Free Fatty Acid Receptors and Their Role in Regulation of Energy Metabolism

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Abstract The free fatty acid receptor (FFAR) is a G protein-coupled receptor (GPCR) activated by free fatty acids (FFAs), which play important roles not only as essential nutritional components but also as signaling molecules in numerous physiological processes. In the last decade, FFARs have been identified by the GPCR deorphanization strategy derived from the human genome database. To date, several FFARs have been identified and characterized as critical components in various physiological processes. FFARs are categorized according to the chain length of FFA ligands that activate each FFAR; FFA2 and FFA3 are activated by short chain FFAs, GPR84 is activated by medium-chain FFAs, whereas FFA1 and GPR120 are activated by medium- or long-chain FFAs. FFARs appear to act as physiological sensors for food-derived FFAs and digestion products in the gastrointestinal tract. Moreover, they are considered to be involved in the regulation of energy metabolism mediated by the secretion of insulin and incretin hormones and by the regulation of the sympathetic nerve systems, taste preferences, and inflammatory responses related to insulin resistance. Therefore, because FFARs can be considered to play important roles in physiological processes and various pathophysiological processes, FFARs

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have been targeted in therapeutic strategies for the treatment of metabolic disorders including type 2 diabetes and metabolic syndrome. In this review, we present a summary of recent progress regarding the understanding of their physiological roles in the regulation of energy metabolism and their potential as therapeutic targets.

Contents

1 Introduction

Metabolic diseases, such as diabetes and obesity, are a threat to global health and are one of the most important health problems that impact our quality of life at great expense to society. Obesity is associated with chronic inflammation and induces insulin resistance which in turn causes type 2 diabetes. Diabetes is as a metabolic disorder, the incidence of which has reached epidemic proportions: 150 million people worldwide are estimated to be afflicted by diabetes, and this number is expected to double within 20 years (Wild et al. 2004). Type 2 diabetes results from the body's inability to produce sufficient amounts of insulin and from resistance of the body's fat and muscle cells to insulin. Elevated blood glucose levels are a hallmark of diabetes. There is currently no cure for diabetes, although treatment

options using medicines and dietary approaches are available that can help patients to prevent symptoms. Unfortunately, these approaches have many limitations; therefore, the development of alternative therapeutic targets and new anti-diabetic agents is desirable.

Free fatty acids (FFAs) are an important energy source for most body tissues, and are categorized by the length of their aliphatic tails; short-chain fatty acids (SCFAs) have less than 6 carbons, medium-chain fatty acids have 6–12 carbons, and long-chain fatty acids have 12 or more carbons. In addition to functioning as an energy source, FFAs exhibit a broad range of function such as modulation of receptor signaling, gene expression and whole body fuel energy homeostasis under various physiological conditions (Hara et al. 2011). As physiological sensors of FFAs, members of the intracellular or nuclear lipid binding protein families including fatty acid binding proteins and peroxisome proliferator-activated receptors (PPARs) are considered functional receptors that contribute to the regulation of numerous physiological and pathophysiological processes (Chawla et al. 2001). However, because some of the physiological observations induced by FFAs cannot be explained by functions that are mediated through these sensors, other mechanisms, perhaps involving plasma membrane receptors, may be expected to mediate some of the biological processes of FFAs.

The human genome contains 800 G protein-coupled receptors (GPCRs) that belong to large gene families. GPCRs are seven-transmembrane receptors that activate heterotrimeric G proteins and thus provide a wide variety of therapeutic targets for numerous diseases (Lagerstrom and Schioth 2008). To date, approximately 350 GPCRs, with the exception of the olfactory receptors, have been characterized in the human genome using bioinformatics analyses based on their sequence similarities. Approximately 260 of these GPCRs have already been characterized as deorphaned receptors after identification of the respective endogenous ligand; however, approximately 70 orphan GPCRs remain (Civelli et al. 2013). Therefore, these receptors have yet to be explored as potential drug targets. Several groups, including our own, have identified a series of orphan receptor, which are activated by FFAs and its derivatives. To date, five free fatty acid receptors (FFARs) have received considerable attention as a result of their physiological importance in various biological processes (Table 1 and Fig. 1). Among the FFARs that have been identified, FFA1 (GPR40) and GPR120 are activated by medium- and long-chain FFAs, and GPR84 is activated by medium-chain FFAs. In contrast, FFA2 (GPR43) and FFA3 (GPR41) are activated by SCFAs (Hara et al. 2012; Ichimura et al. 2009). Therefore, each FFAR may act as a sensor for FFAs with selectivity for a particular FFA carbon chain length that is derived from food or food-derived metabolites. Physiological functions of FFARs have been reported to include the secretion of insulin and incretin hormones, adipocyte differentiation, anti-inflammatory effect, nerve activation, and taste preferences. Accordingly, these physiological functions of FFARs could be considered to regulate energy metabolism. Therefore, FFARs have received considerable attention as potential therapeutic targets for energy metabolism disorders. In this review, we focus on recent advances regarding the understanding of FFARs in conjunction with our observations.

Table 1 Characteristics of FFARs

Nomenclature	Natural agonist	Synthetic agonist	Antagonist	G-protein coupling	Expression	Physiological functions	Chromosomal location	mRNA and protein (human)
FFA1 (GPR40)	Medium to long FFAs	GW9508	GW1100	Gq/11	Pancreatic β-cell	Insulin secretion	19q13.1	NM_005303
	Conjugate linoleic acid	MEDICA16			Intestine	Incretin secretion		NP_005294
		Thiazolidinediones			Tongue	Fat preferences		(300 aa)
		TAK-875						
		AMG837, AMG1638, AMG6226,						
		TUG424, Compound 7						
FFA2 (GPR43)	Short chain FFAs	Propiolic acid	Compound 3 and 4 (orthosteric antagonist)	Gq/11	Adipose tissue	Leptin production	19q13.1	NM_005306
		Angelic acid		Gi/o		Adipogenesis		NP_005297
		Compound 1 and 2 (orthosteric agonist)				Lipolysis		(330 aa)
		Phenylacetamides 1 and 2 (allosteric agonist)			Intestine	Incretin secretion		
						Intestinal motility		
					Immune cells (neutrophil)	Inflammatory response		

FFA3 (GPR41)	Short chain FFAs	Compound 5	Compound 6	Gi/o	Intestine	PYY, GLP-1	19q13.1	NM_005304
			β-hydroxybutyrate	Gβγ	Sympathetic nerve system	Sympathetic activation		NP_005295 (346 aa)
GPR84	Medium chain FFAs	Diindolylmethane		Gi/o	Adipose tissue	Adiponectin	12q13.13	NM_020370
					Macrophage Microglia			NP_065103 (396 aa)
GPR120	Medium to long FFAs	NCG21		Gq/11	Intestine	GLP-1 secretion	10q23.33	NM_181745
	Grifolic acid (partial agonist)	Compound 8		β-arrestin	Adipocytes	Differentiation		NP_859529
		Compound 9			Macrophage Tongue	Anti-inflammatory Fat preferences		(377 aa)

