IDENTIFICATION OF CELLULAR SENSORS FOR UNSATURATED FATTY ACIDS

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DEDICATION

I would like to dedicate my thesis to Hyunjin Kim, my lovely wife.

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In mammalian cells fatty acids (FAs) are required for the synthesis of membrane phospholipid components and energy generation. However, overaccumulation of FA is toxic. Accumulation of FAs prevents their further synthesis by stabilization of Insulin-induced gene 1(Insig-1), an ER membrane protein that inhibits proteolytic activation of sterol regulatory element binding protein-1 (SREBP-1), a transcription factor that activates all genes required for FA synthesis. This regulatory reaction is stimulated by unsaturated but not saturated FAs. Unsaturated FAs stabilize Insig-1 through disrupting the interaction between Insig-1 and UBX domain-containing protein 8 (Ubxd8), which recruits p97, a protein required for degradation of endoplasmic-reticulum (ER) membrane proteins, to Insig-1. Here, we identified Ubxd8, a protein that does not contain any previously recognized domains that bind FAs, as a sensor for unsaturated FAs. In cultured cells, we demonstrated that unsaturated FAs but not saturated FAs stimulated polymerization of Ubxd8 through bimolecular fluorescence complementation (BiFC) assays. *In vitro*, unsaturated but not saturated FAs also stimulated polymerization of purified recombinant Ubxd8. The effect of different FAs and their derivatives on polymerization of Ubxd8 *in vitro* correlated with their

effect on stabilization of Insig-1 in cultured cells. Ubxd8 contains 3 domains, namely ubiquitin X (UBX), ubiquitin associated (UBA) and ubiquitin associating (UAS) domain. Deletion analysis showed that UAS domain was necessary and sufficient for polymerization of the protein in response to unsaturated FAs. Point mutations in UAS domain that disrupted its interaction with unsaturated FAs *in vitro* also impaired the ability of full length Ubxd8 to stabilize Insig-1 in response to unsaturated FAs in cultured cells. In addition to Ubxd8, the only other protein expressed in mammalian cells that contains a UAS domain similar to that of Ubxd8 is Fas-associated factor 1 (FAF1). We showed that unsaturated FAs also specifically induced polymerization of FAF1, and this polymerization was mediated by the UAS domain. The identification of UAS domain as a motif polymerizing upon interaction with unsaturated FAs should provide more insights into cellular responses to FAs.

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**equal contribution*

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAA, ATPase associated with various cellular activities
ACC, Acetyl CoA carboxylase
ATGL, Adipose triglyceride lipase
ATK, Arachidonyl trifluoromethyl ketone
BiFC, Bimolecular fluorescence complementation
β-Trcp, F-box/WD repeat-containing protein 1A
CHO, Chinese hamster ovary
CoA, Coenzyme A
DAG, Diacylglycerol
DGAT, Diacylglycerol acyltransferase
E1, Ubiquitin activating enzyme
E2, Ubiquitin conjugating enzyme
E3, Ubiquitin ligase
ER, Endoplasmic-reticulum
ERAD, Endoplasmic-reticulum-associated protein degradation
FA, Fatty acid
FAF1, Fas-associated factor1
FAS, Fatty acid synthase
FCS, Fetal calf serum
FPLC, Fast protein liquid chromatography
IκB, Inhibitor of κB
IKK, IκB kinase
Insig-1, Insulin-induced gene 1
Lro1, Phospholipid:diacylglycerol acyltransferase
NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells
NP-40, Nonidet P-40
PAGE, Polyacrylamide gel electrophoresis
SCAP, Sterol regulatory element-binding protein cleavage-activating protein
SCD, Stearoyl CoA desaturase
SREBP, Sterol regulatory element-binding protein
TG, Triacylglycerol
UAS, Ubiquitin associating
Ub, Ubiquitin
UBA, Ubiquitin associated
UBX, Ubiquitin regulatory X
Ubx2, UBX domain-containing protein 2
Ubxd8, UBX domain-containing protein 8

CHAPTER ONE:

Introduction

1.1 ERAD

Endoplasmic-reticulum-associated protein degradation (ERAD) is a cellular pathway which targets proteins in endoplasmic reticulum (ER) for ubiquitination and subsequent degradation by proteasome, a protein-degrading complex (1). It eliminates misfolded or unassembled proteins from the ER. Molecular chaperones and associated factors recognize and target substrates for retrotranslocation to the cytoplasm, where they are degraded by the ubiquitin-proteasome pathway machinery (2, 3). ERAD substrates are selected by molecular chaperones that identify proteins that might be unable to fold, that fold slowly or contain a misfolded domain, or that lack specific protein partners (2, 4). ERAD substrates are recognized, targeted, retrotranslocated, polyubiquitylated and then degraded by the 26S proteasome (5). Nearly all ERAD substrates are modified with ubiquitin (Ub), a 76 amino-acid peptide that helps target proteins to the proteasome (2). Ub is covalently attached to ε–amino groups of lysine residues on target proteins in a cascade of enzymatic reactions catalyzed by an Ub-activating enzyme (E1), Ub-conjugating enzymes (E2s), and Ub ligases (E3s) (2). In addition to the ubiquitination and proteasomes, ERAD requires p97, which extracts ubiquitinated proteins from the ER membrane to make them accessible to proteasomes (1). P97 is a member of the ATPase associated with various cellular activities (AAA) ATPase family (6). p97 extracts ubiquitinated proteins from the ER membrane to make them accessible for proteasomal degradation (7, 8).

1.2 ERAD of Insig1

In addition to degrade unfolded proteins, ERAD also plays an important role to degrade certain functional ER proteins in a manner that is regulated by small molecule metabolites. One such protein is

Insulin-induced gene-1 (Insig-1), an ER membrane protein that plays a crucial role in feedback regulation of cholesterol and FA synthesis (9). When sterols build up in cells Insig-1 reduces cholesterol synthesis by binding to Sterol regulatory element-binding protein cleavage-activating protein (Scap), a polytopic membrane protein (9). Binding to Scap prevents the activation of Sterol regulatory element-binding proteins (SREBPs), membrane-bound transcription factors that enhance transcription of genes required for cholesterol and FA synthesis and uptake (10, 11). Scap is an escort protein that transports SREBPs from ER to Golgi where the SREBPs are cleaved to release NH_2-terminal fragments from the membrane, allowing them to enter the nucleus to activate their target genes. When Insig-1 binds to Scap, the Scap-SREBP complex is trapped in the ER, and transcription of SREBP target genes declines, leading to a reduction in cholesterol and FA synthesis and uptake (12) (Figure 1.1). In the absence of sterol, gp78, a membrane-bound E3 ubiquitin ligase, binds and add polyubiquitin chains to Insig-1 (13). UBX domain-containing protein 8 (Ubxd8) is a membrane-bound protein that recruits p97 to Insig-1 through its bridging interaction with both proteins (8). p97 complex extracts ubiquitinated proteins from the ER membrane to make them accessible for proteasomal degradation (8). In the presence of sterol, Insig-1 binds to SCAP, which displaces gp78 from Insig-1, thereby inhibiting ubiquitination of Insig-1 (13, 14). However, sterols do not block the interaction between Insig-1 and Ubxd8/p97 complex because the ubiquitination of Insig-1 is not required for this interaction (8). In the presence of unsaturated FAs, interaction between Insig-1 and Ubxd8 is blocked (8). Consequently, p97 dissociates from Insig-1 so that Insig-1 is stabilized. Excess Insig-1 binds to SCAP, blocking the SREBP pathway (8) (Figure 1.2). Mammalian cells also contain a close relative of Insig-1, namely Insig-2 (15). In comparison with Insig-1, Insig-2 has a much longer half-life, and its degradation is not subject to sterol regulation (16, 17). In cultured fibroblasts, Insig-1 is by far the predominant Insig isoform (16). The block in the membrane extraction is attributable to the disruption of the complex between Insig-1 and p97. The complex is disrupted because unsaturated FAs trigger the dissociation of Insig-1 from Ubxd8, a protein that mediates

complex formation between Insig-1 and p97. In addition to sterols, unsaturated FAs have been shown to decrease the proteolytic processing of one isoform of SREBP, namely SREBP-1 (18). Unsaturated FAs block the degradation of Insig-1 so that SREBP cleavage is inhibited by increasing the amount of Insig-1 protein (8). This inhibition occurs at a postubiquitination step (8). Indeed in the presence of unsaturated FAs Insig-1 is ubiquitinated but not extracted from the ER membrane, and therefore it is not degraded (8). The block in the membrane extraction is attributable to the disruption of the complex between Insig-1and p97 (8). The complex is disrupted because unsaturated FAs trigger the dissociation of Insig-1 from Ubxd8, a protein that mediates complex formation between Insig-1 and p97 (8).

1.3 Ubxd8

Ubxd8 was first cloned from CD3-positive blood cells of patients with atopic dermatitis, an inflammatory skin condition characterized by an elevation of eosinophils due to an inappriate immune response (19). Ubxd8 belongs to a family of proteins that contain a ubiquitin regulatory X (UBX) domain, which binds to p97 (20). Some of these proteins (UBXD7, UBXD8, FAF1, SAKS1, and p47) also contain an ubiquitin-associated (UBA) domain at their N termini (21). The UBA domain is known to bind to polyubiquitin chain (20, 21). These proteins are essential components in proteasomal degradation (21). For example, Ubxd7 is known to accelerate proteasomal degradation of hypoxia-inducible factor 1α (HIF-1α) by recruiting p97 to ubiquitinated HIF1-α (21). Likewise, Ubxd8 has been reported to be involved in ERAD (8). In addition to facilitating degradation of Insig-1, Ubxd8 is known to be required for human cytomegalovirus US11 protein-mediated membrane extraction and proteasomal degradation of Class I major histocompatibility complex heavy chains (22). Ubxd8 is also reported to be required for degradation of neurofibromin and ApoB (23, 24).

Ubxd8 appears to play a very important role in regulating triglyceride (TG) metabolism. In cells depleted of FAs, Ubxd8 inhibits TG synthesis by blocking the activity of diacylglycerol acyltransferase

(DGAT) (25). This reaction prevents diversion of FA into TG in cells deprived of FA. In cells exposed to excess FAs, Ubxd8 inhibits the activity of Adipose triglyceride lipase (ATGL), the lipase for TGs, by promoting its dissociation from its coactivator CGI-58 (26). This reaction ensures excess FAs can be converted to TGs for storage in lipid droplets (LDs). In *Saccharomyces cerevisiae*, Ubx2, the yeast homolog of Ubxd8, is important to maintain LDs by increasing TG synthesis through phospholipid:diacylglycerol acyltransferase (Lro1), one of the two TG synthesizing enzymes in *Saccharomyces cerevisiae* (27). This function of Ubx2 may be unique to yeasts as mammalian cells synthesize TGs through DGATs instead of enzymes homologous to Lro1.

In previous study, our lab showed that Ubxd8 is required for the degradation of Insig-1through a process known as ERAD (8). Ubxd8 recruits p97 to Insig-1 through its bridging interaction with both proteins (8). Unsaturated FAs but not saturated FAs block the interaction between Insig-1 and Ubxd8 (8). Consequently, p97 dissociates from Insig-1, and Insig-1 is stabilized (8). The excess Insig-1 binds to Scap, blocking the proteolytic activation of SREBP-1 so that FA synthesis declines (8). However, we don't know the biochemical mechanism by which unsaturated FAs influence the behavior of Ubxd8.

1.4 Scope of the current study

Interaction between Insig-1 and Ubxd8 is regulated by unsaturated FAs even when both proteins are overexpressed (8). This observation suggests that no other protein is limiting for the regulation. Since Ubxd8 also plays an important role in regulating TG metabolism, we hypothesize that Ubxd8 could be a sensor for the FAs. This study aimed to test this hypothesis.

In Chapter 2 of this thesis, I describe the results of the interaction of Ubxd8 with unsaturated but not saturated FAs in a way that correlates with its loss of activity.

In Chapter 3 of this thesis, I identify UAS domain in Ubxd8 as an unsaturated FA-binding domain. In addition, I show that long-chain unsaturated FAs interact with FAF1, another mammalian protein that contains a UAS domain.

