et al., 2003).

### 1.1.1.3 Types of Proteasomes

Besides the constitutively expressed, catalytically active β-subunits β1c, β2c, and β5c that form the constitutive proteasome, there are inducible β-subunits. Upon incorporation of the inducible subunits β1i (LMP2), β2i (MECL-1), and β5i (LMP7) into the 20S CP instead of β1c, β2c, and β5c, immunoproteasomes are formed (Brown et al., 1991; Glynne et al., 1991; Kelly et al., 1991; Martinez & Monaco, 1991; Ortiz-Navarrete et al., 1991; Aki et al., 1994; Groettrup et al., 1996a; Nandi et al., 1996). Interestingly, despite the inducibility of the immunoproteasome subunits, constitutive expression of β1i, β2i, and β5i can be found in tissues of the immune system, such as spleen and thymus, and specifically in immune cells, such as dendritic cells (DCs) (Stohwasser et al., 1997; Macagno et al., 1999, 2001; Barton et al., 2002; DeBruin et al., 2016). In immunoproteasomes, the subunit β1i displays a chymotrypsin-like activity as opposed to β1c that harbours a caspase-like activity. Therefore, the exchange of these subunits alters the cleavage specificities and enhances production of peptides with hydrophobic and basic residues at their C-terminus (Figure 1C, 2nd proteasome from right) (Driscoll et al., 1993; Gaczynska et al., 1993, 1994; Boes et al., 1994; Groettrup et al., 1995; Toes et al., 2001), while the rate of degradation is unaffected and, remarkably, is similar to the degradation rate of the constitutive proteasome (Nathan et al., 2013). Crystallisation of the immunoproteasome and the constitutive proteasome revealed that structural features within the subunits β1i and β5i are responsible for the enhanced generation of more suitable MHC class I peptides. Yet, structural variations cannot explain the differences between the β2c- and β2i-subunit, which are evident in the
phenotype of mice deficient for β2i subunit, since these subunits have an identical substrate binding channel (Basler et al., 2012, 2013; Huber et al., 2012). In line with this, the formation of immunoproteasomes is crucial for the processing of specific antigens (Basler et al., 2013). Notably, the presence of mixed or intermediate proteasomes, which contain constitutive and inducible subunits (Figure 1C, 2nd & 3rd proteasome from left), further enlarges the pool of peptides presented by MHC class I molecules (Guillaume et al., 2010). Apart from antigen processing, the immunoproteasome has only recently been assigned additional functions. It was shown to be necessary for the proper production of pro-inflammatory cytokines, it is involved in the differentiation of pro-inflammatory T cells, and it emerges as an attractive therapeutic target for autoimmune diseases and cancer due to its involvement in the pathogenesis of these diseases (Basler et al., 2015b; Koerner et al., 2017; Vachharajani et al., 2017). However, mechanistically it is not clear how the immunoproteasome and the constitutive proteasome exert different functions. One explanation might be by differential interaction of either proteasome types with the regulatory particles as has been shown by Fabre and colleagues. They found equal binding of the 19S RP and PA28γ to both types of proteasome, preferred binding of PA28αβ and PA200 to the immunoproteasome, and that PI31 preferably interacts with the constitutive proteasome (Fabre et al., 2015). Yet, in vitro studies find equal binding and activation of PA28αβ to the constitutive proteasome and the immunoproteasome (Stohwasser et al., 2000; Schmidtke et al., 2017). Therefore, Schmidtke and colleagues suggested that the binding of the regulatory particles in cells to the different 20S CPs might be also influenced by post-translational modifications, which have been observed at several subunits with varying outcomes (Livneh et al., 2016; Collins & Goldberg, 2017).

Additionally, another β-subunit, β5t, which is constitutively and specifically expressed in cortical thymic epithelial cells (TECs) has been identified giving rise to the so-called thymoproteasome (Figure 1C, right proteasome) (Murata et al., 2007). The thymoproteasome is necessary for proper positive selection of CD8+ T cells (Kincaid et al., 2016).

### 1.1.2 Ubiquitin

Ubiquitin has been discovered already over 40 years ago as a small polypeptide of 8.5 kDa that showed a broad spectrum of expression in diverse species (Goldstein et al., 1975; Ciechanover et al., 1978). The structure of ubiquitin is globular and defined by two α-helices and five β-strands (Vijay-Kumar et al., 1985, 1987). The five β-strands appear to grasp the central α-helix and this structural feature of ubiquitin came to be known as the ‘β-grasp’ fold (Figure 2) (Overington, 1992). Besides the other ubiquitin-like modifiers known in eukaryotes, this
‘β-grasp’ fold is also present in prokaryotic ubiquitin-like proteins, such as ThiS, MoaD, and small archaeal modifier proteins (SAMPs). Thus, all these ‘β-grasp’ fold containing proteins very likely evolved from a common ancestor protein, which gave rise to the different but similar conjugation systems present in all domains of life (Burroughs et al., 2007; Zuin et al., 2014). In eukaryotes, ubiquitin is expressed as a precursor from four different genes. Two of the genes, UBA52 and UBA80, encode each one moiety of ubiquitin together with a ribosomal protein and the other two genes, UBB and UBC, encode for several ubiquitin moieties that are expressed as a polyubiquitin peptide. These precursors are processed by de-ubiquitylating enzymes (DUBs) to free the single ubiquitin molecules and expose the C-terminal glycine residue (Komander et al., 2009; Grou et al., 2015). Soon after ubiquitin’s discovery, it has been shown that it is highly conserved in eukaryotic cells and can be attached covalently to other proteins (Schlesinger & Goldstein, 1975; Goldknopf & Busch, 1977; Hunt & Dayhoff, 1977; Zuin et al., 2014).

![Ubiquitin](image)

**Figure 2: The ubiquitin structure.** Ribbon diagram of the ubiquitin structure with its five β-sheets (purple) and the two α-helices (turquoise). The typical β-grasp fold is formed by the β-sheets and the central α-helix. The image is adapted from (Groettrup et al., 2008).

### 1.1.2.1 Ubiquitin Conjugation and De-Conjugation

In 1980, Ciechanover and colleagues observed ATP-dependent degradation of proteins mediated by covalent attachment of a small protein that turned out to be ubiquitin (Ciechanover et al., 1980; Wilkinson et al., 1980). The C-terminal diglycine motif, inherent to ubiquitin and most ubiquitin-like modifiers (ULMs) (van der Veen & Ploegh, 2012; Cappadocia & Lima, 2018), is necessary for conjugation of ubiquitin to the ε-amino group of lysine residues within
the targeted substrate proteins (Goldknopf & Busch, 1977; Hershko et al., 1980). Apart from internal lysine residues, ubiquitin can also be attached to the N-terminal α-amino group, the hydroxyl group of serine and threonine residues, and the thiol group of cysteine residues (Stewart et al., 2016). The process of ubiquitin conjugation or ubiquitylation (Figure 3) is mediated by an enzymatic cascade involving one of the two known E1 activating enzymes, UBA1 (Handley et al., 1991) and UBA6 (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007), one of the approximately 40 known E2 conjugating enzymes, and one of the over 600 hundred E3 ligases or E3 ligating enzymes (Pickart & Eddins, 2004; Hutchins et al., 2013; Cappadocia & Lima, 2018).

At first, the E1 activating enzyme binds ATP, magnesium, and a ubiquitin moiety which results in the formation of a ubiquitin adenylate at the C-terminal glycine residue and the release of pyrophosphate. This high energetic ubiquitin-adenylate, which is bound to the adenylation site on the E1 activating enzyme, then reacts with the active site cysteine of the E1 activating enzyme yielding a thioester bond between the C-terminal glycine residue and the active site cysteine and releasing AMP. Following this reaction, an additional ubiquitin moiety becomes adenylated by and bound to the adenylation site of the E1 activating enzyme with the already thioester-linked ubiquitin. The E1 activating enzyme is now fully loaded, and ubiquitin is activated for transfer and conjugation (Schulman & Harper, 2009). Subsequently, the E1 activating enzyme mediates the transfer of the thioester-linked ubiquitin molecule onto the active site cysteine of an E2 conjugating enzyme in the so-called transthiolation reaction. The entire group of E2 conjugating enzymes possesses a catalytic ubiquitin-conjugating (UBC) domain, which harbours the active site cysteine and enables the interaction with E1 activating enzymes and E3 ligases (Stewart et al., 2016).

In the final step, E3 ligases help to transfer the ubiquitin molecule onto the substrate protein or another ubiquitin moiety forming an isopeptide bond. The mechanism of this transfer differs between the three main classes of E3 ligases but involves the binding of the E2-ubiquitin thioester complex and the substrate protein. The class of the ‘really interesting new gene’ (RING) domain containing E3 ligases, which include U-box domain E3 ligases, mediate proximity for the transfer of ubiquitin onto the substrate. On the contrary, the classes of ‘homologous to E6AP C-terminus’ (HECT) domain and ‘RING-between-RING’ (RBR) domain E3 ligases harbour an active site cysteine which accepts ubiquitin from the E2 enzyme in another transthiolation reaction and then transfer the ubiquitin onto the substrate (Buetow & Huang, 2016; Morreale & Walden, 2016).
Introduction

Figure 3: The ubiquitin conjugation and de-conjugation pathway. Initially, an E1 activating enzyme activates ubiquitin in an ATP-dependent manner resulting in the formation of a ubiquitin-adenylate. The ubiquitin-adenylate reacts with the active site cysteine of the E1 activating enzyme forming a thioester bond. After a second ubiquitin-adenylation, the E1 activating enzyme interacts with an E2 conjugating enzyme leading to the transthiolation of the ubiquitin moiety to the active site cysteine of the E2 conjugating enzyme. Finally, an E3 ligase enables the transfer of the ubiquitin moiety from the E2 conjugating enzyme onto a lysine residue within the substrate protein. Multiple rounds of conjugation of ubiquitin onto one of the seven lysine residues within the previously attached ubiquitin facilitates the formation of polyubiquitin chains. The attachment of ubiquitin and chain formation can be reversed or edited by de-ubiquitylating enzymes (DUB). This provides a means of recycling ubiquitin prior to substrate degradation by the proteasome and maintaining the ubiquitin pool of the cells. Image has been re-drawn from (Hochstrasser, 2009).

The conjugation of ubiquitin is reversible by means of DUBs (Figure 3) (Matsui et al., 1982; Hershko et al., 1984). In humans there are approximately 80-110 DUBs that are either cysteine proteases or zinc metalloproteases. DUBs are divided into five families. The ubiquitin C-terminal hydrolases (UCHs), the ubiquitin-specific proteases (USPs), the ovarian tumour proteases (OTUs), and the Josephins are cysteine proteases. The JAB1/MPN/MOV34 metalloenzymes (JAMMs) are the only family representing zinc metalloproteases (Hutchins et al., 2013; Clague et al., 2015). DUBs free single ubiquitin moieties from their precursor polypeptides, as mentioned above, and edit or trim existing polyubiquitin chains on substrates. Additionally, DUBs remove single ubiquitin moieties or entire polyubiquitin chains from modified substrates and counteract the ubiquitin signalling imposed by the modification, such as proteasomal degradation, and recycle ubiquitin from substrates that are being degraded as well as from free polyubiquitin chains. Therefore, DUBs regulate ubiquitin signalling and maintain the pool of free ubiquitin in the cell (Komander et al., 2009).
1.1.2.2 Types of Ubiquitin Modifications

The initial transfer of ubiquitin onto its substrate results in monoubiquitylation. The subsequent transfer of ubiquitin to different lysine residues within or to the N-terminal α-amino group of the same substrates leads to multi-monoubiquitylation. Alternatively, ubiquitin can be attach to the previously covalently-linked ubiquitin through one of the seven lysine residues within ubiquitin, which are K6, K11, K27, K29, K33, K48, and K63, or through the N-terminal methionine (M1) of ubiquitin. Thereby, polyubiquitin chains can be formed on the substrate by multiple rounds of conjugation. Polyubiquitin chains display a large variety, which is achieved though the diversity of the different E2 enzymes, the possible interactions of E2 enzymes with different E3 ligases, PTMs of ubiquitin, such as phosphorylation, and by chain editing through DUBs (Komander & Rape, 2012; Kulathu & Komander, 2012). In recent years, it has become evident that polyubiquitin chains are more complex as was known so far. Thus, apart from homotypic chains made of ubiquitin moieties attached to each other through the same lysine residue, there are polyubiquitin chains consisting of mixed linkages, harbouring branched chains, and even chains with other ubiquitin-like modifiers (Swatek & Komander, 2016; Yau & Rape, 2016). The functional consequences of mono-, multi-mono-, and polyubiquitylation are as diverse as the possible combinations and compositions of these modifications on substrate proteins.

The simplest modification by ubiquitin is monoubiquitylation and multi-monoubiquitylation. Yet, modification of proteins with only one ubiquitin moiety at one or several lysine residues is involved in a broad range of functions, such as transcriptional regulation, endocytosis, nuclear import and export, as well as DNA damage repair and replication (Nakagawa & Nakayama, 2015). The major type of linkage involved in proteasomal degradation is K48-linked ubiquitin chains (Chau et al., 1989; Finley et al., 1994). Besides that, K11- and K63-linked polyubiquitin chains as well as branched chains consisting of K11/K48-linked ubiquitin can lead to proteasomal degradation (Saeki et al., 2009; Bremm & Komander, 2011; Wickliffe et al., 2011; Meyer & Rape, 2014; Min et al., 2015). Thrower and colleagues defined the dogma that a polyubiquitin chain must consist of at least four ubiquitin moieties to be degraded efficiently by the proteasome (Thrower, 2000). But, this dogma is being questioned through recent studies showing that multi- and monoubiquitylation, as well as modification with two di-ubiquitin chains can also induce proteasomal degradation (Dimova et al., 2012; Shabek et al., 2012; Lu et al., 2015). Considering these findings and the upcoming of branched polyubiquitin chains that lead to proteasomal degradation, a ‘ubiquitin threshold’ model has been proposed. In this model, it is not the linkage type of ubiquitin but rather the
amount of polyubiquitin linked to the substrate that destines the substrate for proteasomal degradation (Swatek & Komander, 2016). K63-linked polyubiquitin chains entail proteolytic and non-proteolytic functions, such as DNA repair (Doil et al., 2009; Stewart et al., 2009), protein sorting (Lauwers et al., 2009; Huang et al., 2013), regulation of innate immune response (Gack et al., 2007), mitophagy (Cunningham et al., 2015; Ordureau et al., 2015), and autophagy (Olzmann & Chin, 2008; Tan et al., 2008; Ferreira et al., 2015). Polyubiquitin chains formed at M1 and mixed chains consisting of M1/K63-linked polyubiquitin chains are involved in several immune signalling pathways, most notably the regulation of NF-κB activation (Walczak et al., 2012; Emmerich et al., 2013; Iwai et al., 2014). Ubiquitin chains linked through K6 have been shown to regulate DNA damage response (Wu-Baer et al., 2003; Morris & Solomon, 2004; Elia et al., 2015) and mitochondrial homeostasis (Durcan et al., 2014; Ordureau et al., 2014; Cunningham et al., 2015). Similarly, K27-linked ubiquitin chains are involved in recruitment of proteins to enable DNA damage repair (Gatti et al., 2015) and protein secretion (Palicharla & Maddika, 2015), as well as to increase autophagic flux (Liu et al., 2014b). Interestingly, ubiquitin chains built on K29 likely prevent proteasomal degradation in response to stress (Besche et al., 2014). Moreover, this linkage type seems to be involved in epigenetic regulation (Licchesi et al., 2012; Kristariyanto et al., 2015; Michel et al., 2015; Jin et al., 2016). Furthermore, K33-linked ubiquitin chains are involved in intracellular trafficking (Yuan et al., 2014b) and were shown to dampen TCR signalling (Huang et al., 2010). Finally, polyubiquitin chains can be modified with other ubiquitin-like modifiers such as SUMO and NEDD8. However, the functional roles of these types of chains have yet to be identified (Swatek & Komander, 2016). In conclusion, the diversity of ubiquitin modifications, which have been identified so far, signals a plethora of different functions. Nonetheless, further research is necessary to unravel the entire complexity and functions of the multi-faceted ubiquitin network, which has become obvious in the recent years.

1.1.3 Ubiquitin-Like Modifiers

As mentioned above, the globular structure of ubiquitin known as the ‘β-grasp’ fold (Figure 2) is shared by many proteins (Burroughs et al., 2007; Zuin et al., 2014). These proteins are summarised in the class of ubiquitin-like (UBL) proteins that in turn combines two groups of proteins, the ubiquitin-like modifiers (ULMs) and the ubiquitin-domain proteins (UDPs). UDPs are proteins where the ubiquitin-like structure is present as a domain of a larger protein. In these proteins, the ubiquitin-like structure is not conjugated but rather used for protein-protein interactions. ULMs are small proteins that essentially consist of the ubiquitin-like ‘β-grasp’ fold
(Jentsch & Pyrowolakis, 2000; Groettrup et al., 2010a). Up to date 11 ULMs have been identified that share common characteristics apart from the ‘β-grasp’ fold. The ULMs are listed in table 1, which was adapted from (Herrmann et al., 2007). Most of the ULMs are expressed as precursor polypeptides and need to be processed to free the C-terminal diglycine motif that is necessary for their conjugation to target proteins. For conjugation, most ULMs utilise a distinct conjugation machinery that works similar to that of ubiquitin (Kerscher et al., 2006).

### Table 1: The known ubiquitin-like modifiers.

<table>
<thead>
<tr>
<th>ULM</th>
<th>Ubiquitin-like modifier</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISG15</td>
<td>Interferon-stimulated gene 15</td>
<td>(Farrell et al., 1979)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Korant et al., 1984)</td>
</tr>
<tr>
<td>MNSFβ</td>
<td>Monoclonal nonspecific suppressor factor β</td>
<td>(Michiels et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>(FUB1, Fubi)</td>
<td>(Nakamura et al., 1995)</td>
</tr>
<tr>
<td>NEDD8</td>
<td>Neural precursor cell-expressed, developmentally downregulated 8</td>
<td>(Kumar et al., 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Kamitani et al., 1997)</td>
</tr>
<tr>
<td>FAT10</td>
<td>HLA-F locus associated transcript 10</td>
<td>(Fan et al., 1995, 1996)</td>
</tr>
<tr>
<td>SUMO1-3</td>
<td>Small ubiquitin like modifiers-1, -2, -3</td>
<td>(Matunis et al., 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Mahajan et al., 1997)</td>
</tr>
<tr>
<td>ATG12</td>
<td>Autophagy related gene 12</td>
<td>(Mizushima et al., 1998a, b)</td>
</tr>
<tr>
<td>ATG8</td>
<td>Autophagy related gene 8</td>
<td>(Mann &amp; Hammarback, 1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Kabeya, 2000)</td>
</tr>
<tr>
<td>URM1</td>
<td>Ubiquitin-related modifier-1</td>
<td>(Furukawa et al., 2000)</td>
</tr>
<tr>
<td>UBL5 (HUB1)</td>
<td>Ubiquitin-like 5 (Homology to ub-1)</td>
<td>(Friedman et al., 2001)</td>
</tr>
<tr>
<td>UFM1</td>
<td>Ubiquitin-fold modifier-1</td>
<td>(Komatsu et al., 2004)</td>
</tr>
<tr>
<td>MUB</td>
<td>Membrane anchored ubiquitin-fold protein</td>
<td>(Downes et al., 2006)</td>
</tr>
</tbody>
</table>

Aside of the common features there are differences between the ULMs, for example, FAT10 (Bates et al., 1997), ATG12 (Mizushima et al., 1998b), and URM1 (Furukawa et al., 2000) are synthesised as mature proteins and do not need to be processed by de-conjugating enzymes prior to conjugation (Kerscher et al., 2006). Furthermore, UFM1, ATG8 and ATG12 do not contain the conserved diglycine motif and only have one glycine residue at the C-terminus (Mizushima et al., 1998b; Kabeya, 2000; Komatsu et al., 2004). MUB represent a unique group of ULM since they contain a conserved CaaX (cysteine-aliphatic-aliphatic-any amino acid) motif at their C-terminus with which MUBs can be anchored to membranes upon prenylation. Therefore, MUB is likely not a substrate modifying ULM. Additionally, the structure of MUB
shows that there are pronounced N- and C-terminal extensions as well as external loops protruding from the ‘β-grasp’ fold (Downes et al., 2006). Another atypical ULM is UBL5 since it lacks C-terminal glycine residues completely and has a conserved di-tyrosine motif at its C-terminal end instead (Dittmar et al., 2002). Interestingly, this di-tyrosine motif is dispensable for UBL5 function and followed by a non-conserved amino acid, which is not cleaved. Therefore, UBL5 exerts its function only by noncovalent interaction and independent of ATP hydrolysis (Lüders et al., 2003; McNally et al., 2003; Yashiroda & Tanaka, 2004; Mishra et al., 2011). In line with this, the UBL5 and MUB ‘conjugation’ pathway lacks E1 activating and E2 conjugating enzymes as well as E3 ligases and de-conjugating enzymes (Vierstra, 2012).

The conjugation pathway of URM1 differs from the canonical E1-E2-E3 cascade in that its E1 activating enzyme UBA4 leads to the formation of a thiocarboxylate at the C-terminal glycine. Following activation, the URM1-thiocarboxylate likely conjugates to substrates without further enzymatic assistance by E2 conjugating enzymes and E3 ligases (Goehring et al., 2003; Leidel et al., 2009; Van der Veen et al., 2011). Due to this characteristic of the URM1 conjugation pathway, it represents the evolutionary link between ubiquitin or ULM protein conjugation pathways in eukaryotes and ubiquitin-like sulphur transfer pathways present in prokaryotes (Schmitz et al., 2008; van der Veen & Ploegh, 2012; Vierstra, 2012). ISG15 and FAT10 consist of two ubiquitin-like domains, and together with MNSFβ are a group of cytokine-inducible ULMs (Reich et al., 1987; Kessler et al., 1988; Nakamura et al., 1992, 1996; Liu et al., 1999; Raasi et al., 1999). Moreover, ISG15 and MNSFβ can be secreted from cells acting in a cytokine-like manner (Knight & Cordova, 1991; Nakamura et al., 1992, 1996). Apart from these differences, the conjugation pathways of several ULMs intersect. FAT10 and ubiquitin both can be activated by UBA6 and transferred onto USE1 (Chiu et al., 2007; Aichem et al., 2010) and likewise the E2 conjugating enzyme UBE2L6 is bispecific for ISG15 and ubiquitin (Tatham et al., 2001). Additionally, ATG8 and ATG12 use the same E1 activating enzyme ATG7 but preserve their own E2 conjugating enzyme (Geng & Klionsky, 2008). Similarly, the SUMO1-3 paralogs are activated by the heterodimeric E1 activating enzyme SAE1/2 and are transferred onto UBC9, the only known E2 conjugating enzyme of the SUMO conjugation pathway (Tatham et al., 2001; Stewart et al., 2016). Notably, SUMO1-3 and NEDD8 can form polymers in a ubiquitin-like manner whereas the other modifiers have only been found as a single modification of one or more lysine residues on their substrates (van der Veen & Ploegh, 2012).

Modifications with ULMs implicate a range of different outcomes. Among others, ULMs are involved in proteasomal or autophagosomal targeting, in protein trafficking, in signal
transduction and T cell activation. Furthermore, modification by certain ULMs regulates E3 ligase activity, transcription, and stress responses, as well as influences RNA splicing. Further details about the functions of the different ULMs can be found in (Cajee et al., 2012; van der Veen & Ploegh, 2012; Vierstra, 2012). A detailed description of the ULM FAT10 will be presented in the following chapters.

1.2 The Ubiquitin-Like Modifier FAT10

The ubiquitin-like modifier FAT10 has been discovered by sequencing of the HLA-F locus within the human MHC class I region, therefore its name ‘HLA-F adjacent transcript 10’ (Fan et al., 1995, 1996). Further analysis showed that FAT10 resembles a di-ubiquitin protein (Fan et al., 1996) and consists of two ubiquitin-like domains (Bates et al., 1997). The two domains are arranged in a tandem head to tail conformation and are joined by a short linker of five amino acids. Interestingly, the N- and C-terminal ubiquitin-like domains of human FAT10 share 29% and 36% sequence identity to ubiquitin, respectively, but only a 20% sequence identity to each other indicating the development of distinct functions for both domains (Bates et al., 1997). The two ubiquitin-like domains of murine FAT10 are less similar to ubiquitin with a sequence identity of the N- and C-terminal domain of 26% and 33%, respectively, and again both domains are less conserved compared to each other with only 18% sequence identity than compared to ubiquitin. But a comparison of both domains of murine and human FAT10 shows a similar level of 69% sequence identity, which led Raasi and colleagues conclude that both domains contribute equally to the function of FAT10 (Liu et al., 1999; Raasi et al., 1999).

Although murine and human FAT10 share a high sequence identity, the lysine residues corresponding to K27, K33, K48, and K63 within the ubiquitin sequence are only conserved in both domains of human FAT10 (Figure 4, upper panel) (Bates et al., 1997). Only lysine K48 of ubiquitin is conserved in both domains of murine FAT10 indicating that the lysine residues K27, K33, and K63 are dispensable for its function (Raasi et al. 1999).

Up to date only the structure of the N-terminal ubiquitin-like domain of FAT10 is known and the structure of the full-length protein remains hypothetical based on the ubiquitin and ISG15 structure (Figure 4, lower panel) (Groettrup et al. 2008). The N-terminal domain, as has been shown by Theng and colleagues, consists of a typical ubiquitin ‘β-grasp’ fold with five β-strands and one α-helix, but its electrostatic potential surface is different to that of ubiquitin. They also conclude that the linker connecting the N- and C-terminal domain of FAT10 is flexible and allows for free movement of both domains against each other (Theng et al., 2014). Furthermore, it has been suggested that the linker might be crucial for the function of FAT10.
since its sequence and the surrounding amino acids are highly conserved between the species (Liu et al., 1999; Raasi et al., 1999).

<table>
<thead>
<tr>
<th>Conserved lysine</th>
<th>hFAT10-N</th>
<th>mFat10-N</th>
<th>Ubiquitin</th>
<th>hFAT10-C</th>
<th>mFat10-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>K27</td>
<td>MAPNASCLCVHVR-EEENDMTFDANPYDSDVKKTVKSTKVPQDVQVLGSLKLKRRRsslSYYGIDIEKTlHlTLKVKP</td>
<td>MASVRCTV-...VRQRPMLTFETTEDKVKINEHRQSVQVSQHILLLSDSGLKHRLSSYGIDETTTIHLTLKVKP</td>
<td>MQIF-...-VAT-LSNKTITLVEPSTNIEV/AKTDK...EGIPNDQQLFAGQLEDGRNLDVNYIQESTLHLVLRLRGG</td>
<td>PLFL-----...VESEDEAKHRLLQVRSSSSVQVRAMIEETKIIPEIQTIVCTNGKRLDGGMADYGRIGNLFLACYCIGG</td>
<td>PLFL-----...VSEKNESQVRLVRSSSSVQVRKMEIESTSVIPKQVNCNGKLEDGTMADYNIKGSGLLFLTTHCTGG</td>
</tr>
</tbody>
</table>

**Figure 4:** Sequence alignment of FAT10 and ubiquitin and FAT10 structural model. Upper panel: Sequences of the N- and C-terminal domain of murine FAT10 (mFat10) and human FAT10 (hFAT10) separately as well as ubiquitin were aligned. The linker sequence ‘SDEEL’ was omitted from the alignment. Lysine residues conserved in both domains of human or murine FAT10 are shown above the alignment. Lysine residues K27, K33, and K63 (shown in magenta) of ubiquitin are conserved in both domains of human FAT10. Lysine residue K48 (highlighted in red) of ubiquitin is conserved in both domains of murine and human FAT10. Lysine residues of ubiquitin that are not conserved (K6 & K29) and conserved in only one domain of human and murine FAT10 are shown in green (K11 in the C-terminal domain of human FAT10; K27 in the C-terminal domain and K63 in the N-terminal domain of murine FAT10). Overall conserved, conservative, and semi-conservative amino acids are shown below the alignment. Asterisk = conserved amino acid; Colon = conservative amino acid change; Period = semi-conservative amino acid change. The alignment was calculated using the multiple sequence alignment tool MAFFT on the EMBL-EBI website (https://www.ebi.ac.uk/Tools/msa/mafft/) on 3rd March 2018. For the alignment, the ‘ClustalW’ output format was chosen and the ‘Blosum30’ matrix was used for calculation. Lower panel: Ribbon diagram of the proposed structural model of human FAT10 showing both domains with the characteristic ubiquitin ‘β-grasp’ fold consisting of four β-strands and one α-helix. The structural model is adapted from (Groettrup et al., 2008).

Apart from modifying other proteins, FAT10 itself has been shown to be the target of post-translational modifications. It has been reported that lysine residues within FAT10 can become ubiquitylated (Hipp et al., 2005; Buchsbaum et al., 2012a) and acetylated (Kalveram et al., 2008). Moreover, threonine and serine residues of FAT10 can be phosphorylated (Raasi et al., 2001; Hipp, 2005).
1.2.1 \textit{FAT10} Expression

Basal expression of murine and human \textit{FAT10} is most prominently in tissues of the immune system and organs harbouring mucosa-associated lymphoid tissues. Accordingly, basal expression of \textit{FAT10} in humans and mice is observed in adult and foetal thymus, spleen, and liver, as well as lymph nodes, tissues of the gastrointestinal tract, lung, and kidney, but also in the immune-privileged reproductive organs (Liu et al., 1999; Lee et al., 2003; Hase et al., 2005; Canaan et al., 2006; Lukasiak et al., 2008). While there is comprehensive data about tissue-specific expression of \textit{FAT10}, the identity of the cells that express \textit{FAT10} in these tissues has not been addressed fully. So far, expression of \textit{FAT10} is described for non-activated and activated CD34\textsuperscript{+}-derived and monocyte-derived dendritic cells (MoDCs) and B cells (Bates et al., 1997; Lukasiak et al., 2008). The high level of \textit{FAT10} expression observed in thymus is almost only caused by medullary thymic epithelial cells (TECs) and to a marginal extent by cortical TECs (Buerger et al., 2015). Furthermore, Ocklenburg and colleagues could show that \textit{FAT10} is expressed in long-term cultures of human regulatory T cells (Tregs) (Ocklenburg et al., 2006).

Besides basal expression, \textit{FAT10} expression can be induced by various inflammatory stimuli or by infection with specific viruses. Therefore, \textit{FAT10} is expressed in many mature B cell lines that were transformed with the Epstein-Barr virus (EBV) but not in transformed precursor B cell lines (Fan et al., 1996; Bates et al., 1997). \textit{FAT10} expression is similarly upregulated in the human proximal tubular cell line (HPT-1) upon transduction with mutant, VSV-G pseudotyped HIV-1 (Ross et al., 2006), in the human respiratory epithelial cell line A549 upon influenza virus infection (Zhang et al., 2016), and in human dermal microvascular endothelial cells infected with Kaposi sarcoma-associated herpesvirus (Hong et al., 2004). Apart from this, \textit{FAT10} expression is inducible in many human and murine cell types using pro-inflammatory cytokines. Notably, a synergistic effect of the cytokines IFN-\(\gamma\) and TNF (Liu et al., 1999; Raasi et al., 1999; Lukasiak et al., 2008; Oliva et al., 2010), IL-6 and TNF (Choi et al., 2014), as well as IFN-\(\gamma\) and extracellular HSPB5, which acts as TLR2 agonist, (Bsibsi et al., 2014) can be observed. Interestingly, the cytokines IL-1\(\beta\) and IFN-\(\gamma\) induce \textit{FAT10} in human and rat beta cells but without any synergistic effect (Brozzi et al., 2016). Furthermore, induction of \textit{FAT10} expression is reported after IL-1\(\beta\) stimulation of human keratinocytes (Rebane et al., 2014) and by different cytokine cocktails consisting of several pro-inflammatory cytokines in rat neuronal cell cultures and human monocyte-derived dendritic cells (Ebstein et al., 2009; Lisak et al., 2011). Additionally, \textit{FAT10} is induced by the Toll-like receptors (TLR) ligands lipopolysaccharide (LPS) and polyI:C, as well as CD40L alone or in combination with
prostaglandin E2 (PGE2) in human \textit{in vitro}-generated dendritic cells and mature B cells (Bates et al., 1997; Lukasiak et al., 2008; Ebstein et al., 2009). Moreover, \textit{FAT10} expression is upregulated in human keratinocytes treated with the TLR1/2 ligand Pam3CSK4 (Rebane et al., 2014), in villous epithelium of RANKL-treated mice (Kobayashi et al., 2012), and in human MCF-7 breast carcinoma cells incubated with retinoic acid and fenretinide, a synthetic retinoic acid derivate (Dokmanovic et al., 2002). The inducibility of \textit{FAT10} by this diverse range of stimuli can be explained by studies that have investigated the promoter and the 5’ untranslated region of the murine and human \textit{FAT10} gene. These regions contain recognition sites targeted by transcription factors, such as STAT-1, STAT-3, AP-1, NF-κB, IRF-1, IRF-2, and MZF-1, which are involved in the signalling of the above-mentioned stimuli (Canaan et al., 2006; Zhang et al., 2006; Choi et al., 2014; Gao et al., 2015).

Besides that, expression of \textit{FAT10} is upregulated in graft-versus-host disease after transfer of allogeneic T cells together with syngeneic bone marrow cells into irradiated mice (Ichiba et al., 2003; Sugerman et al., 2004). In graft-versus-host disease in human and rat skin explants \textit{FAT10} is upregulated and has even been identified as risk gene by expression profiling (Novota et al., 2011). Opposite to this, by means of a comprehensive integrated meta-analysis of publicly available gene expression data sets of transplantation studies, \textit{FAT10} expression has been identified as a signature gene in acute rejection of solid organ transplants (Choi et al., 2017).

Although not fully understood, regulation of \textit{FAT10} expression has been observed on several levels. First, the promoter region of \textit{FAT10} contains a p53 recognition site by which p53 negatively regulates \textit{FAT10} expression (Zhang et al., 2006). In line with this, overexpression of \textit{FAT10} in gastric cancer tissue was positively correlated with mutant p53 expression suggesting that the \textit{FAT10} expression level could serve as prognostic marker for gastric cancer (Ji et al., 2009). Both FAT10 and p53 are acting in a feedback loop, where p53 reduces \textit{FAT10} expression and FAT10 modifies p53 and its activity (Figure 7). Yet, controversial results have been published regarding the effect of \textit{FAT10} overexpression on p53 activity, which either increased or decreased (Li et al., 2011; Choi et al., 2014), however, endogenous \textit{FAT10} expression induced by cytokines results in a reduction of p53 activity (Choi et al., 2014). Secondly, Liu and colleagues have reported that the 5’ untranslated region of both the murine and human \textit{FAT10} mRNA contains untranslated open reading frames, which can prevent translation of FAT10 from the proper start codon (Liu et al., 1999). Finally, the microRNA, miR-146a, can suppress IFN-γ-induced \textit{FAT10} expression in keratinocytes and skin biopsies of patients with atopic dermatitis by targeting the NK-κB pathway (Rebane et al.,
2014), and microRNA, miR-24-1-5p, has been shown to directly target \textit{FAT10} and suppress its expression (Xiao et al., 2017).

Interestingly, reports on the stability of mRNA have found that murine \textit{FAT10} mRNA is very stable with a stability of at least 12 h (Hao and Baltimore 2009 supplementary table 1; Sharova et al. 2009 supplementary table 1). In the same study they clustered murine \textit{FAT10} into a group of genes with a progressively increasing expression that follow the kinetic of induction reported for human \textit{FAT10} (Aichem et al., 2012). The high stability of \textit{FAT10} mRNA and the short half-life of \textit{FAT10} protein of approximately one hour (Raasi et al., 2001; Hipp et al., 2005; Aichem et al., 2014; Schmidtke et al., 2014) suggests a very tight regulation of \textit{FAT10} expression at both the transcriptional and the translational level. This might allow for a rapid response to environmental changes and prevent deleterious effects of excessive translation of \textit{FAT10} protein. Accordingly, a genome-wide study on mRNA and protein levels and half-lives, as well as transcription and translation rates has assigned genes producing stable mRNAs and unstable proteins to biological processes in which \textit{FAT10} is involved, such as proteolysis, defence response, and regulation of cell proliferation (Schwanhäusser et al., 2011).

\subsection*{1.2.2 \textit{FAT10} Conjugation}

Like ubiquitin, \textit{FAT10} uses an enzymatic cascade comprising an E1, E2, and possibly E3 enzymes, which mediate covalent attachment of \textit{FAT10} to its substrates (Figure 5). \textit{FAT10} shares its activating enzyme ‘ubiquitin-like modifier-activating enzyme 6’ (UBA6) with ubiquitin (Chiu et al., 2007). Thioester formation of UBA6 with ubiquitin is preferred as has been shown in an \textit{in vitro}-conjugation assay, and thioester formation with \textit{FAT10} has been observed only at increasing concentrations of \textit{FAT10} suggesting no difference in the affinity of UBA6 for both ULMs (Chiu et al., 2007). Despite that, Gavin and colleagues determined the affinity of UBA6 for \textit{FAT10} and ubiquitin and found a higher affinity of UBA6 for \textit{FAT10} than for ubiquitin. Additionally, they observe that the initial adenylation of \textit{FAT10} by UBA6 is slower than the UBA6-ubiquitin adenylate formation (Gavin et al., 2012). In summary, both studies suggest that the increase of the \textit{FAT10} level upon induction shifts the ratio of the levels of ubiquitin and \textit{FAT10} towards \textit{FAT10} and eventually switches on the UBA6-dependent \textit{FAT10} conjugation pathway.