Molecular, biochemical and functional characteristics of FFARs are shown

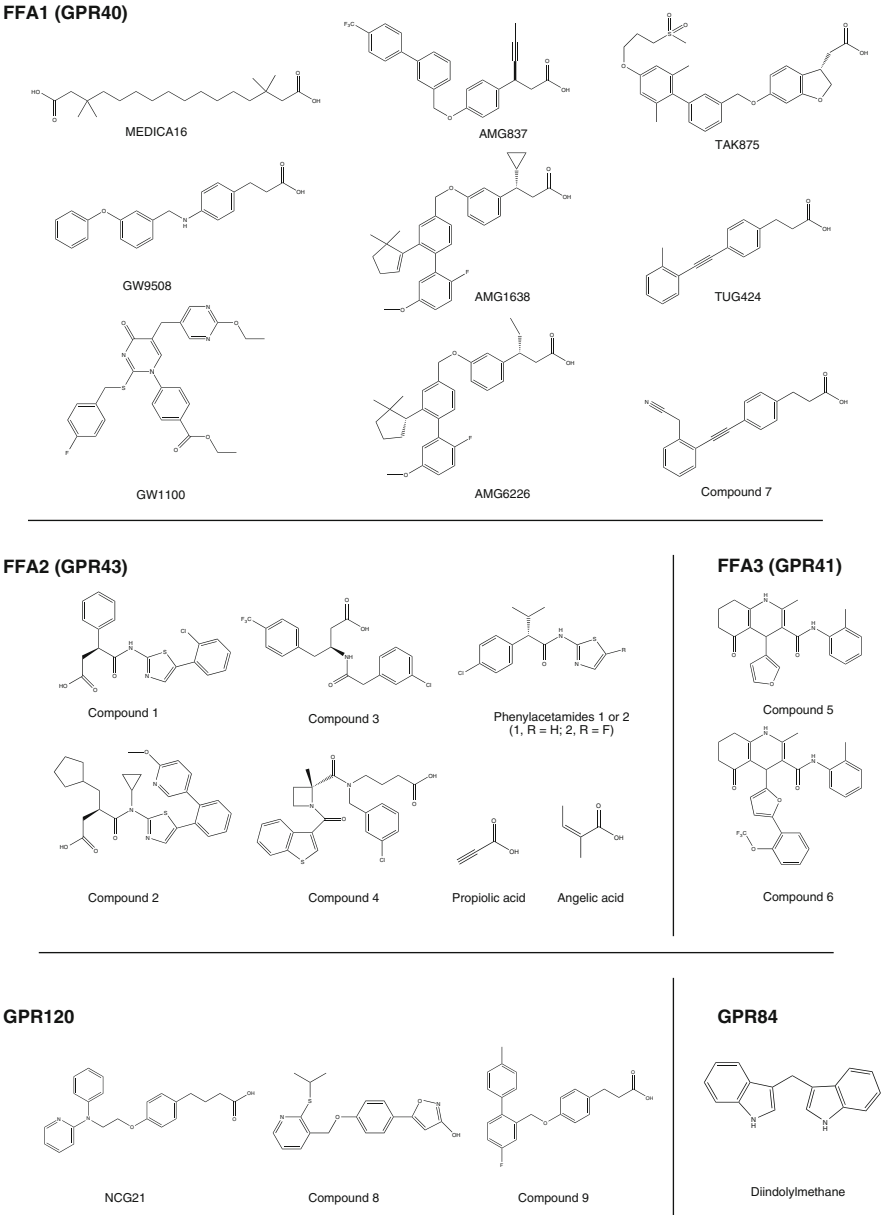

Fig. 1 Chemical structures of synthetic FFARs ligands. The name of each synthetic compound is also described in Table 1

2 FFA2 (GPR43)

2.1 Ligands

FFA2 was identified as a receptor for SCFAs and was de-orphaned in 2003. During a routine ligand bank screening with bioactive compounds, termed ligand fishing, it emerged that FFA2 was activated by acetate using Ca^{2+} mobilization assays in transfected mammalian cells (Brown et al. 2003; Nilsson et al. 2003). FFA2 was activated in vitro by physiological concentrations (micromolar range) of other SCFAs such as formate, propionate, butyrate, and pentanoate. The rank order of potencies with respect to activation of FFA2 was as follows: acetate = propionate > butyrate, whereas that of FFA3 was as follows: propionate > butyrate > acetate (Brown et al. 2003; Le Poul et al. 2003). The 100-fold lower activation potency of acetate to FFA3 can be used diagnostically to distinguish FFA3 from FFA2 (Brown et al. 2003). Interestingly, SCFA ligands showed different rank order of potency between species orthologs of FFA2 and FFA3 (Hudson et al. 2012). Acetate is selective for human FFA2 compared to human FFA3 however, this selectivity was not observed among the mouse orthologs. In addition, although propionate did not show selectivity between human orthologs, this ligand selectively activated mouse FFA3 compared to mouse FFA2. These results might be caused by high constitutive activity of human FFA3 and mouse FFA3 which was monitored by [^{35}S]-GTPγS binding assay. The molecular basis analysis suggested that the single negatively charged residues that were not conserved between species might regulate constitutive activity of FFA2 and FFA3.

2.2 Selective Compound

According to the physiological function of FFA2 (as described under Sect. 2.4), activation of colonic and adipose FFA2 by SCFA or pharmacological compounds is considered a promising therapeutic target for the treatment of obesity. Several selective agonists such as propiolic acid and angelic acid that showed 10–100-fold selectivity for human FFA2 compared to FFA3 were reported (Schmidt et al. 2011). A series of compounds that showed agonistic or antagonistic activities were reported as the patented compounds (Brantis et al. 2011; Hoveyda et al. 2010; Saniere et al. 2012; Ulven 2012). Compound 1 dose dependently increased glucose uptake in 3T3-L1 adipocyte cell line. On the other hand, Compound 2 increased GLP-1 secretion from NCI-H716 cell line. Further Compound 3 and 4 showed antagonistic activity for FFA2, with IC_{50} values of 20 and 100 nM, respectively. Since radiolabeled Compound 3 was displaced by natural agonist propionate, these synthetic compounds were considered to act as orthosteric ligands. Also, Phenylacetamide 1 and 2 are novel synthetic allosteric agonists for FFA2 that have pharmaceutical potential. Lee et al. (2008) demonstrated greater activation potency by phenylacetamides 1 and 2 compared with SCFAs by high-throughput screening

and showed that these compounds activate both G_q and $G_{i/o}$ pathways (Lee et al. 2008). These compounds may serve as useful tools for further elucidation of the physiological functions of the receptor and its involvement in various diseases. Results from in vivo studies using these compounds are eagerly awaited.

2.3 Signal Transduction

Although FFA2 and FFA3 have similar endogenous ligands, their respective G-protein signaling mechanisms differ: The pertussis toxin-sensitive $G_{i/o}$ pathway is activated by FFA2 and FFA3, whereas the $G_{q/11}$ pathway is activated by FFA2 (Le Poul et al. 2003). Stimulation of FFA2 by SCFAs resulted in inhibition of cAMP production; activation of the extra cellular regulated kinase 1/2 (ERK1/2) cascade through interactions with the $G_{i/o}$ family of G proteins; elevation of $[Ca^{2+}]_i$ and activation of the ERK1/2 cascade via interactions with the G_q family of G proteins. However, the physiological significance of this dual-coupled signaling mechanism associated with FFA2 is still unclear.

2.4 Expression and Physiological Functions

The expression of FFA2 in adipose and gastrointestinal tissues suggests that FFA2 may be involved in energy regulation (Sleeth et al. 2010). Reverse transcription polymerase chain reaction (RT-PCR) anatomical profiling in murine tissue revealed that *FFA2* was expressed in white adipose tissue and in the gastrointestinal tract (Hong et al. 2005). The presence of FFA2 in adipose tissues may imply a role in obesity and energy accumulation because *FFA2* mRNA is expressed in white adipose tissues such as subcutaneous, perirenal, epididymal, 3T3-L1-derived adipocytes, and mature adipocytes (Hong et al. 2005). The presence of FFA2 in the intestine may imply a role in appetite regulation because *FFA2* mRNA is expressed in both rat whole-wall and separated mucosa from the distal ileum and colon (Karaki et al. 2006). These findings were confirmed by Dass et al. (2007) and also reported for whole-walls of the human colon (Karaki et al. 2008). Moreover, recent quantitative real-time PCR anatomical profiling in murine tissue revealed that *FFA2* was abundantly expressed in immune cells such as neutrophils and in immune tissues such as the spleen and bone marrow (Maslowski et al. 2009). Therefore, FFA2 may be involved in various physiological processes (Fig. 2).