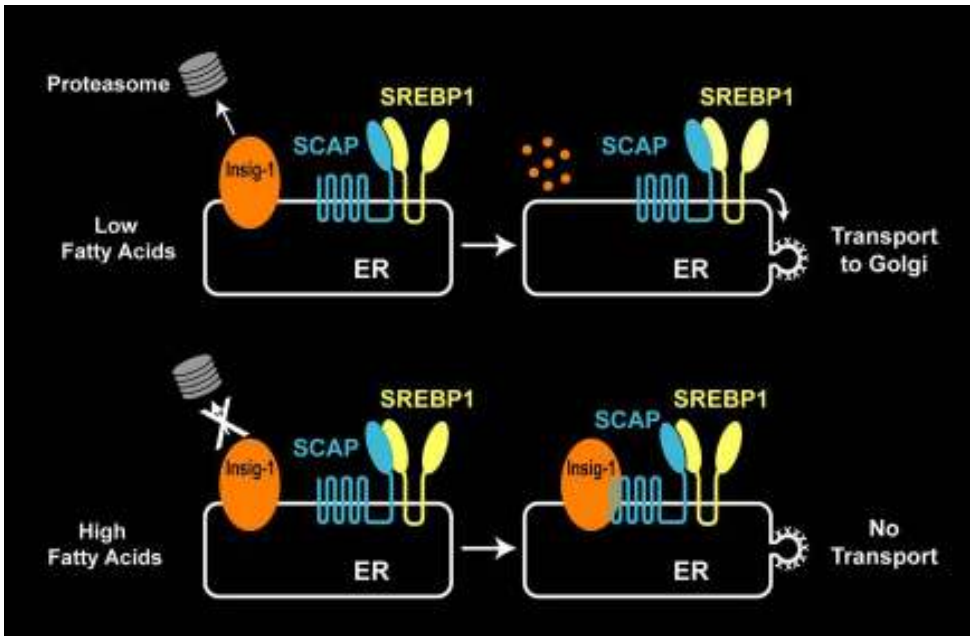


Figure1.1 Feedback Inhibition of SREBP activation Is regulated by Proteasomal degradation of Insig-1. In low cholesterol and FAs, Insig-1 is degraded and the SREBP/SCAP complex moves to the Golgi and SREBP get activated. In high cholesterol or FAs, Insig-1 is stabilized and inhibits SREBP pathway

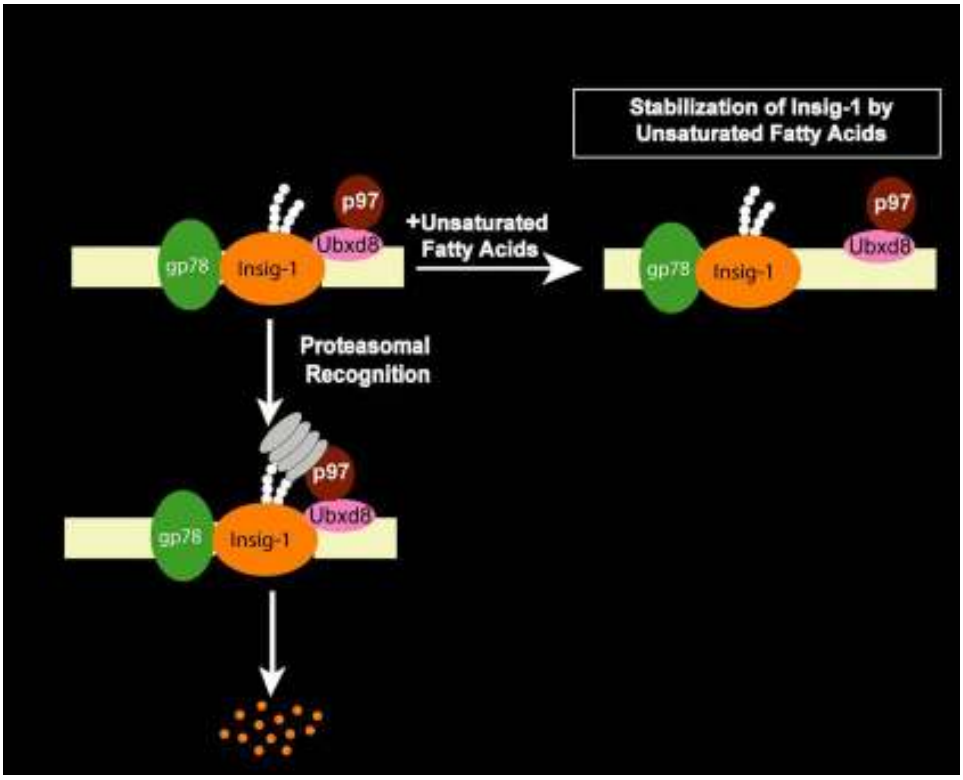


Figure1.2 Unsaturated FAs inhibit degradation of Insig-1. Feedback inhibition of FAs synthesis is achieved at least in part by unsaturated FAs-mediated inhibition of the proteolytic activation of SREBP-1. Unsaturated FAs achieve this regulatory action by blocking degradation of Insig-1. In cells depleted of unsaturated FAs, Insig-1 is ubiquitinated by gp78 and binds to Ubxd8 that mediates the interaction between Insig-1 and p97. Attachment of polyubiquitin chains and recruitment of p97 allow Insig-1 to be recognized and subsequently degraded by proteasomes. This degradation stimulates FAs synthesis by activating proteolytic activation of SREBP-1. Treatment of cells with unsaturated but not saturated FAs blocks the interaction between Insig-1 and Ubxd8, leading to dissociation of p97 complex from Insig-1. Consequently, degradation of Insig-1 is inhibited. The excess Insig-1 inhibits cleavage of SREBP-1, thereby inhibiting FA synthesis. Thus, FA-regulated degradation of Insig-1 plays an important role in feedback inhibition of FA interaction between Ubxd8 and Insig-1.

CHAPTER TWO:

Identification of Ubxd8 as a specific cellular sensor for unsaturated fatty acids

2.1 Abstract

When FAs accumulate in cells, they prevent their further synthesis by inhibition of the proteolytic activation of SREBP-1 which activates all genes required for FAs synthesis. This regulatory action is mediated by unsaturated but not saturated FAs achieved by blocking the degradation of Insig-1, an ER membrane protein that inhibits cleavage of SREBP-1. Unsaturated FAs stabilize Insig-1 by disrupting its interaction with Ubxd8, which recruits p97 to Insig-1. Without p97, Insig-1 is unable to be extracted from membranes and degraded by proteasome. However, the biochemical mechanism by which unsaturated FAs influence the behavior of Ubxd8 remains a mystery. Here, we identify Ubxd8 as a sensor for unsaturated FAs. *In vitro*, unsaturated but not saturated FAs alter the structure of purified recombinant Ubxd8 in a way that correlates with its loss of activity, as monitored by changes in thermal stability, trypsin cleavage pattern and oligomerization state of the protein. In cultured cells, unsaturated FAs but not saturated FAs stimulate polymerization of Ubxd8 as demonstrated by bimolecular fluorescence complementation (BiFC) assays. These results suggest that Ubxd8 is the specific cellular sensor for unsaturated FAs.

2.2 Introduction

In mammalian cells FAs are required for the synthesis of the phospholipid components of membranes and generation of energy. However, overaccumulation of FAs is toxic. When FAs accumulate in cells, they exert regulatory action to prevent their further accumulation (18, 28, 29). They inhibit their own synthesis (18, 28, 29). In general, these regulatory functions are carried out by unsaturated but not saturated FAs (8, 18, 30). The mechanism by which cells specifically sense the level of unsaturated FAs and orchestrate their responses is not understood. Feedback inhibition of FA synthesis is achieved at least

in part by unsaturated FA-mediated inhibition of the proteolytic activation of SREBP-1 (18), a transcription factor that activates all genes necessary to synthesize FAs (11). SREBP-1 is a membrane-bound transcription factor that must be transported from the ER to the Golgi complex where it is proteolytically cleaved so that the NH_2-terminal domain of the protein is able to enter the nucleus to activate its target genes (9, 10). Transport of SREBP-1 is regulated by two ER membrane proteins: 1) Scap, an escort protein that binds SREBP-1 and carries it to the Golgi (31-33); and 2) Insigs, proteins that bind to Scap and retain the Scap·SREBP-1 complex in the ER (12, 15). By blocking Scap/SREBP transport, Insigs are negative regulators of FA synthesis.

Unsaturated FAs regulate FA synthesis by blocking the degradation of Insig-1, the major Insig isoform in cultured cells (16). In FA-depleted cells, Insig-1 is rapidly degraded through a process known as ERAD (1, 2). This degradation requires Ubxd8, a membrane-bound protein that recruits p97 to Insig-1 through its bridging interaction with both proteins (8). Recruitment of p97 is necessary for Insig-1 to be recognized and degraded by proteasomes (34). Unsaturated but not saturated FAs block the interaction between Insig-1 and Ubxd8 (8). Consequently, p97 dissociates from Insig-1 and Insig-1 is stabilized. The excess Insig-1 binds to Scap, blocking the proteolytic activation of SREBP-1 (8).

Our previous studies in cultured cells defined Ubxd8 as a key mediator by which unsaturated FAs inhibit their own synthesis (8). However, the biochemical mechanism by which unsaturated FAs influence the behavior of Ubxd8 remains a mystery. In the current study we develop *in vitro* assays to demonstrate that unsaturated FAs alter the structure of Ubxd8 in a way that correlates with its loss of activity. In cultured cells we also provide direct evidence that long-chain unsaturated FAs also induce polymerization of full length Ubxd8. These results suggest that Ubxd8 is the specific cellular sensor for unsaturated FAs in regulation of Insig1 degradation.

2.3 Materials and Method

Purification of Ubxd8(Δ90-118)

To generate recombinant baculovirus-encoding Ubxd8(Δ90-118) with a His_6-tag at the NH_2 terminus, 2 μg of pAcHLT-Ubxd8(Δ90-118) and 0.5 μg of linealized BaculoGold DNA were transfected into Sf9 cells with Cellfectin reagent (Invitrogen) according to the manufacturer's protocol. Cells were incubated for 5 days at 27°C. Viral amplification was performed twice to obtain a high viral titer, and the recombinant virus stock solution was stored at 4°C.

To purify the protein, 1 liter of sf9 cells at 1×10^6 cells/ml in Sf-900 II SFM insect medium (Invitrogen) was infected with 2 ml stock solution of baculovirus encoding Ubxd8(Δ90-118) for 72 hr at 27°C. All of the procedures following harvest of the cells were carried out at 4°C. Cells collected from the culture were resuspended with buffer A (25 mM Tris-HCl at pH 7.2, 0.15 M NaCl, 1 mM DTT), homogenized, and centrifuged at 100,000 g for 1 hr. The supernatant was applied to a column packed with 2 ml Ni-NTA agarose beads (Qiagen) previously equilibrated with buffer A. The column was washed with 150 ml of buffer A containing 40 mM imidazole. Ubxd8(Δ90-118) was eluted from the column with 4 ml buffer A containing 250 mM imidazole. Imidazole in the eluted material was removed by a disposable PD-10 desalting column (GE Healthcare).

CD Spectroscopy

The CD spectra of Ubxd8 (Δ90-118) were recorded by Aviv model 62DS CD spectrometer (Aviv Associates Inc., Lakewood, N.J.). Ellipticity was measured in 0.5 ml of buffer A containing 2.5 μM of the protein at 222 nm using a 2 mm cell. Thermal denaturation was determined by CD recorded as a temperature scan with temperature ranging from 25 to 95 °C.

Blue Native PAGE

Ubxd8 (Δ90-118) (0.7 μg) was incubated with 100 μM of indicated FAs in buffer A at room temperature for 5 min (final volume, 18 μl). After receiving 2μl of a 10x loading buffer (5mM Bis-Tris,

pH 7.0, 60% glycerol, 0.5 μg/ml Coomassie G250, and 10 mg/ml 6-aminohexanoic acid), the samples were subjected to 4-12% blue native gel electrophoresis for 2 h at room temperature as previously described (35).

Gel Filtration Analysis

Ubxd8 (Δ90-118) (0.4 mg) incubated with or without 100 μM Na Oleate (final volume, 0.4ml) was applied to FPLC using a Superose 6 size exclusion column (Amersham) pre-equilibrated with buffer A supplemented with or without 100 μM Na Oleate at a flow rate of 0.4 ml/min at 4 °C. Absorbance at 280 nm was monitored continuously to identify position of elution of the protein.

Live cell fluorescent microscopy

On day 0, SRD-13A/pUbxd8-Venus cells were set up at 6×10^4 cells per 35-mm glass bottom dish. On day 1, cells were switched to medium A supplemented with 5% delipidated FCS. On day 2, fluorescent images were acquired with EX 492/EM 535nm filter of Deltavision RT microscope in a 37°C chamber before and after incubation for 6 h with the indicated FAs in the same medium. ImageJ (http://rsbweb.nih.gov/ij) was used for quantification of fluorescent intensity. Relative fluorescent intensity was calculated by dividing the intensity after the FA treatment by that before the treatment. The statistical analysis was performed with one tailed paired t-test.

2.4 Results

Unsaturated FAs but saturated FAs alters the structure of purified Ubxd8(Δ90-118)

Ubxd8 is inserted into membranes via a stretch of hydrophobic amino acid residues located between the UBA and UAS domain (amino acid residues 90-118) that forms a hairpin loop in membranes (Figure 2-1A) (29). Surprisingly, deletion of the membrane localization domain from Ubxd8 did not affect interaction between Ubxd8 and Insig-1 in cells deprived of FAs, and this interaction was still inhibited by unsaturated FAs (29). Thus, Ubxd8 (Δ90-118) became a facile tool to study the interaction of

Ubxd8 with FAs *in vitro* because the assay can be performed in the absence of detergents that frequently interfere with the *in vitro* binding assays with FAs. We used the baculovirus expression system in sf-9 insect cells to express human Ubxd8 (Δ90-118) with a His_6-tag at the NH_2 terminus. The recombinant protein was purified to homogeneity using Ni-chromatography. To measure the interaction between FAs and Ubxd8, we first determined whether unsaturated FAs altered the thermal stability of Ubxd8. We analyzed thermal stability by incubating the protein at temperatures ranging from 25-95 °C and measuring circular dichroism (CD) at 222 nm. CD signals recorded at 222 nm are inversely correlated with the secondary structure content of a protein. In the absence of FAs, Ubxd8 (Δ90-118) became completely denatured at ~70 °C (Figure 2-1B, the black line). Incubation with the unsaturated FAs oleate (C18:1) or arachidonate (C20:4) stabilized Ubxd8, shifting the denaturation curve to the right by ~10 °C (Figure 2-1B, red and blue lines). In contrast to unsaturated FAs, the saturated FA palmitate (C16:0) failed to stabilize the protein (Figure 2-1B, the green line).