Remarkably, transthioylation of activated \textit{FAT10} by UBA6 is more specific than that of activated ubiquitin by UBA6. Transfer of \textit{FAT10} is observed only onto one known E2 conjugating enzyme, namely UBA6-specific E2 conjugating enzyme 1 (USE1) (Aichem et al., 2010). In line with this, the E2 conjugating enzymes UBC5, UBC13 (Chiu et al., 2007), and
UBCH8 (Aichem et al., 2010), do not accept FAT10 but ubiquitin from UBA6. Activated ubiquitin on the contrary can be accepted by many E2 conjugating enzymes from UBA6 (Jin et al., 2007). Like UBA6, USE1 is bispecific for FAT10 and ubiquitin and accepts both modifiers only from UBA6 (Gu et al., 2007; Jin et al., 2007; Aichem et al., 2010). Similar to the slower adenylation of FAT10, the transfer of FAT10 onto USE1 is slower than the transfer of ubiquitin (Gavin et al., 2012). A recent study determined the structure of USE1 and thereby gained insight into how the conjugation pathway of FAT10 achieves its specificity. They show that the four amino acids before the C-terminal diglycine motif, i.e. CYCI, determine most of the specificity of FAT10 towards UBA6 and USE1 in terms of activation and transthiolation. When these amino acids are replaced by LRLR, the corresponding four amino acids in ubiquitin, the specificity of FAT10 is abolished and FAT10 can be activated by UBE1 as well as transferred onto several E2 conjugating enzymes. Likewise, the velocity of the transfer from UBA6 onto USE1 increases to the same level observed for ubiquitin. Besides that, the authors suggested that apart from the CYCI peptide the specificity of the FAT10 conjugation pathway is achieved by specific interactions between UBA6, USE1, and FAT10 (Schelpe et al., 2016). Noteworthy, the specificity inferred from the CYCI peptide probably applies only to the human FAT10 conjugation system, since the amino acid stretches in murine and rat FAT10 are THCT and AHCI, respectively, thus are not well conserved. But, the conjugation enzymes UBA6 and USE1 in rat (rattus norvegicus; taxid:10116) and mouse (mus musculus; taxid:10090) are 89% and 99% identical to their human orthologs, respectively (protein blast search using human UBA6 [accession number: A0AVT1.1] and USE1 [accession number: Q9H832]), suggesting that in rat and mouse the specificity might be determined mostly by structural features and interactions between FAT10, UBA6, and USE1.

USE1 is also one of the few confirmed substrates of FAT10, since USE1 FAT10ylates itself in cis (Figure 5, right side) (Aichem et al., 2010). This auto-modification also represents a means of negatively regulating the FAT10 conjugation pathway by accelerating degradation of USE1. Interestingly, FAT10ylated USE1 still can be loaded with an additional FAT10 or ubiquitin molecule, thus FAT10 modification does not affect USE1 activity. Therefore, Aichem and colleagues hypothesised that FAT10ylation of USE1 determines substrate specificity or helps to discriminate between ubiquitin- and FAT10-specific E3 ligase (Aichem et al., 2014). Another means of regulating the FAT10 conjugation pathway has been suggested by Wu and colleagues. They have seen that the LIM domain only protein 2 (LMO2), which is involved in transcriptional regulation, can prevent the interaction between UBA6 and USE1, and consequently FAT10ylation (Wu et al., 2016). Likewise, it has been shown that aryl
hydrocarbon receptor interacting protein-like 1 (AIPL1), which, if mutated, is the cause of the inherited blindness Leber's congenital amaurosis, interacts with UBA6 changing the profile of FAT10ylated proteins and competes with FAT10 for binding to UBA6 (Bett et al., 2012).

Figure 5: The FAT10 conjugation pathway and auto-FAT10ylation of USE1. FAT10 becomes activated by UBA6 through adenylation of the C-terminal glycine residue in an ATP-dependent manner. The resulting FAT10-adenylate reacts with the active site cysteine of UBA6 and forms a thioester bond. Subsequently UBA6 transfers the FAT10 moiety onto the active site cysteine of USE1 in a transthiolation. Right pathway: USE1 auto-FAT10ylates itself leading to proteasomal degradation, which represents a means of limiting or regulating FAT10ylation of substrates. Left pathway: FAT10-loaded USE1 can transfer FAT10 onto lysine residues within substrate proteins. So far unknown E3 ligases (E3?) very likely assist during FAT10 transfer onto substrates. No chain formation has been observed for FAT10, but mono-FAT10ylation serves already as signal for proteasomal degradation. FAT10 is degraded by the proteasome along with its substrates. In line with this, no FAT10-specific protease (DUB?) that cleaves FAT10 from its substrates has been identified.

Although it is assumed that there are E3 ligases for FAT10, which help to transfer FAT10 onto its substrates, and potential E3 ligases have been identified in a mass spectrometry analysis of induced cells (Aichem et al., 2012) no FAT10-specific E3 ligase has been confirmed so far (Figure 5, left side). For formation of FAT10 conjugates, E3 ligases seem to be irrelevant since \textit{in vitro} conjugation is successful in presence of UBA6 and USE1 alone (Aichem et al., 2012; Bialas et al., 2015). It has been suggested by Bialas and colleagues that the close proximity of the proteins in \textit{in vitro} assays, which is due to the high amount of protein used, can already lead
to efficient transfer and conjugate formation, which would normally be mediated by E3 ligases (Stewart et al., 2016). Yet, cellular protein levels are much lower than in in vitro assays, thus formation of FAT10 conjugates in cells still could require E3 ligases (Bialas et al., 2015). Moreover, it is conceivable that FAT10 might employ E3 ligases that are again bispecific for ubiquitin and FAT10, as is the case for UBA6 and USE1.

Intriguingly, FAT10 seems to be degraded along with its substrates rather than being recycled (Hipp et al., 2005; Aichem et al., 2014; Schmidtke et al., 2014; Bialas et al., 2015). Furthermore, FAT10 does not require a de-conjugating enzyme for processing prior to conjugation since it is synthesised already with a free diglycine motif at its C-terminus (Bates et al., 1997). Despite this observed redundancy of a de-conjugating enzyme specific for FAT10, five potential de-conjugating enzymes have been identified (Aichem et al., 2012) and since not all substrates of FAT10 are known it is possible that there is a de-conjugating enzyme regulating FAT10-mediated degradation (Figure 5, left side). If existent, this FAT10-specific de-conjugating enzyme does not seem to be associated with the 26S proteasome, since no evidence for de-conjugation prior to degradation has been observed so far (Hipp et al., 2005; Schmidtke et al., 2014). In conclusion, further research on the identification of FAT10-specific E3 ligases and de-conjugating enzymes is necessary and currently ongoing.

Contrary to ubiquitin, which is known to form diverse polyubiquitin chains, this feature has not been observed for FAT10. Most proteins become modified covalently with only one FAT10 molecule as has been shown for UBE1 (Rani et al., 2012; Bialas et al., 2015), USE1 (Aichem et al., 2010), AIPL1 (Bett et al., 2012), p53 (Li et al., 2011), and luminal domain-like LAP1 (LULL1) (Buchsbaum et al., 2012b). Albeit less often, modification with several FAT10 molecules, i.e. multi-monoFAT10ylation, is found on the autophagy adaptor p62/SQSTM1 that becomes FAT10ylated at three to four lysine residues (Aichem et al., 2012), on mutant Huntingtin (Nagashima et al., 2011), and on leucine-rich repeat flightless-interacting protein 2 (LRRFIP2), which positively regulates NF-κB activity upon TLR4 stimulation (Buchsbaum et al., 2012b). Remarkably, the choice of the lysine residue that becomes FAT10ylated seems to be random with only minor preferences for specific lysine residues, since mutation of the lysine residues, for example, in USE1 and LRRFIP2 does not largely affect FAT10ylation of these substrates (Buchsbaum et al., 2012b; Aichem et al., 2014). Additionally, although not observed so far it cannot be excluded that there are heterologous chains consisting of FAT10 and ubiquitin, since the lysine residues K27, K33, K48, and K63 of ubiquitin are conserved in human FAT10 and K48 in murine FAT10 (Bates et al., 1997; Raasi et al., 1999). Moreover, modification of FAT10 with several ubiquitin molecules has been described (Hipp et al., 2005;
Nagashima et al., 2011; Buchsbaum et al., 2012a) and one study has shown modification of ubiquitin with a single FAT10 molecule (Nagashima et al., 2011).

Several studies performed large proteomic analyses to identify FAT10 substrates or interacting proteins (Aichem et al., 2012; Merbl et al., 2013; Leng et al., 2014). Functional classification of these proteins displays a broad range of biological functions suggesting that FAT10 is involved in many different rather than only specific cellular processes. In addition, Leng and colleagues could not identify a specific consensus sequence around FAT10ylated lysine residues, however, hydrophilic amino acids are observed frequently within six amino acids up-and downstream of the modified lysine residue (Leng et al., 2014). This is in line with the observation that FAT10 does not discriminate considerably between the lysine residues that are targeted (Aichem et al., 2012; Buchsbaum et al., 2012b). Moreover, FAT10 is conjugated to newly synthesised proteins. Nevertheless, newly synthesised proteins do not represent the main portion of FAT10ylated substrates, as their FAT10ylation is not as prominent as observed for ISG15 or ubiquitin suggesting that only a certain group of newly synthesised proteins becomes FAT10ylated. Most likely, these proteins represent defective ribosomal products (DRiPs), which are only a fraction of all co-translationally modified and rapidly degraded proteins (RDPs) (Spinnenhirn et al., 2017).

1.2.3 Functions of FAT10

First speculations about the functions of FAT10 have been made by Sherman Weissman’s group, who identified FAT10 as a di-ubiquitin. Due to its expression in EBV-transformed B cells and its localisation in the HLA-F locus in the MHC class I region, they suggested that FAT10 might be involved in antigen processing, apoptosis, or oncogenesis (Figure 7 & 8) (Fan et al., 1996). These hypothesised functions have set the agenda for the following years of research on FAT10, which has been reviewed recently (Basler et al., 2015a; Aichem & Groettrup, 2016). The first functional implications were made be Liu and colleagues, seeing an accumulation of endogenous FAT10 in cells treated with proteasome inhibitor. They also observe that FAT10 can interact non-covalently with mitotic arrest deficient 2 (MAD2), yet they fail to detect covalent conjugation of FAT10 (Liu et al., 1999). The first covalent interaction was described by Raasi and colleagues in a murine fibroblast cell line stably expressing murine FAT10 using a Tet-Off system. They observe a covalent interaction with an unknown protein depending on FAT10’s C-terminal diglycine motif and, again, they see accumulation upon proteasomal inhibition (Raasi et al., 2001). Thus, both studies identified the
main mechanisms by which FAT10 exerts its functions, namely by covalent conjugation with subsequent proteasomal degradation and non-covalent interaction.

Regarding proteasomal targeting, FAT10 is unique among the known ULMs since it is the only other ULM apart from ubiquitin that targets its substrates directly for proteasomal degradation and in a ubiquitin-independent manner (Hipp et al., 2005; Schmidtke et al., 2009). As mentioned above ubiquitylation of FAT10 and vice versa has been observed, but ubiquitylation does not seem to be necessary for degradation of FAT10ylated substrates as well as FAT10 itself (Hipp et al., 2005). In the study of Hipp and colleagues, a FAT10-GFP fusion protein has been shown to become polyubiquitylated, but the low amount of polyubiquitylation of this fusion protein does not reflect its degradation rate suggesting that the degradation is mediated by FAT10 and occurs independent of ubiquitin. Using a lysine-less FAT10 mutant that does not become modified with ubiquitin, they can further support their finding and show that this mutant has the same degradation rate compared to wild type (WT) FAT10 (Hipp et al., 2005). To confirm the ubiquitin-independent degradation of FAT10ylated substrates an in vitro degradation assay using a FAT10-dihydrofolate reductase (DHFR) fusion protein shows again ubiquitin-independent degradation (Schmidtke et al., 2009). Despite this evidence, Buchsbaum and colleagues questioned the ubiquitin-independent degradation capability of FAT10 because they have seen that degradation of FAT10 and a FAT10-GFP fusion protein requires ubiquitylation. Yet, they also observe degradation of the same FAT10-GFP fusion protein in presence of a non-conjugatable ubiquitin, i.e. ubiquitin-independent degradation, thus the authors could not exclude degradation mediated by FAT10 alone (Buchsbaum et al., 2012a).

The mechanism how FAT10 is degraded by the proteasome involves interaction of FAT10 with the 19S RP and its subunit RPN10. The degradation of FAT10 can be accelerated by interaction with NEDD8 ultimate buster 1-long (NUB1L) (Hipp et al., 2004), and the 19S RP of the 26S proteasome (Figure 6). An interaction of FAT10 with the subunit RPN10 and an interaction of NUB1L with the subunits RPN10 and RPN1 of the 19S RP is reported. Additionally, a trimeric complex exists consisting of FAT10, NUB1L, and RPN10. Based on these findings, Rani and colleagues suggest two different models of how the accelerated degradation of FAT10 by NUB1L can be explained. In the ‘transfer’ model NUB1L serves as a soluble receptor that binds FAT10 and subsequently transfers FAT10 and its conjugates onto RPN10 after binding to RPN1 allowing for fast degradation. The ‘facilitator’ model proposes that binding of NUB1L to RPN1 causes conformational changes in the subunits RPN1 and RPN10 and thereby facilitates faster degradation of FAT10, which binds to RPN10, and its
substrates. Similarly, FAT10 can bind RPN10 in absence of NUB1L, but this slows down degradation of FAT10 and its substrates (Rani et al., 2012).

**Figure 6: The different proposed mechanisms of FAT10 degradation by the proteasome.** Degradation without NUB1L: In absence of NUB1L, FAT10 is bound by RPN10 and degraded by the 26S proteasome. Transfer model: NUB1L binds FAT10 with or without substrate. Then, NUB1L binds to RPN1 and subsequently transfers FAT10 onto RPN10 to enhance degradation. Facilitator model: Upon binding of NUB1L, RPN1 and RPN10 undergo conformational changes that allow for faster degradation of FAT10 after binding to RPN10. Models are depicted as proposed by Rani and colleagues (Rani et al., 2012).

AIPL1 has been shown to influence not only the FAT10ylation profile, as mentioned above, but also counteracts NUB1L-mediated degradation of FAT10ylated proteins. It has been proposed that AIPL1 first interacts with FAT10 bound to its substrate and then forms a ternary complex together with NUB1L to prevent degradation (Bett et al., 2012). Interestingly, the degradation rate of FAT10 by the constitutive proteasome and the immunoproteasome is the same. This indicates that both proteasome types bind the 19S RP equally well (Schmidtke et al., 2017).

### 1.2.3.1 Functions in Disease and Pathological Implications

Apart from its immunological functions, FAT10 is involved in several biological processes that are disease-related. Most prominently FAT10 is involved in cancer development that recently has been summarised comprehensively (Aichem & Groettrup, 2016) and will be discussed only briefly below. *FAT10* is expressed in a wide variety of different types of cancer. These include cancers of the gastrointestinal and urogenital tract, hepatocellular carcinomas, osteosarcoma, glioma, as well as lung, kidney, and breast cancer (Lee et al., 2003; Lukasiak et al., 2008; Ji et al., 2009; Yan et al., 2010; Yuan et al., 2012, 2014a; Liu et al., 2014a; Sun et al., 2014; Han et
al., 2015; Dai et al., 2016; Dong et al., 2016; Ma et al., 2016; Xue et al., 2016). Remarkably, among all the types of cancer in which FAT10 is expressed there have been no reports about cancers of the haematopoietic and lymphoid system expressing FAT10, so far. High FAT10 expression has been associated with cancer progression, poor prognosis, and a higher risk of relapse in several types of cancer (Ji et al., 2009; Yan et al., 2010; Yuan et al., 2012; Liu et al., 2014a; Sun et al., 2014; Han et al., 2015; Zhao et al., 2015; Dong et al., 2016; Ma et al., 2016; Xue et al., 2016). Additionally, several studies showed that FAT10 increases cancer cell proliferation and promotes formation of metastasis by enhancing the migratory capacity of cancer cells (Liu et al., 2014a; Yuan et al., 2014a; Dai et al., 2016; Dong et al., 2016; Ma et al., 2016; Xue et al., 2016). How FAT10 is involved in the formation of cancers is not fully understood, but there are studies supporting the idea that FAT10 may act as a proto-oncogene (Chen et al., 2014; Gao et al., 2014; Xue et al., 2016) and FAT10 may serve as an epigenetic marker in liver neoplasia (Oliva et al., 2008). It has been suggested that FAT10 induces the malignant properties of tumours through inflammation-induced expression that may persist due to a positive feedback loop between FAT10 expression and NF-κB activation. Additionally, four pathways have been summarised by Aichem and colleagues that are affected by aberrant FAT10 expression promoting the formation of malignant tumours (Figure 7) (Aichem & Groettrup, 2016), since then two additional mechanisms have been identified (Dong et al., 2016; Liu et al., 2016).

One mechanism is the induction of chromosomal instability by expression of FAT10 that has been observed in several studies (Figure 7, red circle) (Ren et al., 2006; Theng et al., 2014; Gao et al., 2015). This chromosomal instability is likely the result of aberrant high expression of FAT10 during mitosis at which stage of the cell cycle FAT10 levels are usually low (Lim et al., 2006; Liu et al., 2014a), and the subsequent increased non-covalent interaction of FAT10 with MAD2 (Liu et al., 1999). By interacting with MAD2, FAT10 prevents binding of MAD2 to the kinetochores (Ren et al., 2006, 2011) disturbing the proper alignment of the chromosomes at the spindle equator and the mitotic checkpoint (Theng et al., 2014). Another mechanism leading to carcinogenesis can be an imbalance in the regulatory feedback that is existing between FAT10 expression and p53 activity, as described above (Figure 7, yellow circle). Here, a mechanism has been suggested that under non-inflammatory conditions p53 represses the expression of FAT10 (Zhang et al., 2006), but, under persistent inflammatory conditions high levels of FAT10 are reached that in turn reduce p53 transcriptional activity (Choi et al., 2014). The reduced p53 activity then leads to downregulation of p53-regulated genes necessary for cell cycle arrest and DNA repair or the induction of apoptosis (Levine & Oren, 2009). Hence,
causing chromosomal instability by the enhanced interaction of FAT10 with MAD2 and preventing apoptosis eventually initiating carcinogenesis (Aichem & Groettrup, 2016). Likewise, mutations in p53, which lead to decreased transcriptional activity, could cause abnormal expression of FAT10 leading to the same outcome (Zhang et al., 2006). In line with this, Ji and colleagues found a positive correlation with FAT10 and mutant p53 expression levels at both the mRNA and protein level (Ji et al., 2009). Furthermore, FAT10 has been implicated in the regulation of the WNT signalling pathway in hepatocellular carcinoma (HCC) and osteosarcoma leading to the stabilisation of β-catenin (Figure 7, blue circle) (Liu et al., 2014a; Yuan et al., 2014a; Ma et al., 2016), which is involved in metastasis of HCC (Lai et al., 2011). In this pathway, FAT10 is involved at two steps. On the one hand, FAT10 directly interacts with β-catenin thereby decreasing its ubiquitylation and proteasomal degradation. Then, the stabilised β-catenin leads to increased induction of genes resulting in cancer development and enhanced metastasis formation (Yuan et al., 2014a). On the other hand, FAT10 increases the active form of RAC-alpha serine/threonine-protein kinase (AKT). The active AKT, then, inactivates glycogen synthase kinase-3 beta (GSK3β) by dephosphorylation. Through inactivation of GSK3β, β-catenin is stabilised consequently which normally becomes phosphorylated by GSK3β and subsequently ubiquitylated and degraded (Liu et al., 2014a). Additionally, a less well understood mechanism by which FAT10 can exert tumourigenic effects upon its upregulation has been identified in glioma, where high FAT10 expression levels have been associated with poor prognosis and enhanced formation of metastasis (Figure 7, green circle) (Yuan et al., 2012; Dai et al., 2016). In glioma, FAT10 influences the transforming growth factor beta-1 (TGF-β1) signalling pathway by increasing levels of phosphorylated SMAD2 (pSMAD2) and elevating levels of cancer stem cell markers and pSMAD2 target genes, such as BMI-1, CD133, nestin, and OCT4 (Dai et al., 2016).

The last two identified mechanisms have been identified in the lab of Jianghua Shao and involve the stabilisation of proteins by FAT10 through inhibiting ubiquitylation (Dong et al., 2016; Liu et al., 2016), like the stabilisation of β-catenin (Yuan et al., 2014a). The second stabilised protein implicated in FAT10-mediated tumourigenesis is eukaryotic elongation factor 1 alpha-1 (eEF1A-1). This stabilisation of eEF1A-1 by FAT10 leads to increased tumour proliferation (Liu et al., 2016). Survivin, also known as baculoviral inhibitor of apoptosis repeat-containing protein 5 (BIRC5), represents the third protein that is stabilised by increased FAT10 expression in bladder cancer (Dong et al., 2016). The mechanisms by which survivin promotes cancer development comprise, for example, inhibition of apoptosis by preventing caspase activation, but most notably its involvement in pathways such as the p53, WNT, and
TGF-β signalling pathways (Chen et al., 2016). This suggests that FAT10 uses a complex network of mechanisms to mediate tumourigenesis that might be interconnected. Further research will be necessary to determine how exactly these mechanisms might be intertwined relating to FAT10 and its functions in promoting cancer development.

![Image: Regulation of FAT10 expression and FAT10-regulated pathways during tumourigenesis.](image)

**Figure 7: Regulation of FAT10 expression and FAT10-regulated pathways during tumourigenesis.** The cancer-promoting properties of FAT10 are provoked by persistent FAT10 expression in tumours and the tumour microenvironment induced by pro-inflammatory cytokines, such as TNF and IFN-γ. On the one hand, FAT10 regulates pathways such as NF-κB-, AKT-, and WNT-signalling and, on the other hand, modulates downstream targets, such as β-catenin, SMAD2, MAD2, and p53 within these pathways by direct interaction. Thereby, FAT10 is involved in several pathways that promote cancer development and increases resistance to apoptosis as well as promotes proliferation, formation of metastasis, and invasion of cancer cells. (+) positive regulation by FAT10; (-) negative regulation by FAT10. The image is reprinted from (Aichem & Groettrup, 2016).

Despite the anti-apoptotic functions of FAT10 described above, there are reports showing that FAT10 induces apoptosis upon HIV-1-induced expression and after overexpression in several cell lines (Liu et al., 1999; Raasi et al., 2001; Ross et al., 2006; Snyder et al., 2009). In HIV-1 infected renal cells FAT10 is induced by the viral protein VPR and this supposedly leads to apoptosis, which was confirmed by knockdown of FAT10 what prevented VPR-induced apoptosis (Snyder et al., 2009). Alternatively, Li and colleagues suggested that overexpression of FAT10 induces apoptosis by increasing p53 activity (Li et al., 2011).
Moreover, \textit{FAT10} deficiency in mice is associated with an extended life span and less age-associated infirmities. These effects are likely the result of the increased metabolism in skeletal muscle and the low amount of fat tissue present in \textit{FAT10}-deficient mice. Therefore, it has been suggested that \textit{FAT10} favours the development of obesity and dampens the fat metabolism thereby adversely affecting the aging process (Canaan et al., 2014).

\textit{FAT10} displays a certain propensity to co-localise with its interacting proteins in cytosolic spots as has been observed with its non-covalent interaction partners LLRFIP2 (Buchsbaum et al., 2012b) and eEF1A-1 (Yu et al., 2012). Similarly, \textit{FAT10} localises to protein aggregates in the cytosol, such as p62 bodies together with its interaction partner p62 (Aichem et al., 2012) and to aggresomes in a largely HDAC6-dependent manner under proteasome inhibition (Kalveram et al., 2008), suggesting indirect targeting of \textit{FAT10}ylated substrates into the autophagy pathway, as mentioned above. Additionally, formation of aggresome-like Mallory-Denk bodies, which are formed during several diseases in liver cells (French et al., 2016), depends on \textit{FAT10} since \textit{FAT10}-deficient mice did not form Mallory-Denk bodies \textit{in vivo} (French et al., 2012). \textit{FAT10} also interacts with mutant, still soluble huntingtin and sends it for degradation by the proteasome thereby reducing huntingtin aggregation (Nagashima et al., 2011).

Single nucleotide polymorphisms (SNPs) within the \textit{FAT10} locus have been associated with the pathogenesis of several human diseases. In coeliac disease, which is an autoimmune disease especially of the small intestine, a regulatory SNP located downstream of the coding sequence of \textit{FAT10} has been associated with high \textit{FAT10} expression and increased risk of disease development (Castellanos-Rubio et al., 2010). Another study analysed the association between SNPs in inflammatory genes and the risk of contracting colorectal cancer. In this study a SNP in \textit{FAT10} was identified that is associated with late stages of colorectal cancer and with a higher risk of contracting colorectal cancer below 65 years of age (Frank et al., 2010). Furthermore, a genome-wide association study (GWAS) identified nine loci that have an effect on male fertility. Among these genes they found \textit{FAT10} and a SNP downstream of it that is associated with several traits of male fertility (Kosova et al., 2012). Interestingly, Aly and colleagues found SNPs in the region of the \textit{FAT10} locus that relate to T1D (Aly et al., 2008), which is in line with the finding that \textit{FAT10} in rats is a susceptibility gene for T1D and that \textit{FAT10}-deficient rats are protected from contracting virus-induced T1D (Cort et al., 2014). On the contrary, Brozzi and colleagues observed an anti-apoptotic effect of \textit{FAT10} expression on cytokine-induced beta cells (Brozzi et al., 2016). Exposure of beta cells to pro-inflammatory cytokines induces the unfolded protein response (UPR) likely contributing to apoptosis of beta
cells in T1D (Eizirik et al., 2013). In this scenario, induction of FAT10 in beta cells reduces UPR-mediated apoptosis by reducing the activation of the IRE1α/JNK pro-apoptotic pathway (Brozzi et al., 2016).

### 1.2.3.2 Immunological Functions

FAT10 is encoded in the HLA-F locus of the MHC class I region and since its discovery it has been proposed that it might be involved in antigen presentation (Figure 8) (Fan et al., 1996). But, initial experiments using murine FAT10 have not found evidence for a role of FAT10 in antigen processing and presentation on MHC class I. In this report no influence of FAT10 on surface expression of at least three different MHC class I molecules is observed. Additionally, presentation of specific epitopes of the 89-kDa phosphoprotein (pp89) of murine cytomegalovirus (MCMV) and the nucleoprotein (NP) of the lymphocytic choriomeningitis virus (LCMV) occurs independent of FAT10 expression. Despite these setbacks the authors suggest that more epitope need be tested to allow for a final conclusion on the role of FAT10 in antigen presentation (Raasi et al., 2001). After the discovery that FAT10 can serve as a ubiquitin-independent signal for proteasomal degradation (Hipp et al., 2005; Schmidtke et al., 2009) the initial hypothesised function of FAT10 in antigen presentation has been fuelled again.

In fact, subsequent reports show that murine and human FAT10 can efficiently destabilise viral proteins and enhance antigen presentation via MHC class I (Ebstein et al., 2012; Schliehe et al., 2012). Both studies used fusion proteins, where murine and human FAT10 was N-terminally linked to viral proteins. In doing so, Ebstein and colleagues see FAT10-mediated direct presentation and cross-presentation of an epitope of the 65-kDa phosphoprotein (pp65) of the human cytomegalovirus (HCMV). Here, the FAT10 fusion proteins reach levels comparable to that of the ubiquitin fusion protein, which is significantly above the presentation level of the untagged pp65 protein (Ebstein et al., 2012). In addition, Schliehe and colleagues used murine FAT10 and ubiquitin that have been N-terminally linked to the LCMV-NP and show that the fusion of either FAT10 or ubiquitin markedly destabilises the otherwise stable LCMV-NP. Subsequent in vitro presentations assays reveal that the FAT10-NP and the ubiquitin-NP fusion protein increase presentation of epitopes to a similar extent. Yet, this enhanced presentation of the fusion proteins does not improve the cytotoxic T cell (CTL) response in vivo rather the untagged stable LCMV-NP shows a higher CTL response than both the FAT10 and ubiquitin fusion protein. Interestingly, the FAT10-NP fusion protein elicits an intermediate CTL response. These results suggest that in vivo priming of NP-specific CTL responses occur via cross-presentation, and FAT10 somehow stabilises the LCMV-NP and
prevents premature degradation (Schliehe et al., 2012). In summary, these studies show that FAT10 can target antigenic proteins for proteasomal degradation with subsequent presentation by the MHC class I pathway (Figure 8, left side). It should be mentioned here that fusion proteins of FAT10 with viral proteins were used and evidence that covalent conjugation of FAT10 to viral proteins still lacks.

Figure 8: Role of FAT10 in antigen presentation. FAT10 is conjugated to substrate proteins by UBA6, USE1, and likely by unknown E3 ligases. The MHC class I presentation pathway: Following conjugation, FAT10ylated substrates are recognised by RPN10 in the 19S regulatory particle of the 26S proteasome and subsequently FAT10 together with its bound substrate are degraded. The resulting peptides are translocated into the ER-lumen by transporter associated with antigen processing (TAP) and loaded onto MHC class I molecules. Subsequently, the peptide-loaded MHC class I complexes are exported to the cell surface by the ER-Golgi network and presented to cytotoxic CD8+ T cells. The MHC class II presentation pathway: In theory, FAT10ylated substrates can be directed into the autophago-lysosomal degradation pathway upon interaction of FAT10 with the autophagy adapter p62. p62 in turn interacts with LC3 and thereby might shuttle the FAT10ylated substrate into a maturing autophagosome. After complete maturation of the autophagosome and the fusion with a lysosome, FAT10 and the substrate are degraded by lysosomal proteases in this autophagolysosome. At the same time, MHC class II molecules bearing an invariant chain, which blocks the peptide-binding groove, are transported through the ER-Golgi network ending up in vesicles. Subsequently, these vesicles fuse with autophagolysosomes where lysosomal proteases cleave the invariant chain to free the peptide-binding groove of the MHC class II molecule, which now can bind peptides generated from the digested FAT10ylated substrate. Finally, to facilitate presentation to CD4+ T cells, the peptide-MHC complexes are transported to the cell surface. The image is reprinted from (Basler et al., 2015a).
Besides that, involvement of FAT10 in the MHC class II presentation pathway via feeding into autophagy is debatable, yet conceivable, since FAT10 covalently modifies the autophagy marker p62/SQSTM1 and localises into p62 bodies (Aichem et al., 2012). Moreover, FAT10 co-localises especially with p62/SQSTM1, but also the autophagy-related protein light chain 3B (LC3B), ubiquitin, and to a lesser extent with the nuclear dot protein 52 (NDP52) on cytosolic Salmonella that are targeted for autophagy (Spinnenhirm et al., 2014). Finally, FAT10 has been identified recently as a susceptibility gene for type 1 diabetes (T1D) induced by virus infection in rats which have a permissive MHC class II haplotype (Cort et al., 2014). Taken together, these studies all support the notion that FAT10 is involved in antigen processing with subsequent MHC class II presentation (Figure 8, right side).

By means of antigen presentation, FAT10 can influence negative selection by medullary TECs in the thymus in an antigen-dependent manner leading to an altered TCR repertoire. Furthermore, although not influencing MHC class I surface expression (Raasi et al., 2001), a mass spectrometry analysis of peptides eluted from MHC class I molecules from wild type HEK cells and HEK cells stably overexpressing FAT10 shows differences in the peptides presented (Buerger et al., 2015).

Another function of FAT10 has been proposed by Ocklenburg and colleagues who have seen FAT10 expression in long-term cultures of Tregs. After overexpression of FAT10 in conventional T cells, these cells acquire a Treg-like phenotype indicating that FAT10 expression is involved in the regulation of T cell anergy as well as contributes the anergic phenotype of Tregs (Ocklenburg et al., 2006).

As of late, FAT10 has been assigned a function in clearance of microbial pathogens by autophagy. FAT10 co-localises together with several autophagy markers to cytosolic Salmonella in infected human cells, as mentioned above, and FAT10 deficiency in mice leads to an increased susceptibility to Salmonella infection. Despite that, knockdown and over-expression of FAT10 in vitro has no influence on the replication of Salmonella. Since direct targeting of FAT10 into autophagy has not observed (Aichem et al., 2012), FAT10 might serves as linker between the FAT10ylated Salmonella and the autophagy marker feeding the pathogen indirectly into autophagy. However, it still needs be clarified whether FAT10 association with Salmonella is dependent on conjugation or dependent on recruitment of p62 or other autophagy markers. If it is covalent conjugation, it will be interesting to identify the substrates that are targeted. Notably, the survival and the body weight of FAT10-deficient mice is reduced compared to FAT10-proficient mice after infection with Salmonella. Accordingly, the bacterial burden is increased in mesenteric lymph nodes from FAT10-deficient mice at 14 days post-
infection. The delayed effect manifesting in FAT10-deficient mice after infection with *Salmonella* could also indicate a function of FAT10 in adaptive immunity (Spinnenhirn et al., 2014).

FAT10 has been associated with the regulation of the NF-κB pathway after LPS and TNF stimulation and virus infection in several studies (Figure 7) (Gong et al., 2010; Buchsbaum et al., 2012b; Gao et al., 2014; Nguyen et al., 2016), but the reported observations are contradictory. On the one hand, FAT10 has been shown to boost NF-κB activity in human and murine cells, as well as cancer cells very likely by impairing IκBα-inhibition of NF-κB by reducing the IκBα protein level, without affecting ubiquitylation and phosphorylation of IκBα (Gong et al., 2010; Gao et al., 2014, 2015). On the other hand, Buchsbaum and colleagues found that FAT10 is conjugated covalently to LRRFIP2, which is implicated in the activation of NF-κB after TLR4 stimulation. After being modified with FAT10, LRRFIP2 is relocated into cytoplasmic aggregates and thereby FAT10 can modulate NF-κB activation (Buchsbaum et al., 2012b). In a similar fashion, FAT10 interacts with retinoic acid inducible gene 1 (RIG-I) after influenza virus infection, renders RIG-I insoluble, and leads to subsequent proteasomal degradation that is counteracted by TRIM25. Thus, FAT10 inhibits RIG-I signalling and downstream NF-κB activation (Nguyen et al., 2016). Similar contrasting observations have been observed in mice. FAT10-deficient mice display higher sensitivity towards LPS-induced sepsis than FAT10-proficient mice arguing in favour of a FAT10-mediated negative regulation of the NF-κB pathway (Canaan et al., 2006). As opposed to this, in aged mice the level of IL-10 is increased in skeletal muscle of FAT10-deficient mice indicating a positive effect of FAT10 on NF-κB activation, since IL-10 is known to down-regulate NF-κB signalling (Canaan et al., 2014). Given these opposing findings, Gong and colleagues suggested that the effects of FAT10 on NF-κB activation are cell type- or tissue-specific (Gong et al., 2010), which is underlined by the fact that FAT10-deficient mice display only a marginal phenotype and are otherwise fertile and healthy (Canaan et al., 2006). Considering the observations made in FAT10-deficient mice, it might be possible that FAT10 regulates the NF-κB pathway differently in immune and non-immune cells. Nevertheless, the role of FAT10 in NF-κB signalling needs further investigation to come to a satisfactory conclusion.

Recent reports suggest that FAT10 acts anti-inflammatory in response to viral infection and even promote viral replication (Nguyen et al., 2016; Zhang et al., 2016). Nguyen and colleagues found that FAT10 attenuates RIG-I signalling very likely to regulate the strength of the antiviral immune response. On the downside, due to sustained suppression of RIG-I signalling by FAT10 in the case of chronic infections this can enable the establishment of
persistent infection that might ultimately lead to cancer development (Nguyen et al., 2016). Another study observed FAT10-dependent increase of viral replication in A549 cells infected with influenza virus H5N1. Furthermore, FAT10 inhibits expression of type I interferons and STAT-1 phosphorylation, thus negatively regulating the antiviral immune response (Zhang et al., 2016). In summary, FAT10 is upregulated during RNA virus infection and negatively regulates immune responses against these viruses, most likely to prevent an excessive immune response and deleterious effects thereof, such as sepsis.