2.4.1 Adipose Tissues

FFA2 may be involved in obesity and related disorders such as metabolic syndrome. Metabolic syndrome is a combination of medical disorders and increases the risk of developing obesity-related cardiovascular disease and diabetes, impaired

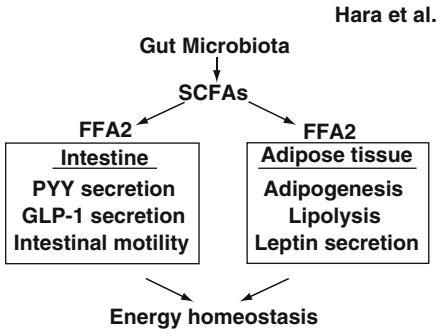

Fig. 2 Schematic diagram of the metabolic regulation by FFA2 via gut microbiota. SCFAs are produced in gut by bacterial fermentation of dietary fiber followed by the stimulation of FFA2 expressed in intestine and adipose tissues. FFA2 regulates incretin secretion (such as PYY and GLP-1) and motility in intestine. On the other hand, FFA2 also regulates adipogenesis, lipolysis and leptin secretion in adipose tissues. These functions induced by the FFA2 stimulation contribute to the energy homeostasis of the whole body

glucose tolerance, dyslipidemia, and hypertension (Grundy et al. 2004). Excess fat mass, characterized by both adipocyte hyperplasia and hypertrophy, is cause to exhibit the various symptoms of the metabolic syndrome. Adipose tissue is considered not only an energy reservoir for lipids and glucose but also a complex endocrine organ. It secretes hormones and inflammatory cytokines, some of which act as peripheral sensors for energy balance (Tilg and Moschen 2006).

2.4.2 Adipogenesis

Based on the *FFA2* expression in adipose tissues and adipocytes, Hong et al. (2005) performed a series of studies to elucidate the physiological role of FFA2 in adipocytes. They first showed that expression of *FFA2* in adipose tissues from high-fat diet-induced obesity mice was significantly greater than that of normal chow-fed mice. After induction of adipogenesis in 3T3-L1 cells, the expression of mRNA for *FFA2* and *Pparγ2* (a marker of mature adipocytes) was increased by treatment with the SCFAs, acetate and propionate. A reduction of *FFA2* mRNA by small interfering RNA (siRNA) in 3T3-L1 cells blocked adipogenesis. Collectively, these results suggested that activation of FFA2 by SCFA may be critical for the differentiation of adipocytes, resulting in promotion of fat accumulation.

2.4.3 Lipolysis

Following the ingestion of a meal, insulin stimulates the uptake of nutrients such as glucose into specialized tissues, and also potently inhibits lipolysis in adipocytes. However, cells that have become insulin resistant do not respond to this cue,

resulting in an outflow of lipids to plasma and ensuing dyslipidemia. FFA2 may exhibit the protective effect against this metabolic disruption by inhibiting lipid release from adipocytes. In 3T3-L1-derived adipocytes, acetate and propionate suppressed isoproterenol-induced lipolysis in a dose-dependent manner (Hong et al. 2005). Similarly, it was reported that the SCFAs, acetate and propionate, produced by long term intervention of resistant starch rich diet suppressed release of non-esterified fatty acid and glycerol from subcutaneous abdominal adipose tissues (Robertson et al. 2005). Moreover, Ge et al. (2008) demonstrated that these effects were mediated by FFA2 using *FFA2* knockout (KO) mice. It was reported that acetate produced a dose-dependent suppression of lipolysis and the release of glycerol in adipocytes from wild-type (WT) mice in vitro. Furthermore, intraperitoneal injection of sodium acetate to WT mice elicited a simultaneous peak in plasma acetate concentration, and instantly reduced plasma non-esterified fatty acids after injection in vivo (Ge et al. 2008). These effects were abolished in *FFA2* KO mice. Thus activation of FFA2 by SCFAs directly leads to inhibition of lipolysis and suppression of plasma FFAs.

2.4.4 Leptin Secretion

Activation of FFA2 by SCFAs potentially leads to the promotion of adipogenesis and inhibition of lipolysis, which may lead to the improvement of metabolic syndrome. This may partly explain the beneficial effects of dietary fiber supplementation on glucose control and dyslipidemia that have been observed in some studies (Chandalia et al. 2000). Another significant finding of SCFA receptor function in adipose tissues was reported by several groups. Brown et al. (2003) demonstrated the presence of FFA3 mRNA in human adipose tissue by quantitative RT-PCR. Xiong et al. (2004) reported that murine FFA3 was expressed in white adipose tissue and that propionate increased the concentration of leptin in the culture medium of primary white adipose tissue from mice. Further, leptin secretion was increased according to the overexpression of exogenous FFA3 and was decreased by siRNA-mediated knockdown of FFA3 in adipocytes. However, contradictory reports state that FFA3 expression has not been confirmed in murine adipose tissue (Hong et al. 2005; Kimura et al. 2011). Thus, Hong et al. (2005) reported that they could not detect FFA3 mRNA in differentiated 3T3-L1 cells nor in murine subcutaneous, perirenal, mesenteric, or epididymal fat pads, despite using the same PCR primers as those used in the study by Xiong et al. (2004). We were also unable to detect FFA3 mRNA expression in murine adipose tissues by quantitative RT-PCR or by in situ hybridization analysis (Kimura et al. 2011). In contrast, Hong et al. (2005) demonstrated the presence of FFA2 mRNA in murine adipose tissues, and Zaibi et al. (2010) reported that FFA2, rather than FFA3, was expressed in murine adipose tissue. They showed that acetate, rather than butyrate, stimulated leptin secretion in mesenteric adipocytes from WT mice but not in adipocytes from FFA3 KO mice, suggested that this effect was mediated by FFA2, whose expression was reduced in FFA3 KO mice compared with WT mice. Nevertheless, in the

presence of adenosine deaminase to suppress $G_{\alpha\ (i/o)}$ signaling through the adenosine A1 receptor, SCFA stimulated leptin secretion by adipocytes from WT but not FFA3 KO mice (Zaibi et al. 2010). Pertussis toxin prevented stimulation of leptin secretion by propionate in epididymal adipocytes, thus implicating $G_{\alpha\ (i/o)}$ signaling mediated by FFA2 in SCFA-stimulated leptin secretion (Al-lahham et al. 2010). Leptin is secreted from white adipose tissue and is a potent anorexigenic hormone and a long-term dynamic marker of body adiposity. This suggests that SCFA supplementation may also act on appetite through an FFA2-mediated response. Furthermore, to investigate the relationships between FFA3 expression and the effect of SCFAs on leptin secretion in adipose tissues, additional experiments using an FFA3-specific modulator, and/or adipose tissue-specific FFA3 KO mice are required.

2.4.5 Intestine

The intestines play a critical role in energy homeostasis because they are associated with nutrient absorption, and secretion of gut hormones that are involved in appetite control. Karaki et al. (2006) demonstrated that FFA2-expressing cells completely co-localize with peptide YY (PYY)-expressing enteroendocrine L cells of the gastrointestinal tract by immunohistochemistry (Karaki et al. 2006). Although both neural and hormonal factors stimulate the release of PYY, it is also likely that nutrients in the luminal environment induce secretion of this peptide and other peptides from L cells; for example, glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) encoded by the proglucagon gene (Greeley et al. 1989). The expression of FFA2 in L cells in both rats and humans may suggest that activation of FFA2 by SCFA regulates PYY and GLP-1 secretion (Karaki et al. 2006). As the source of the SCFA, gut microbiota played important role in producing SCFA through fermentation processes (Owira and Winter 2008). In addition, colonic fermentation-derived SCFAs might regulate body weight and incidence of diabetes (Sleeth et al. 2010).

2.4.6 PYY Secretion

FFA2 which is reported to be expressed in enteroendocirne L cells, contributed to the secretion of PYY (Karaki et al. 2006, 2008).