We also characterized the interaction of unsaturated FAs with Ubxd8 (Δ90-118) by subjecting the protein to partial trypsin digestion in the absence and presence of various FAs. In the absence of FAs, trypsin reduced the amount of the full length protein (58 kDa) with concomitant generation of a protected band at ~35 kDa (Figure 2-1C, lane 2). Incubation with the saturated FAs palmitate (C16:0) or stearate (C18:0) did not change this cleavage pattern (Figure 2-1C, lanes 3-8). In contrast, the cleavage pattern was altered by incubation with the unsaturated FA oleate (C18:1) or arachidonate (C20:4): The amount of full length Ubxd8 (Δ90-118) and 35-kDa fragment was reduced while a new protected band at ~30 kDa appeared (Figure 2-1C, lanes 9-14).

Unsaturated FAs induce the oligomerization of purified Ubxd8(Δ90-118)

To further define the specificity of the interaction between FAs and Ubxd8, we tested the ability of arachidonyl alcohol and arachidonyl trifluoromethyl ketone (ATK) to influence trypsin cleavage of Ubxd8 (Δ90–118). Neither of these compounds changed the trypsin cleavage pattern of Ubxd8 (Δ90–118)

(Fig. 2-2A). In agreement with this *in vitro* result, arachidonate but not arachidonyl alcohol stabilized Insig-1 in cultured cells (Fig. 2-2B). We were unable to determine the *in vivo* effect of ATK because the compound was toxic to cells. These results suggest that in addition to acyl chains, the carboxyl groups of unsaturated FAs also determine their specificity to bind Ubxd8.

To further define the influence of FAs on the structure of Ubxd8, we performed blue native polyacrylamide gel electrophoresis (PAGE), a technique that allows detection of protein complexes in their native state (25, 35). In the absence of FAs, Ubxd8 (Δ90-118) migrated as a band at ~100 kDa (Figure 2-3A, lane 1). Incubation with C12:1(Δ11) or stearate (C18:0) did not change the migration of the protein (Figure 2-3A, lanes 2-3). In the presence of oleate (C18:1) or arachidonate (C20:4), Ubxd8 (Δ90-118) migrated as a broad band at ~700-1000 kDa (Figure 2-3A, lane 4, 5). The calculated molecular mass for Ubxd8 (Δ90-118) is 58 kDa. Thus, the result shown in Figure 2-3A suggests that the protein forms a dimer in the absence of FAs and polymerizes into oligomers that contain at least 12 Ubxd8 (Δ90-118) molecules when treated with unsaturated FAs. This result was confirmed by gel filtration analysis. In the absence of oleate, Ubxd8 (Δ90-118) emerged from the column as a 100 kDa species (Figure 2-3B, blue line). In the presence of oleate, the protein eluted as broad species of ~700 kDa (Figure 2-3B, red line). Oleate did not affect the filtration of molecular weight marker proteins, indicating that the effect of oleate on Ubxd8 (Δ90-118) was specific to this protein. FAs are activated by Co-enzyme A (CoA) before they are incorporated into various lipids. We thus examine the effect of acyl-CoA on oligomerization of Ubxd8 (Δ90–118). As shown in Figure 2-3C, only oleate but not oleoyl-CoA, stearate, or stearoyl-CoA stimulated oligomerization of Ubxd8 (Δ90–118). This result agrees with our earlier observation that the carboxyl groups in unsaturated FAs are important for their interaction with Ubxd8.

Unsaturated FAs induce polymerization of full length Ubxd8 in cultured cells

We then determined whether unsaturated long-chain unsaturated FA induces the polymerization of full length Ubxd8 in cultured cells. In order to perform the experiments, we had to first develop an

assay to measure such polymerization. We chose the approach of BiFC (36) to investigate long-chain unsaturated FA-induced polymerization of Ubxd8. For this purpose, we generated two plasmids encoding full length Ubxd8 fused at its COOH-terminus with either NH_2-terminal or COOH-terminal half of the Venus protein, a variant of yellow fluorescent protein (36) (Figure 2-4A). Cells in which Ubxd8 is not polymerized should not be fluorescent because the NH_2 and COOH-terminal halves of the Venus protein are separated from each other (Figure 2-4A). Polymerization of Ubxd8 should bring the NH_2 and COOH-terminal halves of the Venus protein to close proximity to reconstitute the fluorescent activity of the Venus protein (Figure 2-4A). To use this approach to determine long-chain unsaturated FA-induced polymerization of Ubxd8, we stably transfected SRD-13A cells with these two plasmids and selected a clone of the cells (SRD-13A/pUbxd8-Venus) in which the amount of expression of the Ubxd8 fusion proteins was no more than that of endogenous Ubxd8 (Figure 2-4B). We chose SRD-13A cells because they are mutant Chinese hamster ovary (CHO) cells auxotrophic for unsaturated FAs so that they can be easily depleted of these FAs by incubating them in medium free of FAs (25, 37). These cells were rarely fluorescent when they were incubated in the absence of FAs (Figures 2-4C and D*,* panel 1). Supplementation of the incubation medium with long-chain unsaturated FAs such as oleate (C18:1) or arachidonate (C20:4) dramatically enhanced the fluorescent intensity of the cells (Figures 2-4C and D, panels 2 and 3). Quantification of the fluorescent signal in these images showed that these long-chain unsaturated FAs increased the fluorescent intensity by 6-7 fold (Figure 2-4E). This effect was specific to long-chain unsaturated FAs, as medium-chain unsaturated FA C12:1 and saturated FA palmitate (C16:0) failed to produce the same result (Figures 2-4*C* and D, panels 4 and 5, and 4E).

As a control, we also examined the effect of long-chain unsaturated FAs on interaction between bFos and bJun analyzed through the same bimolecular fluorescence complementation approach (36). SRD-13A cells were transfected with a plasmid encoding NH_2-terminal half of the Venus protein fused with bJun (bJun-VenusN) and a plasmid encoding COOH-terminal half of the Venues protein fused with

either wild type bFos (bFos-VenusC) or a mutant version of the protein in which the bzip domain required for interaction with bJun was deleted (bFos(Δzip)-VenusC) (36). The fluorescent intensity of the cells cotransfected with the plasmid encoding the mutant bFos fusion protein was only 2% of that cotransfected with the plasmid encoding the wild type bFos fusion protein (Figure 2-5A). Treatment with oleate did not affect the fluorescent intensity of the cells expressing the wild type bFos fusion protein (Figure 2-5B). This result suggests that oleate does not enhance fluorescent intensity of the reconstituted Venus protein. Oleate also had no effect on fluorescent intensity of the cells expressing the mutant bFos fusion protein (Figure 2-5C). This observation suggests that oleate does not induce reconstitution of the Venus protein nonspecifically.

2.5 Discussion

The current study reveals that Ubxd8 functions as a sensor for long-chain unsaturated FAs in mammalian cells. Previous study showed that Ubxd8 facilitates the degradation of Insig-1 in cells deprived of FAs, thereby activating FA synthesis by promoting proteolytic processing of SREBP-1 (8). Ubxd8 also blocks TG synthesis by limiting the conversion of DAGs to TGs in these cells as well (25). Thus, in cells deprived of FAs, the concerted regulatory actions of Ubxd8 make FAs available for incorporation into phospholipids by limiting their diversion into TGs (25). When long-chain unsaturated FAs are supplied externally, these FAs change the structure of Ubxd8, promoting its polymerization, and inhibiting its activity. Consequently, Insig-1 is stabilized and FA synthesis decreases. TG synthesis increases so that excess exogenous FAs are stored as TGs in lipid droplets.

The strongest piece of evidence that Ubxd8 is a sensor for long-chain unsaturated FAs comes from the observations: 1) long-chain unsaturated FAs alter the structure of purified Ubxd8 (Δ90-118). Using various techniques, we show that unsaturated FAs alter the thermal stability, trypsin cleavage pattern, and oligomerization state of the protein. This effect is specific to long-chain unsaturated FAs as

saturated FAs, medium-chain unsaturated FAs, or long-chain unsaturated alcohols do not alter the structure of the protein; and 2) long-chain unsaturated FAs not saturated FAs induces the polymerization of full length Ubxd8 in cultured cells. Using BiFC assay, we show that unsaturated FAs enhance the fluorescent intensity of the cells which were stably transfected with Venus fused Ubxd8. This effect is specific to Ubxd8 because oleate does not induce reconstitution of the Venus protein nonspecifically.

Previous study indicates that Ubxd8 inhibits TG synthesis by blocking the conversion of DAGs to TGs and this inhibition is inhibited by unsaturated FAs (25). Conversion of DAGs to TGs is catalyzed by DGAT enzymes (38). DGAT1 and DGAT2 are the only DGAT enzymes characterized in mammalian cells (38). Inasmuch as Ubxd8 is a sensor for unsaturated FAs as well as a protein that facilitates ERAD, we postulate that unsaturated FAs might act through Ubxd8 to regulate degradation of a DGAT or proteins that control its activity. In addition to TG synthesis, Ubxd8 may also control degradation of proteins involved in other metabolic pathways. Thus, identification of proteins whose degradation is regulated by unsaturated FAs through Ubxd8 will help us to better understand cellular responses to FAs.

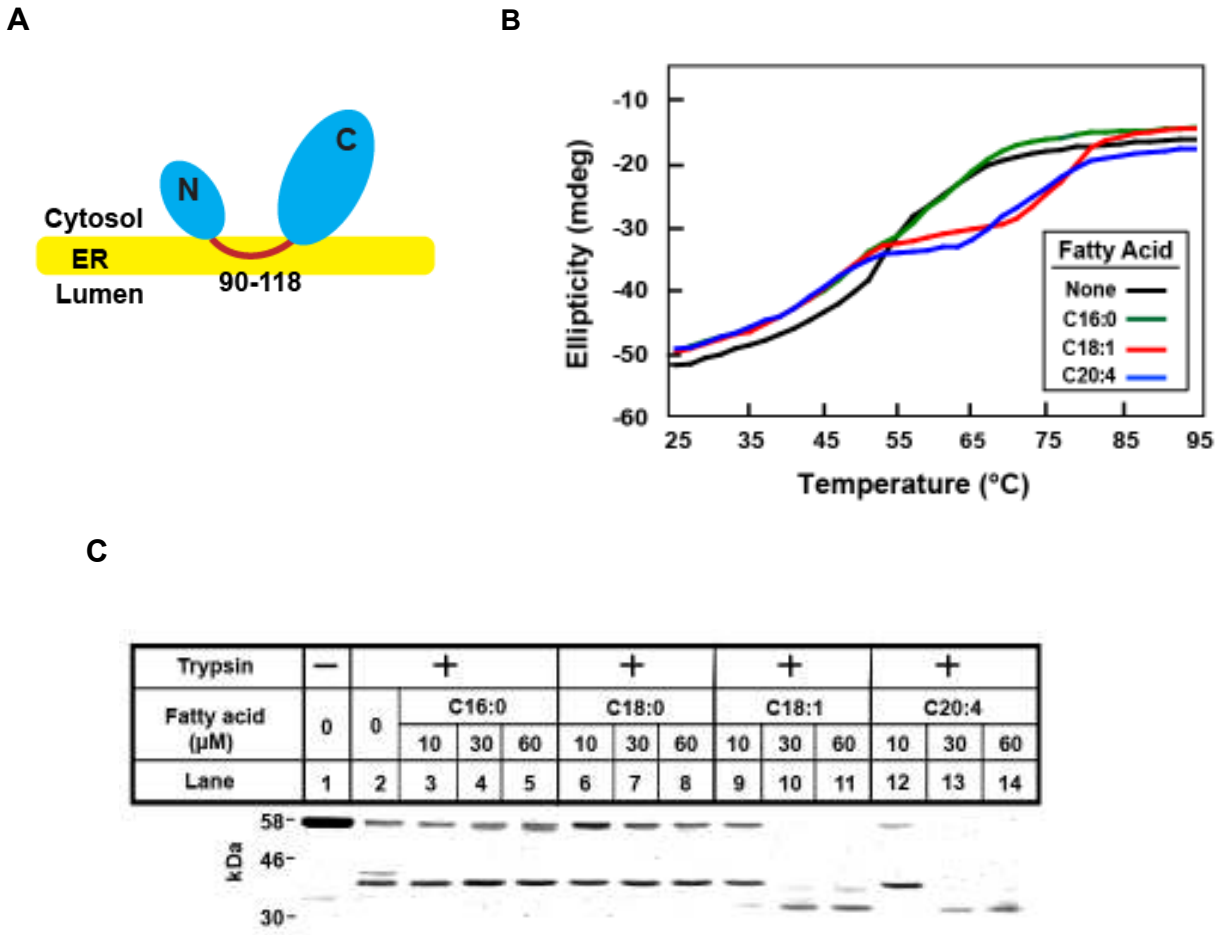


FIGURE 2.1 Unsaturated but not saturated FAs interact with Ubxd8. (A) Proposed membrane topology of Ubxd8. (B) Thermal denaturation of Ubxd8 (Δ90-118) was measured by CD at 222 nm in the absence or presence of 60 μM of indicated FAs added as stock solutions dissolved in ethanol. (C) Purified Ubxd8 (Δ90-118) (7 μg) was incubated with the indicated FAs added as stock solutions dissolved in dimethyl sulfoxide (DMSO) in 0.2 ml buffer A. The protein was then treated with 0.1 μg/ml of trypsin for 20 min at 25 °C, subjected to SDS-PAGE, and visualized by Coomassie blue staining.