1.3 Dendritic Cell Aggresome-Like Induced Structures

Dendritic cells (DCs) belong to the group of professional antigen-presenting cells (APCs), such as macrophages and B cells. DCs have been discovered in the early 1970s by Steinman and Cohen, who also coined the term ‘dendritic cells’ from the Greek ‘dendron’ for tree due to their tree-like morphology (Steinman & Cohn, 1973, 1974). Since then DCs have become a highly heterogenic group of immune cells, and classification of DCs into distinct subsets has proven difficult because of overlapping functional and phenotypic characteristics of the DC subsets among themselves as well as with monocytes and macrophages. Nevertheless, five major distinct DC subsets can be defined, i.e. two subsets of ‘classical’ or myeloid DCs, plasmacytoid DCs, monocyte-derived DCs and Langerhans cells, that display certain functional specialisation and can be distinguished based on phenotypic markers (Merad et al., 2013; O’Keeffe et al., 2015; Lutz et al., 2017; Collin & Bigley, 2018). DCs are present in most tissues in an immature state and function as so-called sentinels of the immune system by continuously scanning the environment. Upon detection and phagocytosis of pathogens or dead/damaged cells, DCs start to mature and migrate to secondary lymphoid tissues, where they present the detected potential threat in form of antigens to T cells. DCs thereby either elicit an immune response or help to maintain tolerance, which likely depends on the type of maturation signals the DCs received in the periphery. Therefore, DCs are crucial for the regulation of immunity and tolerance (Steinman, 2007; Mellman, 2013; Austyn, 2016). Maturation is a highly complex process during which DCs initially and transiently increase endocytosis, which then is drastically downregulated. Moreover, maturation is accompanied by increased surface expression of so-called maturation markers that include immunostimulatory molecules, such as MHC class I and II, and co-stimulatory molecules, such as CD86 and CD40. Furthermore, the maturational process enhances the antigen processing abilities of DCs and the generation of stable peptide-MHC complexes on the cell surface. In addition, DCs start secreting cytokines, which might be pro- or anti-inflammatory depending on the maturation stimulus. Lastly, dendritic
cells undergo a morphological change and start forming dendrites, which might improve interaction with T cells, and change their surface expression profile of chemokine receptors, most notably CCR7, to enable migration towards secondary lymphoid tissue. In these tissues, DCs localise to T cell zone and interact with T cells in order to trigger immune responses or tolerance (Mellman & Steinman, 2001; Trombetta & Mellman, 2004; Hammer & Ma, 2013; Mellman, 2013).

Due to the low number of DCs in vivo, in vitro-cell culture systems have been developed that allow the generation of high number of DCs from progenitor cells, such as bone marrow and monocytes, using cytokines, such as granulocyte-macrophage colony-stimulating factor and interleukin-4 (Inaba et al., 1992; Romani et al., 1994; Sallusto & Lanzavecchia, 1994; Lutz et al., 1999). Although one should be aware that these cell culture system are not identical to dendritic cells in vivo and results need to be carefully interpreted and transferred into DC biology, these culture systems led to great advances not only in DC biology but also in cancer immunotherapy (Radford et al., 2014; Lutz et al., 2017). Using such cell culture systems and primary DCs from spleen and epidermal sheets, Lelouard and colleagues observed and characterised an additional phenotypic change during DC maturation, namely the transient accumulation of polyubiquitylated protein aggregates. These structures turned out to be similar, but yet distinct from aggresomes and were, therefore, named dendritic cell aggresome-like induced structures or short DALIS (Lelouard et al., 2002).

1.3.1 Characteristics, Formation and Clearance

DALIS differ in several characteristics from aggresomes as described by Johnston and colleagues (Johnston et al., 1998). DALIS do not localise to the microtubule organising centre (MTOC) as shown by lack of co-localisation with γ-tubulin and are not encaged by the intermediate filament vimentin. Furthermore, DALIS formation is independent of a functional microtubule network and is neither dependent on nor influences proteasome activity. Most notably, DALIS accumulate transiently during DC maturation, whereas aggresomes are stable during impaired proteolysis. Apart from this, DALIS formation and maintenance relies on active protein synthesis, and incorporation of ubiquitylated proteins into DALIS displays a certain specificity. For example, IκBα is not targeted to DALIS under normal physiological conditions (Lelouard et al., 2002), since IκBα needs to be degraded to allow for NF-κB nuclear translocation which is essential for DC maturation and survival (Rescigno et al., 1998). Remarkably, proteasomes do not localise to DALIS suggesting that accumulated proteins in DALIS are protected from unspecific proteasomal degradation (Canadien et al., 2005; Herter
et al., 2005; Szeto et al., 2006), although enzymes of the ubiquitin-conjugation system are present in DALIS (Lelouard et al., 2004; Clausen et al., 2010; Kettern et al., 2011; Wenger et al., 2012). Another intriguing feature of DALIS is the motility of the protein aggregates within the cytosol (Lelouard et al., 2004) that depends on a functional microtubule network contrary to their formation (Canadien et al., 2005, supplementary video 2). This movement might be facilitated by spartin, which was shown to localise to DALIS (Karlsson et al., 2014) and is able to interact with microtubules (Lu et al., 2006).

DALIS are not restricted to DCs and can be found in macrophages and non-immune cells, such as HeLa cells and mouse embryonic fibroblasts (MEFs), where these structures have been termed ALIS (Canadien et al., 2005; Szeto et al., 2006; Seifert et al., 2010). Notably, ALIS are not found in B cells as opposed to other professional APCs (Cenci et al., 2006). Furthermore, DALIS can be induced by other stimuli than the initially reported LPS (Lelouard et al., 2002), and they can form in response to heat-shock (DeFillipo et al., 2004; Szeto et al., 2006), IFN-γ treatment (Canadien et al., 2005; Seifert et al., 2010; Nathan et al., 2013), and by various pharmacological treatments (Szeto et al., 2006). Formation of DALIS can also be observed upon infection with influenza virus and Coxsackie virus B3 (CVB3) (Herter et al., 2005; Rahnefeld et al., 2011). Infection of macrophages with Salmonella typhimurium and Listeria monocytogenes likewise have been reported to induce DALIS (Canadien et al., 2005), contrary to infection of macrophages with Legionella pneumophila that interfere with DALIS formation (Ivanov & Roy, 2009).

DALIS are transient protein accumulations and it is striking how the cells manage to first accumulate and eventually dispose of DALIS during DC maturation. It has been proposed that formation and clearance of DALIS in DCs is induced and regulated by an initial increase and a subsequent decrease in protein translation that also eventually leads to DALIS clearance by lack of newly translated protein (Lelouard et al., 2007; Pierre, 2009). Concomitantly, a decrease of autophagy that occurs upon triggering DC maturation by LPS promotes DALIS formation (Wenger et al., 2012; Terawaki et al., 2015). During DALIS induction defective ribosomal products (DRiPs) are produced that constitute the major component of DALIS. DRiPs are rapidly and constantly incorporated into DALIS and thereafter slowly released with a retention of up to 16 hours (Lelouard et al., 2002, 2004). DRiPs are recognised co-translationally by HSC70 and subsequently ubiquitylated very likely by UBC4/5 E2 conjugating enzyme family members facilitated by the E3 ligase ‘carboxy terminus of HSP70-interacting protein’ (CHIP). Finally, the co-chaperone ‘bcl-2 associated athanogene 1’ (BAG-1), the autophagy adaptor p62, and the ‘autophagy-linked FYVE protein’ (ALFY), and spartin ensure that the ubiquitylated
DRiPs are deposited in DALIS. Following this aggregation phase, DALIS are cleared via autophagy as well as the proteasome and fed into the MHC class I and II presentation pathways. In this situation, chaperone-mediated degradation via the proteasome occurs involving BAG-1 and BAG-6 in concert with HSC/HSP70. At the same time, chaperone-mediated autophagy by means of BAG-3 and HSC/HSP70, and selective autophagy that involves ALFY, the autophagy adaptors ‘neighbor of BRCA1 gene 1’ (NBR1) and p62, and ATG8 direct DALIS into the lysosomal degradation pathway (Szeto et al., 2006; Clausen et al., 2010; Fujita et al., 2011; Kettern et al., 2011; Wenger et al., 2012; Karlsson et al., 2014; Terawaki et al., 2015; Argüello et al., 2016).

1.3.2 Function in Antigen Processing and Presentation

Interestingly, only endogenously expressed proteins seem to accumulate in DALIS. This has been observed using the model antigen ovalbumin (OVA). Exogenously added OVA, which has been fed to immature DCs before inducing maturation, can be found in the cytosol, but does not accumulate in DALIS that have formed concurrently (Lelouard et al., 2002). Yet, OVA expressed from an adenovirus in infected RAW309 cells, a macrophage cell line, accumulates in DALIS during maturation (Kettern et al., 2011). Thus, as has been proposed DALIS limit processing and presentation of endogenous self-peptides and allow for prioritised MHC class I presentation of exogenous antigens, i.e. cross-presentation, (Lelouard et al., 2004; Canadien et al., 2005; Argüello et al., 2016), at stages when DCs are most capable of cross-presentation (Alloatti et al., 2016). This is in line with the observation that DALIS most prominently form in human and murine primary DCs that are specialised in cross-presentation (Mintern et al., 2015). At the same time, it was found that apart from DRiPs viral proteins are found in DALIS. Herter and colleagues have shown that antigen presentation in DCs infected with influenza is delayed compared to non-professional APCs and that this delay is accompanied by DALIS formation and the storage of influenza NP in DALIS (Herter et al., 2005). Similarly, it has been reported that in a mouse model of chronic CVB3-myocarditis, which shows defects in DC maturation and reduced cross-presentation, viral antigens are trapped in DALIS due to lack of clearance (Rahnefeld et al., 2011). In summary, these findings together with the observed retention of DRiPs (Lelouard et al., 2002, 2004) further support the concept that DALIS serve as antigen storage sites. This allows DCs to acquire a fully mature phenotype, including expression of co-stimulatory molecules and cytokine production, and to reach secondary lymphoid tissues before antigen presentation to T cells, which eventually elicits an immune response while preventing peripheral tolerance (Herter et al., 2005; Pierre, 2005; Faßbender et
al., 2008; Argüello et al., 2016). Additionally, through linking of DALIS with the autophagy pathway, as described above, antigens stored in DALIS can also be presented on MHC class II molecules. Interestingly, the fate of antigens within DALIS seems to be convertible to some extent from proteasomal to autophagolysosomal degradation and vice versa (Wenger et al., 2012; Terawaki et al., 2015). This conversion could be achieved through on-going ubiquitylation and de-ubiquitylation within DALIS, which is supported by the presence of different ubiquitin linkage types, such as K63- and K48-linked polyubiquitin chains, and the ubiquitin-conjugating system (Lelouard et al., 2004; Kettern et al., 2011; Wenger et al., 2012; Karlsson et al., 2014). Considering this, Argüello and colleagues suggested that DALIS could represent temporal sorting compartments that regulate the feed of substrates into both degradation pathways. But, this hypothesis needs further investigation since it has not been demonstrated in professional APCs (Argüello et al., 2016).
**Aim of This Study**

*FAT10* is most prominently expressed in tissues of the immune system and, in particular, during maturation of dendritic cells. Moreover, there is evidence that FAT10 is involved in antigen processing and presentation. Concurrently, DCs form DALIS during maturation, which partially depends on the FAT10 interaction partner p62 and which very likely represent antigen storage sites. Notably, FAT10 has already been shown to localise to cytosolic aggregates, such as aggresomes under proteasome inhibition in a HDAC6-dependent manner and to p62 bodies in HeLa cells. Due to these findings the pending question arose whether FAT10 is involved in the formation, maintenance, and clearance of DALIS during maturation of dendritic cells, whose investigation was the main aim of this study.

At first, it should be demonstrated whether FAT10 localises to DALIS in murine and human dendritic cells and whether this is dependent on covalent conjugation or non-covalent interaction. Due to the lack of an antibody that recognises murine FAT10, two approaches for detection of murine FAT10 in DALIS were pursued. The first approach was to establish a lentiviral vector system for introduction murine FAT10 into bone marrow-derived DCs and the second was the generation of a polyclonal antibody serum in rabbits reactive against murine FAT10. Additionally, it should be addressed whether *FAT10* deficiency alters the formation, maintenance, and clearance of DALIS. Apart from this, the effect of *HDAC6* deficiency on DALIS formation and clearance during DC maturation should be assessed since it has been shown that HDAC6 is necessary for the proper formation of aggresomes.

It has been reported repeatedly that overexpression of FAT10 enhances and that a reduction of FAT10 attenuates the migratory capacities of tumour cells. Thus, in the second part of this study the question whether FAT10 has also an influence on the migration of DCs should be addressed. For clarification of this issue, migration assays should be performed with human monocyte-derived DCs under conditions of FAT10 overexpression and knockdown. Likewise, migration of *FAT10*-deficient and -proficient bone marrow-derived DCs should be compared.

In the third part, the expression profile of *FAT10* in primary immune cell subsets should be determined because cell type-specific expression of *FAT10* has only been determined unsatisfactory, contrary to the comprehensive knowledge of tissue-specific expression of *FAT10*. Therefore, the different leukocyte subsets were purified and *FAT10* mRNA levels
analysed in a naïve state and after stimulation with pro-inflammatory cytokines and endotoxin challenge.

In the last part, the epitope peptide of the anti-FAT10 antibody 4F1 should be defined. The use of peptides for elution of proteins after immunoprecipitations can reduce nonspecific background in downstream applications and enable identification of low abundant interacting partners. Furthermore, epitope peptides allow for sequential immunoprecipitations under normal physiological conditions and can be used as additional controls for antibody detection.
2 Material and Methods

Unless otherwise stated all cell culture media, cell culture supplements, and fluorescently labelled secondary antibodies were ordered from Thermo Fisher Scientific. All cytokines were purchased from PeproTech. Primers used in conventional PCR and quantitative real-time RT-PCR were ordered from and synthesised by Microsynth. Special equipment and antibody-conjugated microbeads necessary for magnetic-activated cell sorting (MACS) were obtained from Miltenyi Biotec. Cell culture material was purchased from Techno Plastic Products (TPP), Greiner Bio-One, or Sarstedt.

2.1 Cell Culture

The murine fibroblast cell line B8 (Groettrup et al., 1995) and human embryonic kidney (HEK) 293T cells were cultured in complete Iscove’s Modified Dulbecco’s Medium (IMDM). HeLa cells were maintained in complete Dulbecco’s Modified Eagle Medium (DMEM). Primary mouse cells were cultured in complete Roswell Park Memorial Institute 1640 (RPMI 1640) medium. Murine cells were supplied additionally with 50 µM β-mercaptoethanol. Primary human cells were cultured in complete AIM-V and were isolated from peripheral human blood from healthy donors. The ethics committee of the University of Konstanz approved blood donations for research purposes, and individual donors gave written consent. Endogenous expression of FAT10 was induced using 400 U/mL tumour necrosis factor (TNF) and 200 U/mL interferon-γ (IFN-γ) for the indicated time points. Peripheral blood mononuclear cells (PBMCs) used for immunoprecipitation and immune-detection of endogenous human FAT10 were cultured in RPMI1640, supplemented with 2% human AB serum for 30 h in presence or absence of 5000 U/mL human TNF and 2500 U/mL human IFN-γ. All cell lines were cultured at 37°C and 5% CO₂. Aliquots of 2x10⁶ cells of each cell line were frozen gradually in 90% iFCS with 10% DMSO (Sigma-Aldrich) in freezing containers at -80°C overnight and finally transferred to a -150°C deep-freezer for long-term storage. Buffers and media used for maintaining and isolating cells are summarised below.
**Material & Methods**

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</table>

### 2.2 Mouse Strains

All mice were purchased from the animal facility at the University of Konstanz and were housed under specific pathogen-free conditions. C57BL/6 (H-2ᵇ) mice were originally purchased from Charles River Laboratories. A. Canaan and S. M. Weissman (Yale University School of Medicine, New Haven, CT) kindly provided FAT10-deficient mice (Canaan et al., 2006). P. Matthias (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland) kindly provided hind legs of HDAC6-deficient mice (Zhang et al., 2008) and corresponding age-matched wild type C57BL/6 mice. C57BL/6 FOXP3-GFP reporter mice were kindly provided
by H.C. Probst (Lahl et al., 2007). For isolation of primary cells, mice were sex- and age-matched and 8-12 weeks of age. Isolation of primary cells from mice was performed in accordance with the German Animal Welfare Act and approved by the animal welfare officer of the University of Konstanz or if applicable by the review board of the Regierungspräsidium Freiburg.

### 2.3 Generation and Cultivation of Primary Dendritic Cells

#### 2.3.1 Bone Marrow-Derived Dendritic Cells

Murine bone marrow-derived dendritic cells (BMDCs) from *HDAC6*-deficient, *FAT10*-deficient, and wild type C57BL/6 mice were generated as described before (Lutz et al., 1999). Briefly, the bone marrow was rinsed from femurs and tibiae and washed with PBS/EDTA. Contaminating erythrocytes were lysed in ACK buffer for 2-5 min at room temperature (RT). Finally, the bone marrow cells were washed, counted, and seeded at 2x10^5 cells/mL in bacteriological 10 cm petri dishes (day 0) in 10 mL of complete RPMI 1640 supplemented with 200 U/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF). On day 3 of culture, 10 mL of fresh complete RPMI 1640 were added. On days 6 and 8, half of the culture medium was removed, non-adherent cells pelleted, resuspended in fresh complete RPMI 1640, and 10 mL per petri dish transferred back to the original culture dishes. Fully differentiated BMDCs were obtained on day 10. For experiments, non-adherent BMDCs were collected and loosely adherent BMDCs detached by incubation with PBS/EDTA at RT for 10 min and gentle pipetting. Maturation of BMDCs and *FAT10* expression were induced with 1 µg/mL lipopolysaccharide (LPS, from *E. coli* O111:B4) (Sigma-Aldrich) or 200-400 U/mL murine TNF and 100-200 U/mL murine IFN-γ in fresh complete RPMI 1640 with 200 U/mL murine GM-CSF. In some experiments, BMDCs were treated with 10 µM MG132 (MG; inhibition of the proteasome), 10 µg/mL cycloheximide (CHX; inhibition of protein biosynthesis), and 2 µM wortmannin (WTN; inhibition of autophagy) (all from Sigma-Aldrich) for 2 h before fixation and immunostaining. For migration assays, BMDCs were stimulated for 24 h or 48 h with 1 µg/mL LPS (from *E. coli* O111:B4) (Sigma-Aldrich), 400 U/mL murine TNF, and 200 U/mL murine IFN-γ in fresh complete RPMI 1640 supplemented with 200 U/mL murine GM-CSF.

#### 2.3.2 Monocyte-Derived Dendritic Cells

Human monocyte-derived dendritic cells (MoDCs) were differentiated from human CD14+ monocytes. Monocytes were isolated by MACS from peripheral blood. Peripheral blood
mononuclear cells (PBMCs) were enriched by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare) at 800 g and 18°C for 20 min in a swinging-bucket rotor with the acceleration set to 6 and the deceleration set to 3. Enriched PBMCs were washed several times with MACS buffer to remove as many platelets as possible. Remaining erythrocytes were lysed in ACK buffer for 2-5 min at RT. After a final washing step with MACS buffer, PBMCs were subjected to MACS, and the CD14+ monocytes enriched using anti-CD14-conjugated microbeads and the manual MidiMACS Separator according to the manufacturer’s instructions. Purified monocytes were cultured at 2x10⁶ cells/mL in complete AIM-V supplemented with 500 U/mL human GM-CSF and 250 U/mL human interleukin-4 (IL-4). The next day (day 1), the same volume complete AIM-V with the cytokines was added. On day 6, monocytes were fully differentiated into MoDCs. MoDCs were harvested by incubation of the cells at RT for 10 min with PBS/EDTA and pipetting. To induce maturation of MoDCs and FAT10 expression, the cells were stimulated with 1 µg/mL LPS (from *E. coli* O111:B4) (Sigma-Aldrich) and a cytokine cocktail in complete AIM-V supplemented with 500 U/mL human GM-CSF and 250 U/mL human IL-4. The cytokine cocktail included 200 U/mL human interleukin-6 (IL-6), 10,000 U/mL human interleukin-1β (IL-1β), 400 U/mL human TNF, and 200 U/mL human IFN-γ. MoDCs used for migration assays were induced with 1 µg/mL LPS (from *E. coli* O111:B4) (Sigma-Aldrich) alone or in combination with 1 µg/mL prostaglandin E2 (PGE2) (Cayman Chemical/Biomol) in complete AIM-V supplemented with 500 U/mL human GM-CSF and 250 U/mL human IL-4 for 48 h.

### 2.4 Generation of Competent Bacteria

An aliquot of 5 mL of lysogeny broth (LB) was inoculated with the desired *E. coli* strain and grown overnight at 37°C and 250 rpm. A 1:100 dilution of this pre-culture was used to inoculate fresh LB that was grown further at 37°C and 250 rpm. The OD₆₀₀nm was determined with the SmartSpec Plus spectrophotometer (Bio-Rad). As soon as the OD₆₀₀nm reached 0.5, the bacterial culture was placed on ice for 15 min and was kept on ice during the following procedures. The bacterial culture was centrifuged successively at 4,000 g and 4°C for 10 min in 50 mL tubes. The bacteria were resuspended carefully in 50 mL of 0.1 M ice-cold sterile MgCl₂ (Acros Organics), centrifuged again, and resuspended carefully in 50 mL of 0.1 M ice-cold sterile CaCl₂ (Roth), in which they were incubated on ice for 20 min. Following a last centrifugation step, the bacteria were resuspended carefully in a total volume of 5 mL of sterile CaCl₂/Glycerol. Finally, aliquots of 200 µL of the chemically competent bacteria were stored
Material & Methods

at -80°C. Media and buffers used to generate chemically competent bacteria and to transform bacteria are summarised below.

**Lysogeny broth (LB)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% (w/v) tryptone (BD Biosciences)</td>
<td></td>
</tr>
<tr>
<td>1% (w/v) NaCl (Roth)</td>
<td></td>
</tr>
<tr>
<td>0.5% (w/v) yeast extract (BD Biosciences)</td>
<td></td>
</tr>
<tr>
<td>in ddH₂O, autoclaved</td>
<td></td>
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</tbody>
</table>

**Super optimal broth (SOB)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% (w/v) tryptone (BD Biosciences)</td>
<td></td>
</tr>
<tr>
<td>0.5% (w/v) yeast extract (BD Biosciences)</td>
<td></td>
</tr>
<tr>
<td>0.05% (w/v) NaCl (Roth)</td>
<td></td>
</tr>
<tr>
<td>0.0186% (w/v) KCl (Roth)</td>
<td></td>
</tr>
<tr>
<td>0.095% (w/v) MgCl₂ (Acros Organics)</td>
<td></td>
</tr>
<tr>
<td>in ddH₂O, autoclaved</td>
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</tbody>
</table>

**LB agar**

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1.5% (w/v) agar (BD Biosciences)</td>
<td></td>
</tr>
<tr>
<td>in LB, autoclaved</td>
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**CaCl₂/Glycerol**

<table>
<thead>
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<th>Component</th>
<th>Description</th>
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<tr>
<td>86 mM CaCl₂ (Roth)</td>
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</tr>
<tr>
<td>10% (v/v) glycerol (VWR)</td>
<td></td>
</tr>
<tr>
<td>in ddH₂O, sterile-filtered</td>
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</tr>
</tbody>
</table>

**SOB with catabolite repression (SOC)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% (v/v) 50% glucose</td>
<td></td>
</tr>
<tr>
<td>in SOB, sterile-filtered</td>
<td></td>
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</tbody>
</table>

**50% glucose**

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 g/L glucose (Merck)</td>
<td></td>
</tr>
<tr>
<td>in ddH₂O, sterile-filtered</td>
<td></td>
</tr>
</tbody>
</table>

### 2.5 Transformation of Bacteria

Chemically competent bacteria were thawed on ice and aliquots of 50 µL distributed into tubes. Approximately 100 ng of pure plasmid vector DNA or ligation reactions were added and mixed with the bacteria. The pUC18 plasmid vector was used as positive control, and ddH₂O was used as negative control when novel plasmid vectors were transformed. This mixture was incubated on ice for 30 min followed by a heat shock at 42°C for 45 s and an incubation on ice for 2 min. Then, 500 µL of SOC medium were added, and the bacteria were grown for 45-60 min at 37°C and 250 rpm. A volume of 50-500 µL of bacterial culture was streaked onto pre-warmed LB agar plates containing 100 µg/mL ampicillin (Roth). The LB agar plates were incubated upside down at 37°C overnight. Single colonies, i.e. individual clones, were used to inoculate aliquots of 5 mL of LB supplemented with 100 µg/mL ampicillin (Roth), which were again grown at 37°C and 250 rpm overnight. Several clones were analysed when novel plasmid vectors were transformed. For long-term storage and re-use, 800 µL of the bacterial culture were mixed with 200 µL of sterile glycerol (VWR) and stored at -80°C. For large-scale purifications of plasmid vector DNA, 150-200 mL of bacterial cultures was prepared in LB.
with 100 µg/mL ampicillin (Roth). Plasmid vector DNA was isolated using the NucleoSpin Plasmid kit or the NucleoBond PC 500 kit (both from Macherey-Nagel).

2.6 Plasmid Vectors and Standard Cloning Procedure

All plasmid vectors were maintained and amplified in the *E. coli* strains XL10-Gold (Agilent Technologies) or TOP10F’ (Thermo Fisher Scientific). Plasmid vector sequences were verified by sequencing (GATC Biotech). Table 2 summarises the plasmid vectors that have been used.

<table>
<thead>
<tr>
<th>Name</th>
<th>Published</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMal-MBP-6xHis</td>
<td>Gift from Prof. Dr. T. U. Mayer</td>
<td>Group of T. U. Mayer, University of Konstanz</td>
</tr>
<tr>
<td>pMal-MBP-mFAT10-6xHis</td>
<td>Gift from Dr. S. Bürger</td>
<td>Group of M. Groettrup, University of Konstanz</td>
</tr>
<tr>
<td>pcDNA3.1-HA-mFAT10</td>
<td>(Raasi et al., 2001)</td>
<td>Group of M. Groettrup, University of Konstanz</td>
</tr>
<tr>
<td>pcDNA3.1-6xHis-3xFLAG-hFAT10</td>
<td>(Chiu et al., 2007)</td>
<td>Group of M. Groettrup, University of Konstanz</td>
</tr>
<tr>
<td>pMD2.G</td>
<td>Gift from Prof. Dr. D. Trono (Addgene plasmid #12259)</td>
<td>Group of E. May, University of Konstanz</td>
</tr>
<tr>
<td>pSAX2</td>
<td>Gift from Prof. Dr. D. Trono (Addgene plasmid #12260)</td>
<td>Group of E. May, University of Konstanz</td>
</tr>
<tr>
<td>pCDH-EF1α-MCS-IRES-copGFP</td>
<td>Commercially available</td>
<td>System Biosciences/BioCat</td>
</tr>
<tr>
<td>pCDH-EF1α-hFAT10-IRES-copGFP</td>
<td>(Schmidtke et al., 2017)</td>
<td>Group of M. Groettrup, University of Konstanz</td>
</tr>
<tr>
<td>pCDH-EF1α-hFAT10ΔGG-IRES-copGFP</td>
<td>Unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pCDH-EF1α-mFAT10-IRES-copGFP</td>
<td>Unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pCDH-EF1α-mFAT10ΔGG-IRES-copGFP</td>
<td>Unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pCDH-EF1α-Ub-IRES-copGFP</td>
<td>Unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pCDH-EF1α-UbΔGG-IRES-copGFP</td>
<td>Unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pCDH-CMV-MCS-EF1α-copGFP</td>
<td>Commercially available</td>
<td>System Biosciences/BioCat</td>
</tr>
<tr>
<td>pCDH-CMV-mFAT10ΔGG-EF1α-copGFP</td>
<td>Unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pCDH-CMV-mFAT10-EF1α-copGFP</td>
<td>Unpublished</td>
<td>This study</td>
</tr>
<tr>
<td>pCDH-CMV-hFAT10-EF1α-copGFP</td>
<td>Unpublished</td>
<td>This study</td>
</tr>
</tbody>
</table>
Plasmid vectors were generated by restriction enzyme cloning. For this, cDNA inserts were amplified using the Phusion High-Fidelity PCR Kit (New England Biolabs) according to the manufacturer’s instruction. The amplified cDNA inserts were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Following this, the cDNA inserts and backbone plasmid vectors were digested with restriction enzymes (all from New England Biolabs) according to the double-digest recommendations on the website of New England Biolabs. After restriction enzyme digest, backbone plasmid vectors were dephosphorylated using Antarctic Phosphatase (New England Biolabs) according to the manufacturer’s instruction. Digested cDNA inserts and backbone plasmid vectors were separated on agarose gels and were subsequently purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Ligations were done using the T4 DNA ligase (New England Biolabs) according to the manufacturer’s instruction at molar ratios ranging from 1:3 to 1:10 backbone plasmid vector to cDNA insert.

2.7 Cloning of Lentiviral Plasmid Vectors

The cDNA of human \(\text{FAT10 (hFAT10)}\) with an N-terminal \(6\times\text{His}-3\times\text{FLAG}\) tag was amplified from pcDNA3.1-6xHis-3xFLAG-hFAT10 (Chiu et al., 2007) and inserted into the \(NheI\) and \(NotI\) sites of the HIV-based lentiviral plasmid vector pCDH-EF1\(\alpha\)-MCS-IRES-copGFP (System Biosciences/BioCat) to get pCDH-EF1\(\alpha\)-hFAT10-IRES-copGFP (Schmidtke et al., 2017). The \(6\times\text{His}-3\times\text{FLAG}-\text{hFAT10}\) cDNA was inserted additionally into the HIV-based lentiviral plasmid vector pCDH-CMV-MCS-EF1\(\alpha\)-copGFP (System Biosciences/BioCat) by restriction enzyme cloning using \(NheI\) and \(NotI\). The cDNA of the non-conjugatable form of human \(\text{FAT10 (hFAT10}\Delta\text{GG})\) was excised from pcDNA3.1(+)\(-6\times\text{His}-3\times\text{FLAG-}\text{FAT10AV}\) (Aichem et al., 2010) and inserted into the \(EcoRI\) and \(NotI\) sites of the lentiviral plasmid vector pCDH-EF1\(\alpha\)-hFAT10-IRES-copGFP to obtain the lentiviral plasmid vector pCDH-EF1\(\alpha\)-hFAT10\(\Delta\text{GG}\)-IRES-copGFP. The cDNA of murine \(\text{FAT10 (mFAT10)}\) was amplified from pGEX-2TKS-GST-6xHis-mFAT10 (Rani, 2011), and the cDNA of non-conjugatable murine \(\text{FAT10 (mFAT10}\Delta\text{GG})\) was amplified from pBK-CMV-mFAT10AV-GFP (kindly provided by PD Dr. Gunter Schmidtke, University of Konstanz). The amplified cDNAs were inserted into the \(EcoRI\) and \(NotI\) sites of the lentiviral plasmid vector pCDH-EF1\(\alpha\)-hFAT10-IRES-copGFP to obtain the lentiviral vector plasmids pCDH-CMV-mFAT10-IRES-copGFP and pCDH-CMV-mFAT10\(\Delta\text{GG}\)-IRES-copGFP. In addition, the \(6\times\text{His}-3\times\text{FLAG-mFAT10}\) and \(6\times\text{His}-3\times\text{FLAG-mFAT10}\Delta\text{GG}\) sequences were excised by restriction enzyme digest using \(NheI\) and \(NotI\) and inserted into the HIV-based lentiviral plasmid vector pCDH-CMV-MCS-EF1\(\alpha\)-
copGFP (System Biosciences/BioCat) to get the lentiviral vector plasmids pCDH-CMV-mFAT10-EF1α-copGFP and pCDH-CMV-mFAT10ΔGG-EF1α-copGFP. The *ubiquitin (Ub)* cDNA was amplified from pcDNA3.1(+) HA-Ubiquitin (kindly provided by PD Dr. Michael Basler, University of Konstanz), and the cDNA of non-conjugatable *ubiquitin (UbΔGG)* was amplified from pRSET-C-6xHis-Xpress-UbiquitinAV-GFP (kindly provided by PD Dr. Gunter Schmidtke, University of Konstanz). Both cDNA inserts were ligated into the *EcoRI* and *NotI* sites of the lentiviral plasmid vector pCDH-EF1α-hFAT10-IRES-copGFP. As a result, the following lentiviral plasmid vectors with an N-terminal 6xHis-3xFLAG tag sequence were obtained: pCDH-EF1α-Ub-IRES-copGFP, and pCDH-EF1α-UbΔGG-IRES-copGFP. Vehicle (GFP only) control lentiviral particles were generated using the plasmid vector pCDH-CMV-MCS-EF1α-copGFP since the *copGFP* gene is under the direct control of the *EF1α* promoter.

### 2.8 Transient Transfection

For transient overexpression, cells were transfected using the TransIT-LT1 transfection reagent (Mirus Bio), polyethylenimine (PEI; linear; MW 25,000) (Polysciences) or FuGENE HD transfection reagent (Promega). Prior to transfections, cells were cultured to reach a density of 70% when being transfected with TransIT-LT1 reagent or FuGENE HD reagent and a confluency of 80-90% when PEI was used. The culture medium was replenished with fresh complete medium 2-4 h before transfections. In case of PEI, antibiotics were omitted from the culture medium. HEK293T cells were transfected at a ratio of 3 µg of PEI or 3 µL of TransIT-LT1 reagent to 1 µg of plasmid vector DNA. B8 cells were transfected at a ratio of 5 µg of PEI to 1 µg of plasmid vector DNA. HeLa cells were transfected at a ratio of 3 µg of FuGENE HD reagent to 1 µg of plasmid vector DNA. Plasmid vector DNA was diluted in serum-free medium first, and then transfection reagent was admixed. This mixture was incubated at RT for 15 min and finally added dropwise to the cells. The TransIT-LT1 and FuGENE HD reagent could be left on the cells until further processing. If PEI was used, the medium had to be changed after 8 h or at the latest after overnight incubation due to its toxicity. Transfected cells were incubated at 37°C for at least 20 h prior to further processing.

### 2.9 Production and Purification of Lentiviral Vectors

Lentiviral particles were produced by transient co-transfection of a lentiviral expression vector, the lentiviral envelope vector pMD2.G, and the lentiviral packaging vector psPAX2 into HEK293T cells using PEI. For transfections, lentiviral vector DNA was mixed at a ratio of
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1 µg pMD2.G : 1.84 µg psPAX2 : 2.1 µg lentiviral expression vector in serum-free IMDM. After 8–16 h, the medium was removed and the cells were replenished with lentivirus harvest medium. Supernatant containing lentiviral particles was harvested 48 h and 72 h post-transfection.

To purify lentiviral particles, contaminating plasmid vector DNA was digested by adding 1 µg/mL DNase I (Roche) and 1 mM MgCl₂ (Acros Organics), and incubating at 37°C for 20 min. Subsequently, the supernatant was sterile-filtered (0.45 µm, polyethersulfone membrane) (TPP), and aliquots of 34 mL of the supernatant were distributed into 50 mL tubes. For precipitation of the lentiviral particles, 8.5 mL of 50% PEG6000 (final concentration 8.5% (w/v)) and 2.8 mL of 5 M NaCl (Roth) (final concentration 0.3 M) were added. Finally, the tubes were filled up to 50 mL with PBS, mixed by inverting, and incubated at 4°C overnight. Precipitated lentiviral particles were pelleted by centrifugation at 4,600 g and 4°C for 25 min. The cleared supernatant was discarded, and each pellet was dried for 2 min at RT upside down on autoclaved paper towels. To dissolve each pellet, 100-200 µL of cold PBS were added, and pellets were incubated at 4°C overnight with occasional shaking. On the next day, the pellets were resuspended carefully and combined. Finally, aliquots of 100 µL were prepared on ice in pre-cooled PCR tubes, snap-frozen in liquid nitrogen, and stored at -80°C. Lentiviral stocks were titrated in HEK293T cells using tenfold serial dilutions ranging from 10⁻¹ to 10⁻⁵. After at least 3 days, the percentage of GFP-expressing cells was determined by flow cytometry. For calculating the functional titre, only dilutions resulting in 1-25% GFP-positive cells were used. The titre was determined according to equation (1) and given as transducing units (TU)/mL:

\[
\text{Titre} \left[ \frac{\text{TU}}{\text{mL}} \right] = \frac{\text{Number of cells at time of transduction} \times \frac{\text{Percentage GFP}^+\text{cells}}{100}}{\text{Volume of lentivirus stock of respective dilution [mL]}}
\]

The composition of the lentiviral harvest medium and the 50% PEG6000 are shown below.