2.4.7 GLP-1 Secretion

Enteroendocrine L cells are a major source of GLP-1 and GLP-2. These peptides are co-stored and co-secreted with PYY from enteroendocrine L cells (Kim et al. 2005). Therefore, SCFA may also stimulate L cell secretion of GLP-1 and other products of the pre-proglucagon gene through FFA2. Incretins are intestinal

hormones that act to increase insulin secretion. GLP-1 is a potent anorexigenic hormone and incretin. GLP-1 secretion by ileal L cells is dependent on the presence of nutrients in the lumen of the small intestine (Elliott et al. 1993). Acute intracerebroventricular administration of GLP-1 to fasted rats is associated with a decline in short-term energy intake (Turton et al. 1996) and a significant reduction in body weight in response to repeated administration (Davis et al. 1998). Moreover, GLP-1 as an incretin promotes insulin secretion through the GLP-1 receptor, which is expressed in pancreatic islets (Bullock et al. 1996). Studies of non-digestive fiber supplementation have indicated that administration of SCFA was associated with increased colonic proglucagon mRNA expression and increased circulating plasma GLP-1 levels (Delzenne et al. 2005; Keenan et al. 2006). It was reported that SCFAs resulted in the secretion of GLP-1 through FFA2 in mixed colonic cultures and in vivo. Quantitative PCR revealed enriched expression of FFA2 and FFA3 in GLP-1-secreting L cells, and SCFAs raised cytosolic Ca^{2+} through G_q signaling pathways in L cells in primary culture. Mice lacking FFA2 or FFA3 exhibited reduced SCFA-triggered GLP-1 secretion in vitro and in vivo and a parallel impairment of glucose tolerance (Tolhurst et al. 2012). Thus, the potential for dietary or pharmacological manipulation to increase satiety could be used in the treatment of obesity. Furthermore, with specific relevance to GLP-1, incretin action may be useful for potentiating insulin secretion in patients with type 2 diabetes. The anorexigenic neural circuits involving PYY and GLP-1 are subsequently activated to reduce food intake and increase energy expenditure to restore the body back to neutral energy balance. Hence, regulation of PYY and GLP-1 secretion through FFA2 may control energy intake and could be applied to the treatment of obesity and metabolic syndrome.

2.4.8 Intestinal Motility

Gastrointestinal motility enhances digestion and nutrient absorption, resulting in increased energy intake. Moreover, gastrointestinal motility may be partially controlled by FFA2. Recent findings have indicated that FFA2 may also affect the release of gastrointestinal 5-hydroxytryptamine (5-HT or serotonin). 5-HT, or serotonin, is a neurotransmitter in the central nervous system (CNS) known to modulate mood, behavior and appetite (Berger et al. 2009) because of co-localization with PYY. 5-HT is found peripherally in the gastrointestinal tract, primarily in enterochromaffin cells but also in 5-HT-containing mucosal mast cells (Kim and Camilleri. 2000). It is released in a dose-dependent manner in response to mechanical and chemical stimulation, including SCFA, during nutrient ingestion in the gut (Zhu et al. 2001; Fukumoto et al. 2003). It was reported that FFA2-expressing cells co-localize with 5-HT-containing mucosal mast cells of the rat distal ileum and colon and human colon (Karaki et al. 2006, 2008). Therefore, SCFA activation of FFA2 may mediate the release of 5-HT in the gut, thus constituting gastric motility-mediated appetite regulation that is independent of PYY.

3 FFA3 (GPR41)

3.1 Ligands

The FFA3 gene was found downstream of CD22 on human chromosomal locus 19q13.1 by PCR using degenerate primers based on conserved sequences in encoding transmembrane domains of the rat galanin receptor 1 (GALR1), GALR2, and human GALR1 (Sawzdargo et al. 1997). Several groups have performed screenings to identify ligands for FFA3. In 2003, two distinct groups reported that FFA3 can be activated by SCFAs, particularly by propionate, butyrate, and valerate (Brown et al. 2003; Le Poul et al. 2003). On the other hand, several synthetic compounds are reported as FFA3 agonist (Compound 5) or antagonists (Compound 6) (Leonard et al. 2006).

3.2 Signal Transduction

Brown et al. (2003) measured SCFA-induced GTPγS binding using HEK293T cells expressing human or rat FFA3. Le Poul et al. (2003) examined SCFA-mediated cAMP inhibition in CHO-K1 cells expressing human FFA3. The following year, Xiong et al. (2004) confirmed these results using human FFA3-expressing *Xenopus melanophore* cells. Le Poul et al. (2003) also showed that SCFAs induced the release of $[Ca^{2+}]_i$ and the phosphorylation of ERK1/2. Both these responses were completely abolished by pertussis toxin (PTX) treatment, suggesting the unique coupling of FFA3 to $G_{\alpha(i/o)}$.

3.3 Expression and Physiological Functions

3.3.1 Adipose Tissue

The involvement of FFA3, which was expressed in adipose tissue, in the release of leptin is still a lot of debate. To understand the physiological function of FFA3 related to leptin secretion, the study using the adipose tissue-specific FFA3 KO mice might be required (refer to Sect. 2.4.4 in paragraph of FFA2).

3.3.2 Intestine

FFA3 is reported to regulate host energy absorption by modulating gut motility. Samuel et al. (2008) showed that quantitative RT-PCR was used to demonstrate that FFA3 mRNA is expressed in the distal small intestine and colon of the mouse.

Tazoe et al. (2009) found that human FFA3 is also expressed in PYY-containing enteroendocrine cells, and Samuel et al. (2008) used in situ hybridization analysis to demonstrate that FFA3 mRNA is expressed in enteroendocrine cells. Samuel et al. (2008) showed that the body weight and fat pad weight of FFA3 KO mice were significantly lower than those of WT mice and that these differences were abolished in germ free mice. In addition, fasting plasma leptin level was reduced in FFA3 KO mice compared to WT mice raised in conventional condition. Co-colonization of human gut-derived *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii* in germ-free mice led to significantly increased levels of circulating PYY, which regulates gut motility. This effect was significantly suppressed in FFA3 KO littermates, although FFA3 deletion did not affect food intake. These results indicated that the function of FFA3 depends on SCFAs produced by bacterial fermentation. Also, the intestinal transit rate was significantly faster in FFA3 KO mice compared with WT littermates, and this effect was abolished in germ-free mice. The fecal content of SCFA in FFA3 KO mice was significantly higher than of WT mice. These results led the authors to suggest that the deceased PYY levels in FFA3 KO mice increased gut motility, leading to reduced SCFA absorption as an energy source, which resulted in the lean phenotype (Samuel et al. 2008). In contrast, Bellahcene et al. (2012) reported that male but not female FFA3 KO mice have a higher body fat mass than their WT littermates when they are fed with either a low- or high-fat diet. Genotype had no effect on the amount of food intake by either sex regardless of maintenance on a low- or high-fat diet. This included mice of the same age (10 weeks) as those used in the report by Samuel and co-workers (2008). This observation was supported by Zaibi et al. (2010) stated that FFA3 KO mice exhibit obesity in an unpublished observation. In the high-fat-fed condition, male but not female FFA3 KO mice had lower energy expenditure than WT mice (Bellahcene et al. 2012). Low fat diet-fed male FFA3 KO mice also displayed lowered liver TG and plasma FFA concentrations, elevated plasma adiponectin and impaired glucose tolerance. On the other hand, HFD-fed male FF3 KO mice displayed elevated plasma glucose and leptin and reduced lean body mass. The differences in the effects of sex hormones on adipose tissue distribution and on the central regulation of metabolism could explain why female FFA3 KO mice have comparable energy expenditure to WT mice. Nevertheless, we cannot exclude the possibility that reduced SCFA absorption by increased gut motility alleviated obesity in FFA3 KO mice used in the study by Bellahcene et al. (2012). Further, energy expenditure was reduced in male but not female FFA3 KO mice compared to WT mice, which might be caused by reduced sympathetic activity (refer to Sect. 3.3.3). Therefore, the obese phenotype in male FFA3 KO mice could be explained by reduced energy expenditure. Consistent with reports by Samuel et al. (2008) and Tazoe et al. (2009), Tolhurst et al. (2012) demonstrated FFA3 mRNA in intestinal L cells, which secrete PYY and GLP-1. Glucose-stimulated GLP-1 secretion was reduced in primary colonic cultures obtained from FFA3 KO mice compared with cultures from WT mice. Consistent with these findings, oral glucose tolerance was impaired in FFA3 KO mice (Tolhurst et al. 2012). Blunted GLP-1 secretion in response to SCFA in colonic cultures from FFA3 KO mice was

suggested to result from the reduced expression of FFA2 because SCFAs stimulated the release of GLP-1 even when $G_{i/o}$ protein signaling was inhibited by pretreatment with PTX.