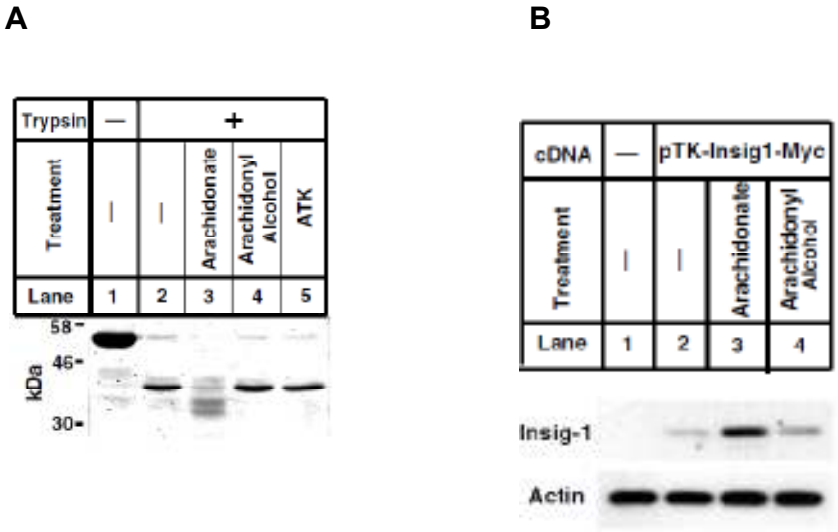


FIGURE 2.2 Specificity for the interaction between long-chain unsaturated FAs and Ubxd8 (Δ90-118). (A) Trypsin digestion of Ubxd8 (Δ90-118) in the presence or absence of indicated FAs was performed and analyzed as described in Figure 1-1C. (B) SRD-13A cells were seeded on day 0 and transfected with 0.4 μg of pTK-Insig1-myc on day 1. Total plasmid concentration was adjusted to 2 μg/dish by using the empty vector pcDNA3.1. Following incubation for 8 h, cells were switched to medium A supplemented with 5% delipidated FCS. On day 2, cells were treated with 60 μm of indicated FAs in medium supplemented with 5% delipidated FCS for 6 h. Detergent lysates of these cells were subjected to SDS-PAGE followed by immunoblot analysis with anti-Myc IgG-9E10 (against Insig-1) and polyclonal anti-actin.

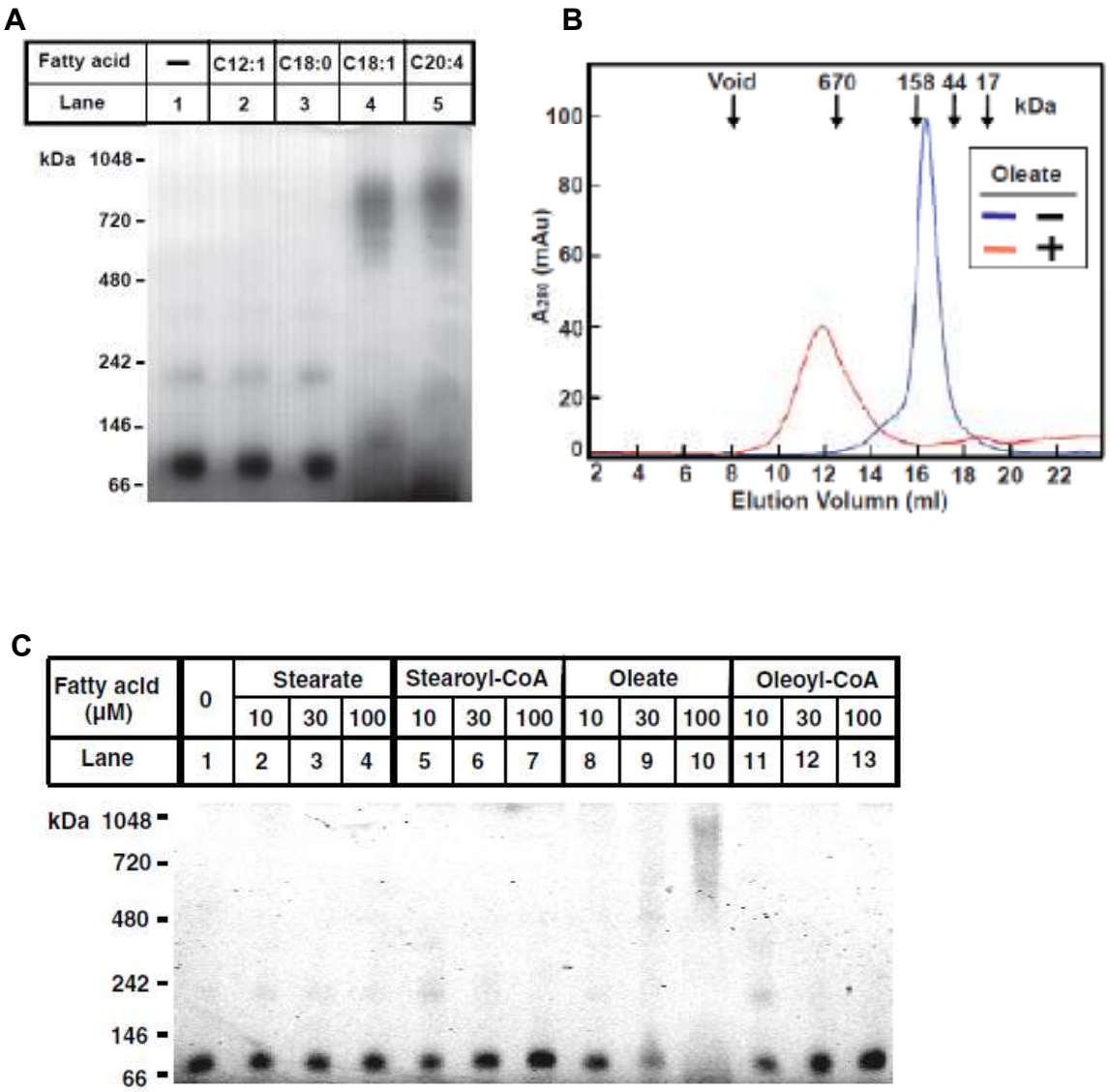


FIGURE 2.3 Long-chain unsaturated FAs induce oligomerization of Ubxd8. (A) Ubxd8 (Δ90-118) (0.7 μg) was incubated with 100 μM of indicated FAs added as stock solutions dissolved in ethanol, subjected to blue native PAGE, and visualized with Coomassie blue staining. Molecular weights for protein standards are indicated. (B) Ubxd8 (Δ90-118) (0.4 mg) incubated with or without 100 μM Na oleate was applied to FPLC using a Superose 6 size exclusion column. Absorbance at 280 nm was monitored continuously to identify position of elution of Ubxd8 (Δ90-118). Standard molecular weight

markers (thyroglobulin, Mr 670,000; ferritin, Mr 440,000; γ globulin, Mr 158,000; and myoglobin, Mr 17,000) were chromatographed on the same column under identical buffer conditions and eluted at the positions shown by arrows. (C) Oligomerization of Ubxd8 (Δ90-118) in the presence of indicated FAs or their derivatives was determined by blue native PAGE as described in Figure 2-3 A.

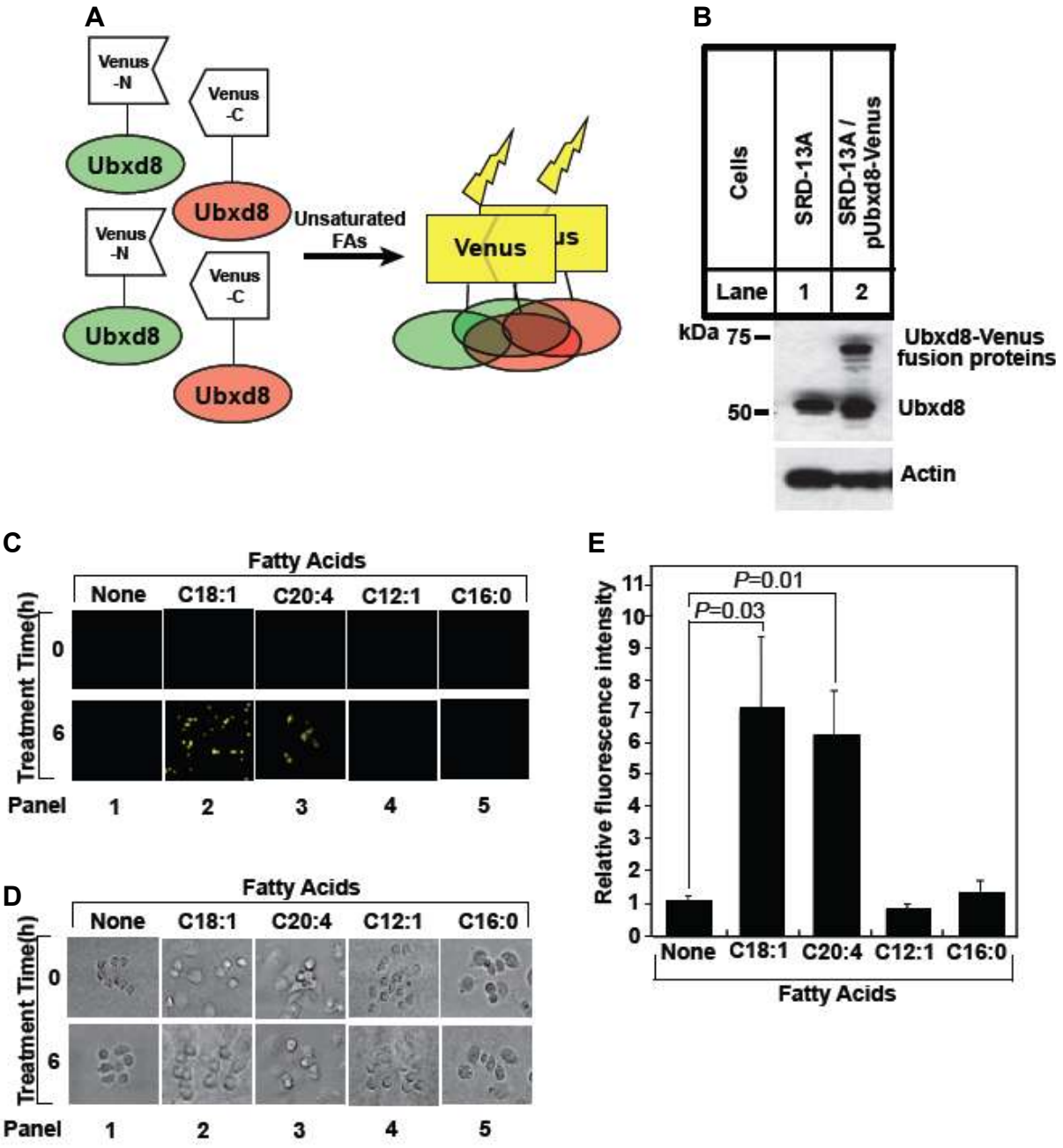


FIGURE 2.4 Long-chain unsaturated FAs induce polymerization of full length Ubxd8 in cells. (A) Principle of using bimolecular fluorescence complementation to analyze unsaturated FA induced polymerization of Ubxd8. (B) Immunoblot analysis of indicated cells. (C and D) Fluorescent (C)

and bright field images (D) of SRD-13A /pUbxd8-Venus cells before and after 6 h treatment with 100 μM of indicated FAs. (E) Relative fluorescent intensity calculated by dividing fluorescent intensity after the FA treatment by that before the treatment shown in c. Results were reported as mean ± S.E. from three independent experiments.