<table>
<thead>
<tr>
<th>Lentivirus harvest medium</th>
<th>50% PEG6000</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% (v/v) iFCS</td>
<td>50% (w/v) polyethylene glycol (PEG) with</td>
</tr>
<tr>
<td>100 U/mL penicillin</td>
<td>an average molecular weight of 6,000 Da</td>
</tr>
<tr>
<td>100 µg/mL streptomycin</td>
<td>(Sigma-Aldrich)</td>
</tr>
<tr>
<td>in IMDM with GlutaMAX</td>
<td>in ddH₂O, autoclaved</td>
</tr>
</tbody>
</table>
2.10 Transduction of Cells

2.10.1 Bone Marrow-Derived Dendritic Cells

Murine BMDCs were transduced at a multiplicity of infection (MOI) of 50 by spinfection on day 4 of differentiation. The BMDCs were harvested, and 2x10^6 cells were distributed into bacteriological 6-well plates (Roth) in complete RPMI 1640. Lentiviral particles were mixed with protamine sulphate (MP Biomedicals), and then this transduction mix was added to the BMDCs. The volume was adjusted to 2 mL with complete RPMI 1640 to get a final concentration of 50 µg/mL protamine sulphate (MP Biomedicals). Mock-transduced cells received complete RPMI 1640 with protamine sulphate (MP Biomedicals) instead of lentiviral particles. Finally, the 6-well plates with the BMDCs in the transduction mix were centrifuged at 1,500 g and 32°C for 90 min. After centrifugation, the BMDCs were resuspended and transferred back into bacteriological 10 cm petri dishes. The BMDCs were cultured further in a final volume of 10 mL of complete RPMI 1640 supplemented with 200 U/mL murine GM-CSF. On day 6, 10 mL of complete RPMI 1640 with 200 U/mL murine GM-CSF were added, and half of the culture medium was refreshed on day 8. On day 10, the BMDCs were seeded appropriately for experiments. Transduced BMDCs were treated with 5 µM MG132 (Sigma-Aldrich) for 4 h prior to processing for confocal microscopy to enrich the proteins expressed from the lentiviral vectors. Successful transduction was tested by flow cytometry measuring the percentage of GFP-positive cells. Transduction efficiency was determined prior to seeding of the cells for experiments on day 10 post-transduction.

2.10.2 Human Monocyte-Derived Dendritic Cells

Human MoDCs were transduced by spinfection after 3 days of culture in complete AIM-V supplemented with 500 U/mL human GM-CSF and 250 U/mL human IL-4. The cells were harvested and distributed into 6-well plates at 1x10^6 cells/mL in 2-3 mL and left to adhere for 30 min at 37°C. Lentiviruses were added to give an MOI of a 100. After 10 µg/mL protamine sulphate (MP Biomedicals) was added, everything was mixed gently. Finally, the cells were centrifuged at 1,500 g and 32°C for 60 min, and afterwards incubated further at 37°C in a CO₂ incubator. After 48 h, 1-2 mL of fresh complete AIM-V supplemented with 500 U/mL human GM-CSF and 250 U/mL human IL-4 were added. On day 6, maturation of MoDCs was induced. Transduction efficiency of MoDCs was determined after 48 h of maturation by measuring GFP-positive cells by flow cytometry.
2.10.3 HeLa Cells

HeLa cells (1x10^6) were transduced at an MOI of 25-30 in 10 cm dishes. Again, lentiviral particles were mixed with protamine sulphate (final concentration 50 µg/mL) (MP Biomedicals), and subsequently the transduction mix was added to the cells. The Hela cells were incubated with the transduction mix for 3 days. On day 3, the HeLa cells were seeded appropriately for use in confocal microscopy and left to adhere overnight. To induced aggresome formation, HeLa cells were treated with 10 µM MG132 (Sigma-Aldrich) for 6 h prior to fixation. To test successful transduction, the percentage of GFP-positive cells was measured by flow cytometry. Transduction efficiency was determined prior to seeding of cells for experiments on day 3 post-transduction.

2.11 Migration Assay

Migrations assays were performed using 6.5 mm Transwell inserts with a polycarbonate membrane and a pore size of 5 µm in 24-well plates (Corning), i.e. Boyden chambers. Mature BMDCs and mature MoDCs were harvested and counted using the Cellometer Auto 2000 cell viability counter (Nexcelom BioScience/Cenibra). Cells were stained with the Cellometer ViaStain AOPI solution (Nexcelom BioScience/Cenibra) to determine the number of viable cells. The cell concentration of BMDCs and MoDCs was adjusted to 1x10^6 viable cells/mL in complete RPMI 1640 supplemented with 200 U/mL murine GM-CSF and complete AIM-V supplemented with 500 U/mL human GM-CSF and 250 U/mL human IL-4, respectively. To induce migration of murine and human DCs, the chemokines CCL19 and CCL21 alone or in combination were used. The chemokines were diluted in appropriate complete medium at 0.5 µg/mL, and aliquots of 600 µL were transferred into the lower compartments of the 24-well plates. The Transwell inserts were placed back carefully on top of the medium without trapping air bubbles between the insert membrane and the medium. Finally, 100 µL of the DC suspension were distributed into the Transwell inserts at 1x10^5 cells/insert. Wells used to determine input numbers of DCs received 500 µL of respective complete medium and 100 µL of DC suspension (1x10^5 cells/well) without Transwell insert. To account for random migration, chemokines were omitted from the medium in the lower compartment of respective wells. MoDCs and BMDCs could migrate at 37°C for 3 h and 4 h, respectively. Afterwards the inserts were discarded, and the migrated cells in the lower compartments were resuspended similarly to recover the cells equally. The cells were transferred and filtered into 5 mL filter-cap round-bottom tubes. The wells were incubated once with PBS/EDTA for 10 min at 37°C.
to recover as many cells as possible. To avoid loss of cells, the cells were counted without prior washing using 123count eBeads counting beads (Thermo Fisher Scientific) according to the manufacturer’s instructions on the BD LSRFortessa cell analyser with the BD FACSDiva software (both from BD Biosciences). Dead cells were excluded by staining with 1 µM (1:1,000 dilution) SYTOX Blue Dead Cell Stain (Thermo Fisher Scientific). Specific migration was determined using equation (2):

\[
\text{Specific migration} = \frac{\text{Number of migrated cells} - \text{Number of randomly migrated cells}}{\text{Number of input cells}}
\]  

(2)

### 2.12 Isolation of Immune Cell Subsets

Different human leukocyte populations were purified from PBMCs from healthy donors and were enriched by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare) as describe above. Enriched PBMCs were washed several times with MACS buffer to remove contaminating platelets. Finally, remaining erythrocytes were lysed in ACK buffer for 2-5 min at RT. Granulocytes were isolated from the fraction containing polymorphonuclear cells (PMNCs) and erythrocytes after density centrifugation. To remove as many erythrocytes as possible, the cells were lysed twice consecutively in ACK buffer. Prior to sorting, the cells were incubated with human Fc receptor (FcR) blocking reagent (Miltenyi Biotec) according to the manufacturer’s instructions. Sorting of cells was performed using the manual MidiMACS Separator according to the manufacturer’s instructions. The different murine leukocyte populations were isolated from spleen of C57BL/6 wild type and FOXP3-GFP reporter mice. The MACS kits used for isolation of the different immune cell types are listed in table 3.
Table 3: MACS kits used to isolate immune cell subsets.

<table>
<thead>
<tr>
<th>MACS Kit</th>
<th>Cell Type</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD15 MicroBeads</td>
<td>Granulocytes</td>
<td>Human</td>
</tr>
<tr>
<td>CD14 MicroBeads</td>
<td>Monocytes</td>
<td>Human</td>
</tr>
<tr>
<td>CD19 MicroBeads</td>
<td>B cells</td>
<td>Human</td>
</tr>
<tr>
<td>CD56 MicroBeads</td>
<td>Natural killer &amp; natural killer T cells</td>
<td>Human</td>
</tr>
<tr>
<td>Blood Dendritic Cell Isolation Kit II</td>
<td>Dendritic cells</td>
<td>Human</td>
</tr>
<tr>
<td>Pan T Cell Isolation Kit</td>
<td>Pan-T cells</td>
<td>Human</td>
</tr>
<tr>
<td>CD4+ CD25+ Regulatory T Cell Isolation Kit</td>
<td>CD4+, CD8+, &amp; regulatory T cells</td>
<td>Human</td>
</tr>
<tr>
<td>anti-Ly-6G MicroBead Kit</td>
<td>Granulocytes</td>
<td>Mouse</td>
</tr>
<tr>
<td>CD11b MicroBeads</td>
<td>Macrophages &amp; monocytes</td>
<td>Mouse</td>
</tr>
<tr>
<td>CD19 MicroBeads</td>
<td>B cells</td>
<td>Mouse</td>
</tr>
<tr>
<td>CD11c MicroBeads</td>
<td>Dendritic cells</td>
<td>Mouse</td>
</tr>
<tr>
<td>CD4 MicroBeads</td>
<td>CD4+ T cells</td>
<td>Mouse</td>
</tr>
<tr>
<td>CD8 MicroBeads</td>
<td>CD8+ T cells</td>
<td>Mouse</td>
</tr>
<tr>
<td>CD4+ T Cell Isolation Kit</td>
<td>Regulatory T cells</td>
<td>Mouse</td>
</tr>
</tbody>
</table>

Human PBMCs were sorted sequentially as illustrated in figure 9. Before and after each sorting step the cell number and cell viability were determined using the Cellometer Auto 2000 cell viability counter (Nexcelom BioScience/Cenibra). For this, the cells were stained with the Cellometer ViaStain AOP1 solution (Nexcelom BioScience/Cenibra), which contained acridine orange (AO) and propidium iodide (PI). Human and murine regulatory T cells (Tregs) were purified further by fluorescence-activated cell sorting (FACS) (see next chapter). The isolated human immune cell subsets were distributed into four wells and cultured for 24 h in complete AIM-V without stimulus, with 1 µg/mL LPS (from *E. coli* O111:B4) (Sigma-Aldrich), and with 400 U/mL human TNF and 200 U/mL human IFN-γ. The sorted mouse immune cell subsets were cultured for 24 h in complete IMDM. The murine cells were left untreated or stimulated with 1 µg/mL LPS and 400 U/mL murine TNF and 200 U/mL murine IFN-γ. One well of unstimulated cells from each population was used to determine MACS efficiency and viability by flow cytometry.
Figure 9: Isolation procedure of the different human leukocyte populations. After density centrifugation (1), the PBMCs were sorted successively for monocytes (2), B cells (3), NK & NKT cells (4), and DCs (5) using respective MACS kit. To remove remaining unwanted cell types prior to the purification of T cell sub-populations, pan-T cells were isolated (6). Finally, the human T cell sub-populations, i.e. regulatory T cells, CD8+ T cells, and CD4+ T cells, were purified using the CD4+CD25+ Regulatory T Cell Isolation Kit (7 & 8). In the first step, the CD8+ T cells were purified by positive selection (7). In the second step, CD25+ cells were selected positively to retain CD4+CD25+ Tregs on the MACS column and obtain pure CD4+ T cells in the flow-through (8). Granulocytes were purified from the PMNC fraction and were sorted separately (9). Tregs are shown faded since they were sorted from different donors and in addition by FACS. The MACS kits are italicised. The markers used to assess the purity of the isolated cell types by flow cytometry are indicated in brackets behind the respective cell type.
2.13 Isolation of Regulatory T Cells

Human regulatory T cells were first sorted from PBMCs by MACS on the autoMACS Pro Separator as described above. After washing with MACS buffer once, the cells were resuspended at 5x10^6 cells/mL in sorting buffer, and were incubated with antibodies and a life/dead staining dye at 4°C for 30 min. Dead cells were excluded by staining with Fixable Viability Dye eFluor 520 (1:1,000 dilution) (Thermo Fisher Scientific). To isolate FOXP3-GFP positive cells from mice, splenocytes from C57BL/6 FOXP3-GFP reporter mice were first sorted using autoMACS Pro Separator and the enriched CD4^+ T cells stained in FACS buffer at 4°C for 20 min. The antibody panel used to discriminate human and murine Tregs is summarised in table 4.

<table>
<thead>
<tr>
<th>Table 4: Antibodies used to identify human and murine Tregs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Host (Isotype)</strong></td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td>Mouse (IgG2b, κ)</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
</tr>
<tr>
<td>Mouse (IgG2a, κ)</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
</tr>
<tr>
<td>Rat (IgG2b, κ)</td>
</tr>
</tbody>
</table>

To remove excess antibodies and life/dead staining dye the human and murine cells were washed three times in sorting buffer and FACS buffer, respectively. Sorting was performed on a BD FACS Aria IIIu cell sorter (BD Biosciences) and the cells were collected in 15 mL tubes filled with 2 mL of collecting buffer. The sorting strategy used for FACS-isolation of human and murine Tregs is shown in figure 10. After sorting, the human and murine Tregs were washed with complete AIM-V and IMDM, respectively, and distributed into three wells at equal numbers. The Tregs were stimulated for 24 h with 1 µg/mL LPS (from *E. coli* O111:B4) (Sigma-Aldrich), and with 400 U/mL TNF and 200 U/mL IFN-γ in complete AIM-V or IMDM. Control cells were cultured in complete AIM-V or IMDM without stimulus. The sorting efficiency was determined by re-analysis of untreated Tregs.
Figure 10: Sorting strategy for isolation of human Tregs. (A): MACS pre-sorted human Tregs were stained for CD4, CD25, CD127, GITR, and viability, and then were subjected to FACS (1). First, total cells were identified (2), followed by exclusion of doublets (3) and dead cells (4). Then, cells were gated on CD4+ cells (5), following CD4+CD25+ cells (6). Finally, human Tregs were sorted from the gate including CD127low GITR+/- cells (7). To assess successful sorting, the cells were stained for intracellular FOXP3 (8). (B): MACS pre-sorted murine CD4+ T cells from splenocytes of FOXP3-GFP reporter mice were stained with anti-CD4 antibodies. After washing cells were subjected to FACS. First, total splenocytes were identified (1) followed by CD4+ cells (2). Finally, murine Tregs were identified and sorted from the gate including FOXP3-GFP+ cells (3). Successful purification of murine Tregs was assessed by FACS re-analysis of the sorted population in the sorting gate (B, right plot). Numbers next to gates indicate percentage of positive cells of parent populations. SSC, side scatter; FSC, forward scatter; A, area; W, width; H, height.


2.14 Flow Cytometry

The phenotype and the proper maturation of human and murine DCs were assessed by cell surface staining. Murine BMDCs were stained for CD11c, CD86, and MHCII (I-A/I-E), and human MoDCs were stained for CD11c, CD83, and CD86. For this, the cells were washed three times with FACS buffer, and were incubated with Fc receptor (FcR) block at 4°C for 10 min to prevent unspecific binding of antibodies. Murine BMDCs were incubated with self-made FcR block (supernatant of hybridoma 2.4G2) and human MoDCs with commercially available human FcR blocking reagent (Miltenyi Biotec). Subsequently the cells were pelleted, resuspended in 200 µL of FACS buffer, and without washing incubated with antibodies and isotype controls at 4°C for 30 min using a 1:200 dilution. After three washing steps with FACS buffer, fluorescent signals were acquired on a flow cytometer. MoDCs were stained additionally with TO-PRO-3 (Thermo Fisher Scientific) to assess the viability of the cells prior to measuring on a flow cytometer.

Dendritic cells used for migrations assays were stained for CCR7 to assess their migration potential towards the chemokines CCL19 and CCL21. Transduced human MoDCs were stained only for CCR7, and murine BMDCs were stained for CCR7, CD11c, and CD86. Prior to staining, the cells were washed three times with FACS buffer, and Fc receptors were blocked using appropriate FcR block as described above. Transduced MoDCs were incubated with 10 µL/10^6 cells anti-CCR7 antibodies at RT for 30 min in 200 µL of FACS buffer. Murine BMDCs were stained in 100 µL of FACS buffer with anti-CD11c and anti-CD86 antibodies using a 1:200 dilution, and with anti-CCR7 antibodies or the corresponding isotype control at 1:20 dilution. Murine BMDCs were incubated at 4°C for 60 min. After washing the cells three times with FACS buffer, 1 µM (1:1,000 dilution) SYTOX Blue Dead Cell Stain (Thermo Fisher Scientific) was added to assess viability of human and murine DCs.

To test successful purification of the different immune cells subsets by MACS, the cells were re-analysed by flow cytometry after overnight culture at 37°C. Only human B cells had to be assessed on the same day of isolation by anti-CD19 staining because this marker was found to be downregulated after overnight incubation in the first experiments. The cells were incubated with antibodies against cell type-specific markers in 100 µL of MACS buffer at 4°C for 30 min using a 1:100 dilution. Prior to analysis one sample was stained with 5 µL of propidium iodide solution (Thermo Fisher Scientific) at 4°C for 15 min to assess cell viability.

Purity of human Tregs was judged by measuring the percentage of FOXP3+ cells (Figure 10A, step 8). Intracellular FOXP3 was stained directly after sorting using the ‘Foxp3 Transcription Factor Staining Buffer’ Kit (Thermo Fisher Scientific) according to the
manufacturer’s instructions, except that incubation with anti-FOXP3 antibodies at 4°C was prolonged to overnight. Efficient isolation of murine Tregs was judged by re-analysis of FOXP3-GFP+ cells (Figure 10B, right plot). Antibodies and isotype controls used for flow cytometry are listed below in table 5 and table 6, respectively.

Table 5: Antibodies used for flow cytometry.

<table>
<thead>
<tr>
<th>Host (Isotype)</th>
<th>Target</th>
<th>Clone</th>
<th>Label(s)</th>
<th>Target Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster (IgG1, λ1)</td>
<td>anti-CD11c</td>
<td>HL3</td>
<td>APC</td>
<td>Mouse</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Hamster (IgG2, λ1)</td>
<td>anti-CD11c</td>
<td>N418</td>
<td>BV421 Mouse</td>
<td>BioLegend</td>
<td></td>
</tr>
<tr>
<td>Rat (IgG2a, κ)</td>
<td>anti-CD86</td>
<td>GL1</td>
<td>PE, APC</td>
<td>Mouse</td>
<td>BD Biosciences, Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Rat (IgG2b, κ)</td>
<td>anti-MHCII (I-A/I-E)</td>
<td>M5/114.15.2</td>
<td>PE</td>
<td>Mouse</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Rat (IgG2a, κ)</td>
<td>anti-CCR7</td>
<td>4B12</td>
<td>PE</td>
<td>Mouse</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Mouse (IgG2a, κ)</td>
<td>anti-CCR7</td>
<td>150503</td>
<td>APC</td>
<td>Human</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
<td>anti-CD11c</td>
<td>3.9</td>
<td>FITC</td>
<td>Human</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
<td>anti-CD83</td>
<td>HB15e</td>
<td>PE</td>
<td>Human</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
<td>anti-CD86</td>
<td>2331 (FUN-1)</td>
<td>PE</td>
<td>Human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
<td>anti-FOXP3</td>
<td>236A/E7</td>
<td>PE</td>
<td>Human</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Mouse (IgG2a, κ)</td>
<td>anti-CD3</td>
<td>HIT3a</td>
<td>FITC</td>
<td>Human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
<td>anti-CD4</td>
<td>RPA-T4</td>
<td>APC, FITC</td>
<td>Human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
<td>anti-CD8</td>
<td>SK1</td>
<td>APC</td>
<td>Human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse (IgG2a, κ)</td>
<td>anti-CD14</td>
<td>TÜK4</td>
<td>FITC</td>
<td>Human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse (IgM, κ)</td>
<td>anti-CD15</td>
<td>HI98 (HIM1)</td>
<td>FITC</td>
<td>Human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
<td>anti-CD19</td>
<td>HIB19</td>
<td>APC</td>
<td>Human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse (IgG1, κ)</td>
<td>anti-CD56</td>
<td>B159</td>
<td>APC</td>
<td>Human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse (IgG2a, κ)</td>
<td>anti-HLA-DR</td>
<td>G46-6</td>
<td>FITC</td>
<td>Human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Mouse (IgG2a, κ)</td>
<td>anti-CD303</td>
<td>201A</td>
<td>APC</td>
<td>Human</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>
Table 6: Antibody isotype controls used for flow cytometry.

<table>
<thead>
<tr>
<th>Target</th>
<th>Clone</th>
<th>Label</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-hamster IgG1, λ1</td>
<td>G235-2356</td>
<td>APC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-rat IgG2a, κ</td>
<td>eBR2a</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-mouse IgG1, κ</td>
<td>P3.6.2.8.1</td>
<td>PE</td>
<td>Thermo Fisher Scientific</td>
</tr>
</tbody>
</table>

The fluorescent signals were acquired using a BD Accuri C6, a BD FACSCalibur, a BD LSRFortessa, or a BD LSR II cell analyser. The purity of the sorted human Tregs was assessed on the BD FACSaria Illu cell sorter. All flow cytometers were from BD Biosciences. Flow cytometry data was analysed with the FlowJo software version 8.8.7 (TreeStar).

### 2.15 Confocal Microscopy

Coverslips (Menzel-Gläser, diameter 13 mm, thickness #1) (VWR) were sterilised by baking at 180°C for 6 h. Coverslips used for HEK293T cells and BMDCs were coated with 100 µg/mL poly-L-lysine in ddH$_2$O (sterile-filtered) (Sigma-Aldrich). For HeLa cells coverslips were coated with 2 mg/mL porcine gelatine in ddH$_2$O (autoclaved) (Sigma-Aldrich). For coating, coverslips were covered completely with coating solution and incubated for at least 30 min at 37°C in 24-well plates. Coating solution was re-used, and coated coverslips were dried at RT. Cells were seeded onto coated coverslips and allowed to adhere for at least 8 h. At indicated time points, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in D-PBS at RT for 15 min. Fixed cells were incubated first with quenching buffer at 4°C for 10 min and then with permeabilisation buffer at RT for 5 min. After each step, cells were washed two to three times with D-PBS. To block unspecific binding sites, the cells were incubated with blocking buffer at RT for 30-60 min. Primary antibodies were incubated at RT for 60 min or at 4°C overnight. Antibodies used for confocal microscopy are listed in table 7. After washing with D-PBS, cells were incubated with 4 µg/mL of appropriate secondary antibodies at RT for 60 min. Secondary antibodies were F(ab')2-goat anti-rabbit or -mouse IgG fragments coupled to Alexa Fluor-488, -546 or -568, and -647. All antibodies were diluted in blocking buffer. DAPI Fluoromount-G (SouthernBiotech) was used to mount coverslips onto microscope slides (SuperFrost Plus, Menzel-Gläser) (VWR).

Images were acquired using a Zeiss LSM510 Meta and LSM880 confocal laser-scanning microscope (both from Carl Zeiss) with a 40x or 63x plan-apochromat, oil-immersion objective (both with NA 1.4).
Table 7: Primary antibodies used for immunocytochemistry.

<table>
<thead>
<tr>
<th>Target species</th>
<th>Target</th>
<th>Host</th>
<th>Clone</th>
<th>Concentration</th>
<th>Supplier/Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>FAT10</td>
<td>Mouse</td>
<td>4F1</td>
<td>0.5-1 µg/mL</td>
<td>(Aichem et al., 2010)</td>
</tr>
<tr>
<td>Human &amp; Mouse</td>
<td>Ubiquitin</td>
<td>Mouse</td>
<td>FK2</td>
<td>1 µg/mL</td>
<td>Enzo Life Sciences</td>
</tr>
<tr>
<td>Human &amp; Mouse</td>
<td>Ubiquitin</td>
<td>Rabbit</td>
<td>10H4L21</td>
<td>2.5 µg/mL</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Human &amp; Mouse</td>
<td>p62</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1 µg/mL</td>
<td>H-290; Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>/</td>
<td>FLAG</td>
<td>Mouse</td>
<td>M2</td>
<td>0.5 µg/mL</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

Images were analysed using ImageJ FIJI software version 1.51s (Schindelin et al., 2012) and its feature ‘analyse particles’. To avoid false positives by nuclei, the DAPI signals were depleted prior to analysis. For counting, the intensity threshold of the image was set as high as possible to avoid noise. Additionally, the threshold-adjusted images were compared with the original images to avoid false-positive DALIS. In this way, particles that were counted by the software, but were positioned outside of the cells, were excluded from analysis. Only DALIS larger than 0.15 µm² were counted. For this, the size cut-off for counting DALIS was set to 0.15-infinity µm². The buffers used to prepare microscopy samples are specified below.

**D-PBS**

0.1 mM CaCl₂ (Roth)
1 mM MgCl₂ (Acros Organics)
2 mM NaN₃ (Merck)
in 1x PBS, sterile filtered

**Quenching buffer**

50 mM NH₄Cl (Roth)
in D-PBS, prepared freshly

**Permeabilisation buffer**

0.2% (v/v) Triton X-100 (Sigma-Aldrich) in D-PBS, prepared freshly

**Blocking buffer**

1x Roti-ImmunoBlock (Roth) or
0.2% fish-skin gelatine (Sigma-Aldrich) in D-PBS, prepared freshly

**2.16 Quantitative Real-Time RT-PCR**

To evaluate gene expression total RNA was extracted with the RNeasy Plus Mini Kit in combination with the RNase-Free DNase Set (both from Qiagen) according to the manufacturer’s instruction. cDNA was synthesised from total RNA using the Reverse
Transcription System (Promega) according to the manufacturer’s instruction. After synthesis, the cDNA concentration was adjusted to 10 ng/µL with nuclease-free ddH₂O. To set up PCR reactions, the LightCycler FastStart DNA Master SYBR Green I Kit (Roche) was used according to the manufacturer’s instructions. PCR reactions were run with 2 µL (=20 ng) of cDNA and 0.5 µM of each primer. The primers used for quantitative real-time RT-PCR (qPCR) are listed in table 8.

Table 8: Primer pairs and corresponding properties used for qPCR.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward (5’ to 3’ Reverse) (5’ to 3’)</th>
<th>Annealing temperature</th>
<th>Elongation time (Amplicon length)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(HUMAN) FAT10old</td>
<td>AATGACCTTGTAGTGGGCAACC GGCCTATTCCTGCACATTCT</td>
<td>60°C</td>
<td>16 s (391 bp) (only used for qPCR in chapter 3.1.1)</td>
</tr>
<tr>
<td>(HUMAN) FAT10new</td>
<td>CTGGTGCTAGTCCGTCGCTGCAA GGGTATGGGTAGGCTTCTCT</td>
<td>64°C</td>
<td>9 s (209 bp)</td>
</tr>
<tr>
<td>(HUMAN) GAPDH</td>
<td>GAAGGTGAAGGTGCAAGCTGGAAGATGATGATGAGGATGTTCTC</td>
<td>60°C</td>
<td>10 s (226 bp)</td>
</tr>
<tr>
<td>(HUMAN) SDHA</td>
<td>GATTACCTCAAGCCCATCCA CACCTCAGCCTGCTTCAAA</td>
<td>60°C</td>
<td>7 s (151 bp)</td>
</tr>
<tr>
<td>(HUMAN) TBP</td>
<td>GTAAGTTAAGACATTGC CTGTTCCTCCATTGCTTCTCT</td>
<td>60°C</td>
<td>7 s (161 bp)</td>
</tr>
<tr>
<td>(HUMAN) RPL13A</td>
<td>CTACAGAAACAGTTGAAGTACTG ATGCCGTCACAAACACTGGAG</td>
<td>60°C</td>
<td>8 s (183 bp)</td>
</tr>
<tr>
<td>(MOUSE) FAT10_1</td>
<td>GCTTCCTTCGCCACCTGTTGT TGGGGCTTGAGGATTTTGGAGTCT</td>
<td>68°C</td>
<td>7 s (167 bp)</td>
</tr>
<tr>
<td>(MOUSE) FAT10_2</td>
<td>GGAGGTGACAAAGAAACACTA TTCACAACCTGCTTCTAGG</td>
<td>62°C</td>
<td>8 s (200 bp) (only used for qPCR in chapter 3.3.2)</td>
</tr>
<tr>
<td>(MOUSE) UBA6</td>
<td>GATTCCTTCTCCCAACAAACT TTGCCAAAACAAACTCCGCAAT</td>
<td>64°C</td>
<td>11 s (269 bp)</td>
</tr>
<tr>
<td>(MOUSE) USE1</td>
<td>AAACGTGACAAACGGGCAA TGCTGTCTCTGATGATGTTT</td>
<td>64°C</td>
<td>9 s (217 bp)</td>
</tr>
<tr>
<td>(MOUSE) p62</td>
<td>GCTGGCCTATAACCCACATCT CGCCTTCCATCCAGAAA</td>
<td>62°C</td>
<td>4 s (94 bp)</td>
</tr>
<tr>
<td>(MOUSE) HDAC6</td>
<td>TCAACACAACTTTATGGAGTGG CCACTAGTGGACAACCTGCTTC</td>
<td>64°C</td>
<td>4 s (101 bp)</td>
</tr>
<tr>
<td>(MOUSE) ACTB</td>
<td>GACCTCTATGCCAACACAGT ACTCATGCTACTCTGCCTGTT</td>
<td>66°C</td>
<td>9 s (220 bp)</td>
</tr>
<tr>
<td>(MOUSE) HPRT</td>
<td>CCACAGGGTACCAAGAAAATAT AAGACGGACGGACGAAGACTGG</td>
<td>59°C</td>
<td>5 s (111 bp)</td>
</tr>
<tr>
<td>(MOUSE) TBP</td>
<td>ATCTGTGCTTAACCTGACC ATTGTTCCTCCCTCGCTTGC</td>
<td>60°C</td>
<td>7 s (171 bp)</td>
</tr>
<tr>
<td>(MOUSE) RPL13A</td>
<td>TGAAGGCACTCAACATTTCTGG GGTAGGAAACCTCTGCTGATG</td>
<td>57°C</td>
<td>12 s (299 bp)</td>
</tr>
<tr>
<td>FLAG-X-IRES</td>
<td>GATGCCAGCTACAAAGACCATGC AACATAGACAAAGCCACACC</td>
<td>62°C</td>
<td>16 s (401 bp; FLAG-Ubiquitin-IRES) 26 s (663 bp; FLAG-hFAT10-IRES)</td>
</tr>
</tbody>
</table>
PCR efficiency was determined at least once for each primer pair, except for the primer pairs of human FAT10 old and GAPDH. To determine PCR efficiencies, a tenfold serial dilution ranging from 20 ng to 0.2 ng of cDNA from one sample was prepared and each dilution measured by qPCR. The level of expression was determined by the LightCycler instrument with the LightCycler Software Version 3.5 (both from Roche) or the TOptical Gradient 96 Real-Time PCR-Thermocycler and the qPCRsoft V3.1 software (both from Analytik Jena). The PCR programmes were set up using the LightCycler software version 3.5 and the qPCRsoft V3.1 software as described in table 9 and table 10, respectively.

Raw data, including PCR efficiencies, was inserted into the Excel-based relative expressions software tool with a multiple condition solver (REST-MCS) version 2 which automatically calculated the relative expression level according to the Pfaffl method (Pfaffl et al., 2002). Expression of human genes was normalised to the housekeeping genes GAPDH, SDHA, TBP, or RPL13A, and expression of murine genes was normalised to the housekeeping genes ACTB, TBP, HPRT, or RPL13A.

<table>
<thead>
<tr>
<th>Table 9: General PCR programme using the LightCycler software version 3.5.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>Polymerase activation</td>
</tr>
<tr>
<td>Amplification (40 cycles)</td>
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<tr>
<td>Melting curve</td>
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<tr>
<td>Cooling</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 10: General PCR programme using the qPCRsoft V3.1 software.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>Polymerase activation</td>
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<tr>
<td>Amplification (40 cycles)</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Melting curve</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
2.17 Expression and Purification of Recombinant Proteins

Recombinant proteins were expressed in the *E. coli* strain B834 arctic express (B834 AE). This *E. coli* strain was obtained from the strain B834 (DE3) pLys (Merck) after transformation of a plasmid encoding cold adapted chaperones (kind gift from Prof. Dr. Martin Scheffner, University of Konstanz) which allow for better folding and solubility of expressed proteins at low temperatures, hence the name ‘arctic express’. Proteins expressed in bacteria had an N-terminal maltose binding protein (MBP) tag and a C-terminal 6xHis tag for purification. For recombinant expression, plasmid vectors were always transformed freshly into bacteria. The entire transformation was spread onto LB agar plates containing 85 µg/mL chloramphenicol (Sigma-Aldrich), 20 µg/mL gentamicin (Thermo Fisher Scientific), and 100 µg/mL ampicillin (Roth), and the plates were incubated at 37°C overnight. All grown colonies were used to inoculate a starter culture of 25 mL in terrific broth (TB) and the antibiotics mentioned above. After overnight incubation at 37°C and 250 rpm, the started culture was diluted 1:100 into fresh TB supplemented with respective antibiotics and grown further at 37°C and 220 rpm. The OD$_{600nm}$ was measured using the SmartSpec Plus spectrophotometer (Bio-Rad) until an OD$_{600nm}$ of 0.4-0.5 was reached. Prior to induction of protein expression with 0.3 mM IPTG (Roth), the bacterial culture was cooled on ice for 15 min. The induced bacteria were cultured at 16°C and 220 rpm for 16 h. The bacterial culture was harvested and lysed directly.

For purification of proteins, the bacteria were resuspended in 30 mL of resuspension buffer by pipetting, and then were sonicated three times for 20 s with 5 cycles at 70% intensity using the Sonoplus sonicator (Bandelin) for complete resuspension and physical lysis. Subsequently the bacteria were lysed enzymatically by addition of 1 mg/mL lysozyme (Sigma-Aldrich) and 100 µg/mL DNase I (Roche). The bacterial suspension was incubated with the enzymes further on ice for 30 min followed by a single sonication step. The lysed bacteria were centrifuged for 10 min at 4°C and 14,000 rpm in the fixed-angle Sorvall rotor SS-34 (Thermo Fisher Scientific). The cleared supernatant was transferred into a fresh tube and was incubated with 400 mg Protino Ni-IDA nickel beads (Macherey Nagel) at 4°C overnight under rotation. After incubation, the nickel beads were pelleted, and the supernatant was discarded. The beads were washed once with 20 mL of resuspension buffer, resuspended in 10 mL of resuspension buffer, and transferred into a 5 mL column (Thermo Fisher Scientific). The column was washed once with 10 mL of resuspension buffer, once with 10 mL of resuspension buffer without Triton X-100, and finally with 5 mL of resuspension buffer without Triton X-100 supplemented with 10 mM imidazole (Merck). The protein was eluted by adding 2 mL of 250 mM imidazole (Merck), incubating for 2 min, and collecting the eluate. The elution was repeated five times.
The eluted samples were pooled and concentrated to 1.5-2 mL using an Amicon Ultra-15 centrifugal filter unit (Merck) with a molecular weight cut-off (MWCO) of 10 kDa according to the manufacturer’s instructions. Finally, concentrated protein samples were dialysed against 2 L of 1x PBS at 4°C overnight using a Slide-A-Lyzer G2 dialysis cassette (Thermo Fisher Scientific) for 0.5-3 mL of sample volume and a MWCO of 10 kDa. The protein sample was recovered and stored at 4°C on ice until further usage.

Control samples were removed before induction and after incubation of induced bacteria, before and after the pull-down with the nickel beads, after the wash with resuspension buffer without Triton X-100, and after the wash with resuspension buffer without Triton X-100 supplemented with imidazole. Additionally, 2% of the sample volume was kept as control sample prior to and after concentrating the sample, and after the dialysis. Control samples and a BSA standard were boiled with 2x SDS sample buffer and separated by SDS-PAGE. Successful expression and purification of proteins was judged by staining with sensitive Coomassie and by detecting the 6xHis tag using immunoblotting. The signals of the purified recombinant protein and the BSA standard were quantified using ImageJ FIJI software version 1.51s on a sensitive Coomassie-stained gel (Schindelin et al., 2012). Finally, the total amount of the recombinant protein in the sample was calculated using the BSA standard. The medium, the buffers, and the solutions necessary for expression and purification of recombinant proteins are listed below.

**Terrific broth basis**
- 1.33% (w/v) peptone (BD Biosciences)
- 2.67% (w/v) yeast extract (BD Biosciences)
- in ddH₂O, autoclaved

**10x phosphate buffer**
- 720 mM K₂HPO₄ (Sigma-Aldrich)
- 170 mM KH₂PO₄ (Roth)
- in ddH₂O, autoclaved

**Terrific broth (TB)**
- 90% (v/v) TB basis
- 10% (v/v) 10x phosphate buffer
- 0.8% (v/v) 50% glucose
- prepared freshly

**Resuspension buffer, pH 8**
- 50 mM NaH₂PO₄ (Sigma-Aldrich)
- 300 mM NaCl (Roth)
- 0.5% (v/v) Triton X-100 (Fluka)
- 1x protease inhibitor cocktail (complete Mini EDTA-free) (Roche)
- in ddH₂O, prepared freshly
2x SDS sample buffer (Laemmli)

- 125 mM TRIS-HCl, pH 6.8 (Roth)
- 20% (v/v) glycerol (Roth)
- 4% (w/v) SDS (Serva)
- 0.2% bromophenol blue (AppliChem)

in ddH2O, aliquots stored at -20°C

Sensitive Coomassie staining solution

- 0.02% (w/v) Coomassie G-250 (Serva)
- 5% (w/v) Al2(SO4)3 x 14-18H2O (Sigma-Aldrich)
- 10% (v/v) ethanol, p.a. (Roth)
- 2% (v/v) H3PO4 (Roth)

in ddH2O, stored in the dark

Sensitive Coomassie destaining solution

- 10% (v/v) ethanol, p.a. (Roth)
- 2% (v/v) H3PO4 (Roth)

in ddH2O

2.18 Immunisation of Rabbits

To raise antibodies for detection of murine FAT10, three rabbits were immunised with recombinant MBP-mFAT10-6xHis, which was expressed and purified as described above. Rabbits were housed in the animal facility of the University of Konstanz and handled only by the staff of the animal facility. The rabbits were immunised in accordance with the general notification ‘Immunisierung von Kaninchen zur Erzeugung von Antikörpern’ (I-13/02), which was approved by the review board of the Regierungspräsidium Freiburg.