3.3.3 Sympathetic Nervous System (SNS)

FFA3 regulates host energy balance by modulating sympathetic nerve activity. In 2011, we reported that murine FFA3 mRNA was abundantly expressed in sympathetic ganglia in murine tissues from the embryonic to the adult stage and in human sympathetic ganglia. This was confirmed by in situ hybridization and quantitative RT-PCR analysis (Kimura et al. 2011). FFA3 KO mice exhibited a significantly reduced density of sympathetic innervation and a reduced level of tyrosine hydroxylase (TH; rate limiting enzyme for catecholamine biosynthesis) protein in the heart, suggesting that FFA3 is involved in sympathetic nerve growth. However, further studies are needed to clarify the role of FFA3 in sympathetic nerve differentiation and growth. In adult WT or FFA2 KO mice, energy expenditure was reflected by propionate-induced increase in heart rate and oxygen consumption, whereas these effects were abolished in FFA3 KO mice. The effect of propionate on heart rate is inhibited by pretreatment with the β-adrenergic receptor blocker propranolol but not by the nicotinic acetylcholine receptor blocker hexamethonium. This indicates that propionate activates SNS through FFA3 at the ganglionic level (Kimura et al. 2011). This function of FFA3 in sympathetic ganglia is consistent with the lower energy expenditure and obese phenotype of FFA3 KO mice reported by Bellahcene et al. (2012). We further clarified the signaling mechanism for sympathetic activation using primary cultured murine sympathetic ganglion neurons. Pharmacological and knockdown experiments showed that propionate increased the release of tritium-labeled noradrenaline from sympathetic neurons through the FFA3-$G_{\beta\gamma}$-phospholipase C (PLC) β3-ERK1/2-synapsin 2 pathway (Kimura et al. 2011; Inoue et al. 2012). The synapsins are a family of synaptic vesicle-associated phosphoproteins. Serine 426 of synapsin 2b is phosphorylated by activated ERK1/2 in response to propionate stimulation in murine sympathetic neurons (Inoue et al. 2012).

During assessment of the effects of SCFAs and ketone bodies in FFA3-expressing HEK293 cells, we found that β-hydroxybutyrate (β-HB) had a potent antagonistic effect on FFA3-mediated ERK1/2 phosphorylation and cAMP inhibition (Kimura et al. 2011). β-HB is a ketone body, which can be produced in the liver under ketogenic conditions such as fasting, low-carbohydrate dietary intake, and diabetes. In contrast, another major ketone body, acetoacetate, had no significant effect. We further showed that β-HB suppressed propionate-induced sympathetic activation in primary cultured sympathetic neurons, and in mice (Kimura et al. 2011; Inoue et al. 2012). These findings suggest that FFA3 functions as an energy sensor in sympathetic ganglia to maintain energy homeostasis (Fig. 3). Under fed conditions, SCFAs are produced in the gut by bacterial fermentation of dietary fiber. SCFAs activate SNS by stimulating FFA3, leading to an increase in energy expenditure to consume excess

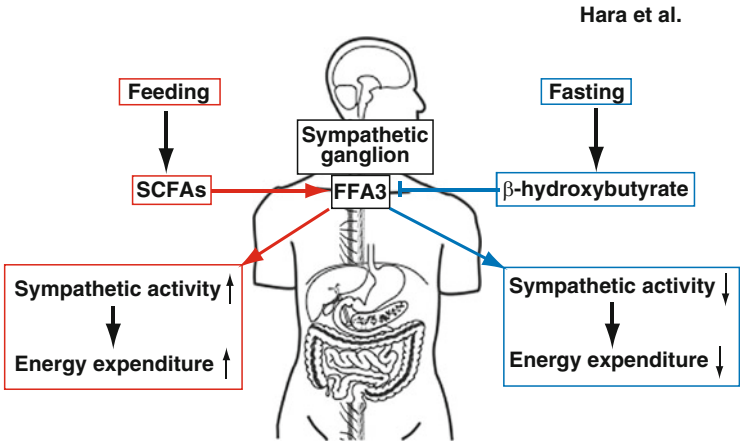

Fig. 3 Schematic diagram of the metabolic regulation by FFA3 on sympathetic ganglion. Under fed condition, SCFAs are produced in gut by bacterial fermentation of dietary fiber followed by the increase of sympathetic activity through FFA3 expressed in sympathetic ganglion. Sympathetic activation leads to the increase in energy expenditure. On the other hand, under fasting condition, β-hydroxybutyrate is produced as energy sources in liver and decreases sympathetic activity through inhibition of FFA3 in sympathetic ganglion. Accordingly, β-hydroxybutyrate suppresses energy expenditure through FFA3

energy. In contrast, β-HB is produced in the liver under fasted conditions. β-HB suppresses SNS by inhibiting FFA3, leading to a reduction in energy expenditure to preserve energy. Thus, these monocarboxylic metabolites appear to control energy balance by directly regulating FFA3-mediated sympathetic activation at the ganglionic level.

4 FFA1 (GPR40)

4.1 Ligands

In 2003, three groups almost simultaneously reported that medium to long-chain FFAs activated FFA1 by monitoring either $[Ca^{2+}]_i$ or by reporter assay (Briscoe et al. 2003; Itoh et al. 2003; Kotarsky et al. 2003). Since FFAs are involved in various physiological properties, stably expressing cell lines were primarily used in high-throughput screening to examine the effects of FFAs on FFA1. Numerous FFAs were reported to act as agonists for FFA1 in the micromolar concentration range, and the rank order of the agonistic activities of FFA ligands measured by $[Ca^{2+}]_i$ response were as follows: docosahexaenoic acid (DHA, C22:6) > α-linolenic acid (α-LA) (C18:3) = oleic acid (C18:1) > palmitic acid (C16) = lauric acid (C12) > capric acid (C10) > caprylic acid (C8). These results indicated

that both saturated and unsaturated FFAs could be agonists for FFA1. Carbon chain length was important for agonistic activities of saturated fatty acids but not for unsaturated fatty acids (Itoh et al. 2003). Interestingly, SCFA such as acetic acid (C2), butyric acid (C4), caproic acid (C6), and methyl linoleate did not show any response, suggesting that carbon chain length and the presence of carboxylate group in the structure appeared to be critical for the activation of FFA1. In addition to the essential FFAs, several compounds of clinical interest such as conjugated linoleic acid, which is known as a dietary component associated with anticarcinogenic effects, and 9-hydroxyoctadecadienoic acid (9-HODE), which is associated with arteriosclerosis, were revealed to activate FFA1 in a dose-dependent manner.