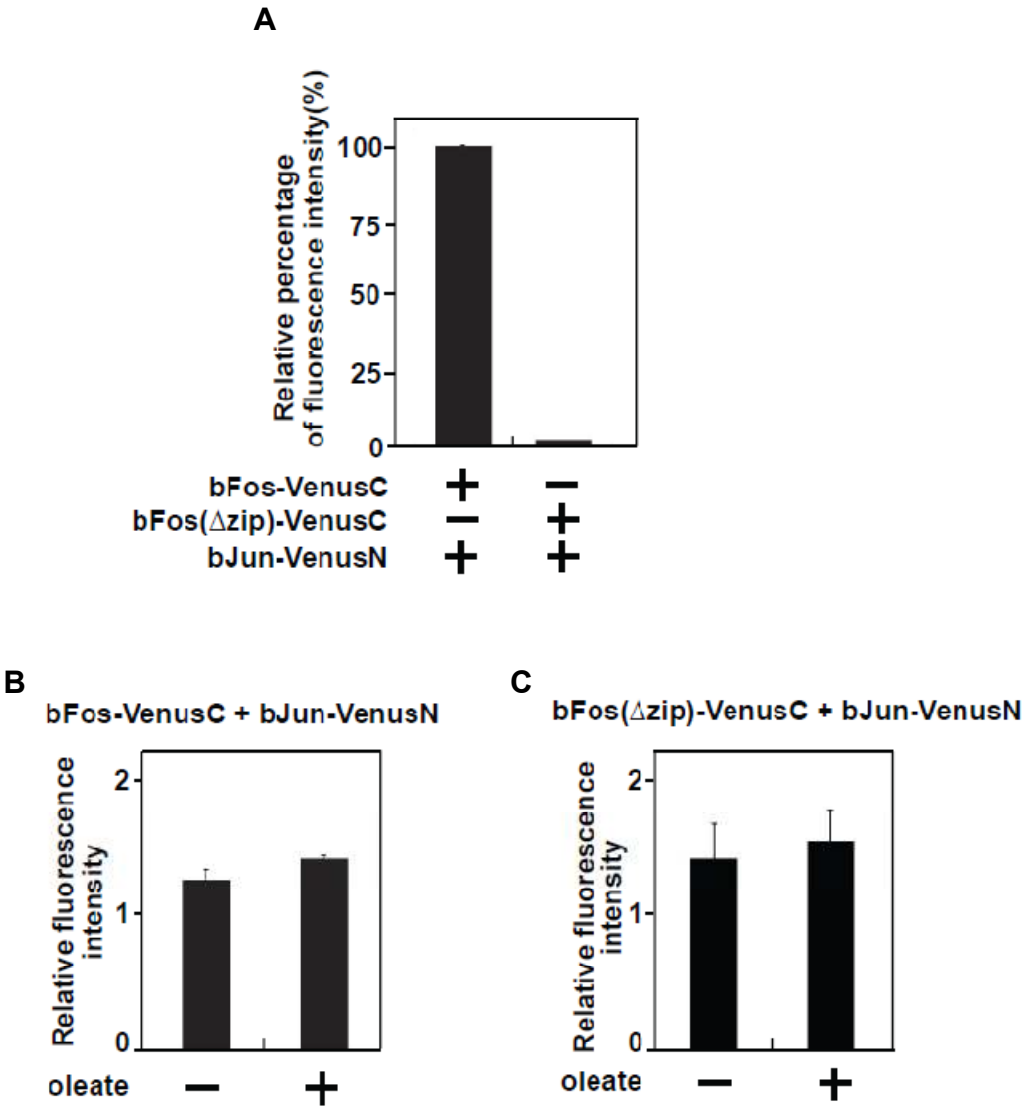


FIGURE 2.5 Oleate does not affect bimolecular fluorescence complementation between bFos and bJun. (A) Fluorescent intensity of SRD-13A cells transiently transfected with indicated plasmids. The fluorescent intensity of the cells transfected with the plasmids encoding bJun-VenusN and bFos-VenusC is set to 100%. (B and C) The effect of oleate on relative fluorescent intensity of the cells transfected with the indicated plasmids was quantified as described in Figure 2-4 E.

CHAPTER THREE:

Identification of UAS Domain as a Motif Polymerizing upon Interaction with Unsaturated FAs

3.1 Abstract

Ubxd8 has been reported to play a crucial role to maintain cellular homeostasis of FAs. The protein functions as a sensor for unsaturated FAs because it polymerizes upon interaction with these FAs. However, the molecular mechanism involved in this polymerization remains unclear. Here we report that this polymerization is mediated by the Ubiquitin associating (UAS) domain, a motif present in Ubxd8 without previously identified functions. We show that a positively charged surface area in the domain is required for the reaction. Mutations changing the positively charged residues in this area to glutamates prevented unsaturated FAs from inducing oligomerization of Ubxd8 and from regulating the activity of Ubxd8 in cultured cells. Unsaturated FAs also induced polymerization of FAF1, another mammalian protein containing a UAS domain. These results suggest that proteins containing a UAS domain may subject to regulation by unsaturated FAs.

3.2 Introduction

FAs are crucial nutrients for cell survival yet their overaccumulation is toxic to cells. Thus, cells develop multiple pathways to maintain their homeostasis. One of the pathways is feedback inhibition in FA synthesis. We have previously identified Ubxd8 as a key regulator for the reaction (25). In cells depleted of FAs, Ubxd8 facilitates proteasomal degradation of Insig-1, a membrane protein of the endoplasmic reticulum (ER), through its interaction with the protein (8). Depletion of Insig-1 triggers proteolytic activation of SREBP-1, a transcription factor synthesized as a membrane-bound precursor 1, (12). This activation allows the NH_2-terminal domain of SREBP-1 to activate all genes required for FA synthesis (11). Excessive unsaturated FAs block the interaction between Ubxd8 and Insig-1, leading to

stabilization of Insig-1 (8). Consequently, proteolytic activation of SREBP-1 is inhibited and transcription of genes involved in FA synthesis declines (8, 11, 39).

Ubxd8 belongs to a family of proteins that contain a UBX domain (Figure3.1A) (19), which interacts with p97 (20), a protein required for proteasomal degradation of ER-associated membrane proteins (40). In addition to the UBX domain, Ubxd8 also contains a UBA domain that is known to bind polyubiquitin chains (20), and a UAS domain with unknown function (Figure3.1A). Ubxd8 is inserted into membranes via a stretch of hydrophobic amino acid residues located between the UBA and UAS domain (amino acid residues 90-118) that forms a hairpin loop in membranes (Figure3.1A) (25). Surprisingly, deletion of the membrane localization domain from Ubxd8 did not affect interaction between Ubxd8 and Insig-1 in cells deprived of FAs, and this interaction was still inhibited by unsaturated FAs (25). Thus, Ubxd8 (Δ90-118) became a facile tool to study the interaction of Ubxd8 with FAs *in vitro* because the assay can be performed in the absence of detergents that frequently interfere with the *in vitro* binding assays with FAs. This analysis revealed that long-chain unsaturated FAs stimulated polymerization of purified recombinant Ubxd8 (Δ90-118) (25). This effect was specific to long-chain unsaturated FAs as long-chain saturated or median-chain unsaturated FAs failed to produce the same effect (25). This specificity matches the specificity of FAs to stabilize Insig-1 in cultured cells (8, 25). Thus, the *in vitro* characterization performed with Ubxd8 (Δ90-118) is likely to be a good indication of the full length Ubxd8 protein inside cells.

In the current study, we demonstrate that polymerization of Ubxd8 is mediated through the UAS domain present in Ubxd8. Moreover, we show that long-chain unsaturated FAs also specifically stimulate polymerization of FAF1, another mammalian protein that contains a UAS domain. These observations suggest that proteins containing UAS domains may subject to regulation by FAs.

3.3 Materials and Method

Materials

We obtained FAs from NU-CHEK-PREP, INC.; FA-free bovine serum albumin from Roche Applied Science ; MG132 and Nonidet P-40 alternative (Nonidet P-40) from Calbiochem; monoclonal anti-T7 from Novagen; horseradish peroxidase-conjugated donkey anti-mouse and anti rabbit IgGs (affinity-purified) from Jackson ImmunoResearch Laboratories; and Ni-NTA agarose from Qiagen. Hybridoma cells producing IgG-9E10, a mouse monoclonal antibody against Myc tag, were obtained from the American Type Culture Collection. FCS was prepared from newborn calf serum by n-butyl alcohol and isopropyl ether extraction method (18). All FAs added into culture media were conjugated to bovine serum albumin (18). For *in vitro* assays, FAs dissolved in ethanol were added into the in vitro assays. The final concentration of ethanol in the reaction mixture was 1% for these assays.

Plasmid constructs

The following plasmids were described in the indicated references: pCMV-Myc-Ubxd8 encoding human Ubxd8 with a c-Myc tag at its NH_2.terminus under control of the CMV promoter (8); pCMV-Insig1-T7 encoding human Insig-1 followed by three tandem copies of a T7 epitope tag under control of the CMV promoter (17); and pAcHLT-Ubxd8 (Δ90–118) used for producing Ubxd8 (Δ90–118) in sf9 cells through recombinant baculovirus (25). pAcHLT-Ubxd8 (Δ90-118, Δ360-445), pAcHLT-Ubxd8 (Δ90-118, Δ278-445), pAcHLT-Ubxd8 (122-277), pAcHLT-Ubxd8 (1-66), pAcHLT-FAF1 and pAcHLT-FAF1 (325-491) was generated to produce indicated fragments of Ubxd8 or FAF1 in sf9 cells through recombinant baculovirus as previously described (25). pCMV-5myc-Ubxd8-VenusN encodes human Ubxd8 tagged at the NH_2-terminus with 5 tandem copies of a c-Myc tag fused at the COOH-terminus with the NH_2-terminal fragment of the Venus protein under control of the CMV promoter, and a neomycin resistant gene. pCMV-flag-Ubxd8-VenusC encodes NH_2-terminal flag epitope-tagged human

Ubxd8 fused at the COOH-terminus with the COOH-terminal fragment of the Venus protein under control of the CMV promoter, and a hygromycin resistant gene. The NH_2- and COOH-terminal fragments of the Venus gene were amplified through PCR from pBiFC-VN155 (I152L) and pBiFC-VC155 (Addgene), respectively. pBiFC bFos-VC155 encoding bFos fused with the COOH-terminal fragment of the Venus protein, pBiFC bJun-VN155 (I152L) encoding bJun fused with the NH_2-terminal fragment of the Venus protein, and pBiFC bFos(Δzip)-VC155 encoding a mutant bFos in which the bZIP domain was deleted fused with the NH_2-terminal fragment of the Venus protein were all acquired from Addgene.

Cell culture

All cells were maintained at 37 °C in 8.8% CO_2. SRD-13A cells are a clone of mutant CHO cells deficient in Scap (37). They were maintained in medium A (1:1 mixture of Ham's F-12 medium and DMEM, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate) supplemented with 5% (v/v) FCS, 5 μg/ml cholesterol, 1 mM sodium mevalonate, and 20 μM sodium oleate. SRD-13A/pUbxd8-Venus cells were generated by stably transfecting pCMV-5myc-Ubxd8-VenusN and pCMV-flag-Ubxd8-VenusC into SRD-13A cells. The cells were maintained in medium used to culture SRD-13A cells supplemented with 2.5 mg/ml hygromycin and G418.

Transient transfection

On day 0, SRD-13A cells were set up for experiments at 4.5×10^5 cells per 60-mm dish. On day 1, cells were transiently transfected with the indicated plasmids with FuGENE6 reagent (Roche Applied Science) according to the manufacturer's protocol. Conditions of incubation after the transfection were described in the figure legends.

Immunoblot analysis

Aliquots of the lysate were subjected to SDS/PAGE followed by immunoblot analysis. Antibodies used in the current studies were IgG-9E10 (1 μg⁄ml), a monoclonal anti-T7 (0.4 μg⁄ml), a polyclonal anti-Ubxd8(1 μg⁄ml), a polyclonal anti-actin (1:2000 dilution). Horseradish peroxidase-

conjugated donkey anti-mouse and anti-rabbit IgGs (0.2 μg⁄ml) were used as the secondary antibody in all immunoblot analysis. Bound antibodies were visualized by chemiluminescence using the SuperSignal substrate system (Pierce) according to the manufacturer's instructions.

Immunoprecipitation

Co-immunoprecipitation experiments to determine the interaction between Ubxd8 and Insig-1 was performed as previously described (8). Briefly, the pooled cell pellets from three 60-mm dishes were lysed in buffer A (25 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1% NP-40, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 μg/ml aprotinin, and 25 μg/ml N-acetylleucinal-leucinal-norleucinal), and incubated with 30 μl Anti-c-Myc Affinity Gel (Sigma) to immunoprecipitate transfected Ubxd8. Aliquots of pellet and supernatant fractions of the reaction were subject to SDS-PAGE followed by immunoblot analysis as described in the figure legend.

Live cell fluorescent microscopy

On day 0, SRD-13A/pUbxd8-Venus cells were set up at 6×10^4 cells per 35-mm glass bottom dish. On day 1, cells were switched to medium A supplemented with 5% delipidated FCS. On day 2, fluorescent images were acquired with EX 492/EM 535nm filter of Deltavision RT microscope in a 37°C chamber before and after incubation for 6 h with the indicated FAs in the same medium. ImageJ (http://rsbweb.nih.gov/ij) was used for quantification of fluorescent intensity. Relative fluorescent intensity was calculated by dividing the intensity after the FA treatment by that before the treatment. The statistical analysis was performed with one tailed paired t-test.

Blue native PAGE analysis of purified proteins

Proteins were purified, incubated with indicated FAs, and analyzed by blue native PAGE as previously described (25). Briefly, wild type or mutant Ubxd8 or FAF1 proteins were expressed in sf9 cells and purified to homogeneity with Ni^{2+}-affinity followed by size exclusion chromatography using Superdex 200 (10/300GL) column (GE Healthcare). Purified proteins (0.7 μg) were incubated with FAs

(added as stock solutions dissolved in ethanol) in buffer B (25 mM Tris-HCl at pH 7.2, 0.15 M NaCl, 1 mM DTT) at room temperature for 5 min (final volume, 18 µl). After receiving 2µl of a 10x loading buffer (5mM Bis-Tris, pH 7.0, 60% glycerol, 0.5 µg/ml Coomassie G250, and 10 mg/ml 6-aminohexanoic acid), the samples were subjected to 4-12% blue native gel electrophoresis for 3 h at 4 °C.

Circular Dichroism(CD)

Purified proteins (1.3 µM) in 0.5 ml buffer B were measured for CD spectrum. The spectra were recorded at wavelength ranging from 198-250 nm by a J-815 CD spectrometer (JASCO Inc.).

Structural modeling of the UAS domain in Ubxd8

The structure model of the UAS domain in Ubxd8 was generated by using the NMR structure of the FAF1-UAS domain (PDB ID: 2EC4_A) as the template with programs HHPred (41) and Modeller (42) incorporated at the MPI Bioinformatics Toolkit web server (43).