For immunisations, MBP-mFAT10-6xHis was purified freshly each time and supplied at 0.3 µg/µL or higher. Each rabbit received 500 µL of antigen solution together with 500 µL of adjuvant per immunisation. Pre-immune serum was collected from the rabbits prior to the basic immunisation in the first week (week 0). Rabbits received three booster immunisations at intervals of three weeks (week 3, 6, and 9) and one week before the entire serum was collected (week 11). One week after the second and third booster immunisation, test-serum of each rabbit was collected and each tested by immunoblotting for the presence of anti-mFAT10 antibodies (week 7 and 10). Twelve weeks after the basic immunisation, the rabbits were euthanised and the entire serum collected. Sera collected from the rabbits K1146, K1151, and K1154 were stored at -20°C until further usage. If necessary, the rabbit serum was cross-absorbed against crude E. coli lysate in which MBP-6xHis was expressed. For cross-absorption, the E. coli lysate was coupled to NHS-activated Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer’s instruction. The cross-absorbed serum was concentrated to the original volume.
using Amicon Ultra-15 centrifugal filter unit (Merck) with a molecular weight cut-off (MWCO) of 10 kDa according to the manufacturer’s instructions.

2.19 Cross-linking of Antibodies to Beads

Antibodies were cross-linked to protein A beads to reduce antibody signals on immunoblots and to allow for cleaner elution of proteins after immunoprecipitations. The mouse anti-FAT10 antibody (clone 4F1) and mouse IgG isotype antibodies were cross-linked at 1 mg of antibody per 1 mL of bead slurry. Cross-absorbed rabbit anti-FAT10 polyclonal serum (K1154), which was used to detect murine FAT10, was coupled covalently to beads at 200 µL of serum per 1 mL of affinity gel beads. For cross-linking, the EZview Red Protein A affinity gel (Sigma-Aldrich) or the Protein A Sepharose CL-4B antibody purification resin (GE Healthcare).

To bind antibodies to the protein A beads, an aliquot of the resuspended bead slurry was washed four times with three bead volumes 1x PBS and pelleted at 8,200 g for 60 s. After the last wash, the beads were resuspended in 1 mg/mL BSA (Sigma-Aldrich) in 1x PBS (dilution buffer) at a 1:1 ratio (e.g. 500 µL of dilution buffer to 500 µL of packed beads), mixed gently, and incubated with rocking at 4°C for 10 min. The supernatant was discarded, and the beads resuspended in diluted pure antibody or antibody serum. Antibodies and sera were diluted in dilution buffer and mixed with the beads at a 1:1 ratio. Antibodies could bind at 4°C for 60 min to overnight under rotation. After binding, the supernatant was discarded, and the beads were resuspended in dilution buffer at a 1:1 ratio. The beads were incubated with rocking at 4°C for 5 min following a wash with three bead volumes 1x PBS.

For cross-linking of the antibodies with the protein A, the beads were incubated with dimethyl pimelimidate (DMP) (Sigma-Aldrich). A 13 mg/mL DMP stock solution in 1x PBS was prepared freshly prior to usage due to its instability in solution. This solution was diluted 1:1 with 0.2 M triethanolamine (Merck) in 1x PBS (wash buffer) and vortexed immediately. The pH of this solution was checked and set to pH 8-9, if necessary. Diluted DMP solution was added to the beads and incubated at RT for 30 min under rotation. Subsequently the beads were washed with wash buffer at a 1:1 ratio and incubated at RT for 5 min under rotation. The cross-linking with DMP solution and the washing step were repeated twice. To quench remaining DMP, 50 mM ethanolamine (Merck) in 1x PBS (quench buffer) was added at a 1:1 ratio, and the beads quenched twice consecutively at RT for 5 min under rotation. To remove excess of unlinked antibody, the beads were incubated with rocking at RT for 10 min with 1 M glycine (pH 3) (Roth) in ddH₂O at a 1:1 ratio. The glycine wash was repeated once. Finally, the beads were washed four times at RT for 5 min under rotation with three bead volumes 1x PBS. The
beads, coupled with the antibodies, were stored in 1x PBS supplemented with 0.1% (w/v) NaN₃ (Merck) for long-term storage at 4°C.

Successful binding and cross-linking of the antibodies was assessed by SDS-PAGE and Coomassie staining using control samples. For this, aliquots of 10 µL were removed after each step. Before and after cross-linking additionally 10 µl of the beads were kept as control samples. The bead samples were incubated with 100 µL of 2x SDS sample buffer without reducing agent at 37°C for 30 min. Then, 95 µL of the supernatant fractions were transferred into fresh tubes, and 5 µL of DTT (2 M stock) (Sigma-Aldrich) were added. The bead fractions were resuspended in 100 µL of 2x SDS sample buffer supplemented with 100 mM DTT (Sigma-Aldrich). Finally, all samples were boiled at 95°C for 5 min and subjected to SDS-PAGE following a staining with sensitive Coomassie staining solution. Antibody binding was successful, if antibody content was reduced in the control sample after binding to the beads and decreased or absent in the other samples compared with the control sample of antibodies and sera prior to binding. Antibody cross-linking was successful, if there was no or little antibody in the supernatant fraction and bead fraction of the bead control sample after cross-linking compared with the supernatant fraction and bead fraction of the control sample from before cross-linking.

2.20 Sample Preparation and Immunoprecipitation

All buffers used for cell lysis and immunoprecipitations were supplemented with 5 µM MG132 (Sigma-Aldrich) and 1x protease inhibitor cocktail (complete Mini EDTA-free) (Roche) prior to use. If total cell lysates were analysed, RIPA lysis buffer or NP-40 lysis buffer was used. For immunoprecipitation experiments, cells were lysed only in NP-40 lysis buffer. Cells were lysed in 150 µL of lysis buffer per 6-well or 1 mL of lysis buffer per 10 cm dish. For lysis, cells were resuspended in lysis buffer and incubated on ice for 30 min. Afterwards the cells were sonicated twice for 10 s with 5 cycles at 70% intensity using the Sonoplus sonicator (Bandelin), and the lysates were cleared by ultracentrifugation at 20,000 g and 4°C for 15 min. Finally, protein concentrations were determined using the Pierce BSA protein assay kit (Thermo Fisher Scientific). Protein levels were adjusted to be equal in each sample. Following adjustment of protein concentrations, total cell lysates and load/input samples for immunoprecipitations were boiled with 3x SDS sample buffer at 95°C for 5 min. For load/input samples, 10% of the total sample volume was used.

For immunoprecipitations, the EZview Red Anti-FLAG M2 affinity gel (Sigma-Aldrich), and the protein A beads coupled with antibodies, which were prepared as described above, were
used. Beads were washed twice in bulk with lysis buffer. Pre-equilibrated beads were blocked in bulk at RT for 30-60 min with 1x Roti-Block (Roth) diluted in lysis buffer. After blocking, equal volumes of the blocked bead slurry (15-25 µL per sample) were distributed into single tubes, pelleted, and the supernatant discarded. Then, equal volumes of each cell lysate were added to the beads, and the beads were incubated with rocking at 4°C for 2 h to overnight. After incubation, 10% of the supernatant was removed as control of successful immunoprecipitation and boiled with 3x SDS sample buffer at 95°C for 5 min. Remaining supernatant was discarded and the beads were washed three times with 1 mL of NET-TN wash buffer and then three times with 1 mL of NET-T wash buffer. After the last wash, remaining buffer was aspirated, and the beads were boiled with 2x SDS sample buffer at 95°C for 5 min.

For the detection of human endogenous FAT10 in unstimulated and stimulated PBMCs, cells were lysed in RIPA lysis buffer on ice for 30 min. The lysates were cleared by ultracentrifugation at 20,000 g and 4°C for 10 min. Before immunoprecipitation, load/input samples were removed and equal volumes were subjected to immunoprecipitation using the monoclonal anti-FAT10 antibody 4F1 bound to protein A beads. After incubation at 4°C for 2 h to overnight, the supernatant was removed and beads washed as described above with NET-TN and NET-T washing buffer. Finally, the beads were boiled in 2x SDS sample buffer and subjected to SDS-PAGE and immunoblotting. The composition of the lysis buffers, the wash buffers, and the SDS sample buffer is specified below.

### RIPA lysis buffer
- 50 mM TRIS-HCl, pH 8 (Roth)
- 150 mM NaCl (Roth)
- 1% (v/v) NP-40/Igepal (Sigma-Aldrich)
- 0.5% (w/v) deoxycholate (Sigma-Aldrich)
- 0.1% (w/v) SDS (Serva)
- in ddH₂O

### NP-40 lysis buffer
- 20 mM TRIS-HCl, pH 7.8 (Roth)
- 50 mM NaCl (Roth)
- 10 mM MgCl₂ (Acros Organics)
- 1% (v/v) NP-40/Igepal (Sigma-Aldrich)
- in ddH₂O, prepared freshly

### NET-TN (high salt wash buffer)
- 50 mM TRIS-HCl, pH 8 (Roth)
- 650 mM NaCl (Roth)
- 5 mM EDTA (Roth)
- 0.5% (v/v) Triton X-100 (Fluka)
- in ddH₂O, prepared freshly

### NET-T (low salt wash buffer)
- 50 mM TRIS-HCl, pH 8 (Roth)
- 150 mM NaCl (Roth)
- 5 mM EDTA (Roth)
- 0.5% (v/v) Triton X-100 (Fluka)
- in ddH₂O, prepared freshly
**Material & Methods**

**4x SDS sample buffer (Laemmli)**

250 mM TRIS-HCl, pH 6.8 (Roth)

40% (v/v) glycerol (Roth)

8% (w/v) SDS (Serva)

0.4% bromophenol blue (AppliChem)

in ddH2O, aliquots stored at -20°C, add 20% (v/v) β-mercaptoethanol (Merck)
or 400 mM DTT (Sigma-Aldrich) prior to use to get 3x sample buffer

**2.21 SDS-PAGE and Immunoblotting**

Protein samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions using the Mini-PROTEAN 3 or Tetra cell system (both from Bio-Rad). Gels for SDS-PAGE were prepared and run using the discontinuous Laemmli buffer system (Laemmli, 1970). Separation gels with 10-15% of polyacrylamide were prepared using Acrylamide 4K solution (30%) Mix 37.5:1 (AppliChem) and 4x resolving gel buffer. Stacking gels containing 5% of polyacrylamide were prepared with Acrylamide 4K solution (30%) Mix 37.5:1 (AppliChem) and 4x stacking gel buffer. Polymerisation of gels was induced using 1% (v/v) ammonium persulphate (APS) (Bio-Rad) and 0.1% (v/v) N,N,N',N'-Tetramethylethylendiamin (TEMED) (Sigma-Aldrich). The PageRuler Prestained Protein Ladder (10-180 kDa) (Thermo Fisher Scientific) was used as molecular weight marker. Gels were run in 1x running buffer first at 60 V for 40 min to allow the protein samples to align at the running front and to enter the resolving gel. Subsequently, the voltage was increased to 100-120 V to separate the proteins, and gels were run further for approximately 75 min.

Prior to protein transfer, gels were soaked in pre-cooled transfer buffer at RT for 10 min. Dunn’s blotting buffer (Garfin & Bers, 1989) with a pH of 9.9 was used for transfer of endogenous murine and human FAT10 due to their high isoelectric points (pI) of pI 9.4 and pI 8.9, respectively (calculated using Protein Calculator v3.4; http://protcalc.sourceforge.net/). For transfer of other proteins, Towbin’s blotting buffer (Towbin et al., 1979) was used. Proteins were transferred onto Amersham Protran 0.45 NC nitrocellulose membranes with a pore size of 0.45 μm (GE Healthcare) using a Criterion Blotter or a Mini Trans-Blot cell (both from Bio-Rad). If Towbin’s blotting buffer was used, the proteins were transferred at 110 V for 60-90 min. In case of Dunn’s blotting buffer, protein transfer was done at 80 V for 120-150 min. Transfer buffer was prepared freshly before use and pre-cooled at -20°C.
After transfer, membranes were rinsed with ddH$_2$O and blocked at RT for 30-60 min. Membranes were blocked with 1x Roti-Block (Roth) in 1x PBS or 1x Odyssey TBS blocking buffer (LI-COR Biosciences) in ddH$_2$O. Membranes were incubated with primary antibodies with rocking at RT for 60-120 min or at 4°C overnight. Before and after labelling with HRP- and IRDye-coupled secondary antibodies, membranes were washed three times with PBS-T and TBS-T, respectively, for 10 min at RT under agitation. Membranes were incubated with secondary antibodies at RT for 60-120 min under agitation.

For detection of endogenous human FAT10 from PBMCs after immunoprecipitation, samples were separated on NuPAGE 4-12% BIS-TRIS gels at 80 mA per gel using NuPAGE SDS MES running buffer (both from Thermo Fisher Scientific). After separation, the proteins were blotted using Towbin’s buffer as described above. The membranes were washed once with TBS-T for 5 min, and then blocked for 60 min at RT in 1x Roti Block (Roth) in TBS-T. Blocked membranes were incubated with diluted primary antibodies at RT for 90 min followed by one washing step with TBS-T at RT for 5 min before being incubated with secondary HRP-coupled antibodies at RT for 60 min. Finally, the membranes were washed three times with TBS-T at RT for 10 min each.

Antibodies were diluted in 1x Roti-Block (Roth) in PBS-T or TBS-T, or in 5% (w/v) skim milk powder or 3% (w/v) BSA in TBS-T. Primary and secondary antibodies used for probing membranes are listed below in table 11 and table 12, respectively.

<table>
<thead>
<tr>
<th>Target species</th>
<th>Target</th>
<th>Host</th>
<th>Clone</th>
<th>Concentration/Dilution</th>
<th>Supplier/Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>FAT10</td>
<td>Mouse</td>
<td>4F1</td>
<td>0.2-0.25 µg/mL</td>
<td>(Aichem et al., 2010)</td>
</tr>
<tr>
<td>Human</td>
<td>FAT10</td>
<td>Rabbit</td>
<td>Polyclonal 105(7)</td>
<td>1:1,000</td>
<td>(Hipp et al., 2005)</td>
</tr>
<tr>
<td>Mouse</td>
<td>FLAG(-HRP)</td>
<td>Mouse</td>
<td>M2</td>
<td>0.1-0.2 µg/mL</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Mouse</td>
<td>HA-HRP</td>
<td>Mouse</td>
<td>HA-7</td>
<td>2.5 µg/mL</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Mouse</td>
<td>Penta-His</td>
<td>Mouse</td>
<td>P-21315</td>
<td>0.2 µg/mL</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Human &amp; Mouse</td>
<td>GAPDH-HRP</td>
<td>Mouse</td>
<td>71.1</td>
<td>1:10,000</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Human &amp; Mouse</td>
<td>γ-Tubulin</td>
<td>Mouse</td>
<td>GTU-88</td>
<td>1:10,000</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Human &amp; Mouse</td>
<td>HSP90</td>
<td>Rabbit</td>
<td>C45G5</td>
<td>1:10,000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Human</td>
<td>β-Actin</td>
<td>Mouse</td>
<td>AC-15</td>
<td>1:5,000</td>
<td>Abcam</td>
</tr>
</tbody>
</table>
Table 12: Secondary antibodies used for detection in immunoblotting.

<table>
<thead>
<tr>
<th>Target species</th>
<th>Host</th>
<th>Label</th>
<th>Concentration/Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse or Rabbit IgG</td>
<td>Goat</td>
<td>IRDye800CW</td>
<td>0.1 µg/mL</td>
<td>LI-COR Biosciences</td>
</tr>
<tr>
<td>Mouse or Rabbit IgG</td>
<td>Goat</td>
<td>IRDye680RD</td>
<td>0.1 µg/mL</td>
<td>LI-COR Biosciences</td>
</tr>
<tr>
<td>Mouse or Rabbit IgG</td>
<td>Goat</td>
<td>HRP</td>
<td>0.1 µg/mL</td>
<td>Dako/Agilent Technologies</td>
</tr>
<tr>
<td>Mouse or Rabbit IgG</td>
<td>Goat</td>
<td>HRP</td>
<td>1:5,000</td>
<td>Jackson Immuno Research</td>
</tr>
</tbody>
</table>

To induce signals from HRP-coupled secondary antibodies the SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (both from Thermo Fisher Scientific) were used according to the manufacturer’s instructions. Signals were detected and images were taken using the ChemiDoc XRS system with the Quantity One 1-D analysis software V4.6.1 (both from Bio-Rad). Signals from IRDye-coupled antibodies were detected using the LI-COR Odyssey Fc imaging system and images were taken and analysed with the Image Studio software version 5.2 (both from LI-COR Biosciences) according to manufacturer’s instruction. The PBS-based buffer system was used in combination with HRP-coupled antibodies, and the TBS-based buffer system was applied to IRDye-coupled antibodies. The buffers used for SDS-PAGE and immunoblotting are listed below.

10x running buffer (Laemmli), pH 8.3
250 mM Trizma (Sigma-Aldrich)
1.92 M glycine (Roth)
in ddH₂O

4x resolving gel buffer (Laemmli)
1.5 M Trizma, pH 8.8 (Sigma-Aldrich)
0.4% (w/v) SDS (Serva)
in ddH₂O

1x running buffer (Laemmli)
10% (v/v) 10x running buffer (Laemmli)
0.1% (w/v) SDS (Serva)
in ddH₂O, prepared freshly

Towbin’s blotting buffer, pH 8.3
10% (v/v) 10x running buffer (Laemmli)
20% (v/v) methanol, p.a. (Riedel-de-Haën)
in ddH₂O, prepared freshly

4x stacking gel buffer (Laemmli)
0.5 M Trizma, pH 6.8 (Sigma-Aldrich)
0.4% (w/v) SDS (Serva)
in ddH₂O

10x Dunn’s blotting buffer, pH 9.9
100 mM NaHCO₃ (Roth)
30 mM Na₂CO₃ (Sigma-Aldrich)
in ddH₂O
Material & Methods

1x Dunn’s blotting buffer
10% (v/v) 10x Dunn’s blotting buffer
20% (v/v) methanol, p.a. (Riedel-de-Haën)
0.01% (w/v) SDS (Serva)
in ddH₂O, prepared freshly

PBS-T/TBS-T
0.1% (v/v) Tween 20 (Sigma-Aldrich)
in 1x PBS or 1x TBS

1x TBS, pH 7.4
137 mM NaCl (Roth)
20 mM TRIS-HCl (Sigma-Aldrich)
in ddH₂O, autoclaved

2.22 Antibody Epitope Mapping

2.22.1 Epitope Excision and Extraction

The recombinantly expressed and purified N-terminal domain of cysteine-less human FAT10 (N-hFAT10(C0)) was a kind gift from Nicola Catone (Biotechnology Institute Thurgau at the University of Konstanz). Peptides of N-hFAT10(C0) were obtained by digestion using the endopeptidase Asp-N (sequencing grade), the serine protease Trypsin Gold (mass spectrometry grade) (both from Promega), and the unspecific protease Pronase (from *Streptomyces griseus*) (Roche). The lyophilised enzymes were dissolved and stored according to the manufacturer’s instructions. For in-solution digestion, N-hFAT10(C0) was re-buffered into 50 mM NH₄HCO₃ (pH 7.8) (Sigma-Aldrich) using an Amicon Ultra-4 centrifugal filter unit (Merck) with a MWCO of 3 kDa according to the manufacturer’s instructions. Re-buffered protein was aliquoted, snap-frozen, and stored at -20°C. Samples from epitope extraction and excision experiments were analysed and identified by Nano-LC-ESI-MS/MS and the MaxQuant software (Cox & Mann, 2008) by the staff of the ‘Proteomics Core Facility’ at the University of Konstanz. The number of peptide-spectrum matches (PSM) of the peptides obtained was clustered into specific peptide groups. The peptide groups were defined based on the cleavage profile of Asp-N and trypsin. Due to the unspecific cleavage of Pronase, a modified cleavage profile of trypsin was used to define peptide groups for peptides obtained after Pronase digestion.

For epitope excision, 50-100 µg of N-hFAT10(C0) were bound to 50 µL of packed 4F1-coupled protein A beads in Mobicol ‘classic’ 1 mL columns (MoBiTec) at 4°C overnight under agitation. Protein A beads without 4F1 antibody (empty beads) incubated with N-hFAT10(C0) served as negative control, and 30-50 µg of re-buffered N-hFAT10(C0) was digested in solution and used as positive control. The beads were washed with 10 mL of sterile-filtered 1x PBS and
incubated with 200 µL of diluted enzyme. Samples with Asp-N and trypsin were incubated at 37°C for 3 h, and samples with Pronase were incubated at 40°C for 1 h. Enzymes were diluted in 50 mM NH₄HCO₃ (pH 7.8) (Sigma-Aldrich). Asp-N and trypsin were used at a µg-enzyme to µg-protein ratio of 1:40-200 and 1:20-50, respectively. Pronase was applied to samples at a concentration of 0.5 µg/µL. After digestion, the beads were washed with 30 mL of sterile-filtered 1x PBS and with 30 mL of sterile-filtered ddH₂O. Finally, peptides were eluted twice using 250 µL of 0.1% trifluoroacetic acid (TFA) pH 2.3 (Roth) at RT for 15 min under agitation. The elution fractions were combined, snap-frozen, and stored at -20°C until final analysis.

For epitope extraction, 200 µg of re-buffered N-hFAT10(C0) were digested in a total volume of 750 µL with diluted enzyme as described above. Aliquots of 250 µL of the digested N-hFAT10(C0) were incubated with 50 µL of packed 4F1-coupled protein A beads or empty beads at 4°C overnight under agitation. After binding, the beads were washed and peptides were eluted as described above. A 250 µL aliquot of the digested N-hFAT10(C0) was used as positive control, and directly snap-frozen and stored -20°C.

2.2.2 Peptide Array

A peptide array membrane with synthesised peptides was ordered from INTAVIS Bioanalytical Instruments and stored at -20°C. The cellulose membrane had three times the N-terminal domain of human FAT10 spotted onto it. FAT10 was represented as overlapping 15-mer peptides with a two-amino acid offset. The array membrane was cut into three pieces each with one N-terminal domain of human FAT10 and beginning and end of the peptide spots were marked with a pencil. To rehydrate peptides, the membrane was wetted with 100% methanol (p.a.) (Riedel-de-Haën). After washing three times with 1x TBS, the membrane was blocked with 1x Roti-Block in TBS-T at RT overnight. The blocked membrane was washed once with TBS-T and probed with 4 µg/mL anti-FAT10 antibody (clone 4F1) diluted in 1x Roti-Block in TBS-T at RT for 3.5 h under agitation. The membranes were washed again three times and probed with 0.6 µg/mL HRP-coupled secondary antibody diluted in 1x Roti-Block in TBS-T at RT for 1.5 h under agitation. The probed membrane was washed three times with TBS-T and three times with 1x TBS. Each washing step was performed at RT for 10 min under agitation. Finally, the membrane was developed using chemiluminescence as described above for immunoblots. Signals were quantified using ImageJ FIJI software version 1.51s (Schindelin et al., 2012) and its feature ‘protein array analyzer’.
2.23 Antibody Epitope Peptide Testing

4F1 epitope peptides (Eurogentec) were dissolved in DMSO (Sigma-Aldrich). 3xFLAG peptide (Sigma-Aldrich) was used as random control peptide and dissolved in 1x TBS. Peptide stocks were prepared at 10 µg/µL and stored in aliquots at -20°C.

2.23.1 Peptide Blocking

To block 4F1 antibodies, 2 µg of antibody were incubated with a 1,000-4,000 molar excess of peptide in 500 µL of 1x TBS supplemented with 0.01% (v/v) Tween-20 (Sigma-Aldrich). Antibodies and peptides were incubated at RT for 2 h under rotation. Unblocked antibody control received DMSO instead of peptide. Blocked and unblocked antibodies were used to probe immunoblot membranes and fixed samples for confocal microscopy. Blocked antibodies were diluted to 0.2 µg/mL for immunoblot membranes and 0.5 µg/mL for confocal microscopy and were incubated at 4°C for 2 h to overnight. Samples were further processed and analysed as described above for immunoblotting and confocal microscopy.

2.23.2 Peptide Elution

Cell lysates of endogenously expressed and overexpressed human FAT10 were incubated with 15 µL of packed 4F1- or Isotype-coupled protein A beads as described above. Lysates of mock-transfected cells served as negative control. After the last wash, the beads were incubated with three bead volumes of 500 µg/mL peptide in elution buffer at RT for 4 h under agitation. Eluted protein was transferred into fresh tubes and the beads washed twice with 1 mL of elution buffer. Finally, eluted protein and beads were boiled in 3x SDS sample buffer, and all samples were subjected to SDS-PAGE and immunoblotting as described above. The composition of the elution buffer is specified below.

**Elution buffer**

50 mM TRIS-HCl, pH 8 (Roth)
300 mM NaCl (Roth)
5 mM EDTA (Roth)
0.5% (v/v) Triton X-100 (Fluka)
5 µM MG132 (Sigma-Aldrich)
1x protease inhibitor cocktail (complete Mini EDTA-free) (Roche)
in ddH₂O, prepared freshly
2.24 Statistical Analysis

The GraphPad Prism 6 software version 6.04 (GraphPad Software) was used for statistical analysis. For calculation of the statistical significance, a two-tailed paired t test, a repeated-measures (RM) as well as an ordinary one-way analysis of variance (ANOVA), and a RM two-way ANOVA were applied where applicable. Following the ANOVA, planned multiple comparisons were analysed by the uncorrected Fisher’s least significant difference (LSD) test.
3 Results

3.1 FAT10 in Dendritic Cell Aggresome-Like Induced Structures

FAT10 localises to cytosolic protein aggregates together with its interaction partners HDAC6 and p62 (Kalveram et al., 2008; Aichem et al., 2012). In addition, FAT10 can destabilise viral antigens and directly target them for proteasomal degradation (Ebstein et al., 2012; Schliehe et al., 2012), is encoded in the MHC class I locus (Fan et al., 1995, 1996), and is cytokine-inducible (Liu et al., 1999; Raasi et al., 1999). These properties indicate FAT10 to be involved in antigen presentation (Basler et al., 2015a). Furthermore, FAT10 expression is upregulated upon DC maturation (Lukasiak et al., 2008; Ebstein et al., 2009), which at the same time transiently accumulate ubiquitylated proteins in dendritic cell aggresome-like induced structures (DALIS) that are proposed antigen storage sites (Lelouard et al., 2002; Argüello et al., 2016). These findings prompted the question whether FAT10 also localises to DALIS in maturing DCs and is involved in the accumulation and turnover of DALIS.

3.1.1 FAT10 Localises to DALIS in Human and Murine Dendritic Cells

To test whether human FAT10 localises to DALIS, DCs were generated from CD14+ monocytes isolated from peripheral blood. Maturation of the immature monocyte-derived DCs (MoDCs) was induced on day six with a cytokine cocktail consisting of IL-1β, IL-6, TNF, and IFN-γ (referred to as TNF/IFN-γ or T/I) and with LPS, both in GM-CSF and IL-4 containing medium. Immature and matured MoDCs were stained for ubiquitin or p62 to visualise DALIS and for FAT10 (Fig. 11A-C). The phenotype, proper maturation, and viability of the MoDCs were assessed by surface staining using the pan-DC marker CD11c as well as the maturation markers CD83 and CD86, and by life/dead staining (Fig. 11D & 11E). CD11c was expressed on ~90% of the cells and did not change during maturation. Surface expression of CD83 and CD86 was upregulated during maturation most prominently at 24 h of maturation and did not differ between cytokine and LPS stimulation. The high variances of the median fluorescence intensity were caused by the different flow cytometer used for analysis. The viability was slightly reduced upon maturation (Fig. 11E). Furthermore, upregulation of FAT10 expression during DC maturation, as reported by others (Lukasiak et al., 2008; Ebstein et al., 2009), was confirmed by qPCR (Fig. 11F). Thus, the MoDC preparations showed the proper DC phenotype upon maturation without extensive cell death and upregulation of FAT10 expression.
Figure 11: Human FAT10 localises to DALIS in monocyte-derived DCs. Monocyte-derived DCs (MoDCs) were differentiated from CD14+ peripheral blood monocytes using GM-CSF and IL-4. (A-C): Maturation of MoDCs was induced using IL-1β, IL-6, TNF, and IFN-γ (referred to as TNF/IFN-γ in [A & B] and T/I in [D-F]) and with LPS (C) in GM-CSF and IL-4 containing medium for the indicated time points. After fixation, the cells were stained for FAT10 and ubiquitin (A & C) or p62 (B) prior to microscopic analysis. Inlets highlight regions of interest where FAT10 and ubiquitin or p62 co-localise in DALIS. DAPI was used to stain nuclei. Representative images from two to five different MoDC preparations are shown. Scale bar is 10 µm. (D-E): MoDC preparations were analysed by flow cytometry. (D): Immature (0 h) and mature (16 h, 24 h T/I, & 24 h LPS) MoDCs were stained with antibodies against CD11c, CD83, and CD86 including isotypes controls. (E): Viability of MoDCs was analysed using TO-PRO-3. (F): FAT10 expression levels determined by qPCR were normalised to GAPDH and depicted as Log2-fold change relative to immature MoDCs. Summarised data is shown in (D-F) obtained from two to five different MoDC preparations and is depicted as mean ± SD.
Ubiquitin- as well as p62-positive DALIS that co-localised with endogenous FAT10 were detected at late stages of maturation (16 h and 24 h) after imaging of immature and mature MoDCs. The detection of endogenous FAT10 protein upon maturation was reflected by the expression levels determined by qPCR (Figure 11A-C & 11F). Moreover, FAT10 accumulated in DALIS in cytokine- as well as LPS-induced MoDCs (Figure 11A-C, highlighted by inlets). Interestingly, almost all DALIS identified by ubiquitin and p62 also contained FAT10 suggesting that FAT10 plays a general role in DALIS clearance that takes place at late stages of DC maturation. Unfortunately, only few MoDCs efficiently formed DALIS, therefore, numbers of FAT10- and ubiquitin- or p62-positive DALIS were not quantified. The inefficiency of DALIS formation in human MoDCs has been reported before by Terawaki and colleagues who observed almost no DALIS upon maturation at 8 h post-induction with LPS (Terawaki et al., 2015).

Next, localisation of murine FAT10 (mFAT10) to DALIS in bone marrow-derived dendritic cells (BMDCs) was analysed. Since there was no mFAT10-reactive antibody at hand, two approaches to show localisation of mFAT10 to DALIS in BMDCs were pursued. On the one hand a polyclonal antibody serum in rabbits was generated (see chapter 3.1.5) and on the other hand a lentiviral vector system, which proved more successful, was established. The transduction efficiency was 40-60% of FAT10-deficient (KO BMDC) and -proficient (WT BMDC) BMDCs using vehicle lentiviruses that only express GFP. Moreover, ~20% of KO BMDCs could be transduced with 3xFLAG-mFAT10 encoding lentiviruses, and 10-20% of WT BMDCs could be transduced with 3xFLAG-ubiquitin encoding lentiviruses (Figure 12A). In initial experiments two different lentiviral vector constructs for expression of mFAT10 and mFAT10ΔGG, pCDH-EF1α-mFAT10/mFAT10ΔGG-IRES-copGFP and pCDH-CMV-mFAT10/mFAT10ΔGG-EF1α-copGFP, were tested and no mFAT10 protein could be detected by both constructs (not shown). Nevertheless, the construct with the strong CMV promoter for expression of mFAT10 and mFAT10ΔGG were chosen for further experiments. The failure to detect mFAT10 protein was probably due to its short half-life of approximately one hour (Raasi et al., 2001; Hipp et al., 2005) in combination with a low translation rate, since expression of mFAT10 and ubiquitin from the lentiviruses was detectable by qPCR (Figure 12B, left & right graph). By comparing the level of mFAT10 expression from the lentiviruses in KO BMDCs to endogenous mFAT10 expression in WT BMDCs the level of 3xFLAG-mFAT10 expression was approximately twice as high (Figure 12B, left & middle graph). Since the level of expression from the lentiviruses originates from ~20% of the cells and endogenous mFAT10 from very likely all cells indicate that endogenous mFAT10 is expressed at low levels. Considering the
difficulties in detection of overexpressed 3xFLAG-mFAT10 protein in the transduced cells, protein levels of endogenous mFAT10 protein are likely lower and more difficult to detect given translation from overexpressed and endogenous mFAT10 mRNAs is equal.

Figure 12: Murine FAT10 accumulates in DALIS formed in murine DCs. Bone marrow-derived DCs (BMDCs) were generated from FAT10-deficient (KO BMDC) and -proficient (WT BMDC) mice and transduced with mFAT10- and ubiquitin-encoding lentiviral vectors (C) as well as lentiviral vectors encoding the respective non-conjugatable forms of mFAT10 and ubiquitin (D). Control BMDCs were mock-transduced and transduced with vehicle/GFP only-encoding lentiviral vectors (Supplementary figure 1). Progenitor cells were transduced on day 3, and immature BMDCs stimulated or not on day 10 using 400 U/mL TNF and 200 U/mL IFN-γ. (A): One day prior to stimulation, the transduction efficiency was determined by measuring the percentage of GFP+ positive cells using flow cytometry. (B): Homologous expression of the ubiquitin transgene was determined using IRES-specific primers. For the expression level of the mFAT10 transgene and endogenous mFAT10, mFAT10-specific primers were used. Expression levels were normalised to ACTB and are depicted as log2-fold change relative to immature mock-transduced KO BMDCs (represented as the dotted line in all three graphs). (C & D): Four hours prior to fixation, 5 μM of MG132 was added to enrich the proteins expressed from the lentiviral vectors. Immature (0 h) and mature (24 h) BMDCs were stained for ubiquitin and FLAG-tagged proteins. Nuclei were visualised by DAPI staining and images were taken on a LSM880 confocal microscope at 63x magnification. Inlets are shown to highlight co-localisation of proteins in DALIS. Results of two to four independent experiments are shown as mean ± SD (A & B) or representative images (C & D). Scale bar is 10 μm.
Because of the low 3xFLAG-mFAT10 protein level, all immature and cytokine-matured BMDCs were incubated with the proteasome inhibitor MG132 for four hours prior to processing for confocal microscopy to enrich the proteins expressed from the lentiviral vectors. In doing so, accumulation of mFAT10 together with ubiquitin in DALIS in BMDCs after 24 h of maturation was observed (Figure 12C, 24 h left panel). Proteasome inhibition is known to induce the formation of aggresomes that consist of ubiquitylated as well as FAT10ylated proteins (Johnston et al., 1998; Kalveram et al., 2008). To exclude that proteasome inhibition induced aggresome formation, immature BMDCs were also treated with MG132 for four hours. In immature BMDCs neither 3xFLAG-mFAT10 nor ubiquitin accumulated in protein aggregates indicating that the protein aggregates observed upon maturation were DALIS, which formed during maturation, and not aggresomes, which formed due to the inhibition of the proteasome (Figure 12C, 0 h left panel). Cells were transduced with a lentiviral vector that expressed 3xFLAG-ubiquitin as control confirming that formation of DALIS in transduced cells was unaffected by transduction and overexpression of 3xFLAG-mFAT10 or 3xFLAG-ubiquitin. In turn this suggests that mFAT10 is not involved in the accumulation of DALIS (Figure 12C, right panel). Likewise, DALIS formation did not differ between mock- and vehicle-transduced BMDCs (Supplementary figure 1). As observed for endogenous FAT10 in human MoDCs, almost all DALIS in BMDCs contained 3xFLAG-mFAT10 further highlighting the general targeting of murine and human FAT10 into DALIS (Figure 12C highlighted by inlets).

Since FAT10 can be covalently attached to its substrates, it was of interest to determine whether localisation of mFAT10 into DALIS depends on conjugation. Therefore, BMDCs were transduced with a lentivirus expressing a non-conjugatable form of 3xFLAG-mFAT10 with a mutated diglycine motif at the C-terminus (3xFLAG-mFAT10ΔGG). The 3xFLAG-mFAT10ΔGG accumulated in DALIS in maturing BMDCs indicating that the localisation of mFAT10 to DALIS does not rely on conjugation and its C-terminal diglycine motif (Figure 12D, 24 h left panel). Interestingly, a non-conjugatable form of monomeric ubiquitin with a likewise mutated C-terminal diglycine motif (3xFLAG-ubiquitinΔGG) did not accumulate in DALIS (Figure 12D, 24 h right panel). Again, immature BMDCs were included to exclude unspecific aggregation of the overexpressed proteins upon proteasome inhibition by MG132 (Figure 12D, 0h). This behaviour of FAT10 and ubiquitin was reminiscent of the formation of aggresomes, for which a functional diglycine motif in ubiquitin is required (Ouyang et al., 2012) whereas for FAT10 the diglycine motif is dispensable (Kalveram et al., 2008) (supplementary Figure 2). Since HDAC6 is required for proper formation of aggresomes
(Kawaguchi et al., 2003) and is equally important for transport of FAT10 and ubiquitin to aggresomes (Kalveram et al., 2008) these findings raised the question whether HDAC6 might be involved in DALIS formation. Due to the troublesome detection of mFAT10 even after lentiviral overexpression, the number of mFAT10-positive DALIS were not quantified since that would have yielded data that is not statistically evaluable.