4.2 Selective Ligands

The physiological functions of FFARs appear to be strongly related to metabolic energy regulation such as glucose homeostasis, and secretion of insulin and incretins; thus, studies to develop potent and selective agonists are of global interest. Several research groups including pharmaceutical companies have reported novel series of FFA1 agonists, which were evaluated by in vitro or in vivo studies (Bharate et al. 2009; Garrido et al. 2006; Hu et al. 2009; Humphries et al. 2009; Zhang et al. 2010; Zhou et al. 2010). In particular, an FFA1 selective ligand, GW9508, was used in various studies as a reference compound for FFA1. GW9508 showed 100-fold more potent agonistic activity for FFA1 compared with GPR120. In addition, we reported that PPARγ agonists (thiazolidinediones), also known as antidiabetic drugs, and the experimental anti-obesity compound MEDICA16 could be FFA agonists (Briscoe et al. 2006; Hara et al. 2009b). Furthermore, we developed a synthetic ligand, NCG75, by *in silico* docking simulation using the FFA1 homology model that showed strong activation of ERK1/2 and $[Ca^{2+}]_i$ response. NCG75 promoted insulin secretion from MIN6 (murine insulinoma) cells, which express endogenous FFA1. The potent synthetic compound, TAK-875, has recently entered clinical trials (refer to Sect. 4.8). Results from in vitro and in vivo studies showed that TAK-875 enhanced glucose-stimulated insulin secretion (GSIS) in a glucose-dependent manner in β-cells. The stimulatory effect of TAK-875 was correlated with the $[Ca^{2+}]_i$ response without stimulation of glucagon secretion. Therefore, TAK-875 may be useful as a treatment to control plasma glucose levels without the risk of developing hypoglycemia and β-cell toxicity (Yashiro et al. 2012; Tsujihata et al. 2011). As a partial agonist, AMG837 was identified by optimization of a series of β-substituted phenylpropanoic acids with an EC_{50} value of approximately 0.1 μM (Houze et al. 2012). TAK-875 and AMG837 could act as antihyperglycemic agents, however these two compounds did not improve incretin levels in vivo. Therefore, to investigate novel class of agonist which might improve both insulin and incretin level, chemical modification of AMG837 was conducted and led to the discovery of the potent full agonist AM1638 and AM6226 (Luo et al. 2012). These compounds

stimulated GLP-1 and GIP secretion and increase GSIS. On the other hand, known FFA1 ligands showed lipophilicity responsible for the poor pharmacokinetic properties and toxicity. Christiansen et al. (2013) reported that a novel compound (Compound 7) was developed based on the structure of TUG424 reported as potent agonist with moderate metabolic stability in vitro (Christiansen et al. 2008). This compound showed low lipophylicity, high selectivity, marked bioavailability and efficacy on glucose tolerance. As a result, these compounds are expected to be useful in the future clinical trials and the investigation of FFA1.

4.3 Signal Transduction

Various reports have confirmed that FFAR stimulation promoted the release of $[Ca^{2+}]_i$ and ERK1/2 phosphorylation in cells that both transiently and stably expressed FFA1 (Itoh et al. 2003). The increase in $[Ca^{2+}]_i$ was examined in MIN6 cells and also in primary pancreatic β-cells. Furthermore, since the $[Ca^{2+}]_i$ and ERK1/2 responses did not promote cAMP accumulation, FFA1 could be coupled to a G-protein α-subunit of the G_q family but not from the $G_{i/o}$ or G_s family. Moreover, an endogenous agonist such as linoleic acid promoted PLC activation by the G_q protein through FFA1 expressed in HEK293 cells (Salehi et al. 2005). In contrast, agonist-induced inhibition of voltage-gated K^+ current in pancreatic β-cells could be mediated by cAMP signaling leading to an increase in excitability (Fujiwara et al. 2005; Zhao et al. 2008). Recently, the precise mechanisms of FFA-induced GSIS were further analyzed using islets from FFA1 KO mice. Oleate-induced GSIS in an FFA1-dependent manner and rapid F-actin remodeling was observed in islets from WT but not from KO mice. Moreover, protein kinase D (PKD) phosphorylation induced by oleate stimulation was also observed in WT but not in KO mice. Furthermore, pharmacological inhibition of PKD1 prevented oleate-induced GSIS and F-actin depolymerization. Hence, these results indicated that the signaling pathways leading to insulin secretion in pancreatic islets could be involved in F-actin depolymerization and PKD1 activation (Ferdaoussi et al. 2012). Two different groups reported mechanisms that could regulate the expression levels of FFA1 in pancreatic islet. The paired box 6 (PAX6) protein, a known transcription factor in pancreatic α-cells, could interact with the promoter region of the FFA1 gene which contribute to regulate the expression of FFA1 (Gosmain et al. 2012). On the other hand, pancreatic duodenal homeobox-1 (PDX-1) is involved in glucose-induced FFA1 gene transcription in pancreatic β-cells. Interestingly, PI3K-dependent O-GlcNAcylation of PDX-1 mediated by the association of O-GlcNAc transferase to phosphatidylinositol 3,4,5-triphosphate (PIP_3) is crucial process of regulating genes related to glucose metabolism including FFA1. Therefore, as the compensation mechanism, stimulation of FFA1 gene expression might enhance FFA1 signaling under the condition of excess nutrients, which contribute to normalize blood glucose level (Kebede et al. 2012).

In our previous reports, we mentioned a potential discrepancy between agonist-induced intracellular signaling (ERK1/2 phosphorylation or [Ca] response) and effects on insulin secretion. The selective agonists, NCG75, GW9508, and TAK-875 have been described as potent because they can activate intracellular signalings at lower concentrations compared with the endogenous ligands such as oleic acid, linoleic acid and α-LA however, the effects of these compounds on insulin secretion in the cells that express endogenous FFA1 (INS-1 or MIN6 cells) were similar to those of endogenous ligand (Tsujihata et al. 2011; Briscoe et al. 2006; Takeuchi et al. 2013). In previous studies, insulin secretion was shown to be regulated by the multiple signaling pathways including Ca and ERK (Longuet et al. 2005; Selway et al. 2012). Therefore, FFAs-induced insulin secretion was appeared to be activated by multiple pathways. Together, these discrepancies may be explained by contribution of another signaling mechanism, which could regulate insulin secretion. Further research to address the relationship between signaling pathways and physiological functions are required to investigate the precise mechanisms of FFA1.

4.4 Expression and Physiological Functions

The expression profiles of FFA1 were determined in various tissues by reverse-transcription polymerase chain reaction, immunohistochemistry, and in situ hybridization.

4.4.1 Pancreas

Expression analysis of FFA1 by RT-PCR and immunohistochemistry revealed high levels of expression in insulin-producing pancreatic islet cells (Itoh et al. 2003; Tomita et al. 2006). Itoh et al. reported that the activation of FFA1 by FFA ligands enhanced GSIS from pancreatic β-cells. Several research groups reported a protective effect of FFA1 on β-cells against GSIS using KO and/or transgenic (TG) mice. In addition, FFA1 protein was detected in the periphery of murine islet ⟨-cells and hamster glucagonoma cells (Flodgren et al. 2007). The FFA1 KO mice fed with a regular chow diet did not show any differences in body weight, fasting glucose level, and insulin, triglyceride, and glucose tolerance compared with WT mice. However, KO mice and WT littermates who were fed with high fat diet (HFD) showed fasting hyperglycemia, obesity, glucose intolerance, and insulin resistance. The levels of insulin secretion in response to a mixture of mostly unsaturated fatty acids were reduced by approximately 50 %. Furthermore, although the effects of glucose stimulation on insulin secretion in isolated islets from KO mice were unchanged, fatty acid stimulated-induced insulin secretion was significantly reduced (Lan et al. 2008; Kebede et al. 2008; Latour et al. 2007). Moreover, knockout of FFA1 in mice did not contribute to glucose intolerance,

hyperglycemia, and hypertriglyceridemia. Several groups reported the results of FFA1 TG mice studies. FFA1 overexpression under the mouse PDX-1 promoter exhibited impaired β-cell function (Steneberg et al. 2005). However, other groups could not confirm these observations. FFA1 overexpression under the mouse insulin II promoter did not impair metabolic status, whereas the levels of fasting blood glucose was lower than that of WT mice. Also, insulin secretion and oral glucose tolerance were improved in FFA1 TG mice without affecting insulin tolerance (Nagasumi et al. 2009). These discrepancies were partly explained by differences of the expression levels of FFA1 in each TG mouse and of the promoter subjected to construct TG mice (Alquier and Poitout 2009). Together, these findings indicate that FFA1 contributes to GSIS and the regulation of basal energy metabolism.