3.4 Results

Unsaturated FAs induce polymerization of proteins containing a UAS domain

In order to identify the region in Ubxd8 that is required for long-chain unsaturated FAs to induce polymerization of the protein, we made various deletion mutants in Ubxd8 (Δ90-118) fused with a His_6-tag at the NH_2 terminus, purified the recombinant protein expressed in sf9 cells to homogeneity using Ni^{2+}-affinity followed by size exclusion chromatography, and analyzed the effect of FAs on polymerization of the proteins through blue native PAGE, a technique that allows detection of protein complexes in their native state (25, 35). Deletion of the Ubx domain at the COOH-terminal end of the protein (amino acid residues 360-445) (Figure 3-1A) did not affect oleate (C18:1) and arachidonate (C20:4), two classes of long-chain unsaturated FA, to induce polymerization of Ubxd8 (Δ90-118) (Figure 3-1B, *lanes 4 and 5*). Similar to Ubxd8 (Δ90-118), this polymerization is specific to long-chain unsaturated FAs as medium-chain unsaturated FA dodecenoate (C12:1) and long-chain saturated FA

stearate (C18:0) failed to produce the same effect (Figure 3-1B*, lanes 2 and 3*). This polymerization remained intact when a stretch of 82 amino acids located between the UAS and Ubx domain (amino acid residues 278-359) was also deleted (Figure 3-1C). These results suggest that the remaining two domains in Ubxd8, namely the UBA and UAS domains (Figure 3-1A), may be required for this polymerization. While long-chain unsaturated FAs failed to induce oligomerization of the purified UBA domain (amino acid residues 1-66) (Figure 3-1D), they were effective in stimulating polymerization of the purified UAS domain (amino acid residues 122-277) (Figure 3-1E).

If UAS domain is a motif that polymerizes upon interaction with long-chain unsaturated FAs, proteins other than Ubxd8 that contain a UAS domain should also polymerize in response to these FAs. A database search revealed that human cells express FAF1, a cytosolic protein containing a UAS domain (44) that is 27% identical to that of human Ubxd8 (Figure 3-2A). We thus expressed the UAS domain of FAF1 and the full length FAF1 protein in sf9 cells and purified them to homogeneity. Blue native PAGE analysis indicated that long-chain unsaturated FAs also specifically induced polymerization of the UAS domain of FAF1 (FAF1 (325-491)) (Figure 3-2B) as well as the full length FAF1 protein (Figure 3-2C).

A positively charged surface on the UAS domain is required for unsaturated FAs to induce polymerization of Ubxd8 (Δ90-118)

While the structure of the UAS domain in Ubxd8 remains unsolved, the structure of the same domain in FAF1 has been determined through nuclear magnetic resonance (PDB ID: 2EC4_A/ unpublished). According to the structure, there is a surface area highly enriched in positively charged residues (Figure 3-3A, *left panel*). Structural model built on the homology of the UAS domain between Ubxd8 and FAF1 (Figure 3-3A) predicts that the same positively charged area also exists in Ubxd8, and this area is made by two adjacent loops, each of which contains 3 positively charged residues (*i.e.*, K167, R168 and R171 in one loop and K239, R241, and R242 in the other loop) (Figure 3-3A*, right panel*). Since the negatively charged carboxyl group is required for long-chain unsaturated FAs to interact with

purified Ubxd8 (Δ90-118) (25), the positively charged residues in this area may be important for the interaction. To test this hypothesis, we mutated these lysines and arginines to negatively charged glutamates, and analyzed the effect of the mutations on long-chain unsaturated FA-induced polymerization of Ubxd8 (Δ90-118). Arachidonate was less effective in inducing polymerization of Ubxd8 (Δ90-118, K167E, R168E, R171E) and Ubxd8(Δ90-118, K239E, R241E, R242E) compared to that of Ubxd8(Δ90-118), but the effect was rather modest (Figure 3-3B, *lanes 1 to 6*). This polymerization was more pronouncedly inhibited by combined mutations of all six positively charged residues to glutamates (Figure 3-3B*, lanes 7 and 8*). To further determine the effect of the mutation on long-chain unsaturated FA-induced oligomerization of Ubxd8 (Δ90-118), we incubated Ubxd8 (Δ90-118) and Ubxd8 (Δ90-118, K167E, R168E, R171E, K239E, R241E, R242E) with various concentrations of oleate or arachidonate. Replacing all of the positively charged residues in the surface patch to glutamates not only inhibited polymerization induced by arachidonate (Figure 3-3C) but also that induced by oleate (Figure 3-3D). Ubxd8 (Δ90-118, K167E, R168E, R171E, K239E, R241E, R242E) displayed a CD spectrum identical to that of Ubxd8 (Δ90-118), an observation suggesting that the mutation did not alter the global folding of the protein (Figure 3-3E).

Unsaturated FAs are unable to inhibit degradation of Insig-1 in cells expressing the mutant Ubxd8

To determine the effect of the mutation on functions of the full length Ubxd8 in cultured cells, we made the same mutation (K167E, R168E, R171E, K239E, R241E and K242E) in full length Ubxd8. We stably transfected SRD-13A cells with plasmids encoding the mutant Ubxd8 fused with NH_2 and COOH-terminal fragment of the Venus protein, and selected a clone of the cells (SRD-13A/pUbxd8(mutant)-Venus) in which expression of the mutant Ubxd8 fusion proteins was similar to that of wild type Ubxd8 fusion proteins found in SRD-13A/pUbxd8-Venus cells (Figure 3-4A). We determined the effect of the mutation on long-chain unsaturated FA-induced polymerization of the protein through bimolecular

fluorescence complementation. While arachidonate and oleate markedly enhanced the fluorescent intensity of the cells transfected with the wild type Ubxd8-Venus fusion proteins (SRD-13A/pUbxd8-Venus), these FAs failed to increase the fluorescent intensity of the cells transfected with the mutant Ubxd8-Venus fusion proteins (SRD-13A/pUbxd8(mutant)-Venus) (Figure 3-4B). This result suggests that long-chain unsaturated FAs are unable to induce polymerization of the mutant Ubxd8 in cultured cells.

The lack of the response of the mutant Ubxd8 to long-chain unsaturated FAs may be caused by a specific defect in interaction with these FAs or a global defect in protein folding. If the mutation specifically disrupts the interaction with long-chain unsaturated FAs, the mutant protein should still be able to bind Insig-1and to stimulate its degradation in FA-depleted cells, but the effect can no longer be inhibited by these FAs. On the other hand, if the mutation affects protein folding, the mutant protein should not be functional so that the protein is not expected to bind or degrade Insig-1. To differentiate these possibilities, we first examined the interaction between Insig-1 and the mutant Ubxd8. For this purpose, we transfected SRD-13A cells with a plasmid encoding Insig-1 together with a plasmid encoding either wild type or the mutant Ubxd8, treated the cells with the proteasome inhibitor MG132 to block degradation of Insig-1, and determined their interaction through a co-immunoprecipitation experiment. Insig-1 was co-immunoprecipitated with wild type Ubxd8 in cells depleted of FAs but not those treated with arachidonate (Figure 3-4C*, lanes 3 and 4*). Insig-1 was also co-immunoprecipitated with the mutant Ubxd8 but the amount of Insig-1 co-immunoprecipitated was no longer reduced by treatment with arachidonate (Figure 3-4C*, lanes 5 and 6*).

We then determined the effect of the mutant Ubxd8 on degradation of Insig-1. To this end, we transfected SRD-13A cells with a plasmid encoding Insig-1 with or without cotransfection with a plasmid encoding either wild type or the mutant Ubxd8. Arachidonate increased the amount of Insig-1 protein (Figure 3-4D, *lanes 3 and 4*). This effect was not altered by cotransfection with a plasmid encoding wild type Ubxd8 (Figure 3-4D*, lanes 5 and 6*). In contrast to these results, arachidonate was unable to increase

the amount of Insig-1 protein in cells cotransfected with a plasmid encoding the mutant Ubxd8 (Figure 3-4D*, lanes 7 and 8*), an observation suggesting that the mutant Ubxd8 stimulated degradation of Insig-1 even in cells treated with arachidonate. The results shown in Figure 3-4C and 3-4D suggest that the mutation specifically disrupts the interaction of the protein with long-chain unsaturated FAs without affecting the global folding of the protein.

3.4 Discussion

The current study identifies UAS domain as a motif polymerizing upon interaction with long-chain unsaturated FAs. This observation suggests that proteins containing this domain may subject to regulation by these FAs. Indeed, Ubxd8 is such a protein. We show that long-chain unsaturated FAs induce polymerization of Ubxd8 to prevent the protein from stimulating degradation of Insig-1. This reaction is mediated by the UAS domain in Ubxd8, as unsaturated FAs stimulated polymerization of the purified recombinant UAS domain. The importance of the domain is further demonstrated by the observation that unsaturated FAs were unable to stabilize Insig-1 in cells expressing a mutant version of Ubxd8 in which critical residues in the UAS domain required for these FAs to induce oligomerization of Ubxd8 were mutated.

FAF1 is another human protein containing a UAS domain. We show that long-chain unsaturated FAs also stimulated polymerization of purified recombinant FAF1 through the UAS domain. FAF1 has diverse functions (45), among which is a regulator of NF-κB (46-48), a transcription factor that activates proinflammatory cytokines (49). It was reported previously that saturated but not unsaturated FAs activated production of proinflammatory cytokines through activation of NF-κB (30, 50, 51). The human genome also encodes Ubxd7 that contains a putative UAS domain remotely resembling that of Ubxd8 (21). Ubxd7 facilitates degradation of HIF-1α (21), a transcription factor activating genes in response to hypoxia (52). Ubxd7 also functions as an activator for cullin-RING ubiquitin ligases (53). Exactly how

long-chain unsaturated FAs interact with the UAS domain to drive its polymerization remains unclear. The structure of the UAS domain in FAF1 does not reveal any binding pocket for the FAs. Inasmuch as a positively charged surface area is required for the UAS domain to interact with the FAs, it is conceivable that these FAs may bind to the surface of the domain to increase its hydrophobicity so that the domain can polymerize. Obviously, structural analysis of the UAS domain in the presence of unsaturated FAs is required to address this question. However, such analysis is technically challenging owing to the heterogeneous size of unsaturated FA-induced UAS domain oligomers.

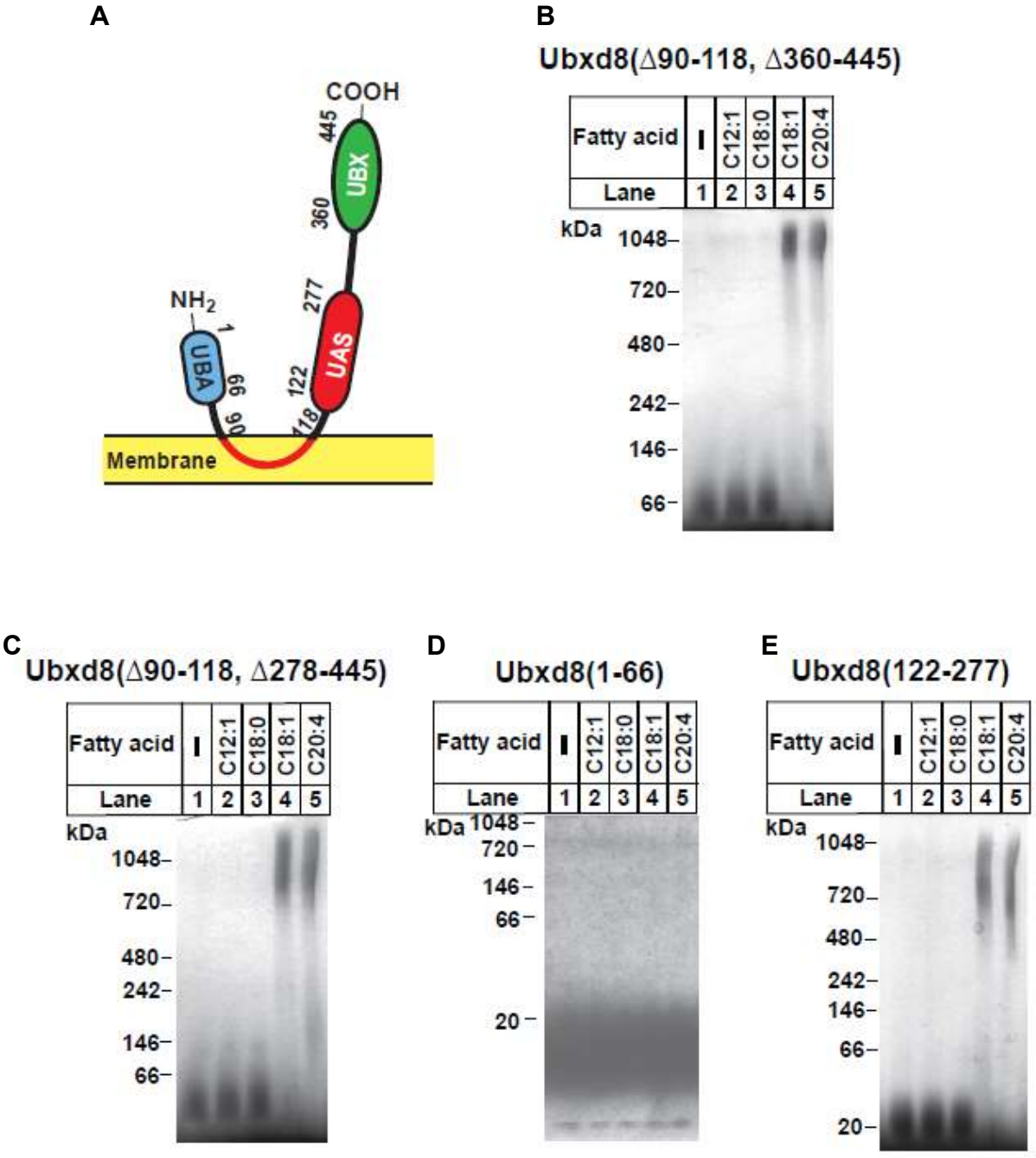


FIGURE 3.1 Long-chain unsaturated FAs induce polymerization of purified UAS domain in Ubxd8. (A) Schematic diagram of the domain structure of Ubxd8. (B-E) Indicated purified proteins were incubated with 100 μM of indicated FAs, subjected to blue native PAGE, and visualized with

Coomassie blue staining as described in Figure 2-2 A.