In summary, FAT10 accumulates in DALIS of human and murine DCs through a general mechanism that leads to the deposition of FAT10 in essentially all DALIS that form during DC maturation. Furthermore, like aggresome formation this targeting mechanism does not depend on conjugation or involves the C-terminal diglycine motif of mFAT10 since the non-conjugatable form of mFAT10 also accumulated in DALIS, contrary to ubiquitin that is targeted to DALIS only when conjugated. Thus, it would be tempting to think that HDAC6 might be involved in this process. Expression of 3xFLAG-mFAT10 at early stages of DC maturation neither prevented nor promoted formation of DALIS. Therefore, it can be concluded that FAT10 does not contribute to the formation of DALIS but is targeted to DALIS at later stages of DC maturation when DALIS need to be degraded. It would be interesting to know the proteins with which FAT10 is interacting or is conjugated to within DALIS. Since DALIS consist of potential antigens (Lelouard et al., 2002, 2004) and FAT10 is strongly linked to antigen processing and presentation (Basler et al., 2015a) it seems reasonable to think that FAT10 is conjugated to antigens within DALIS to facilitate their proteasomal degradation.

### 3.1.2 FAT10 Influences the Accumulation of DALIS

Since no quantifiable data could be obtained from the co-localisation studies in human and transduced murine DCs it was investigated whether the lack of mFAT10 has an influence on DALIS using BMDCs generated from \textit{FAT10}-deficient (KO BMDCs) and -proficient (WT BMDCs) mice. At first, the maturation of KO and WT BMDCs induced by LPS and by the pro-inflammatory cytokines TNF and IFN-\gamma was compared. DALIS were visualised by staining against p62 and ubiquitin which both have been reported to localise to DALIS in BMDCs (Lelouard et al., 2002; Terawaki et al., 2015). By microscopic imaging there were no obvious morphological differences evident upon stimulation between KO and WT BMDCs that were matured with LPS (not shown) and cytokines (Figure 13A). Both KO and WT BMDCs formed DALIS that stained positive for ubiquitin and p62 during a time course of 48 hours upon stimulation with LPS and cytokines (Figure 13A, highlighted by inlets) suggesting mFAT10 is not essential for DALIS formation. Next, induction of \textit{mFAT10} and the expression levels of \textit{UBA6} and \textit{USE1} during maturation of BMDCs induced by LPS and cytokines were analysed.
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(Figure 13B). Induction of mFAT10 by cytokines was detectable already after eight hours and then stayed at the same level until 48 hours. Levels of mFAT10 transcripts after stimulation with LPS increased until 24 hours and then declined slightly until 48 hours. However, level of induction by cytokines was generally higher than by LPS. Expression of the mFAT10 conjugation machinery, i.e. USE1 and UBA6, was not considerably up- or downregulated during BMDC maturation. Likewise, there was no differential regulation of UBA6 and USE1 in KO and WT BMDCs. Since expression of UBA6 and USE1 does not change during DC maturation and it has been reported that protein translation decreases during DC maturation (Lelouard et al., 2007; Ceppi et al., 2009; Argüello et al., 2016) suggests that conjugation of FAT10 is not enhanced during BMDC maturation but rather decreases. p62 upregulation in LPS-stimulated macrophages is crucial for formation of DALIS (Fujita et al., 2011), and p62 is induced upon cytokine treatment in HEK293 cells (Aichem et al., 2012). Therefore, p62 expression levels in cytokine- and LPS-induced BMDCs were examined additionally (Figure 13B, bottom graphs). p62 levels were unchanged in cytokine-induced BMDCs contrary to the results of Aichem and colleagues which is probably due to the different cell types used. However, LPS treatment increased p62 mRNA levels slightly throughout BMDC maturation which is in accordance with the report of Fujita and colleagues (Fujita et al., 2011). There was again no difference of p62 expression in KO and WT BMDCs. If the p62 mRNA levels correlate with p62 protein levels in BMDCs, which was not tested here but has been observed in LPS-induced macrophages (Fujita et al., 2011) and in cytokine-induced HEK293 cells (Aichem et al., 2012), this suggest that DALIS formation in BMDCs induced by cytokines and LPS could be different considering the important role of p62 for DALIS formation.

Additionally, surface expression of CD86, MHC class II and CD11c on BMDCs was examined to confirm proper maturation (Figure 13C). CD86 surface levels increased on cytokine- and LPS-induced BMDCs, however, noticeable upregulation by cytokines seemed to be delayed by approximately 24 hours compared to LPS-induction. Notably, a decrease of CD86 surface expression was observed at 48 hours of maturation, which might be due to increased cell death. Upregulation of MHC class II molecules after cytokine induction reflected CD86 upregulation. The differences in maturation and the high variances of CD86 and MHC class II surface expression levels can be attributed to the different behaviour of independent BMDC preparations and the different flow cytometers used for analysis. Despite that, the percentage of CD11c+ positive cell was constantly around 90% in cytokine-induced BMDC preparations. Nonetheless, an upregulation of maturation markers on BMDCs upon LPS- and cytokine-induction could be observed concluding that BMDCs matured properly.
Figure 13: Characterisation and formation of DALIS in cytokine-induced DCs. BMDCs were generated from FAT10-deficient (KO) and -proficient (WT) mice. On day 10, maturation was induced by 1 µg/mL LPS or 400 U/mL TNF and 200 U/mL IFN-γ. (A): Cytokine- and LPS-induced BMDCs were stained with anti-p62 and anti-ubiquitin antibodies or only secondary antibodies only (2nd AB (8h)) and analysed by confocal microscopy. Nuclei were counterstained with DAPI. Representative images of two to seven independent experiments are shown and inlets are included to highlight co-localisation. Scale bar is 10 µm. (B): Expression levels of murine FAT10, UBA6, USE1, and p62 determined by qPCR in cytokine- or LPS-induced BMDCs. Expression levels were normalised to HPRT only (FAT10) or HPRT and ACTB (UBA6, USE1, & p62) and depicted as log2-fold change relative to immature KO or WT BMDCs (indicated as dotted line). Results of two to six experiments (FAT10) and three to four experiments (UBA6, USE1, & p62) are shown. (C): Cell surface staining of CD86, MHC class II, and CD11c on cytokine- and LPS-induced BMDCs at the indicated time points. Summary of two (LPS) and four to five (TNF / IFN-γ) experiments. Results in (B & C) show the mean ± SD.
Several studies reported that DALIS form transiently with peaking between 8 and 12 hours upon LPS stimulation (Lelouard et al., 2002; DeFillipo et al., 2004; Canadien et al., 2005; Herter et al., 2005; Kettern et al., 2011). Therefore, the number of DALIS was quantified from images as shown in Figure 13A in cytokine- and LPS-induced KO and WT BMDCs. In LPS-induced BMDCs DALIS started to accumulate noticeable after eight hours, increased minimal at 24 hours, and decreased at 48 hours (Figure 14A, right graph). Although DALIS formation did not peak at eight hours after LPS stimulation, these results still can comply with the reported kinetic for DALIS formation since the peak of DALIS formation could have been missed if it was between 8 and 24 hours. Interestingly, the kinetic of DALIS formation upon cytokine stimulation differed from the reported kinetic in that the number of DALIS in KO and WT BMDCs increased strongly after 24 hours and then decreased slightly at 48 hours of maturation suggesting a delayed kinetic in these cells (Figure 14A, left graph). A similar delayed kinetic of ALIS formation has been observed in IFN-γ-treated mouse embryonic fibroblast (Nathan et al., 2013) suggesting that the observed delay is likely due to the cytokine stimulation used for inducing maturation and DALIS formation. By comparing DALIS formation in WT and KO BMDCs at the single time points no differences were found when BMDCs were induced with LPS. However, significantly less DALIS were detected in WT BMDCs at 24 hours and a strong tendency at 48 hours of maturation after cytokine induction suggesting that FAT10 participated in clearance of DALIS at these time points. These findings are underscored by the level of FAT10 expression in these samples (Figure 13B, top graphs), where high expression of FAT10 after cytokine induction was found and low levels of FAT10 transcripts when induced by LPS, which might also explain why there was no FAT10-mediated effect on the kinetic of DALIS in LPS-stimulated BMDCs.

The size of the DALIS did not differ between KO and WT BMDCs at any time point during maturation suggesting that FAT10 neither specifically targets proteins within DALIS for degradation, which should reduce the size of the structures, nor enhances the formation of larger aggregates (Figure 14B). Thus, FAT10 leads to the rapid removal of entire DALIS as observed by the decreased number. Experiments were performed to confirm these results by means of immunoblotting of detergent-insoluble fractions that contain DALIS and detergent-soluble fraction as has been reported by Lelouard and colleagues (Lelouard et al., 2002) but DALIS fractions could not be reproducibly isolated despite testing two different published protocols (Lelouard et al., 2002; Rahnefeld et al., 2011) and one group-internal protocol.
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Figure 14: DALIS accumulation in DCs upon cytokine and LPS stimulation. FAT10-deficient (KO) and -proficient (WT) BMDCs were induced with 400 U/mL TNF and 200 U/mL IFN-γ or 1 µg/mL LPS. At the indicated time points KO and WT BMDCs were stained for ubiquitin following the quantification of the number (A) and the area (B) of DALIS of at least 50 cells using ImageJ software. (A): The number of DALIS was related to the number of total analysed cells. The results were depicted in a before-and-after graph. Lines connect individual data points from the same experiments at the indicated time points. Results are shown for three to seven experiments for cytokine induction and two to five experiments for LPS induction. (B): The total area of ubiquitin-positive DALIS was normalised to the number of DALIS and graphed as mean ± SD. The area of DALIS was determined from two to three experiments after cytokine induction of BMDCs and from two to five experiments of LPS-induced BMDCs. Statistical significance was calculated by two-tailed paired T tests (A & B). * p<0.05.

Next, the effect of FAT10 observed at 24 hours of maturation was further examined. First, the FAT10-mediated decrease of DALIS was confirmed to demonstrate that the observed decrease is mediated by FAT10 and not due to an inherent defect in the FAT10-deficient mice. Therefore, the cytokine-induced KO and WT BMDCs were incubated with cycloheximide two hours prior to fixation and staining for confocal microscopy to inhibit protein neosynthesis thereby detecting FAT10-mediated degradation of DALIS similar to cycloheximide chase experiments followed by immunoblotting. Since murine FAT10 has a half-life of approximately one hour (Raasi et al., 2001; Hipp et al., 2005) it should be possible to see FAT10-mediated degradation of DALIS after two hours of cycloheximide treatment. It can be excluded that the cycloheximide treatment influenced the formation of DALIS at 24 hours since protein synthesis
was shown to be only important at early stages of DALIS formation (Lelouard et al., 2002). After determining the number of DALIS per cell in cycloheximide-treated BMDCs (Figure 15A), surprisingly, a significant increase of DALIS in the KO BMDCs compared to untreated KO BMDCs was evident whereas cycloheximide treatment had no effect on the number of DALIS in WT BMDCs. The sudden increase of ubiquitylated proteins in cycloheximide-treated KO BMDCs was probably caused by increased targeting of long-lived proteins into the aggregates. A similar effect of cycloheximide on ALIS has been observed in mouse embryonic fibroblasts under starvation conditions (Szeto et al., 2006) and in INS1 832/13 β-cells under normal and high glucose conditions (Kaniuk et al., 2007). Since there was no accumulation in WT BMDCs it can be concluded that FAT10 counteracted the increased flux of long-lived proteins into DALIS through proteasomal degradation, which has been shown before for FAT10-fusion proteins (Ebstein et al., 2012; Schliehe et al., 2012) as well as FAT10 substrates (Aichem et al., 2010; Bialas et al., 2015).

In addition, as possible contribution of endogenous FAT10 to the formation of DALIS and to the targeting of DALIS into the autophagolysosomal pathway was investigated. Therefore, autophagy was inhibited using wortmannin, which inhibits the initiation of autophagy
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(Figure 15B), and the proteasome was inhibited using MG132 (Figure 15D). The number of DALIS in KO and WT BMDCs increased equally upon inhibition of autophagy compared to untreated cells (Figure 15B) suggesting that FAT10 does not substantially target DALIS into the autophagolysosomal degradation pathway and that autophagy inhibition does not enhance FAT10-mediated degradation of DALIS by the proteasome. The strong increase of DALIS, however, indicated bulk turnover of DALIS by autophagy in BMDCs induced by cytokines, which is contrary to the proposed degradation mechanisms after LPS stimulation, which suggests that reduced autophagy leads to enhanced proteasomal degradation of DALIS (Argüello et al., 2016). There was no FAT10-specific accumulation of DALIS when the proteasome was inhibited (Figure 15C) confirming that endogenous FAT10 does not contribute to the formation of DALIS, which was also observed after homologous overexpression of FLAG-mFAT10 (Figure 12C).

Taken together, FAT10 contributes to the clearance of DALIS upon cytokine stimulation. Furthermore, FAT10 does not contribute to the formation of DALIS and very likely does not feed DALIS or proteins from DALIS into the autophagolysosomal degradation pathway.

3.1.3 HDAC6 Is Dispensable for the Formation, Maintenance, and Degradation of DALIS

FAT10 and ubiquitin in BMDCs are targeted to DALIS in a manner that is reminiscent of the targeting of both proteins to aggresomes. This raised the question whether HDAC6 might be involved in this process similar to aggresomes. To address this question, the formation of DALIS was monitored in HDAC6-deficient (HDAC6 KO) and -proficient (WT) BMDCs (Figure 16). HDAC6 KO and WT BMDCs were induced with cytokines and accumulation of DALIS was equal in HDAC6 KO and WT BMDCs (Figure 16A). Notably, DALIS accumulation was again delayed as observed before in FAT10-deficient and -proficient BMDCs (Figure 14). Likewise, the average size of DALIS was unaffected by HDAC6 deficiency (Figure 16B). These findings indicate that HDAC6 is not involved in DALIS formation, contrary to aggresomes where HDAC6 is necessary for proper formation (Kawaguchi et al., 2003). Additionally, HDAC6 is highly expressed at a constant level in maturing BMDCs (Figure 16C). The proper maturation and phenotype of the BMDC preparations were assessed and equal upregulation of FAT10 expression upon maturation was confirmed in HDAC6 KO and WT BMDCs (Figure 16D). Furthermore, HDAC6 KO and WT BMDCs were equally viable (Figure 16E) and positive for CD11c as well as expressed similar levels of CD86 and MHC class II on their surface (Figure 16F). The high variations observed for the CD86 and MHC
Results

class II surface levels are due to differences in the BMDCs preparations. There is no explanation so far for the downregulation of CD86 at the 48 hours of maturation. Despite that, it can be concluded that HDAC6 KO and WT BMDCs matured uniformly and showed the same phenotype confirming the validity of the results.

Figure 16: Maturation and DALIS formation of HDAC6-deficient and -proficient DCs. HDAC6-deficient (HDAC6 KO) and -proficient (WT) BMDCs were generated and maturation was induced using 400 U/mL TNF and 200 U/mL IFN-γ. At the indicated time points BMDCs were analysed by confocal microscopy (A & B), qPCR (C & D), and flow cytometry (E & F). (A & B): BMDCs were stained for ubiquitin and DALIS number as well as the area of DALIS was determined using ImageJ software. The average number of DALIS per cell is shown in (A) and depicted using a before-and-after graph. Lines connect individual data points from the same experiments at the indicated time points. The total area of ubiquitin-positive DALIS was normalised to the number of DALIS and is shown in (B). (C & D): Expression level of HDAC6 and FAT10 were determined by qPCR and normalised to HPRT and ACTB. ACTB was excluded from samples at 48 h of maturation due to unstable expression. HDAC6 expression is depicted relative to immature HDAC6 KO BMDCs to see expression level of HDAC6 in immature WT BMDCs. FAT10 expression is shown relative to immature HDAC6 KO or WT BMDCs to see upregulation upon maturation. (E): The viability of BMDCs was assessed at indicated time points using propidium iodide. (F): The percentage of CD11c+ BMDCs and upregulation of CD86 and MHC class II surface expression on BMDCs upon maturation by cytokines
was determined. Results are summarised from three independent experiments and depicted as mean ± SD (except in [A]). Data presented in (A & B) was analysed statistically using a two-tailed paired t tests comparing HDAC6 KO and WT at the individual time points.

In summary, these results indicate that HDAC6 is not involved in the formation, maintenance, and clearance of DALIS. However, some open questions remain, which were not addressed. It is known that DALIS are motile and move along microtubules in the cytoplasm (Lelouard et al., 2004; Canadien et al., 2005), which is the same mechanism that is used by HDAC6 for transport of ubiquitylated and FAT10ylated proteins or protein aggregates to the MTOC, where aggresomes are formed (Kopito, 2000; Kalveram et al., 2008). Thus, it could be possible that HDAC6 is necessary for the movement of DALIS along microtubules. Moreover, HDAC6 could be required to target FAT10 to DALIS because targeting of FAT10 to DALIS and aggresomes is equally independent of FAT10’s diglycine motif and conjugation suggesting a similar mechanism.

3.1.4 Generation and Characterisation of a Polyclonal Rabbit Antibody Specific for Murine FAT10

Up to date there is no sensitive antibody available that recognises endogenous murine FAT10 (mFAT10), despite big efforts that had already been undertaken in the Groettrup group. Due to this, rabbits were immunised with purified recombinant mFAT10 to generate a polyclonal antibody serum. mFAT10 was expressed in E. coli with an N-terminal maltose binding protein (MBP) tag for stabilisation and a C-terminal 6His tag for purification. After purification, the amount of mFAT10 was quantified by densitometry of a Coomassie-stained SDS-PAGE using a BSA standard (Figure 17A). The total amount of purified mFAT10 was used for immunisation of three rabbits K1146, K1152, and K1154. One week after the second and third booster immunisation, test serum was drawn from the rabbits to test for the presence of mFAT10-reactive antibodies. By probing immunoblots of overexpressed mFAT10 with the test sera from rabbit K1154, the presence of mFAT10-reactive antibodies was confirmed in the first (Figure 17B) as well as in the second (Figure 17C) test serum, which were able to detect overexpressed mFAT10. An additional immunoblot was probed with pre-immune serum (0-Serum) and lysates from mock- and vehicle-transfected cells, which were negative for mFAT10, were included confirming the specific detection of mFAT10 by the test sera in the mFAT10-transfected sample. Staining with tag-specific antibodies (anti-FLAG and anti-HA) confirmed presence of overexpressed mFAT10. Two differently tagged expression constructs were used to exclude reactivity of the sera towards the epitope tags. Sera from rabbits K1146
and K1152 also contained specific mFAT10-reactive antibodies (not shown) but serum from rabbit K1154 gave the strongest signals. Therefore, this polyclonal antibody serum was used for further testing. An aliquot of the polyclonal antibody serum K1154 was cross-absorbed against crude *E. coli* lysate in which the empty vector construct containing MBP-6His was expressed (not shown) to reduce unspecific background signals that were observed with the test sera.

**Figure 17:** Generation of a polyclonal rabbit serum for the detection of murine FAT10. (A): Coomassie-stained SDS-PAGE of 1% of one purification of murine FAT10 (mFAT10) with an N-terminal MBP tag and a C-terminal 6His tag used for immunisation of rabbits. A BSA standard was included to quantify the amount of mFAT10 by densitometry using ImageJ software. Asterisk, co-purified contaminating protein; MW, molecular weight marker. (B & C): Immunoblots to detect overexpressed mFAT10 using the first (B) and second (C) test serum obtained after the second and third immunisation of the rabbits, respectively, including the pre-immune serum (0-Serum) as control. Two differently-tagged mFAT10 constructs (3xFLAG-mFAT10 in [B] or HA-mFAT10 in [C]) overexpressed in transfected HEK293T cells including empty vector controls (vehicle) and mock-transfected cells (only in [B]).
Anti-FLAG and anti-HA antibodies were used to test successful overexpression of mFAT10. GAPDH was used as loading control.

The cross-absorbed polyclonal antibody serum K1154 was titrated for detection by immunoblotting and a 1:1,000 dilution resulted in the best signal-to-noise ratio and was used for immunodetection from then on (not shown). Next, it was tested whether the polyclonal serum K1154 is able to immunoprecipitate overexpressed as well as endogenous mFAT10 (Figure 18). The serum K1154 was able to immunoprecipitate overexpressed mFAT10 (Figure 18A, lane 1). In first experiments using ~5x10⁷ cytokine-induced BMDCs, endogenous mFAT10 could not be detected (not shown). Therefore, the cell number was increased by using ~1.5x10⁸ splenocytes or ~1.7x10⁸ thymocytes from five FAT10-deficient and -proficient mice. The isolated splenocytes and thymocytes were induced with TNF and IFN-γ to boost mFAT10 expression and additionally incubated with the proteasome inhibitor MG132 for five hours to enrich mFAT10 protein before lysis (Figure 18A, lanes 2-5). However, endogenous mFAT10 could be detected neither in total cell lysates (load samples) nor after enrichment by immunoprecipitation (Figure 18A, lane 2 & 3), although endogenous mFAT10 expression was confirmed by qPCR (Figure 18B) and loading on immunoblots was similar between the samples as judged from γ-tubulin detection in total cell lysates. In addition to detection by immunoblotting, the polyclonal serum K1154 was used for the detection of endogenous mFAT10 by immunofluorescences (not shown). The antibody serum K1154 successfully detected overexpressed mFAT10 but failed to detect endogenous mFAT10 in induced BMDCs as well as in isolated medullary TECs (not shown), although both cell types were shown to express high levels of mFAT10 by qPCR (Buerger et al., 2015; this study).

In conclusion, the generation of mFAT10-reactive polyclonal antibody was successful. The polyclonal antibodies present in sera from three rabbits can detect overexpressed mFAT10 by immunoblotting. Furthermore, the serum K1154 can be used for detection of mFAT10 by immunofluorescence, as well as for enrichment by immunoprecipitation. Nonetheless, detection of endogenous mFAT10 still failed despite measurable induction by qPCR. This indicates that either the affinity of the mFAT10-reactive antibodies that are present in the serum K1154 could be too low or the actual level of mFAT10 protein in these cells is below the level of detection or both possibilities apply. Although the anti-mFAT10 serum K1154 is not able to detect endogenous mFAT10 it still can be utilised for overexpression studies on mFAT10 as well as in combination with a more sensitive monoclonal or polyclonal mFAT10-reactive antibody that might be generated in the future.
Results

Figure 18: Detection of endogenous murine FAT10 using a polyclonal rabbit serum. (A): Thymocytes and splenocytes from five FAT10-deficient (Fat10 KO) and -proficient (WT) mice were isolated and the cells were induced using 200 U/mL TNF and 100 U/mL IFN-γ for 24 h. Five hours prior to further processing of the cells, 5 µM MG132 was added to prevent proteasomal degradation and accumulate mFAT10. Additionally, 3xFLAG-mFAT10 was overexpressed in B8 cells for 24 h and used as positive control. The cells were lysed and subjected to immunoprecipitation (IP) using K1154-coupled antibody beads following immunodetection (WB) of the IP and load fractions using the polyclonal rabbit serum K1154. γ-Tubulin was used as loading control. Asterisk, antibody heavy and light chain. (B): Expression level of FAT10 in cells used in (A) determined by qPCR. FAT10 expression in WT and Fat10 KO splenocytes and thymocytes was normalised to HPRT, ACTB, and RPL13A. The graph depicts the log2-fold change of expression relative to FAT10 KO cells. Results in (A & B) show one representative experiment of three similar independent experiments.

3.2 Influence of FAT10 on Dendritic Cell Migration

In recent years evidence has accumulated that FAT10 promotes migration and invasion of various cancer types, such as colorectal cancer (Gao et al., 2014), hepatocellular carcinoma (Liu et al., 2014a; Yuan et al., 2014a), triple-negative breast cancer (Han et al., 2015), glioma (Dai et al., 2016), osteosarcoma (Ma et al., 2016), and non-small-cell lung cancer (Xue et al., 2016), and of a hTERT-immortalised hepatocyte cell line (Gao et al., 2014). Since DC migration is initiated upon maturation concurrently with FAT10 expression, the question arose whether FAT10 regulates DC migration. DC migration relies largely on the upregulation of the chemokine receptor CCR7 and its two known ligands CCL19 and CCL21 ( Förster et al., 2008;
Comerford et al., 2013). Interestingly, the CCR7-CCL19/CCL21 axis is also exploited by cancer cells for the formation of metastasis (Legler et al., 2014). Therefore, the effect of FAT10 on the CCR7-dependent migration of human and murine dendritic cells towards the chemokines CCL19 and CCL21 was examined.

3.2.1 Impact of FAT10 Overexpression on Migration of Human Dendritic Cells

The influence of FAT10 overexpression on the migration of dendritic cells was investigated using human monocyte-derived dendritic cells (MoDCs) prepared from peripheral blood mononuclear cells (PBMCs). To overexpress human FAT10 in MoDCs a lentiviral vector system was used. In this system human 3xFLAG-FAT10 and a GFP reporter are translated from one transcript using an IRES sequence for the GFP reporter. Expression of the 3xFLAG-FAT10-IRES-GFP as well as the vehicle control expressing only GFP were under control of the EF1α promoter. The transduction efficiency was determined prior to performing Transwell migration assays. On average 80% and 50% GFP+ cells were obtained after transduction with vehicle and 3xFLAG-FAT10 encoding lentiviruses, respectively (Figure 19A). Maturation of MoDCs was induced by LPS, which induces endogenous FAT10 expression in MoDCs as well (Lukasiak et al., 2008; Ebstein et al., 2009). An additional mock-transduced sample was induced with LPS and PGE2, which greatly enhances migration of MoDCs (Scandella et al., 2002, 2004; Legler et al., 2006), and was used as a positive control for the migration assays. PGE2 was omitted from the remaining samples to prevent that the enhancing effect of PGE2 masks a potential effect of FAT10 on MoDC migration. qPCR analysis was performed to assess the expression of 3xFLAG-FAT10 from the lentiviral vector and to determine how much FAT10 was overexpressed in the MoDCs after transduction compared to mock- and vehicle-transduced cells (Figure 19B). The total level of FAT10 expression was detected using FAT10-specific primers (termed endogenous FAT10). The expression level of FAT10 in 3xFLAG-FAT10-transduced cells was three times higher compared to mock-transduced cells and was twice as high compared to vehicle-transduced and positive control cells (mock+PGE2) (Figure 19B, left graph). Unfortunately, the level of induction of endogenous FAT10 could not be determined since uninduced immature MoDCs lacked for analysis. Additionally, the homologous expression of 3xFLAG-FAT10 from the lentiviral vector was determined using IRES-specific primers relative to the mock-transduced cells. 3xFLAG-FAT10 expression was highly upregulated in FAT10-transduced cells and basically absent from the positive control (mock+PGE2) and vehicle-transduced cells (Figure 19B, right graph). The viability of the mature MoDCs decreased only marginally upon transduction indicating that transduction and
the overexpression of FAT10 had only little effect on MoDC viability (Figure 19C). Likewise, the percentage of CCR7$^+$ cells as well as the level of CCR7 surface expression were similar between the differently-transduced mature MoDCs (Figure 19D).

Taken together, human MoDCs were transduced successfully and transduction as well as overexpression of 3xFLAG-FAT10 has only a minor effect on the viability of MoDCs. Likewise, the upregulation as well as the surface levels of CCR7 were unaffected by transduction and 3xFLAG-FAT10 overexpression.

Figure 19: Characterisation of transduced human DCs. Monocyte-derived DCs (MoDCs) were generated from PBMCs. MoDCs were transduced with lentiviruses encoding only GFP (vehicle), 3xFLAG-FAT10-IRESGFP (FAT10), and mock-transduced. Immature MoDCs were induced for two days with 1 µg/mL LPS alone and in combination with 1 µg/mL PGE2 as positive control for subsequent migration assays. (A): Prior to migration assays, the transduction efficiency of the mature MoDCs was determined by flow cytometry and is given as the percentage GFP$^+$ cells. (B): Endogenous FAT10 expression and homologous FAT10 expression from the lentiviral vectors were determined by qPCR. IRES-specific primers were used to distinguish homologous from endogenous FAT10 expression. Expression levels were normalised to the reference gene SDHA. Normalised expression values are represented as the log2-fold change relative to the FAT10 expression in mock-transduced and LPS-induced MoDCs (shown as dotted line) due to the lack of uninduced samples. (C): The viability of mature MoDCs was evaluated by SytoxBlue exclusion staining and flow cytometric analysis before migration assays. (D): MoDCs were stained with anti-CCR7 antibodies and analysed by flow cytometry to determine the percentage of CCR7$^+$ cells and the levels of surface CCR7 expression indicated by the median fluorescence intensity. Results are shown as mean ± SD for two (B) and four (A, C, & D) independent experiments.
CCR7-dependent migration towards CCL19 and CCL21 was determined using Transwell migration assays followed by flow cytometric enumeration of migrated cells. Specific migration was determined as the number of cells that migrated towards the chemokines minus the number of cells that migrated randomly in absence of chemokines normalised to the number of total input cells. The migration of MoDCs towards CCL19 and CCL21 was similar between the different samples (Figure 20A & 20B). As expected MoDCs induced with LPS and PGE2 showed the highest specific migration of the total cells (Figure 20A). In absence of PGE2 migration was diminished to the same extent and approximately by 5-fold in mock- and vehicle-transduced MoDCs. MoDCs transduced with FAT10 showed a further reduction of specific migration, however, this reduction was not significant compared to the mock- and vehicle-transduced cells (Figure 20A). Specific analysis of the migration of the transduced cells, i.e. the migration of GFP$^+$ cells only, further highlighted a FAT10-mediated decrease of migration (Figure 20B). Interestingly, a negative influence of FAT10 overexpression on migration is contrary to the published results showing FAT10 overexpression increases the migratory ability of cells. However, these were cancer cells or cell lines and a hTERT-immortalised hepatocyte cell line (Gao et al., 2014; Liu et al., 2014a; Yuan et al., 2014a; Han et al., 2015; Dai et al., 2016; Ma et al., 2016; Xue et al., 2016). Yet, this decrease in migration was not significant probably due to the background expression of FAT10 and the high donor-to-donor variation that was encountered. Unfortunately, the viability of the migrated and the input cells at the end of the migration assays was reduced most prominently in transduced MoDCs, indicating that transduction rendered the cells more susceptible to cell death, which likely was caused by unfavourable conditions during sample preparation or analysis (Figure 20C).

In summary, an insignificant but FAT10-mediated reduction of CCR7-dependent migration of MoDCs could be observed, which was very likely diminished by background expression of endogenous *FAT10*. These results could also be falsified by the high cell death after migration assays. Due to these methodological shortcomings and without further investigation it cannot be concluded whether FAT10 truly influences CCR7-dependent migration of human DCs. However, if FAT10 influences migration of human DCs, it most likely regulates downstream signalling of CCR7 since the upregulation and the amount of CCR7 on the surface of MoDCs was unaffected by FAT10 overexpression. An RNAi approach to test the effect of *FAT10* deficiency on human DC migration using lentiviral vectors encoding a *FAT10*-specific short hairpin RNA, which was published by Ross and colleagues (Ross et al., 2006), did not work in this systems (not shown).
Results

Figure 20: Influence of FAT10 overexpression on migration of human DCs. Transduced and mock-transduced MoDCs were matured for two days with 1 μg/mL LPS alone and together with 1 μg/mL PGE2 as positive control. Mature MoDCs were used for Transwell migration assays. The cells were placed into the upper chamber of a Transwell inlet and migration was induced by adding 500 ng/mL CCL19 or CCL21 to the lower compartment (CCL19 & CCL21). Control cells migrated towards medium without chemokine to account for random migration (Random). Samples without Transwell inlet were used to determine the number of input cells (Input). After migration, the cells were harvested and counted on a flow cytometer using fluorescent counting beads. Prior to counting cells were stained with SytoxBlue to exclude dead cells from counting. Living cells from the total cell population were measured and a defined number of beads were counted. From the known volumes of the cell suspension and the beads together with the counted number of cells and beads as well as the concentration of the bead stock suspension, the total number of cells in the sample was calculated. (A): The number of cells was then used to determine the specific migration of the total cells. (B): Additionally, the number of GFP+ cells within the living cells was determined for GFP/vehicle- and FAT10-transduced cells and specific migration of the transduced cells only was calculated. (C): The SytoxBlue staining was also used to assess the viability of the migrated cells. The graphs show the mean ± SD of four experiments. For statistical analysis in (A) a RM one-way ANOVA following the uncorrected Fisher’s LSD test was applied to determine p values for the comparisons of the LPS-induced mock-, vehicle-, and FAT10-transduced samples. p values in (B) were determined using a two-tailed paired t test.
3.2.2 Influence of FAT10 Deficiency on Migration of Murine Dendritic Cells

The effect of FAT10 deficiency on DC migration was assessed in a murine system. For this purpose, bone marrow-derived DCs (BMDCs) from FAT10-deficient (referred to as knockout or KO) and -proficient (referred to as wild type or WT) mice were generated. On day 10 of culture, maturation of BMDCs was induced with LPS and the proinflammatory cytokines TNF and IFN-γ. After 24 h and 48 h of maturation the BMDCs were used for Transwell migration assays. The upregulation of FAT10 expression was tested and surface expression of the co-stimulatory molecule CD86 was measured to confirm BMDC maturation (Figure 21A). FAT10 expression was upregulated in WT BMDCs when compared to immature WT BMDCs at 24 h of maturation and increased further at 48 h of maturation. In comparison, KO BMDCs neither expressed nor upregulated FAT10. CD86 surface expression, which is upregulated during BMDC maturation, was similar between KO and WT BMDCs. CD86 was almost absent on immature BMDCs but gradually increased until 48 h of maturation (Figure 21A). The viability of immature and 24 h-mature BMDCs was equally high (~90%) and decreased after 48 h of maturation (~40%) similar in KO and WT BMDCs (Figure 21B). Approximately 90% of the maturing KO and WT BMDCs were CD11c⁺-positive (Figure 21C, left graph) and reached similar surface levels of CD11c (Figure 21C, right graph).

Finally, CCR7 expression was analysed on KO and WT BMDCs. The number of CCR7⁺-positive cells increased to the same extent in KO and WT BMDCs gradually from ~40% of immature BMDCs to ~85% of 48 h-mature BMDCs, although background binding of the isotype control to approximately 10-15% of the BMDCs was observed (Figure 21D, left graph). The amount of CCR7 molecules on the surface increased substantially upon maturation in KO and WT BMDCs. However, KO BMDCs had more CCR7 molecules on the surface compared to WT BMDCs (Figure 21D, right graph). Taken together, BMDC preparations from KO and WT mice behaved similar upon maturation. Except, KO BMDCs expressed slightly higher levels of CCR7 on the surface than WT BMDCs suggesting that KO BMDCs might be able to migrate better than WT BMDCs.
Figure 21: Analysis of the maturation of murine DCs. BMDCs were generated from FAT10-deficient (KO) and -proficient (WT) mice. On day 10 of culture, BMDCs were stimulated with 5 µg/mL LPS, 400 U/mL TNF, and 200 U/mL IFN-γ for 24h and 48h or left unstimulated. Before cells were used for migration assays, control samples were analysed to evaluate the maturation of the BMDCs (A): To assess maturation of BMDCs, FAT10 expression was measured by qPCR and CD86 surface expression was determined by flow cytometry. FAT10 expression levels were normalised to ACTB and HPRT. Log2-fold changes of FAT10 expression were calculated relative to the immature KO and WT BMDCs (depicted as dotted line). (B): The viability of immature (0 h) and mature (24 h & 48 h) BMDCs was assessed by SytoxBlue exclusion staining on a flow cytometer. (C & D): Surface staining of CD11c (C) and CCR7 (D) on immature (0 h) and mature (24 h & 48 h) BMDCs determined by flow cytometry. Left graphs show the percentage of positive cells and right graphs the level of surface marker given as the median fluorescence intensity. Isotype controls were included in the CCR7 staining due to the high concentration of anti-CCR7 antibodies necessary for staining. Graphs show the mean ± SD of three independent experiments.

Then, the CCR7-mediated migration of KO and WT BMDCs towards the chemokines CCL19 and CCL21 alone as well as in combination was analysed. As described above for human MoDCs, samples to determine the input number of cells (Input) and to account for random migration (Random) were included (Figure 22). The migrated cells were analysed by flow cytometry and the number of cells determined using counting beads as described above. The viability of input and migrated BMDCs was determined by SytoxBlue staining. Total migration as well as specific migration of KO and WT BMDCs was similar at 24 h and 48 h of maturation.
Results

suggesting that *FAT10* deficiency had no effect on the migration of murine BMDCs (Figure 22A). The viability of the migrated KO and WT BMDCs was unaffected and although the input samples showed decreased viability almost no loss of viability was observed for the migrated BMDCs (Figure 22B).

Taken together, migration of *FAT10*-deficient BMDCs compared to *FAT10*-proficient BMDCs did not differ. However, it cannot be ruled out that the slightly increased level of CCR7 surface expression on *FAT10*-deficient BMDCs compared to *FAT10*-proficient BMDCs might have influenced the outcome of the migration assay and that therefore the migration of *FAT10*-proficient BMDCs was underestimated.