4.4.2 Intestine

Edfalk et al. (2008) reported that FFA1 is expressed in endocrine cells of the gastrointestinal tract, including cells expressing the incretin hormones GLP-1 and GIP such as the intestinal L and K cells, respectively. Since FFA1 mediates FFA-induced incretin secretion, the effects of FFAs on insulin secretion are possibly mediated by both direct (through the activation of FFA1 expressed on β-cells) and indirect (through the release of incretin hormone) pathways. Furthermore, FFA1 expression was also confirmed in I cells in the intestine that secretes cholecystokinin (CCK) (Liou et al. 2011). Therefore, FFA1 modulates FFA-induced insulin secretion from β-cells directly and indirectly through regulation of incretin secretion (Edfalk et al. 2008; Latour et al. 2007) (Fig. 4).

4.4.3 Taste Buds

Immunohistochemistry of circumvallate papillae (CV) section using antibodies against FFA1 and GPR120 showed positive signals in type I and type II taste bud cells, respectively. These signals were eliminated in taste tissue from GPR40 KO and GPR120 KO mice (Cartoni et al. 2010). Studies using both FFA1 and GPR120 KO mice revealed that taste nerve responses to fatty acids and preferences for fatty acids, evaluated by short-access tests using a lick meter comparing linoleic acid and xanthan gum, were inhibited in KO mice. There was no difference in preference for other tastes such as bitter, sweet, sour, salty, or umami by FFA1 KO and GPR120 KO mice compared with WT mice. However, recent publication did not confirm the expression of FFA1 in CV, fungiform papillae and taste cell-free lingual epithelium by RT-PCR (Galindo et al. 2012). Matsumura et al. (2007) also reported that GPR120 but not FFA1 could detect in the epithelium of the circumvallate papillae. Therefore, the expression of FFA1 in sensory taste cells is quite controversial. Further research should be necessary to clarify the precise expression profiles of FFA1 and GPR120 in sensory taste cells.

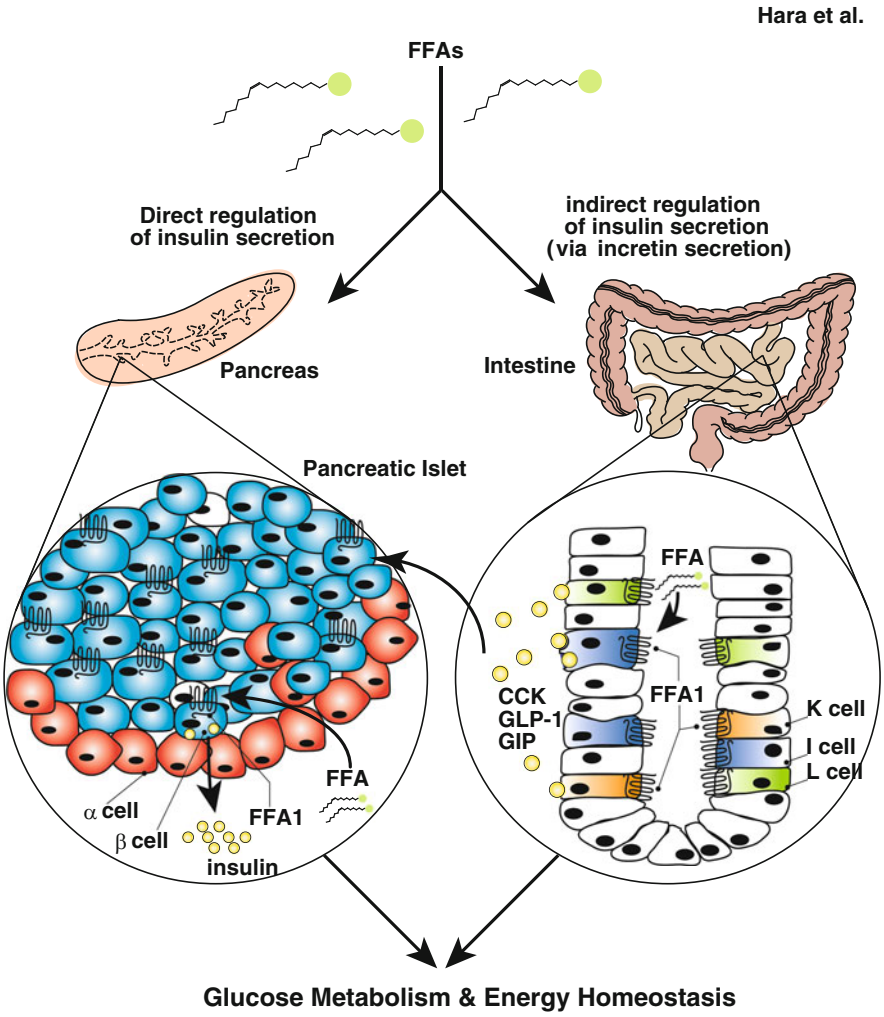

Fig. 4 Schematic diagram of physiological functions of FFA1 in glucose metabolism and energy homeostasis. FFAs stimulate FFA1 expressed in pancreatic β-cells and promote insulin secretion in direct mechanisms. FFAs also stimulate FFA1 expressed in incretin secreting cells and promote insulin secretion in indirect mechanisms. FFA1 partly modulates FFAs-induced insulin secretion from β-cells not only directly but also indirectly mechanisms

4.4.4 Brain

Ma et al. confirmed the expression and distribution profiles of FFA1 in the central nervous system of adult monkeys by immunoblotting and immunohistochemistry. Furthermore, FFAs such as docosahexaenoic acid that could activate FFA1 may play an important role in the regulation of adult hippocampal neurogenesis in primates (Yamashita 2008; Ma et al. 2010).

4.4.5 Mammary Gland

Yonezawa et al. detected FFA1 mRNA in MCF-7 human breast cancer cells, bovine mammary epithelial cells, and bovine mammary gland. These were activated by oleate and linoleate and may be involved in a proliferative effect through $[Ca^{2+}]_i$ response, ERK1/2, and Akt kinase (Yonezawa et al. 2008).

4.5 Polymorphism

Genetic polymorphisms that alter protein expression or function can modify the risk of disease. Polymorphisms have been reported for FFA1: in humans, several single nucleotide polymorphisms identified in the FFA1 coding region are reportedly associated with the function of FFA1. The Gly180Ser polymorphism that was identified in a screening analysis of 734 subjects showed significantly lower levels of insulin secretion in Gly/Gly carriers. In addition, Gly/Ser carriers showed lower levels of plasma insulin and C-peptide in response to a lipid load in a case-control study. Furthermore, these results are supported by an in vitro study in which the Gly/Ser mutant expressed in HeLa cells showed a lower $[Ca^{2+}]_i$ response induced by oleic acid compared with the Gly/Gly mutant (Vettor et al. 2008). The His211Arg polymorphism is considered to be associated with the altered insulin secretion capability and metabolic parameters in Danish Caucasian subjects and Japanese men. In Danish Caucasian subjects, the Arg211His polymorphism showed a similar allele frequency between type 2 diabetic patients and middle-aged glucose-tolerant subjects. Moreover, the levels of insulin release determined by oral glucose tolerance test showed no significant difference between these carriers. Similarly, allele frequencies of this variant in Japanese subjects showed no difference between type 2 diabetic patients and healthy subjects. However, Arg/Arg homozygotes showed a significantly lower homeostasis model of insulin resistance (HOMA-IR) and ®-cell function (HOMA-b) together with serum insulin levels compared to His/His genotypes. In addition, a rare mutation, Asp175Asn was identified during the analysis of Danish Caucasian subjects. The effects of these two variants, Arg211His and Asp175Asn, on inositol phosphate production in response to 5,8,11-eicosatriynoic acid were similar to each other; however, Asp175Asn, but not Arg211His, showed lower maximal efficacy compared to the WT. Further studies addressing the relationships between FFA1 and other genes that control metabolic energy regulation within different ethnicities are required to understand the contribution of these polymorphisms to diabetes and modulation of the function of the FFA1 protein (Hamid et al. 2005; Ogawa et al. 2005).