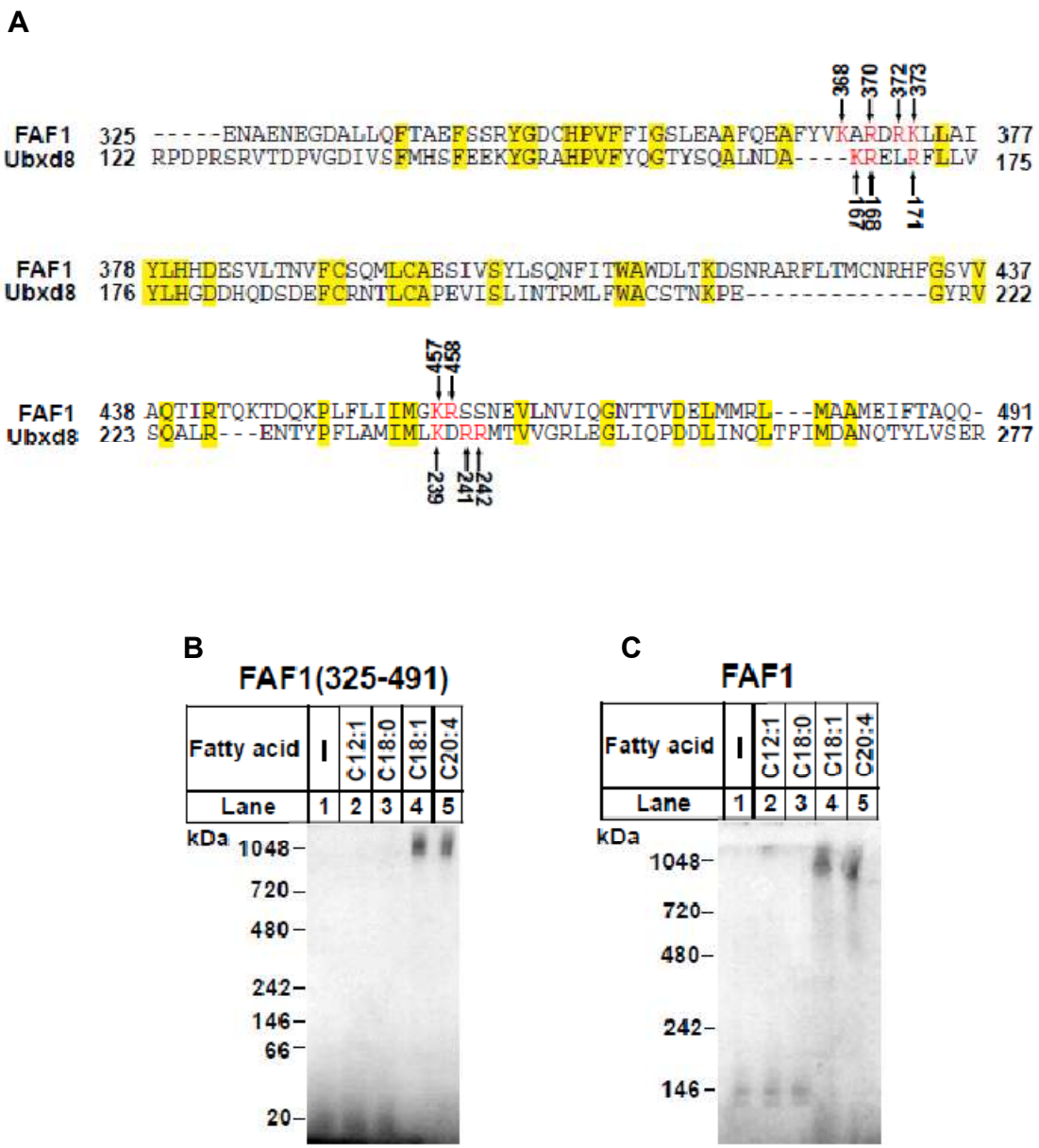
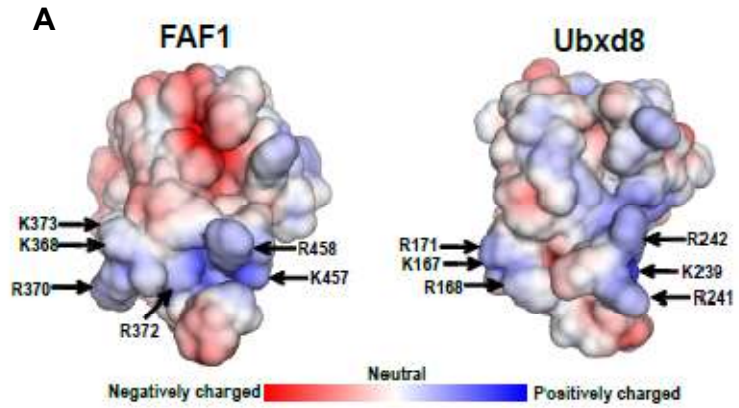
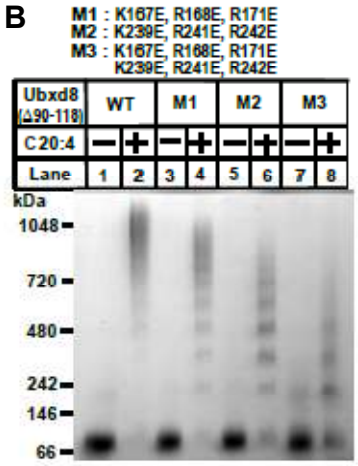
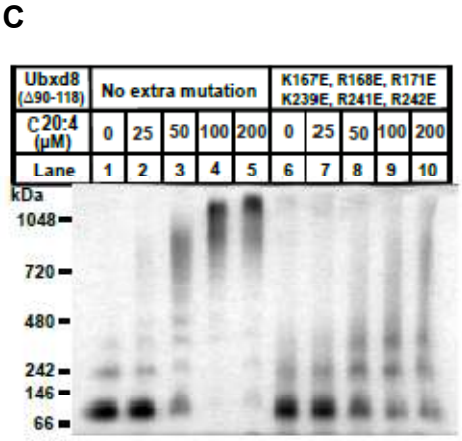
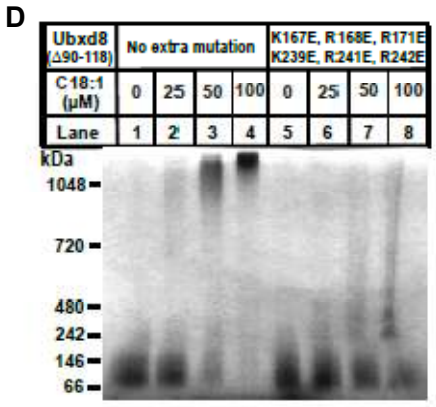


FIGURE 3.2 Long-chain unsaturated FAs induce polymerization of purified FAF1 through its UAS domain. (A) Sequence alignment of UAS domain between Ubxd8 and FAF1. Identical residues were shaded in yellow. Lysines and arginine residues in both proteins constituting the conserved positively charged surface (see Figure 3-3A) were numbered and highlighted in red. (B and C) Blue native PAGE analysis of indicated purified protein was performed as described in Figure 3-1 B.


A
FAF1
Ubxd8
K373
K368
R370
R372
R458
K457
R171
K167
R168
R242
K239
R241
Negatively charged
Neutral
Positively charged


B
M1 : K167E, R168E, R171E
M2 : K239E, R241E, R242E
M3 : K167E, R168E, R171E
K239E, R241E, R242E
Ubxd8 (Δ90-118)
WT
M1
M2
M3
C20:4
Lane
1 2 3 4 5 6 7 8
kDa
1048
720
480
242
146
66


C
Ubxd8 (Δ90-118)
No extra mutation
K167E, R168E, R171E
K239E, R241E, R242E
C20:4 (μM)
0 25 50 100 200 0 25 50 100 200
Lane
1 2 3 4 5 6 7 8 9 10
kDa
1048
720
480
242
146
66


D
Ubxd8 (Δ90-118)
No extra mutation
K167E, R168E, R171E
K239E, R241E, R242E
C18:1 (μM)
0 25 50 100 0 25 50 100
Lane
1 2 3 4 5 6 7 8
kDa
1048
720
480
242
146
66

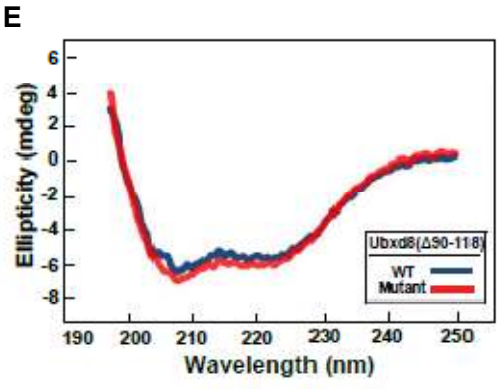

E
Ellipticity (mdeg)
6
4
2
0
-2
-4
-6
-8
190 200 210 220 230 240 250
Wavelength (nm)
Ubxd8(Δ90-118)
WT
Mutant

FIGURE 3.3 Effects of the Mutations in the UAS domain on polymerization of purified Ubxd8 (Δ90-118). (A) The structure of the UAS domain of FAF1 (left, PDB code 2EC4) and that of Ubxd8 predicted by molecular modeling (right). Lysine and arginine residues on the conserved positively charged surface were highlighted. Red and blue denotes negatively and positively charged residues, respectively. (B-D) Blue native PAGE analysis of indicated purified proteins incubated with 100 μM arachidonate (B), indicated concentration of arachidonate (C) or oleate (D) was performed as described in Figure 3-1*B*. (E) CD spectrum of indicated purified proteins.

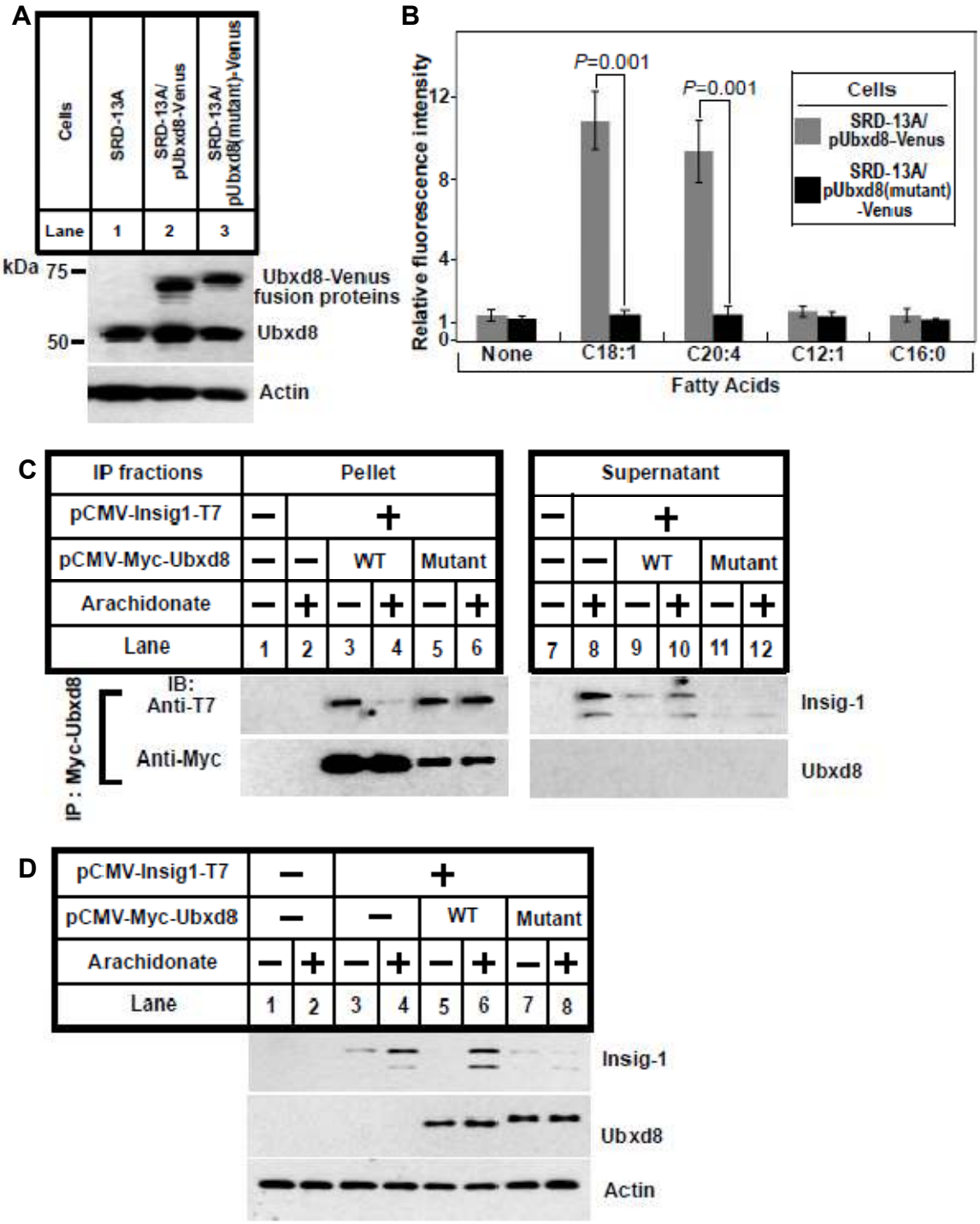


FIGURE 3.4 Effects of the Mutations in the UAS domain on functions of Ubxd8 in cells.