![Figure 22: Migration of *FAT10*-deficient and -proficient murine DCs.](image)

BMDCs were matured with 5 μg/mL LPS, 400 U/mL TNF, and 200 U/mL IFN-γ. After 24 h and 48 h, mature BMDCs were used for Transwell migration assays. For this purpose, an equal number of cells was added into the upper Transwell inlet and migration was triggered by adding 500 ng/mL CCL19, 500 ng/mL CCL21, and both together to the lower compartment (CCL19, CCL21, & CCL19+CCL21). To control for random migration chemokines were omitted in the medium of the lower compartment (Random). Input samples were placed into wells without Transwell (Input). Migrated cells were harvested and counted on a flow cytometer using counting beads. Dead cells were excluded from counting by SytoxBlue staining. For counting, living cells of the total cell population were measured and a defined number of beads was counted. From the known volumes of the cell suspension and the beads together with the counted number of cells and beads as well as the concentration of the bead stock suspension, the total number of cells in the sample could be calculated. (A): The number of cells was used to determine the total (left graphs) and the specific (right graphs) migration. (B): In addition, the SytoxBlue staining was used to check the viability of the migrated cells. The graphs show the mean ± SD of three experiments. The results from the specific migration were tested for statistical significance using a RM two-way ANOVA following the uncorrected Fisher’s LSD test comparing the specific migration of KO and WT samples towards CCL19, CCL21, or both chemokines.
3.3 Expression Profile of \textit{FAT10} in Immune Cell Subtypes

To determine in which of the main leukocyte populations \textit{FAT10} is expressed or inducible by different stimuli the different leukocyte subsets were isolated from human peripheral blood or murine spleen via magnetic sorting (MACS) (Figure 9, only human cells) or in case of regulatory T cells by fluorescence-activated cell sorting (FACS) (Figure 10). The purity of the cell preparations was assessed by flow cytometric re-analysis and varied between 75.7\% and 100\% (Tables 13 & 14). The purified cells were left untreated, stimulated overnight with IFN-\(\gamma\) and TNF, or with LPS. Finally, human and murine \textit{FAT10} expression was assessed by qPCR analysis (Figure 23 & 24) and the human \textit{FAT10} protein level was analysed in bulk leukocytes by immunoblotting after immunoprecipitation (Figure 25).

\begin{table}[h]
\centering
\caption{Purity of the isolated human immune cell subsets.}
\begin{tabular}{|c|c|c|}
\hline
\textbf{Cell Type} & \textbf{Marker} & \textbf{Mean \pm SD} \\
\hline
CD4\(^+\) T cells & CD4, CD3 & 90.2 \pm 8.2 \\
\hline
CD8\(^+\) T cells & CD8, CD3 & 84.8 \pm 4.6 \\
\hline
NK & NKT cells & CD56, CD3 & 82.6 \pm 2.9 \\
\hline
Monocytes & CD14 & 87.3 \pm 10.1 \\
\hline
Granulocytes & CD15 & 90.1 \pm 4.6 \\
\hline
B cells & CD19 & 89.5 \pm 6.7 \\
\hline
Dendritic cells & HLA-DR, CD303 & 75.7 \pm 6.0 \\
\hline
Regulatory T cells & CD25, FOXP3 & 92.6 \pm 2.3 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Purity of the purified murine leukocytes.}
\begin{tabular}{|c|c|c|}
\hline
\textbf{Cell Type} & \textbf{Marker} & \textbf{Mean \pm SD} \\
\hline
CD4\(^+\) T cells & CD4 & 86.2 \pm 0.9 \\
\hline
CD8\(^+\) T cells & CD8 & 93.3 \pm 0.9 \\
\hline
Macrophages & Monocytes & CD11b & 92.8 \pm 1.3 \\
\hline
Granulocytes & Ly-6G & 93.1 \pm 1.0 \\
\hline
B cells & CD19 & 87.6 \pm 1.4 \\
\hline
Dendritic cells & CD11c & 96.3 \pm 1.2 \\
\hline
Regulatory T cells & FOXP3-GFP & 100.0 \pm 0.0 \\
\hline
\end{tabular}
\end{table}
3.3.1 Expression Profile of FAT10 in Human Leukocyte Population

In cells of the adaptive immune system, namely CD4\(^+\) T cells, B cells, and Tregs, FAT10 mRNA was expressed neither in the untreated state nor after induction with LPS (Figure 23A, 23C, & 23D). Interestingly, CD8\(^+\) T cells expressed FAT10 already when not treated, and induction with LPS increased expression of FAT10 slightly but not significantly (Figure 23B). Induction with the pro-inflammatory cytokines IFN-\(\gamma\) and TNF significantly increased FAT10 mRNA levels in CD4\(^+\) and CD8\(^+\) T cells, Tregs, and B cells (Figure 23A-D). The observed expression levels, however, were similar between the cell types ranging from 4.1 to 6.2 log2-fold change meaning that the increase of FAT10 expression under inflammatory conditions was similar between these cell types regardless of the basal expression.

Next, expression of FAT10 in cells of the innate immune system was analysed (Figure 23E-F). Monocytes and granulocytes showed no FAT10 expression when untreated and stimulated with LPS (Figure 23E & 23G). This is in line with a former report showing no expression of FAT10 in unstimulated monocytes isolated freshly from PBMCs and granulocytes derived from CD34\(^+\) haematopoietic progenitors (Bates et al., 1997). Of all human cell types examined, DCs expressed the highest levels of FAT10 already when untreated, which did not increase further upon induction with LPS (Figure 23F). So far, FAT10 expression in DCs was reported for in vitro-generated, mature DCs from CD34\(^+\) haematopoietic progenitors and CD14\(^+\) monocytes which reflected CD1a\(^+\) Langerhans-like DCs and inflammatory DCs, respectively (Bates et al., 1997; Lukasiak et al., 2008; Ebstein et al., 2009). Yet, the dendritic cell population investigated here consisted of plasmacytoid and myeloid DCs, which shows that, despite the lack of inflammatory signals and maturation, DCs have a high basal expression level of FAT10 in vivo. Remarkably, untreated natural killer and natural killer T (NK&NKT) cells displayed high FAT10 expression, which was not enhanced upon LPS stimulation (Figure 23H). This basal expression increased marginally when the cells were treated with the pro-inflammatory cytokines. FAT10 expression was most stable in NK&NKT cells irrespective of treatment ranging from 6.8 (untreated) to 7.8 log2-fold change (cytokine-treated). Pro-inflammatory cytokines up-regulated FAT10 mRNA in monocytes and granulocytes significantly (Figure 23E & 23G), whereas in DCs it increased only marginally (Figure 23F). Here, monocytes showed the most striking increase in FAT10 expression (10.6 log2-fold change), which reached levels similar to DCs (10.9 log2-fold change) (Figure 23E & 23F) and were the highest expression levels observed upon cytokine induction. In cytokine-induced granulocytes FAT10 expression rose to levels (6.9 log2-fold change) similar to CD8\(^+\) T cells (6.2 log2-fold change) (Figure 23B & 23G).
Overall, these results suggest that human FAT10 is not only expressed ubiquitously under inflammatory conditions but also cell type-specific in a non-inflammatory environment. Furthermore, CD8\(^+\) T cells, DCs, NK&NKT cells expressed FAT10 in an untreated state, which only increased insignificantly upon IFN-\(\gamma\) and TNF treatment. This suggests that FAT10 performs functions specifically required in these circulating immune cell types in their naïve, immature, or memory state.

**Figure 23: FAT10 mRNA expression in different human leukocyte populations.** The single immune cell subsets were isolated by MACS (A, B, & D-H) or FACS (C) from the PBMC or PMNC fraction of peripheral blood after density centrifugation. The isolated cells were left untreated, treated with 1 µg/mL LPS, or 400 U/mL TNF and 200 U/mL IFN-\(\gamma\) for 24 h. Subsequently, FAT10 mRNA expression levels were determined by qPCR. Expression levels in each sample were normalised to RPL13a, SDHA, and TBP. TBP was excluded as a reference gene for granulocytes, dendritic cells (PanDC), and regulatory T cells (Treg) due to its unstable expression. Finally, the log2-fold change of expression was calculated relative to unstimulated HEK293T cells that were used as negative control (depicted as dotted line). Graphs display the mean ± SD of three to six independent experiments (n=3 [Treg]; n=4 [CD14\(^+\), CD15\(^+\), & PanDC]; n=5 [CD4\(^+\) & CD56\(^+\)]; n=6 [CD8\(^+\) & CD19\(^+\)]). To determine statistical significance an ordinary one-way ANOVA followed by the uncorrected Fisher’s LSD test was applied only comparing untreated with treated samples. *p<0.05, ***p<0.001, ****p<0.0001.
3.3.2 **FAT10 Expression Pattern in Murine Immune Cell Subsets**

In cells of the adaptive immune system such as CD4$^+$ and CD8$^+$ T cells as well as B cells, FAT10 expression was not detectable in untreated or LPS-treated cells (Figure 24A, 24B, & 24D). Only the cytokines IFN-γ and TNF jointly induced expression of FAT10 in these cell types. The mRNA levels were similar in CD4$^+$ and CD8$^+$ T cells that were 2.3 and 2.5 log2-fold change, respectively, but only increased significantly in CD4$^+$ T cells. In B cells, the level of FAT10 expression was twice as high (5.2 log2-fold change). Notably, Tregs showed basal FAT10 expression, which rose significantly upon LPS treatment (Figure 24C). When treated with cytokines, Tregs expressed FAT10 even stronger attaining levels of 6.9 log2-fold change. High basal expression of FAT10 in Tregs is in accordance with a previous report showing that overexpression of FOXP3 leads to FAT10 upregulation (Ocklenburg et al., 2006). These results infer that all cells of the murine adaptive immune system express FAT10 in an inflammatory setting since the pro-inflammatory cytokines IFN-γ and TNF induced its expression in the aforementioned cell types.

Also, murine granulocytes, macrophages and monocytes (M&M), and DCs, i.e. cells of the innate immune system, were analysed for FAT10 expression (Figure 24E-G). DCs showed low basal FAT10 expression when untreated (Figure 24F) and LPS treatment completely repressed this basal expression. In granulocytes, FAT10 mRNA was not detectable in untreated cells, but FAT10 expression could be stimulated slightly with LPS (Figure 24G). Both cell types up-regulated FAT10 significantly upon cytokine induction (Figure 24F & 24G), where DCs expressed FAT10 at 6.2 log2-fold change and granulocytes at 4.0 log2-fold change. Highest expression levels were found in M&M (Figure 24E). These cells had high basal FAT10 expression, which significantly increased with cytokines but not with LPS. When stimulated with the pro-inflammatory cytokines IFN-γ and TNF, upregulation of FAT10 mRNA expression in DCs and M&M reached similar levels of 6.2 log2-fold change in DCs and 6.4 log2-fold change in M&M (Figure 24E & 24F).

Taken together, these results indicate that upon IFN-γ and TNF treatment, murine FAT10 is expressed in all immune cell subsets, which were analysed. Interestingly, M&M as well as Tregs already express FAT10 in a naïve state or non-inflammatory setting suggesting that FAT10 is necessary for cell type-specific functions unrelated to acute inflammation.
Figure 24: **FAT10** mRNA expression profile of immune cell subsets from mouse spleen. Murine cells were sorted magnetically (A, B, & D-G) or were purified by FACS (C) from spleens of C57BL/6 mice. After isolation, the cells were stimulated with 1 µg/mL LPS or 400 U/mL TNF and 200 U/mL IFN-γ for 24 h or were left untreated. Then, **FAT10** mRNA expression was quantified by real-time RT-PCR. Complementary DNA prepared from total splenocytes of **FAT10**-deficient mice was used as negative control (shown as dotted line). **FAT10** expression was depicted as log2-fold change normalized to **RPL13A** and **ACTB**. Graphs show the mean ± SD of three independent experiments, except for regulatory T cells (Tregs) where two experiments were performed. Statistical significance was determined with an ordinary one-way ANOVA followed by the uncorrected Fisher's LSD test only comparing untreated with treated samples. ** p<0.01, *** p<0.001.

### 3.3.3 **FAT10** Protein Expression is Up-Regulated in Human Leukocytes upon stimulation with IFN-γ and TNF

To test whether **FAT10** protein expression correlated with **FAT10** mRNA expression, human PBMCs were either left unstimulated or were stimulated for 30 h with IFN-γ and TNF \textit{in vitro} before combined immunoprecipitation and western blot analysis was performed. **FAT10** protein was weakly expressed in human leukocytes but was up-regulated upon cytokine stimulation indicating that **FAT10** mRNA and **FAT10** protein expression levels correlated in human leukocytes (Figure 25).
Figure 25: Detection of endogenous FAT10 protein in human PBMCs. Peripheral blood mononuclear cells (PBMCs) from two healthy donors (each ~4x10⁸ cells) were cultivated in medium, supplemented with IFN-γ and TNF to induce endogenous FAT10 expression, or were left untreated, as indicated. After 30 hours of incubation, cells were harvested, lysed, and subjected to immunoprecipitation (IP) using the monoclonal FAT10-reactive antibody 4F1 (Aichem et al., 2010). Endogenous FAT10 in the immunoprecipitated fractions was detected by immunoblotting (IB), using the polyclonal rabbit antibody 105(7) reactive towards FAT10 (Hipp et al., 2005). β-Actin in total lysates prior to immunoprecipitation was used as loading control.

3.4 Characterisation of the Epitope Peptide Recognised by the FAT10-reactive antibody 4F1

Commonly used elution techniques after immunoprecipitation (IP) are boiling the matrix in SDS-containing buffer and eluting the protein of interest at an extreme pH or by increasing ionic strength (Firer, 2001; Bonifacino et al., 2016). However, elution by these methods can lead to co-elution of antibodies and non-specifically bound proteins from the purification matrix, which contaminate down-stream applications, such as SDS-PAGE, in vitro assays, and mass spectrometry (Ten Have et al., 2011). For example, often heavy and light chains of dissociated antibodies are observed as background on immunoblots or stained gels. Additionally, these elution techniques can cause loss of protein due to denaturation of the protein of interest. High background and loss of protein of interest is especially disadvantageous when analysing proteins of low abundance or stability, and protein-protein interactions. To avoid or reduce such contaminations or allow for high recovery of protein after IP, specific elution can be achieved by means of antibody epitope peptides, which is frequently used with epitope tags, such as 1xFLAG, 3xFLAG, and HA (Field et al., 1988; Hopp et al., 1988; Hernan et al., 2000). Regarding FAT10, it has been troublesome to identify and confirm substrates or
interacting proteins of FAT10 at the endogenous level and even after overexpression often due to the relatively small fraction of the entire protein pool that is modified by FAT10 and due to background from contaminating proteins or antibodies (Aichem et al., 2010; Li et al., 2011; Bett et al., 2012; Buchsbaum et al., 2012b; Bialas et al., 2015). Therefore, it was of interest to identify the epitope peptide of the monoclonal FAT10-reactive antibody 4F1, which has been generated by Birte Kalveram in the Groettrup group (Aichem et al., 2010). This will allow for elution of FAT10 with reduced background and increased signal-to-noise ratio, which should simplify the identification of low abundant substrates as well as interacting proteins of FAT10. In addition, the epitope peptide could be applied to small-scale purifications of endogenous or untagged FAT10 along with substrates and interaction partners from continuous and primary cell cultures at conditions ensuring the highest stability of FAT10, which is known to be unstable in solution at higher concentrations (Buchsbaum et al., 2012a; Theng et al., 2014). These purifications can be used for in vitro studies or to further refining the FAT10 interactome by mass spectrometry, which has been done before (Aichem et al., 2012; Leng et al., 2014). Yet, in both studies contaminations from antibody heavy chains prevented analysis of proteins of the same size.

### 3.4.1 Defining the 4F1 Antibody Epitope Peptide

To identify the epitope peptide of the anti-FAT10 antibody 4F1 epitope excision and extractions assays, which are mass spectrometry-based approaches for epitope mapping (Suckau et al., 1990; Macht et al., 1996), were performed. In epitope excision assays or protection assays, FAT10 was first immunoprecipitated using 4F1-coupled beads and then exposed to specific proteases. The proteases degrade FAT10 leaving behind only the epitope sequence that was protected from degradation by binding to the 4F1 antibody. In epitope extraction assays, FAT10 was first digested by specific proteases and the resulting peptides incubated subsequently with 4F1-coupled beads. Thereby, only peptides containing the epitope sequence were immunoprecipitated. Finally, in both setups the peptides were eluted from the beads at low pH and the resulting eluates were subjected to analysis by mass spectrometry. Control experiments included empty beads without 4F1 antibody and in-solution digests of only FAT10. Since it is known that the 4F1 antibody recognises only N-terminal domain of FAT10 (Aichem et al., 2010), a cysteine-less, N-terminal domain of FAT10 (N-FAT10(C0)) was used to determine the epitope peptide. For proteolytic cleavage, first, the proteases trypsin and Asp-N were chosen since these proteases generated completely different peptides and covered the entire primary amino acid sequence of the N-terminal domain of FAT10 (Figure 26A). Due to unspecific and
incomplete cleavage of FAT10 by Asp-N and trypsin, respectively, which was evident by the peptides detected in mass spectrometry (not shown), peptide groups had to be defined into which the different peptides were clustered.

Epitope extraction assays of FAT10 in combination with trypsin showed no specific enrichment of peptides in any peptide group (4F1 beads) when compared to the control sample (empty beads) (Figure 26B). Interestingly, in the epitope excision experiments, in which the epitope was protected from trypsin digestion, peptides in the group ‘SEEWDLMTFDANPYD-SVKKIK’ were enriched in comparison to the control (4F1 beads compared to empty beads) (Figure 26C, red box). In-solution digests with trypsin were performed to see the overall abundance of tryptic FAT10 peptides detectable by mass spectrometry (Figure 26D). The comparison of the in-solution digest with the epitope excision results confirmed specific enrichment of peptides in the amino acid stretch ‘SEEWDLMTFDANDYDSVKKIK’ after trypsin digest. Background binding of peptides to the beads in the amino acid region ‘SKTKVPVQDQLLLGSKILKPR’ in presence and absence of 4F1 antibody was observed (Figure 26B & 26C, upper panel compared to lower panel), which were also present in the in-solution digest control sample (Figure 26D). Notably, no peptides in the group that include the N-terminal region ‘MAPNASSLSVHVR’ and very few peptides in the linker region ‘VVKPSDEELP’ as well as the internal region ‘IKHEVR’ of FAT10 were detectable by mass spectrometry. It should also be noted that the empty bead controls for the epitope extraction and excision assays were performed only once. Nonetheless, these results suggest that the 4F1 epitope is included within the amino acid region ‘SEEWDLMTFDANPYD-SVKKIK’ since peptides within this stretch selectively were enriched in epitope excision assays, in which the epitope is protected from cleavage. In line with this, no enrichment of peptides in this group was detectable in epitope extraction assays likely because the epitope was destroyed by the trypsin digest. Since there are only three lysine residues that are cleaved by trypsin in this peptide region and these are at the C-terminus, this region seems to be crucial for recognition of FAT10 by the 4F1 antibody.
Figure 26: Epitope excision and extraction assays with FAT10 using trypsin and Asp-N. (A): Amino acid sequence of the N-terminal domain of human FAT10, in which all cysteine residues were changed to serine residues (N-FAT10(C0)). Arrow heads above the amino acid sequence indicate cleavage sites of the protease trypsin, which cleaves after arginine (R) and lysine (K) residues. Below the amino acid sequence, the cleavage profile for the protease Asp-N is shown that cuts before aspartic acid (D) residues. Epitope extraction and excision assays performed with N-FAT10(C0) using trypsin (B-D) and Asp-N (E-G). The number of peptides identified by
mass spectrometry were clustered into peptide groups and were plotted as the percentage of total peptides. 
(B, C, E, & F): The upper graphs display the peptides obtained after immunoprecipitation with 4F1 antibody 
(4F1 beads). The lower graphs show the peptides measured in control immunoprecipitations without 4F1 antibody 
(empty beads). Red boxes highlight the peptide groups that show enrichment of peptides in epitope excision assays 
(C & F) and that completely or partially lack enrichment of the same peptides in epitope extraction assays (B & E). 
(D & G): In-solution digest of N-FAT10(C0) using trypsin (D) and Asp-N (G) were performed to see overall 
abundance of peptides after digest with the different enzymes. After clustering into peptide groups, results were 
plotted as percentage of total peptides. Graphs display the mean ± SD. Results from two independent experiments 
are shown in (B, C, E, & F), except for the empty beads controls in epitope excision and extraction assays with 
trypsin that were performed only once (B & C). In-solution digest with trypsin and Asp-N were performed 
independently twice and four times, respectively (D & G).

Similar results were obtained when epitope extraction and excision assays were done with the 
enzyme Asp-N. Epitope extraction revealed enrichment of peptides within the amino acid 
region ‘DANPYDSVKKIKEHVRSKTKVPVQ’ when compared to the ‘empty beads’ control 
(Figure 26E, red box). However, when compared to the total abundance of peptides in the 
in-solution digest (Figure 26G), peptides in this group were only slightly enriched. Notably, the 
empty beads control for the epitope extraction assays was prepared twice, but in one sample no 
peptides were detectable. Specific enrichment of peptides was found after 4F1-IP within the 
peptide group ‘DANPYDSVKKIKEHVRSKTKVPVQ’ in epitope excision assays compared 
to empty beads control (Figure 26F, red box) and the in-solution digest (Figure 26G). Again, 
background binding of peptides to the beads in absence and presence of 4F1 antibody was 
detected (Figure 26E & 26F, upper panel compared to lower panel). Similar to the experiments 
using trypsin the very N-terminal amino acids ‘MAPNASS’ as well as the linker region 
‘PSDEELP’ could not be detected.

Taken together, the epitope of the 4F1 as determined by digestion with Asp-N lies in the 
amino acid region ‘DANPYDSVKKIKEHVRSKTKVPVQ’ due to the selective enrichment in 
the epitope excision assays. Interestingly, in the epitope extraction experiments this stretch was 
only partially enriched suggesting that here the antibody binding peptide was mostly intact. 
Thus, the actual epitope peptide might extend on the N-terminus or the C-terminus of this amino 
acid stretch.
Since rather long amino acid stretches were obtained, within which the 4F1 antibody binds, epitope excision assays were performed using Pronase. Pronase is a mixture of proteases that only leaves behind the protected epitope bound to the antibody. Therefore, Pronase has no specific cleavage profile and specific peptide groups were defined based on the trypsin cleavage profile. Here, a striking enrichment of peptides within the amino acid stretch ‘DANPYDSVKKIKEHVRSKT’ was detected (Figure 27A, red box). Peptides in this group were not enriched in the empty beads control and in the in-solution digest (Figure 27B). Due to the almost complete digest of FAT10 by Pronase, only few peptides were detected in the control samples and were completely lacking in one of the empty beads control samples. In agreement with the epitope excision and extraction assays done with trypsin and Asp-N, the peptide stretch that contains the 4F1 epitope is in the amino acid region ‘DANPYDSVKKIKEHVRSKT’.

![Figure 27: Epitope excision assays with FAT10 using Pronase.](image)

(A): Epitope excision assays performed with N-FAT10(C0) using Pronase (n=2). Peptides identified by mass spectrometry were enumerated and clustered into peptide groups. Graphs show the percentage of total peptides obtained. Results from immunoprecipitation with 4F1 antibody (4F1 beads) followed by mass spectrometry of eluted peptides are displayed in the upper graph and results from control immunoprecipitations in which the 4F1 antibody was omitted (empty beads) in the lower graph. The red box highlights the peptide group that was enriched in 4F1-immunoprecipitations. (B): Percentage of total peptides in one in-solution digest of N-FAT10(C0) using Pronase measured by mass spectrometry and after clustering into peptide groups. The mean ± SD are shown in the graphs.
From the epitope excision and extraction assays three potential 4F1 epitopes of varying length were obtained encompassing the same region within the N-terminal domain of FAT10. These peptides were compared and the amino acid stretch that overlapped was determined. As a result, the peptide that is recognised by the 4F1 antibody is ‘DANPYDSVKKIK’ (Figure 28).

**Figure 28: The 4F1 epitope defined based on the epitope extraction and excision assays.** Alignment of the amino acid sequence of the cysteine-less, N-terminal domain of human FAT10 (N-FAT10(C0)) and the peptides that are recognised by the 4F1 antibody in the epitope excision and extraction assays using trypsin, Asp-N, and Pronase. The 4F1 epitope was defined as the minimal overlapping peptide as shown in the bottom row (epitope peptide).

To analyse the epitope excision and extraction assays peptide groups had to be defined, which might impose bias onto the results. Therefore, a peptide array was performed in addition in order to determine the 4F1 epitope peptide. The peptide array consisted of 15mer peptides of the N-terminal FAT10 domain spotted onto a membrane with an overlap of two amino acids. The peptide array was probed with the 4F1 antibody and developed it using chemiluminescence similar to an immunoblot. The chemiluminescent signals were quantified using ImageJ software and plotted as shown in Figure 29A. The strongest signals were obtained for the peptides from #9-#15 encompassing the region ‘WDLMTFDANPYDSVKKIKEHVRSTKTKV’ where the peptide #11 ‘TFDANPYDSVKKIKE’ gave the highest signal (Figure 29A, red box). Interestingly, low signals in the region ‘DQVLLLGSKILKPRRSSY’ from peptide #24-#27 were obtained suggesting that the 4F1 antibody recognises a partial conformational epitope rather than a linear epitope. But, peptides from the same region showed background binding in the epitope excision and extraction assays (Figure 26 & 27), therefore, these signals were considered background, too. By comparing peptide #11 with the corresponding peptide in murine FAT10 and only partial conservation was found (Figure 29B), which can explain why the 4F1 antibody does not recognise murine FAT10 (Kalveram, 2009).

In conclusion the 4F1 antibody epitope peptide resulting from the epitope excision and extraction assays as well as the peptide array is ‘TFDANPYDSVKKIKE’ since this peptide gave the highest signal in the peptide array and includes the peptide determined by the epitope excision and extraction assays. Yet, it cannot be excluded that the actual 4F1 epitope peptide is smaller since no smaller peptides were tested.
Figure 29: Epitope Mapping of the 4F1 antibody using a peptide array. (A): 15mer peptides of the N-terminal domain of human FAT10 with a two-amino acid overlap (left panel) were spotted onto a membrane, probed with 4F1 antibody, and developed using enhanced chemiluminescence (ECL) (middle panel). ECL signals were detected by ImageJ software and are given as the mean ± SD (right panel). Peptides shown in black gave the strongest signals and the peptide boxed in red the overall strongest signal. Shown are the results of three independent experiments (B): Alignment of the peptide which showed the strongest signal (peptide #11) in human.
FAT10 and the corresponding peptide in murine FAT10. Numbers indicate positions of the first and last amino acids of the peptides within the entire sequence of human and murine FAT10. Conserved amino acids are indicated by the respective letter and conservative changes are indicated by (+), semi-conservative or non-conserved amino acids were left blank.

### 3.4.2 Characterisation and Application of the 4F1 Antibody Epitope Peptide

To verify the 4F1 epitope peptide ‘TFDANPYDSVKIKE’ this peptide was utilised for blocking experiments. For this, the 4F1 antibody was incubated with the peptide and then used in downstream applications to see loss of FAT10 signal.

At first, immunoblots were prepared from total cell lysates from 3xFLAG-FAT10 overexpressed in HEK293T cells and from mock-transfected HEK293T cells. Prior to probing with 4F1 antibody, antibodies were incubated with 4F1 peptide in 1,000, 2,000, and 4,000 molar excess. Unblocked 4F1 antibodies and FLAG antibodies were used as controls for FLAG-FAT10 detection, and 4F1 antibodies incubated with 4,000 molar excess of 3xFLAG peptide served as negative control. After detection with unblocked 4F1 and FLAG antibodies as well as 3xFLAG peptide-blocked 4F1 antibodies, prominent signals for FAT10 monomer and conjugates could be detected in the transfected cells and very low background in the mock controls (Figure 30A & 30B). When the 4F1 antibodies were incubated with 2,000 and 4,000 molar excess of 4F1 peptide the FAT10 signal was almost completely diminished and only minor signals of monomeric FAT10 were still detectable (Figure 30A). No obvious change of FAT10 signal was observed when 4F1 antibodies were incubated with 1,000 molar excess of 4F1 peptide (Figure 30A). FAT10 and mock signals were quantified and normalised to the loading control HSP90. Then, normalised FAT10 signals were related to the background signal from mock controls on the same immunoblot. This quantification showed a significant decrease of FAT10 signals with 4F1 antibodies incubated with 2,000 and 4,000 molar excess of 4F1 peptide whereas the FAT10 signal decreased insignificantly with 4F1 antibodies incubated with 4F1 peptide at 1,000 molar excess (Figure 30C). FAT10 signals from the 4F1 antibodies blocked with 3xFLAG peptide were unaffected (Figure 30C). After an additional normalisation of FAT10 signals to the signals of the unblocked samples, FAT10 signals exhibit a decrease of 26% and 85% at 1,000 molar excess and at 2,000 molar excess or higher of 4F1 peptide, respectively (Figure 30D).
Figure 30: Immunoblotting with peptide-blocked 4F1 antibody. (A): Total cell lysates of 3xFLAG-FAT10- and mock-transfected HEK293T cells were subjected to SDS-PAGE and immunoblot analysis with 4F1 antibody. Prior to antibody probing of immunoblots, 4F1 antibodies were incubated with the 4F1 epitope peptide ‘TFDANPYDSVKKIKE’ at 1,000, 2,000, and 4,000 molar excess. Unblocked and 3xFLAG peptide-blocked (4,000 molar excess) 4F1 antibody were included as controls. (B): Detection of 3xFLAG-FAT10 by FLAG antibodies in 3xFLAG-FAT10 and mock samples was included as control for successful expression. Immunoblots of one representative experiment out of two to three independent experiments are shown. (C & D): Quantification of the signals from immunoblots as shown in (A & B). Signals from mock and 3xFLAG-FAT10 signals were quantified and normalised to HSP90 that served as loading control. 3xFLAG-FAT10 signals were normalised to the mock controls of the same immunoblot (C). Signals in the single experiments were normalised to the unblocked 3xFLAG-FAT10 signal and plotted as percentage of signal relative to unblocked (D). Images were acquired using the LI-COR Odyssey Fc imaging system and analysed with the Image Studio software version 5.2. Results are summarised from two to three independent experiments and depicted as mean ± SD. Statistical significance was determined by ordinary one-way ANOVA followed by multiple comparisons using uncorrected Fisher’s LSD test. Significance was calculated only between the unblocked samples and the 4F1 and 3xFLAG peptide-blocked samples. *** p<0.001, **** p<0.0001.
Results

Figure 31: Immunofluorescent staining using peptide-blocked 4F1 antibody. HEK293T cells were mock-transfected (A), transfected with 3xFLAG-FAT10 (B), and stimulated using 400 U/mL TNF and 200 U/mL IFN-γ (C) for 48 h. Five hours prior to fixation, the cells were incubated with 5 µM MG132 to enrich overexpressed and endogenous FAT10. Cells were stained with unblocked 4F1 and FLAG antibodies, with secondary antibodies only (2nd AB), and with 4F1 antibodies blocked with 2,000 molar excess of 4F1 peptide (4F1 blocked). DAPI was used to counterstain nuclei. Images were acquired on a LSM880 confocal microscope at 63x magnification. Representative images of three independent experiments are shown. Scale bar is 20 µm.

The same blocking approach was applied to immunofluorescence of endogenous and overexpressed FAT10 in HEK293T cells (Figure 31) and in HeLa cells (Supplementary figure 3). HEK293T cells were transfected with 3xFLAG-FAT10 or induced with the cytokines TNF and IFN-γ. Five hours before fixation and staining, the cells were incubated with MG132 to increase the amount of overexpressed and endogenous FAT10. No signals were detected in mock control samples (Figure 31A) and in samples in which the primary antibody was omitted (Figure 31A, 31B, & 31C, 2nd AB panel). Successful transfection and induction of FAT10 was confirmed by fluorescent signals detected by unblocked 4F1 and FLAG antibodies (Figure 31B & 31C). When 4F1 antibodies was blocked with the 4F1 epitope at 2,000 molar excess and then used for staining, no signals of endogenous FAT10 and low remaining signals for overexpressed 3xFLAG-FAT10 were detected indicating successful blocking of 4F1 antibody (Figure 31B & 31C, 4F1 blocked). Taken together, these results confirmed that the 4F1 peptide ‘TFDANPYDSVKKIKE’ includes the entire epitope peptide. Furthermore, this peptide can
bind effectively to the 4F1 antibody and prevent binding to the whole FAT10 protein almost completely. The residual FAT10 detection very likely results from dissociation of the peptide from the antibody during the incubation of the immunoblot or the fixed cells.

Next, it was tested whether the 4F1 peptide could be used to elute FAT10 from 4F1 antibodies. For this purpose, endogenous and overexpressed FAT10 was immunoprecipitated from cell lysates using 4F1-coupled and isotype mouse IgG-coupled beads. Input samples from cleared total cell lysates subjected to immunoblotting confirmed successful FAT10 induction and overexpression (Figure 32B). Elution fractions and bead fractions were subjected to immunoblotting and were quantified from immunoblots shown in Figure 32A. Due to the background from the antibodies in immunoblots of the bead fraction, the signal from the mock 4F1-IP (Figure 32A, lane 2) were subtracted from the 4F1-IPs of the induced and overexpressed FAT10 (Figure 32A, lanes 4, 5, 7, & 8) in all blots. The total signal of FAT10 monomer and FAT10 substrates was defined as the sum of the signals in the elution and bead fractions of the same sample. Thereby, it could be calculated that the 4F1 peptide specifically eluted ~60% of the 3xFLAG-FAT10 monomer, ~50% of the 3xFLAG-FAT10 conjugates, and ~55% of the total 3xFLAG-FAT10 (Figure 32C). Unspecific elution by the 3xFLAG peptide accounted for a maximum of 10% of the 3xFLAG-FAT10 monomer, was not detectable for 3xFLAG-FAT10 conjugates, and less than 10% of the total 3xFLAG-FAT10 signal (Figure 32C). Due to the poor induction of endogenous FAT10, only the monomeric form of FAT10 could be detected. Nonetheless, approximately 50% of the total FAT10 monomer was eluted albeit the elution of endogenous FAT10 was subject to variation (Figure 32D).

Overall, the 4F1 epitope peptide can successfully elute both endogenous and overexpressed FAT10 including interacting partners and substrates. Furthermore, elution using the 4F1 peptide can reduce background and increase the signal-to-noise ratio that might help to easier identify and detect FAT10 conjugates and interaction partners. In addition, this peptide might be suitable for purification of untagged endogenous FAT10 under optimal physiological conditions for the use in in vitro assays or structural studies. However, the elution protocol used here can still be optimised in terms of peptide concentration, elution duration, temperature, and repetitions. An optimised protocol can likely increase the amount of eluted FAT10 above the 55% of total eluted 3xFLAG-FAT10, but also the amount of endogenous FAT10 and its conjugates upon proper induction. The 4F1 peptide can also be used as an additional 4F1 staining control for detection of FAT10 by immunofluorescence, immunoblotting, and potentially by flow cytometry.
Figure 32: 4F1 peptide elution of overexpressed and endogenous FAT10. HEK293T cells were mock-transfected, transfected with 3xFLAG-FAT10, and stimulated with 400 U/mL TNF and 200 U/mL IFN-γ for 24 h. Six hours prior to the harvest and lysis of the cells, 5 µM MG132 was added to enrich induced and overexpressed FAT10. Cell lysates were subjected to immunoprecipitation (IP) by 4F1 or control IgG. (A): The IP-beads were incubated with 500 µg/mL 4F1 peptide or 3xFLAG peptide as negative control. The beads and the eluates were subjected to immunoblotting and FAT10 signals detected by 4F1 antibody. (B): FAT10 signals were detected in cleared total cell lysate of each sample to control proper induction and overexpression. HSP90 was used as loading control. The signal of the 3xFLAG-FAT10 monomer spread into the other lanes of the gel during SDS-PAGE. Images of immunoblots were acquired using the LI-COR Odyssey Fc imaging system (C & D): 3xFLAG-FAT10 (C) and endogenous FAT10 (D) signals were quantified with the Image Studio software version 5.2. The background signal from the mock 4F1-IP (A, lane 2) was subtracted from the FAT10 signals of the 4F1-IPs with FAT10 (A, lane 4, 5, 7, & 8). Signal from the elution and the bead fraction were normalised to the total signal, which was defined as the sum of both signals, to obtain the percentage of FAT10 signal that was eluted or still bound to the beads after elution. Images in (A & B) are representative images of three independent experiments for overexpressed 3xFLAG-FAT10 and two independent experiments for endogenous FAT10. The quantification shown in (C & D) is pooled data from two (endogenous FAT10) to three (overexpressed 3xFLAG-FAT10) independent experiments and is given as mean ± SD.
4 Discussion

4.1 FAT10 Enhances the Degradation of DALIS and DALIS Form Independently of HDAC6

FAT10 expression is upregulated upon DC maturation (Lukasiak et al., 2008; Ebstein et al., 2009) and has been linked to antigen processing and presentation (Ebstein et al., 2012; Schliehe et al., 2012; Basler et al., 2015a). Furthermore, FAT10 localises to aggresomes in a largely HDAC6-dependent manner and with its interaction partner p62 into p62 bodies in HeLa cells (Kalveram et al., 2008; Aichem et al., 2012). Due to these findings the question arose whether FAT10 also localises to dendritic cell aggresome-like induced structures (DALIS) and is involved in DALIS regulation. DALIS are ubiquitylated protein aggregates that form transiently upon maturation of DALIS (Lelouard et al., 2002). It has been proposed that DALIS serve as antigen repositories that store and thereby prevent the presentation of antigens during the maturation process of DCs. This allows DCs to acquire a fully mature phenotype that enables DCs to properly prime T cells and elicit an immune response and prevent tolerance induction (Herter et al., 2005; Pierre, 2005; Faßbender et al., 2008; Argüello et al., 2016). Therefore, co-localisation studies of endogenous FAT10 in human MoDCs and lentivirally overexpressed FAT10 in murine BMDCs were performed. Furthermore, by overexpressing a non-conjugatable form of murine FAT10 it was analysed whether targeting of FAT10 to DALIS is dependent on conjugation and the C-terminal diglycine motif. Moreover, by comparing FAT10-deficient to -proficient murine BMDCs the formation and clearance of DALIS in presence and absence of endogenous murine FAT10 was investigated. Finally, the role of HDAC6 in the formation and clearance of DALIS in murine BMDCs was examined.