(A) Immunoblot analysis of indicated cells. (B) Quantitative analysis of relative fluorescent intensity of

indicated cells treated with indicated FAs was performed as described in Figure 1-4E. (C) On day 0, SRD-13A cells were set up at 4.5 x 10^5 cells/60-mm dish. On day 1, they were transfected with a plasmid encoding T7 epitope-tagged Insig-1 (0.3 μg/dish) and a plasmid encoding wild type or the mutant Ubxd8 fused with the myc epitope tag (0.3 μg/dish), followed by incubation in medium A supplemented with 5% delipidated FCS. On day 2, cells were treated with 100 μm arachidonate as indicated in medium A supplemented with 5% delipidated FCS and 30μm MG132 for 6 h. Detergent lysates of these cells were subjected to immunoprecipitation with anti-Myc to precipitate transfected Ubxd8. Pellets (representing a 0.5 dish of cells) and supernatants (representing a 0.1 dish of cells) of the immunoprecipitation were subjected to immunoblot analysis. (D) SRD-13A cells transfected with indicated plasmids were treated as described in C except that the cells were incubated in the absence of MG132. Cell lysates were subject to immunoblot analysis.

CHAPTER FOUR:

Conclusions and Recommendations

4.1 Overall Conclusions

The current study reveals that Ubxd8 functions as a cellular sensor for unsaturated FAs in mammalian cells. Unsaturated FAs alter the structure of purified Ubxd8 (Δ90-118). This effect is specific to long-chain unsaturated FAs as saturated FAs, medium-chain unsaturated FAs, or long-chain unsaturated alcohols do not alter the structure of the protein. Furthermore, long-chain unsaturated FAs not saturated FAs induce the polymerization of full length Ubxd8 in cultured cells. A previous study showed that Ubxd8 is required for the proteasomal degradation of Insig-1 in cells deprived of FAs, thereby activating FA synthesis by promoting proteolytic processing of SREBP-1 (8). Another effect of Ubxd8 is the regulation of TG synthesis by limiting the conversion of DAGs to TGs in cells depleted of FAs (25). This inhibition is relieved by addition of unsaturated but not saturated FAs (25). These results indicate that Ubxd8 acts as a brake that limits TG synthesis and this brake is released when its structure is altered by exposure to unsaturated FAs. In cells deprived of FAs, the concerted regulatory actions of Ubxd8 make FAs available for incorporation into phospholipids by limiting their diversion into TGs. When long-chain unsaturated FAs are supplied externally, these FAs change the structure of Ubxd8, promoting its polymerization, and inhibiting its activity. Consequently, Insig-1 is stabilized and FA synthesis decreases. TG synthesis increases so that excess exogenous FAs are stored as TGs in lipid droplets (Figure 4-1).

A group of FA binding proteins (FABPs) have been previously identified as cellular proteins that bind to FAs (54). However, the mechanism through which they interact with FAs is different than that used by Ubxd8. Ubxd8 binds to only unsaturated FAs but not saturated FAs while FABPs bind to both FAs (55, 56). FABPs form internalized cavity using β-sheets for binding to FAs (55, 56), while Ubxd8 does not have this cavity. In the current study, two loops with 6 positively charged residues on surface were found to be required for unsaturated FA-induced polymerization of Ubxd8. However it is not clear whether this site is for binding to unsaturated FAs or polymerization in response of unsaturated FAs.

Binding to FAs triggers the conformational change of FABPs but not its polymerization (55, 57) while Ubxd8 is polymerized in the presence of unsaturated FAs. Ubxd8 has lower binding affinity (Kd : 40μM) for FAs than that of FABPs (Kd : 20~4000nM) (25, 55). Importantly, critical miscelle concentration of oleate is over 500μM which is much higher than the concentration we used to study Ubxd8 (25). Thus, this polymerization does not come from spontaneous miscelle formation of oleate but from the interaction of Ubxd8 with unsaturated FAs.

We identify UAS domain as a motif polymerizing upon interaction with long-chain unsaturated FAs. UAS domain is a member of the large thioredoxin-like protein superfamily (58). However, no other members of this superfamily are known to interact with FAs. Positively charged surface patch composed by 6 lysine and arginine residues located at two adjacent loops in UAS domain are important for long-chain unsaturated FAs to induce polymerization of the UAS domain. Thus, long-chain unsaturated FAs may bind to the surface of the UAS domain so that this interaction between UAS domain and unsaturated FAs covers positive charged surface of UAS domain with hydrocarbon of FAs. Such binding may render the protein surface more hydrophobic, a condition that may facilitate self-association of the UAS- FA complexes. The best way to find the binding site is the structural determination of Ubxd8 in the presence of unsaturated FAs. However, polymerized Ubxd8 (Δ90-118) exists as heterogeneous size in the presence of unsaturated FAs. This heterogeneity prevented the structural determination using X-ray crystallography or negative staining electron microscopy.

Identification of UAS domain as a motif polymerizing upon interaction with long-chain unsaturated FAs opened the concept that proteins containing a UAS domain may be subject to regulation by long-chain unsaturated FAs. In this study, we show that long-chain unsaturated FAs induce polymerization of Ubxd8 to prevent the protein from stimulating degradation of Insig-1. FAF1 is another mammalian protein containing a UAS domain. We show that long-chain unsaturated FAs stimulate the polymerization of purified recombinant FAF1.

4.2 Recommendations for Future Studies

Polymerization of FAF1 in response to unsaturated FAs suggests that the function of FAF1 may also be regulated by these FAs. However, none of its functions has been demonstrated to be regulated by FAs. One function of FAF1 is to regulate NF-κB (46-48). NF-κB regulates transcriptional activity of the immune response, inflammatory process, and apoptosis. It was reported previously that saturated but not unsaturated FAs activated production of proinflammatory cytokines through activation of NF-κB (50, 51). These observations suggest that the FA sensor involved in this regulation should tell the difference between saturated and unsaturated FAs. Our *in vitro* analysis indicates that FAF1 meets this requirement. Thus, future work is required to determine whether FAF1 is involved in FA-mediated activation of NF-κB.

FAF1 has been postulated to suppress tumor growth as its reduced expression or genomic loss was found in various carcinoma (59). However, its detailed molecular mechanism had not been known until the binding partner of FAF1 was found (60, 61). Recently, FAF1 was reported to promote β-Trcp-mediated ubiquitination and subsequent degradation of β-catenin by proteasomes (60, 61). β-catenin is one of the main component in response to Wnt to control cell proliferation (62, 63). In normal cells, the level of β-catenin is tightly regulated (62-64). In the absence of Wnt, β-catenin binds to β-Trcp so that β-catenin is ubiquitinated and subsquently degraded by proteasomes (62-64). The interaction between these two proteins is mediated by FAF1 (60, 61). In the presence of Wnt, the interaction between β-catenin and β-Trcp is disrupted (62). As a result, β-catenin is stabilized and translocates into nucleus for transcriptional activation of the genes supporting cell proliferation (62-64). Since unsaturated FAs trigger the polymerization of FAF1 and presumably inactivate the protein, we wonder whether unsaturated FAs disrupt the interaction between β-catenin and β-Trcp. If so, unsaturated FAs may stabilize β-catenin in the absence of Wnt. In cancer cells the ubiquitination of β-catenin is blocked so that the level of β-catenin is abnormally accumulated even in the absence of Wnt (62-65). Since aberrant accumulation of β-catenin is responsible for development of cancers, this hypothesis might explain the observation that cancer cells

have to maintain high level of free unsaturated FAs for their rapid proliferation (66).

To test this hypothesis, we need to determine whether unsaturated FAs regulate the interaction of FAF1 with β-catenin or β-Trcp. This hypothesis can be tested by co-immunoprecipitation assay. We also need to test whether inhibition of unsaturated FA-mediated polymerization of FAF1 blocks the proliferation of cancer cells dependant on β-catenin like colon cancer. In the current study, we showed that mutations in UAS domain of Ubxd8 significantly blocked the polymerization of Ubxd8 and that unsaturated FAs were unable to stabilize Insig-1 in cells expressing this mutant version of Ubxd8. Likewise, we will test whether similar mutations in UAS domain of FAF1 also inhibit the polymerization of FAF1. If so, we will stably transfect cancer cells with mutant version of FAF1 to test whether this mutant version of FAF1 prevents unsaturated FA-mediated stabilization of β-catenin, thereby inhibiting the growth of colon cancer cells that depend on abnormal accumulation of β-catenin for their proliferation (7). Finally, if unsaturated FAs regulate the function of FAF1 in degrading β-catenin like that of Ubxd8 in degrading Insig-1, supplement of unsaturated FAs to cancer cells may stabilize β-catenin. This hypothesis is supported by the observation that 1) feeding with a diet enriched in unsaturated FAs increased the number of polyps and shortened survival span compared to feeding with a low fat diet in mice model of colon cancers (67); 2) SCD-1 protein, an enzyme catalyzing the rate limiting step in synthesis of unsaturated FAs, was found to be highly expressed in colon cancers (68). If this hypothesis is true, depletion of unsaturated FAs may inhibit proliferation of the cancer cells dependant on aberrant accumulation of β-catenin for their proliferation. We will thus test whether we can cure these cancers by depletion of unsaturated FAs.

Previous study showed that Ubxd8 blocks TG synthesis by limiting the conversion of DAGs to TGs in FA-depleted cells (25). In cells exposed to excess FAs, this inhibition is relieved to increase the incorporation of excess FAs to TGs for storage (25). In the same condition, Ubxd8 also inhibits the activity of ATGL to block the release of FAs from TGs so that further accumulation of FAs is blocked in the presence of exogenous excess FAs (26). Ubxd8 inhibits the activity of ATGL by promoting its

dissociation from its coactivator CGI-58 (26). However, we don't know the mechanism by which Ubxd8 inhibits the conversion of DAGs to TGs in FA-depleted cells. This conversion is catalyzed by DGAT enzymes. DGAT-1 and DGAT-2 are the only DGAT enzymes characterized in mammalian cells (38). Inasmuch as Ubxd8 is a sensor for unsaturated FAs as well as a protein that facilitates ERAD, it implies that unsaturated FAs might act through Ubxd8 to regulate degradation of DGAT-1 or DGAT-2. Similar to its action on Insig-1, Ubxd8 may recruit p97 to DGAT enzymes to stimulate their degradation. Previous study in our lab showed that DGAT-2 does not interact with Ubxd8 (unpublished). Thus, it will be interesting to determine whether Ubxd8 binds to DGAT-1, and whether this interaction is regulated by unsaturated but not saturated FAs. It is also necessary to test whether unsaturated but not saturated FAs stabilize the DGAT-1 protein.

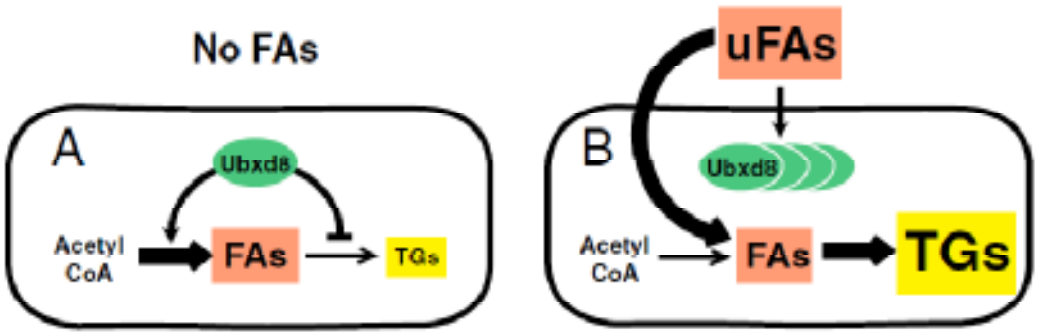


Figure 4.1 Model for Ubxd8-mediated cellular responses to exogenous unsaturated FAs.

(A) Cells incubated in the absence of exogenous unsaturated FAs. In these cells Ubxd8 promotes synthesis of FAs and inhibits incorporation of FAs into TGs. These two regulatory actions allow cells to maintain enough FAs for their survival. (B) Cells incubated in the presence of exogenous unsaturated FAs. In these cells excessive unsaturated FAs inactivate Ubxd8 by promoting its polymerization. In the absence of active Ubxd8, synthesis of endogenous FAs is inhibited and excessive FAs are channeled into TGs. These two regulatory actions prevent toxic overaccumulation of FAs.

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