Both endogenous human FAT10 as well as overexpressed murine FAT10 localised to DALIS. Interestingly, all DALIS contained human or murine FAT10 indicating general targeting to DALIS and a general function of FAT10 in the regulation of DALIS in human as well as murine DCs. It could also be demonstrated that targeting to DALIS does not depend on the diglycine motif of FAT10 and thus also likely not on conjugation. Therefore, it can be assumed that FAT10 is targeted to DALIS independent of conjugation and that FAT10 conjugation takes place within DALIS similar to the conjugation of ubiquitin that can occur within DALIS as indicated by the presence in DALIS of the ubiquitin conjugation machinery, such as the E1 enzyme UBE1 and the E3 ligase CHIP (Lelouard et al., 2004; Kettern et al.,...
However, it is not known whether the FAT10 conjugation enzymes UBA6 and USE1 localise to DALIS, too. This independence of the diglycine motif to localise to DALIS corresponds to the targeting of FAT10 to aggresomes (supplementary Figure 2). Kalveram and colleagues showed that targeting of FAT10 to aggresomes relies mainly on HDAC6, where HDAC6 can interact with both domains of FAT10 and deliver them into aggresomes. Furthermore, they showed that a FAT10-GFP fusion protein with a mutated diglycine motif is likewise targeted to aggresomes (Kalveram et al., 2008). HDAC6 is dispensable for the formation and clearance of DALIS since HDAC6-deficient murine BMDCs showed no difference in the kinetic of DALIS formation compared to HDAC6-proficient BMDCs. Although FAT10-specific transport to DALIS by HDAC6 cannot be excluded, it can be assumed that FAT10 is targeted to DALIS in DCs by another mechanism which parallels the HDAC6-dependent delivery of FAT10 into aggresomes since HDAC6 was not involved in the formation of DALIS and strong localisation of FAT10 to DALIS in murine as well as human DCs was observed. HDAC6 travels along microtubules to dispose of its clients, such as FAT10 and its substrates, into aggresomes at the MTOC (Kopito, 2000; Kalveram et al., 2008). DALIS are also motile within the cytosol and can undergo fusion suggesting that this motility is necessary for the aggregation process (Lelouard et al., 2004). Interestingly, this movement occurs along microtubules since nocodazole treatment instantly abrogates DALIS motility (Canadien et al., 2005, supplementary video 2). Due to this, movement of DALIS along microtubules mediated by HDAC6 can be excluded since otherwise smaller and more aggregates in HDAC6-deficient cells would be expected, which was not the case. Additionally, if HDAC6 was necessary for DALIS motility one would expect that DALIS eventually end up at the MTOC, which they do not, further excluding a role of HDAC6 in this process and in targeting of FAT10 to DALIS. Theoretically, spartin that is involved in cytokinesis and mitochondrial physiology (Renvoise et al., 2010; Joshi & Bakowska, 2011) could be responsible for DALIS motility. Spartin is partially necessary for the formation of DALIS since knockdown of spartin in RAW264.7 macrophages reduced the formation of DALIS (Karlsson et al., 2014). In addition, spartin contains a ‘microtubule interacting and trafficking’ motif used to bind to microtubules (Lu et al., 2006). However, this hypothesis needs further investigation.

Next, the formation of DALIS in FAT10-deficient and -proficient BMDCs was compared upon induction with LPS and cytokines (TNF and IFN-γ). LPS-induced BMDCs formed DALIS as expected and reported before (Lelouard et al., 2002; DeFillipo et al., 2004; Canadien et al., 2005; Herter et al., 2005; Kettern et al., 2011). Contrary to this, the kinetic of the cytokine-induced DALIS formation showed a delay in accumulation that, first, was eminent at 24 hours
with no substantial increase at eight hours. This kinetic is similar to the IFN-γ-induced formation of ALIS in murine embryonic fibroblast observed by Nathan and colleagues who found an increase of the number of ALIS up to 48 hours (Nathan et al., 2013). But, these results are in contrast to the results by Seifert and colleagues who reported the canonical ALIS kinetic with an increase up to eight hours and a subsequent decrease of the number of ALIS (Seifert et al., 2010). In conclusion, the discrepancy between the LPS- and cytokine-induced DALIS kinetic is very likely not due to different maturation stimuli that were used. Furthermore, it can be excluded that this difference is dependent on FAT10 expression since the same kinetics in FAT10-deficient and -proficient cells was observed. To date there is no explanation for the different DALIS kinetics in cytokine- and LPS-induced BMDCs.

At later stages of DC maturation, reduced numbers of DALIS in FAT10-proficient BMDCs compared to FAT10-deficient BMDCs were found suggesting that FAT10 contributed to the degradation of DALIS. Since proteasomes do not localise to DALIS (Canadien et al., 2005; Herter et al., 2005) the transport of FAT10 and FAT10ylated substrates to proteasomes could be mediated by UBL-UBA domain proteins, such as NUB1L and ubiquilins. These proteins serve as soluble ubiquitin receptors and deliver their clients, such as FAT10, to the proteasome enabling degradation according to the proposed ‘transfer model’ of FAT10 degradation (Figure 6) (Rani et al., 2012). The overall effect of FAT10 on the degradation of DALIS was small considering FAT10 is present in almost all DALIS as evident from the co-localisation studies in human and murine DCs (Figure 11 & 12). This suggests that most of the FAT10 that is present in DALIS is not conjugated which is in line with the general observation that most of the cellular FAT10 is present in its monomeric form, despite its conjugation to hundreds of substrates (Aichem et al., 2012). Additionally, conjugation of FAT10 to newly synthesised proteins or puromycin-induced DRiPs, which are the main constituent of DALIS (Lelouard et al., 2002, 2004), occurs at a lower rate than, for example, conjugation of ISG15 and ubiquitin further supporting the finding here (Spinnenhirn et al., 2017). To confirm that the reduced number of DALIS in FAT10-proficient BMDCs at 24 hours was due to degradation of DALIS by FAT10 a cycloheximide chase approach in combination with microscopic quantification of DALIS was utilised. After applying cycloheximide for two hours increased numbers of DALIS in FAT10-deficient BMDCs were observed whereas the number of DALIS in FAT10-proficient BMDCs remained unchanged. Although this confirmed FAT10-mediated clearance of DALIS, this effect was surprising since a decrease of DALIS at 24 hours of maturation was expected where the formation of DALIS should have ceased and clearance should take place as reported in several studies (Lelouard et al., 2002; Canadien et al., 2005;
Herter et al., 2005; Kettern et al., 2011). Yet, an increase of ALIS after cycloheximide treatment has been reported in INS1 832/13 β-cells under normal and high glucose conditions (Kaniuk et al., 2007). The same group reported that ALIS formation in mouse embryonic fibroblasts under starvation conditions was only partially inhibited by cycloheximide treatment at an early stage of ALIS formation (Szeto et al., 2006), which should be dependent on protein synthesis (Lelouard et al., 2002). Both studies concluded that this was the result of an influx of long-lived proteins into DALIS. Relating to the experiments presented in this study it can be concluded that FAT10 counteracted the influx of long-lived proteins by efficient proteasomal degradation. This FAT10-mediated feed of proteins or antigens from DALIS into the proteasome argues in favour of an involvement of FAT10 in antigen processing and presentation via the MHC class I presentation pathway. To address this issue in more detail it would be necessary to elucidate the targets of FAT10 within DALIS. In theory, FAT10 could target pathogenic proteins that accumulate in DALIS for proteasomal degradation and thereby enhance MHC class I presentation of these antigens. In support of this, fusion of FAT10 to viral proteins accelerated their degradation and subsequent presentation on MHC class I (Ebstein et al., 2012; Schliehe et al., 2012). Besides that, accumulation of viral proteins in DALIS in DCs has been observed (Herter et al., 2005; Rahnefeld et al., 2011). In case of the influenza nucleoprotein (NP) it has been shown that the retention of NP in DALIS in BMDCs delayed antigen presentation and that antigen presentation increased concurrently with upregulation of co-stimulatory molecules and MHC class I molecules on BMDCs that then could efficiently prime T cells (Herter et al., 2005). Yet, conjugation of endogenous FAT10 to pathogenic proteins or antigens still awaits discovery.

Furthermore, FAT10-specific formation of DALIS in BMDCs was not seen since the number of DALIS did not change in a FAT10-specific manner upon proteasome inhibition. Likewise, overexpression of 3xFLAG-mFAT10 in BMDCs did not interfere with DALIS formation. This might be explained by FAT10’s short half-life of approximately one hour (Raasi et al., 2001; Hipp et al., 2005; Aichem et al., 2014; Schmidtke et al., 2014) and the high turnover that was evident in the experiments in which overexpressed 3xFLAG-mFAT10 in BMDCs was only detected upon proteasome inhibition (Figure 12) given that FAT10 is mostly present in its non-conjugated monomeric form and not preferentially conjugated to DRiPs (Spinnenhirn et al., 2017) that are stored in DALIS. Interestingly, inhibition of autophagy using wortmannin increased the number of DALIS in FAT10-deficient and -proficient BMDCs indicating that DALIS are targeted substantially into the autophagolysosomal pathway and that targeting into autophagy occurred independent of FAT10. These results are in line with former
findings where FAT10-mediated targeting into autophagy was not detected (Spinnenhirn, 2014). However, FAT10 was shown to positively influence targeting of cytosolic Salmonella into autophagy (Spinnenhirn et al., 2014) and to modify p62 covalently as well as non-covalently (Aichem et al., 2012). Thus, specific pathogenic antigens, which were not present in this study, may be targeted by FAT10 from DALIS into autophagy by interacting with p62 in DALIS and thereby FAT10 might feed antigens into the MHC class II presentation pathway.

In summary, FAT10 localises to DALIS, does not contribute to DALIS accumulation, and might feed antigens from DALIS into the proteasome and maybe into autophagy and thereby might contribute to antigen presentation by MHC class I and class II.

4.2 Insights Into the Role of FAT10 in Dendritic Cell Migration

One hallmark of cancer is the formation of secondary tumours at distant sites in a process called metastasis, which involves tissues invasion and migration of cancer cells (Fouad & Aanei, 2017). In recent years, different groups reported that FAT10 promotes invasion and migration of various cancer cells and cell lines as well as a hTERT-immortalised hepatocyte cell line (Gao et al., 2014; Liu et al., 2014a; Yuan et al., 2014a; Han et al., 2015; Dai et al., 2016; Ma et al., 2016; Xue et al., 2016). Likewise, migration of immune cells and especially of maturing DCs is crucial for establishing an immune response. In DCs, migration is triggered upon sensing danger signals concurrently with DC maturation in the periphery and has been shown to rely for the most part on the chemokine receptor CCR7. CCR7-dependent migration involves the chemokines CCL21 and CCL19 that guide DCs from the periphery into the lymph node and T cell areas ( Förster et al., 2008; Comerford et al., 2013). Therefore, experiments were conducted to determine whether FAT10 influences the CCR7-dependent migration of human and murine dendritic cells towards CCL19 and CCL21 using Transwell migration assays. Contrary to what has been published for cancer cells and cell lines, migration of human MoDCs was not enhanced when FAT10 was overexpressed lentivirally. Instead, in this system the overexpression of FAT10 reduced CCR7-dependent migration although not significantly when compared to control cells and fraught with uncertainty due to increased cell death. Furthermore, FAT10 did not influence the migration of murine BMDCs in Transwell assays.

FAT10 exerted marginal effects on DC migration. Yet, the experiments in this study focussed on CCR7-mediated DC migration in relation to FAT10, thus it cannot be excluded that FAT10 modulates the migration of DCs by means of other receptors that are involved DC migration, too. For example, the chemokine receptors CXCR4 and CCR8 mediate migration of DCs to secondary lymphoid organs similar to CCR7 in response to the chemokines CXCL12
and CCL1, respectively (Qu et al., 2004; Kabashima et al., 2007; Ricart et al., 2011). In support of this, Gao and colleagues reported that overexpression of FAT10 promoted the migration of the hTERT-immortalised hepatocyte cell line NeHepLxHT and the human colorectal cancer cell line HCT116 by increasing CXCR4 and CXCR7 expression through enhancing NF-κB activity (Gao et al., 2014). At the molecular level, it has been proposed that FAT10 promotes the invasion and migration of hepatocellular carcinoma cells via the AKT/GSK3β signalling pathway by enhancing AKT activation and stabilising β-catenin (Liu et al., 2014a). AKT, also known as protein kinase B, is downstream of CCR7 and CXCR4 and activated upon binding of respective chemokines to the receptors (Tilton et al., 2000; Scandella et al., 2004). However, CCR7- and CXCR4-signalling through AKT has different functional outcomes. While CXCR4-mediated migration is dependent on AKT activation CCR7-mediated migration is not (Scandella et al., 2004; Riol-Blanco et al., 2005; Delgado-Martín et al., 2011). Therefore, the different signalling pathways used by CCR7 and CXCR4 to regulate migration of DCs in response to CCL19/CCL21 and CXCL12, respectively, could explain the marginal effects of FAT10 on DC migration observed in this study. Notably, CXCR4 is a homeostatic chemokine receptor and expressed on almost all immune cells (Bachelerie et al., 2014; Griffith et al., 2014). Therefore, it would be of interest to test whether FAT10 can modulate CXCR4-mediated migration of immune cells other than DCs. Most promising in this respect would be human NK & NKT cells and CD8⁺ T cells as well as murine Tregs and CD11b⁺ macrophages and monocytes since high FAT10 expression was detected in these immune cell types even under non-inflammatory conditions.

Another mechanism that is modulated by FAT10 and has been linked to migration of cells is the TGFβ signalling pathway. Migration of glioma cells was shown to be regulated by FAT10, where FAT10 overexpression increased and FAT10 knockdown decreased migration. Investigation into the mechanism revealed that FAT10 regulated the level of phosphorylated SMAD2, which is downstream of TGFβ (Dai et al., 2016). In dendritic cells, TGFβ signalling has the opposite effect and downregulates migration as well as the expression of CCR7 in a RUNX3-dependent manner (Ogata et al., 1999; Fainaru et al., 2005). Thus, by promoting the TGFβ signalling pathway in dendritic cells FAT10 expression could reduce DC migration, which argues in favour of the results obtained from human MoDCs (Figure 20).

In conclusion, these results provide first insights into a potential role of FAT10 in the migration of DCs or immune cells in general, where FAT10 might potentially reduce migration of immune cells to promote lymph node homing, which coincides with high FAT10 expression, and may serve as a starting point for further investigations into this issue.
4.3 The Expression Profile of \textit{FAT10} in Human and Murine Leukocytes Suggests Cell Type-Specific Functions

Here, the expression levels of the cytokine-inducible ubiquitin-like modifier \textit{FAT10} in different subsets of primary immune cells was examined. The purpose was to generate an expression profile of \textit{FAT10} that can guide future \textit{FAT10} research and may help to avoid that cell type-specific functions are overlooked as has occurred in research on the likewise IFN-$\gamma$- and TNF-inducible immunoproteasome. Over the past 20 years, extensive research has been conducted on the function of the immunoproteasome in antigen presentation (Groettrup et al., 2010b). However, researchers became only recently aware that the immunoproteasome subunits LMP2 ($\beta_{1i}$), MECL-1 ($\beta_{2i}$), and LMP7 ($\beta_{5i}$) were highly expressed in T cells (Stohwasser et al., 1997; DeBruin et al., 2016), i.e. poor antigen presenting cells. Otherwise, recently emerging novel functions of the immunoproteasome in pro-inflammatory cytokine regulation and Th17 differentiation and associated therapeutic options to fight autoimmunity might have been discovered much earlier (Basler et al., 2013). Due to the limited numbers of the primary immune cell populations, which could be purified from human peripheral blood, and due to a lack of a sensitive antibody for murine \textit{FAT10}, this study was limited to the quantitative analysis of \textit{FAT10} mRNA expression in purified human and murine immune cell populations and \textit{FAT10} protein expression in bulk human PBMCs. In bulk human leukocytes, \textit{FAT10} protein expression paralleled the low basal and prominent cytokine-inducible expression of human and murine \textit{FAT10} mRNA measured in the different cell types. Moreover, \textit{FAT10} mRNA and \textit{FAT10} protein expression closely correlate as has been shown previously (Aichem et al., 2012), probably because \textit{FAT10}, which serves as a proteasomal degradation signal, is rapidly degraded by the proteasome with a half-life of approximately one hour in the cell types analysed to date (Raasi et al., 2001; Hipp et al., 2005; Aichem et al., 2014; Schmidtke et al., 2014). Therefore, it is reasonable to assume that the amounts of \textit{FAT10} mRNA, observed here, will very likely reflect \textit{FAT10} protein levels in immune cell populations.

Previously, it has been reported that overexpression of \textit{FOXP3}, a specific marker of Tregs, led to \textit{FAT10} upregulation and that \textit{FAT10} expression was found in long-term cultures of CD4$^+$ CD25$^{hi}$-derived human Tregs (Ocklenburg et al., 2006). Ocklenburg and colleagues established long-term cultures of human Tregs by TCR stimulation, IL-2 supplementation, and co-culture with irradiated Epstein-Barr virus (EBV)-transformed B cells. However, in this study Tregs were cultured short-term in the absence of these stimuli and \textit{FAT10} expression was absent in naïve human Tregs. Yet, \textit{FAT10} was inducible in human and murine Tregs by cytokines indicating that Tregs express \textit{FAT10} transiently under inflammatory conditions. Interestingly,
murine FOXP3+ Tregs expressed *FAT10* in a naïve state, which increased significantly upon LPS and cytokine stimulation. It was shown that in Tregs the de-ubiquitylating enzyme USP7 stabilized FOXP3 by de-ubiquitylation, which was necessary for proper Treg function (vanLoosdregt et al., 2013). Besides that, USP7 was identified as a putative substrate of FAT10 in a large-scale mass spectrometry screen (Aichem et al., 2012) indicating that FAT10-mediated degradation of USP7 could negatively regulate FOXP3 and Treg function. Thus, transient FAT10 expression could be involved in fine-tuning Treg function during inflammation. Additionally, Ocklenburg and colleagues showed that *FAT10* overexpression in CD4+ CD25- T cells enhanced CD25 surface expression and reduced *IL-4* and *IL-5* mRNA levels, but also inhibited proliferation and reduced the Ca2+ flux upon TCR stimulation (Ocklenburg et al., 2006). Human and murine CD4+ T cells expressed *FAT10* only in presence of pro-inflammatory cytokines. Therefore, it is conceivable that FAT10 influences the phenotype of CD4+ T cells in the course of an immune response, where the phenotypic change might vary from anergy to subset transition. CD4+ T cells possess a high plasticity enabling them to switch subset-specific phenotypes upon environmental changes (Geginat et al., 2014; Caza & Landas, 2015). This plasticity could account for the Treg-like phenotype observed in CD4+ CD25- T cells overexpressing *FAT10* (Ocklenburg et al., 2006). In line with this, it could be shown that Th17 cells can differentiate into FOXP3- Tregs, which help resolving inflammation (Gagliani et al., 2015). In conclusion, FAT10 could take part in CD4+ T cell anergy or transition into Tregs to help resolving immune responses in order to prevent chronic infections and tumour formation.

Next, human and murine B cells were examined in which no basal expression of *FAT10* was detectable. The lack of FAT10 expression in B cells was surprising as it is contrary to what has been described by Bates and colleagues (Bates et al., 1997). They found that *FAT10* is highly expressed in most EBV-transformed mature B cell lines and not in precursors B cell lines. Consequently, *FAT10* expression appeared to be characteristic for mature B cells as its expression also occurred independent of EBV infection. Yet, not all mature B cell lines expressed *FAT10*. Hence, it cannot be ruled out that the observed *FAT10* expression was an effect of the EBV latency rather than B cell maturity because EBV latency causes NF-κB and STAT-3 activation (Laherty et al., 1992; Li & Bhaduri-McIntosh, 2016), which again can lead to increased *FAT10* expression (Choi et al., 2014). Likewise, *FAT10* expression was induced in the human respiratory epithelial cell line A549 in an NF-κB-dependent manner upon influenza virus infection (Zhang et al., 2016), in the human proximal tubular cell line HPT-1 upon transduction with mutant, VSV-G pseudotyped HIV-1 (Ross et al., 2006), and in human dermal microvascular endothelial cells infected with Kaposi sarcoma–associated herpesvirus (Hong et
In accordance with these results, it can be concluded from data presented here that non-transformed mature B cells do not express FAT10 unless in an inflammatory setting in the presence of IFN-\(\gamma\) and TNF, as observed here, and upon infection with certain viruses.

It is well established that FAT10 is inducible by synergistic stimulation with IFN-\(\gamma\) and TNF (Raasi et al., 1999; Lee et al., 2003; Lukasiak et al., 2008; Oliva et al., 2010), what also could be confirmed in this study. Since FAT10 induction in DCs and B cells by other stimuli was reported, too (Bates et al., 1997; Lukasiak et al., 2008; Ebstein et al., 2009), LPS was chosen as a different stimulus to induce \(FAT10\) in the different leukocyte populations. LPS stimulation did not induce or enhance \(FAT10\) expression in most of the human and murine immune cell subsets, except for murine Tregs and human CD8\(^+\) T cells. The general low induction of \(FAT10\) expression upon LPS stimulation could be due to a negative regulation of NF-\(\kappa\)B signalling as has been reported by Buchsbaum and colleagues (Buchsbaum et al., 2012b). Additionally, differential expression of TLR4 and accessory proteins such as CD14 and MD-2 that are necessary for LPS recognition could account for the apparent unresponsiveness of the immune cell subsets and the lack of \(FAT10\) expression (Vaure & Liu, 2014). Despite that, murine Tregs significantly upregulated \(FAT10\) and in human CD8\(^+\) T cells \(FAT10\) expression increased slightly upon LPS stimulation. Regarding murine Tregs, it is possible that \(FAT10\) expression increases as a direct consequence of LPS stimulation or as consequence of Treg activation mediated by LPS, which has been reported previously (Caramalho et al., 2003). Aside of this, LPS can boost activation of only human effector and memory CD8\(^+\) T cells synergistically together with TCR stimulation or IL-12 (Komia-Koma et al., 2009). Thus, similar to murine Tregs, human CD8\(^+\) T cells could express \(FAT10\) because of LPS-mediated activation. The absence of TCR stimulation or IL-12 in the culture system used here could account for the marginal increase of \(FAT10\) expression in CD8\(^+\) T cells. Furthermore, murine and human DCs neither expressed nor enhanced \(FAT10\) expression upon LPS stimulation, contrary to what has been reported repeatedly for human DC generated \textit{in vitro} from CD14\(^+\) monocytes (Lukasiak et al., 2008; Ebstein et al., 2009), and could be observed in murine bone marrow-derived DCs (this study). This together with the lack of \(FAT10\) upregulation in human monocytes upon LPS stimulation suggests that \(FAT10\) induction in \textit{in vitro}-generated DCs is due to DC maturation rather than mediated by LPS. Upregulation of \(FAT10\) upon LPS stimulation could also be a unique feature of inflammatory DCs, which likely origin from monocytes \textit{in vivo} (Segura & Amigorena, 2013). In support of this, the known \textit{in vitro} DC culture models, in which \(FAT10\) was induced after LPS stimulation, are thought to represent inflammatory DCs (Xu et al., 2007; Segura et al., 2013). To sum up, \(FAT10\) expression is
upregulated in inflammatory DCs during maturation, and can be upregulated in murine Tregs and human CD8\(^+\) T cells upon activation. Thus, \textit{FAT10} upregulation occurs independent of stimulus as it increased further upon cytokine stimulation in these cell types.

\textit{FAT10} is very likely involved in antigen presentation as has already been suggested by Bates and colleagues (Bates et al., 1997) and experimentally supported during studies in which \textit{FAT10} was fused N-terminally to viral antigens (Ebstein et al., 2012; Schliehe et al., 2012). Since then a great effort has been put into investigations to elucidate its role in antigen presentation (Basler et al., 2015a). Hence, it was of special interest to analyse \textit{FAT10} expression in professional antigen presenting cells (APCs), such as B cells, DCs, and M&M. Upon stimulation with pro-inflammatory cytokines, i.e. representing an inflammatory situation, \textit{FAT10} was induced strongly in all APCs further supporting a role of \textit{FAT10} in antigen presentation. In fact, human DCs representing myeloid and plasmacytoid DCs, and murine M&M already showed high basal \textit{FAT10} expression. Although this level of expression did not change upon LPS stimulation, it increased further in the presence of pro-inflammatory cytokines. It will be interesting to find out why these distinct APC subsets already express \textit{FAT10} in the absence of inflammation and the other APC subtypes do not.

Another intriguing finding was that apart from human DCs, human naïve CD8\(^+\) T cells and naïve NK&NKT cells expressed \textit{FAT10} at high basal levels. The expression level changed marginally upon LPS stimulation, and insignificantly increased upon cytokine stimulation. This finding suggests that \textit{FAT10} is very likely involved in functions other than antigen presentation. The potential functions that \textit{FAT10} might fulfil in these cell types may be exerted in all of them. Indeed, CD8\(^+\) T cells and NK&NKT cells bear close resemblance to each other (Narni-Mancinelli et al., 2011; Sun & Lanier, 2011). These similarities are, for example, that both cell types undergo an educational process, exert similar effector functions when activated, and are able to form an immune memory. It also cannot be excluded that these potential new functions of \textit{FAT10} are restricted to the human immune system since \textit{FAT10} was not expressed in murine CD8\(^+\) T cells.

In this study, not only cell type-specific but also species-specific differences in basal \textit{FAT10} expression were observed. Most strikingly, antigen-presenting cells differed between human and mouse regarding \textit{FAT10} expression. Human DCs but not murine DCs expressed high levels of \textit{FAT10} and, on the contrary, \textit{FAT10} expression was readily detectable in murine M&M but was absent in human monocytes. Similarly, murine Tregs and human CD8\(^+\) T cells showed basal \textit{FAT10} expression whereas no expression was detected in their human and murine counterparts, respectively. On the one hand, these differences could point towards a differential
expression pattern of \textit{FAT10} in the different immune cell types depending on their tissue location, since peripheral blood was used as a source for human immune cell subsets and murine leukocytes were isolated from spleen. On the other hand, if the observed cell type-specific expression pattern is not tissue-dependent, the discrepancy between the human and murine immune system could suggest that functions of FAT10 might differ between species. Alternatively, this discrepancy might result from exposure to different pathogens of humans as compared to mice, which are kept in a specific pathogen-free environment.

Coming to know which leukocyte populations in humans and mice express \textit{FAT10}, and to what extent its expression is inducible by LPS and the pro-inflammatory cytokines TNF and IFN-\(\gamma\) will avoid overlooking potential new functions of FAT10 relating to the immune system.
Supplementary figure 1 illustrates that transduction with vehicle lentiviruses and mock-transduction did not influence DALIS formation in BMDCs. This figure is complementary to data presented in subchapter 3.1.1 and in figure 12.

Supplementary figure 1: DALIS formation is not influenced in mock- and vehicle-transduced BMDCs. Bone marrow-derived DCs (BMDCs) were generated from FAT10-deficient (KO BMDC) and -proficient (WT BMDC) mice. The progenitor cells were transduced on day 3 of culture and immature BMDCs stimulated or not on day 10 using 400 U/mL TNF and 200 U/mL IFN-γ. KO and WT BMDCs were transduced with vehicle lentiviruses that only express GFP (A) and were mock-transduced (B). Four hours prior to sample preparation for confocal microscopy, 5 µM of MG132 was added to mock- and vehicle-transduced BMDCs similar to the BMDCs shown in figure 12. Immature (0 h) and mature (24 h) transduced BMDCs were stained with anti-ubiquitin and anti-FLAG antibodies. Nuclei were counterstained using DAPI and images were acquired on a LSM880 confocal microscope at 63x magnification. GFP signals were excluded from the merged images in (B) for clarity. Arrows indicated DALIS that formed in vehicle- and mock-transduced cells upon maturation. Representative images of three to four independent experiments are shown. Scale bar is 10 µm.

Supplementary figure 2 shows localisation of FAT10 to aggresomes independent of conjugation and the dependence of ubiquitin on conjugation for aggresome targeting, which has been reported before (Kalveram et al., 2008; Ouyang et al., 2012). Notably, three to four days after transduction with lentiviruses encoding FLAG-tagged ubiquitin increased cell death was observed that was likely due to the overexpression of ubiquitin as reported before (Crinelli et
Supplementary figure 2: FAT10 localises to aggresomes independent of conjugation. (A & B): HeLa cells were transduced using lentiviral vectors encoding FLAG-FAT10 (A) and the non-conjugatable forms of FAT10 and ubiquitin (FLAG-FAT10ΔGG, FLAG-UbiquitinΔGG) (B). (C): Transduction efficiency was determined on day 3 of culture prior to seeding for confocal microscopy by flow cytometric detection of the GFP-reporter expressed by the lentiviruses. The percentage of GFP+ cells is given as mean ± SD. (D): Control cells were not transduced (Mock) or transduced with lentiviruses expressing only GFP (vehicle). Aggresome formation was induced on day 4 by proteasome inhibition using 10 µM MG132 (+MG132). Control cells were left untreated to exclude overexpression artefacts (-MG132). After fixation, samples were stained for ubiquitin and FLAG-tagged proteins. Control samples were stained with secondary antibodies only (D, right column 2nd AB). Nuclei were counterstained with DAPI. Images were acquired using a LSM880 confocal microscope at 63x magnification. Insets highlight aggresomes of interest. For clarity, the GFP channel was excluded from the merged images in (D). Representative images of three independent experiments are shown. Scale bar is 10 µm.
Supplementary figure 3 shows successful blocking of 4F1 using the 4F1 peptide prior to staining of endogenous and overexpressed human FAT10 in HeLa cells for confocal microscopy. Notably, endogenous FAT10 was detectable only as aggregates which probably represent beginning aggresomes since the cells were incubated for five hours with the proteasome inhibitor MG132 to elevate the FAT10 level for better detection. The figure is supplemental to the data presented in chapter 3.4.2 and shows the same results as depicted in figure 31 in a different cell line.

Supplementary figure 3: Immunofluorescence using peptide-blocked 4F1 antibody in HeLa cells. HEK293T cells were mock-transfected (A), transfected with 3xFLAG-FAT10 (B), and induced using 400 U/mL TNF and 200 U/mL IFN-γ (C) for 48 h. Five hours prior to fixation, the cells were incubated with 5 µM MG132 to enrich overexpressed and endogenous FAT10. Cells were stained with unblocked 4F1 and FLAG (M2) antibody, with secondary antibodies only (2nd AB), and with 4F1 antibody blocked with 2,000 molar excess of 4F1 peptide (4F1 blocked). DAPI was used to counterstain nuclei. Images were acquired on a LSM880 confocal microscope at 63x magnification. Representative images of three independent experiments are shown. Scale bar is 20 µm.
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# Abbreviations

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<tr>
<td>A</td>
<td>area</td>
</tr>
<tr>
<td>ACTB</td>
<td>beta actin</td>
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<tr>
<td>AIPL1</td>
<td>aryl hydrocarbon receptor interacting protein-like 1</td>
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<td>AKT</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
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<td>ALFY</td>
<td>autophagy-linked FYVE protein</td>
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<td>ALIS</td>
<td>aggresome-like induced structures</td>
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<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>acridine orange</td>
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<td>KO</td>
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<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
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<td>MG</td>
<td>major histocompatibility complex</td>
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<td>M&amp;M</td>
<td>macrophages and monocytes</td>
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<td>MNSFβ</td>
<td>monoclonal nonspecific suppressor factor beta</td>
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<td>MoaD</td>
<td>molybdenum cofactor biosynthesis protein D</td>
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<tr>
<td>MoDCs</td>
<td>monocyte-derived DCs</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
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<td>MS/MS</td>
<td>tandem mass spectrometry</td>
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<tr>
<td>MTOC</td>
<td>microtubule organising centre</td>
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<tr>
<td>MUB</td>
<td>Membrane anchored ubiquitin fold protein</td>
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<td>MWCO</td>
<td>molecular weight cut-off</td>
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<td>NBR1</td>
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<td>NDP52</td>
<td>nuclear dot protein 52</td>
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<td>NF-κB</td>
<td>nuclear factor kappa-light-chain enhancer of activated B cells</td>
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<td>NHS</td>
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<td>NK&amp;NKT</td>
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<td>NP</td>
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<td>OD&lt;sub&gt;600nm&lt;/sub&gt;</td>
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<td>OTU</td>
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<td>OVA</td>
<td>ovalbumin</td>
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<td>PA</td>
<td>proteasome activator</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<td>PBS</td>
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<td>PMNC(s)</td>
<td>polymorphonuclear cell(s)</td>
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<td>polyI:C</td>
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<td>65-kDa phosphoprotein</td>
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<td>PTM</td>
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<td>qPCR</td>
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<td>standard deviation</td>
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Abbreviations

- Treg(s) regulatory T cell(s)
- TRIM tripartite motif containing
- TRIS Tris(hydroxymethyl) aminomethane
- TU transducing units
- UBA ubiquitin-associated domain
- UBA6 ubiquitin-like modifier-activating enzyme 6
- UBC ubiquitin-conjugating domain
- UBE1 ubiquitin-activating enzyme 1
- UBL ubiquitin-like
- UBL5 ubiquitin-like 5
- UCH ubiquitin C-terminal hydrolase
- UDP ubiquitin domain protein
- UFM1 ubiquitin-fold modifier-1
- ULM ubiquitin-like modifier
- UPR unfolded protein response
- UPS ubiquitin-proteasome system
- URM1 ubiquitin related modifier-1
- USE1 UBA6-specific E2 conjugating enzyme 1
- USP ubiquitin-specific protease
- V voltage
- VPR viral protein R
- VSV-G vesicular stomatitis virus glycoprotein
- W width
- WT wild type
- WTN Wortmannin

Amino acid | Three letter code | One letter code
---|---|---
Alanine | Ala | A
Arginine | Arg | R
Asparagine | Asn | N
Aspartic acid | Asp | D
Cysteine | Cys | C
Glutamic acid | Glu | E
Glutamine | Gln | Q
Glycine | Gly | G
Histidine | His | H
Isoleucine | Ile | I
Leucine | Leu | L
Lysine | Lys | K
Methionine | Met | M
Phenylalanine | Phe | F
Proline | Pro | P
Serine | Ser | S
Threonine | Thr | T
Tryptophan | Trp | W
Tyrosine | Tyr | Y
Valine | Val | V
References


Bialas J, Groettrup M, Aichem A (2015) Conjugation of the ubiquitin activating enzyme UBE1 with the ubiquitin-like modifier FAT10 targets it for proteasomal degradation. PLoS One 10:


Buchsbaum S, Bercovich B, Ciechanover A (2012a) FAT10 is a proteasomal degradation signal that is itself regulated by ubiquitination. Mol Biol Cell 23:225–232


Choi Y, Kim JK, Yoo JY (2014) NFκB and STAT3 synergistically activate the expression of FAT10, a gene countering the tumor suppressor p53. Mol Oncol 8:642–655


References


References


Ferreira JV, Soares AR, Ramalho JS, Pereira P, Girao H (2015) K6<sup>3</sup>linked ubiquitin chain formation is a signal for HIF1A degradation by Chaperone-Mediated Autophagy. Sci Rep 5:


References


Joshi DC, Bakowska JC (2011) Spg20 protein spartin associates with cardiolipin via its plant-related senescence domain and regulates mitochondrial ca2+ homeostasis. PLoS One 6:


Kabeya Y (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19:5720–5728


Koerner J, Brunner T, Groeßtrup M (2017) Inhibition and deficiency of the immunoproteasome subunit LMP7 suppress the development and progression of colorectal carcinoma in mice. Oncotarget 8:50873–50888


Lim CB, Zhang D, Lee CGL (2006) FAT10, a gene up-regulated in various cancers, is cell-cycle regulated. Cell Div 1:


Rani N, Aichem A, Schmidtke G, Kreft SG, Grootert M (2012) FAT10 and NUB1L bind to the VWA domain of Rpn10 and Rpn1 to enable proteasome-mediated proteolysis. Nat Commun 3:


References