4.6 Structure–Activity Relationships (SARs)

FFA1 is reportedly involved in the activation of insulin release and as such is considered an attractive therapeutic target for the treatment of diabetes. Thus, numerous groups have engaged in the development of potential lead compounds as ligands for the receptor. A deep understanding of the molecular details of ligand recognition and receptor activation is useful for the identification and rational design of ligands that show biological activity and selectivity for a specific receptor. Numerous groups including our own have attempted to develop candidate compounds using docking simulation with homology models for FFA1. Findings have included information concerning the chemical structure of compounds and also the binding mode in the binding cavity of FFA1. The ionic lock at the cytoplasmic surface is known to play an important role in the activation mechanism of class A GPCRs including FFARs. However, the contribution of agonist binding to alteration of the ionic locks remains unclear. The ionic locks in FFA1 are predicted by homology modeling to be located between the second extracellular loop of FFA1 (Glu145 and Glu172) and transmembrane domain residues Arg183 and Arg258. Agonist interaction-induced dissociation of the ionic interactions between Glu–Arg was predicted by simulation of molecular dynamics. Furthermore, the constitutive activation of FFA1 induced by the breakage of these interactions was observed in mutagenesis studies. Therefore, these ionic locks may act as a molecular switch toward receptor activation (Sum et al. 2009). Among the FFA1 agonists, linoleic acid known as a natural polyunsaturated FFA ligand and GW9508 were subjected to examine the molecular determinants of agonist binding to FFA1. Hydrophobic, aromatic and hydrophilic/positively charged amino acid residues were predicted to be potential binding residues in the binding cavity of FFA1. A mutagenesis study revealed that Arg183, Asn244, and Arg258 contributed to the interaction between the carboxylate group present in the structures of linoleic acid and GW9508. In addition, His86, Tyr91, and His137 contributed to the aromatic or hydrophobic interactions in GW9508 binding. Moreover, His147 and His86 may contribute to GW9508-induced receptor activation. Hence, these results may explain why GW9508 could strongly activate FFA1 signaling compared with other FFA ligands (Tikhonova et al. 2007; Sum et al. 2007). Our group also conducted *in silico* docking simulations to search for selective agonists and to investigate the binding modes of these ligands. Consistent with other reports, Arg183 and Arg258 were found to be important for agonist recognition and activation of FFA1. In contrast, our synthetic agonist, NCG75 (refer to section titled “Selective ligand”), and α-LA, a natural agonist, showed a different binding profile compared with GW9508. Thus, Arg 258 and not a combination of Arg183 and Arg258, was deemed to be important for FFA1 activation. This would explain the prediction that the hydrogen bond distance between the ligand and the amino acid residues may reflect agonistic potency. In addition, we confirmed the contribution of other amino acid residues to ligand interaction; His86, Phe87, and Tyr240 formed interactions with pyridine and phenyl ring of

NCG75; Val141, Ala146, and Ala173 stabilized binding through hydrophobic interactions. These findings concerning the critical roles of these residues for ligand interaction are useful for investigating the pharmacology of FFA1 and for further development of potent and selective ligands (Takeuchi et al. 2013).

4.7 Binding Assay

Experimental systems that can monitor direct interactions between ligands and FFARs have not yet been developed because of the relatively weak affinity of natural ligands for their receptors and the lack of potent ligands. Therefore, an experimental system was established using flow cytometry that could detect direct interactions between ligands and FFA1 (Hara et al. 2009a). We designed a fluorescent-labeled ligand (BODIPY-labeled FFA) as a probe, which could activate FFA1. However, we were forced to isolate the FFA1 protein by immunoprecipitation as a result of non-specific binding of the fluorescent probe to cells overexpressing FFA1. Sf9 cells expressing FLAG-tagged FFA1 were solubilized. The lysates containing FFA1 protein were immobilized using immunomagnetic beads and were detected by flow cytometry as the cells that express FFA1. Flow cytometry-based binding assays revealed that fluorescent-labeled FFA specifically interacted with its binding site located on FFA1 in a saturable manner. In addition to the FFA ligands, synthetic ligands including GW9508, MEDICA16, and thiazolidinediones competed with the fluorescent-labeled ligand and bound to the FFA1 protein in a dose-dependent manner. A novel agonist that shows agonistic activity at nanomolar concentrations has recently been used to monitor direct interactions in a conventional radioligand binding assay. This radiolabeled compound is currently unavailable commercially. Therefore, further efforts to develop potent ligands could benefit from successful monitoring of direct interaction with FFA1 or other FFARs. Moreover, these ligands will be useful for further investigation of the physiological and pharmacological functions of FFA1.

4.8 Clinical Trials

The effect of a single oral dose of TAK-875 was evaluated for safety, tolerability, pharmacokinetics, and pharmacodynamics in a phase I, double-blind, placebo-controlled study in healthy volunteers (Naik et al. 2012). The results indicated that TAK-875 appears to be safe and tolerated in healthy subjects after a single oral administration. Two phase II, multicenter, randomized, double-blind, parallel group studies were conducted in Japanese patients with type 2 diabetes. Efficacy and tolerability were demonstrated after administration of TAK-875 for 2 weeks (Araki et al. 2012). In addition, the results of a 12-week dose-ranging study of TAK-875 indicated that TAK-875 showed effective antihyperglycemic properties without severe adverse effects in type 2 diabetes patients whose symptoms were not

adequately controlled by diet and exercise (Kaku et al. 2013). Furthermore, a phase II, randomized, double-blind, placebo-controlled, and active comparator-controlled trial was conducted in type 2 diabetes patients who were non-responders to diet or metformin treatment (Burant et al. 2012). The effect of once daily administration of TAK-875 for 12 weeks revealed an improvement in glycemic control in patients with minimal risk of hypoglycemia. These clinical trials strongly suggest that FFA1 is a crucial drug target, and that FFA1 agonists could be a novel class of therapeutic drugs for the treatment of type 2 diabetes.

5 GPR84

5.1 Ligands

GPR84 was activated by medium-chain FFAs (C9-C14) (Wang et al. 2006). In contrast, neither short- nor long-chain FFAs did not activate GPR84. Among these medium-chain FFA ligands, capric acid (C10:0), undecanoic acid (C11:0) and lauric acid (C12:0) showed the most potent activity for GPR84. The EC_{50} values for these three FFAs were approximately 5–10 μM. As a small molecule for GPR84, diindolylmethane was reported to activate GPR84 with greater potency than FFA agonists. The EC_{50} value of this compound measured by [^{35}S]-GTPγS binding assay was approximately 0.5 μM.

5.2 Signal Transduction

Medium-chain FFAs dose-dependently increased $[Ca^{2+}]_i$ in CHO cells transiently express GPR84. In addition, forskolin-stimulated cAMP production was inhibited by medium-chain FFAs. GPR84 dependent inhibition of cAMP production was reduced by pretreatment with pertussis toxin. Further, medium-chain FFAs increased [^{35}S]-GTPγS incorporation. Therefore, GPR84 was activated by medium-chain FFAs and coupled to $G_{i/o}$ pathway (Wang et al. 2006).

5.3 Expression and Physiological Functions

GPR84 was identified by using an expressed sequence tag (EST) data mining strategy (Wittenberger et al. 2001). GPR84 is reported to be express in various tissues such as heart, lung, kidney, liver and leucocytes. Especially, as related to inflammatory cells, granulocytes, splenic T and B cells express GPR84 (Yousefi et al. 2001; Venkataraman and Kuo 2005). Also, GPR84 is induced by the

stimulation of lipopolysaccharide (LPS) in monocytes (Wang et al. 2006). Furthermore, Nagasaki et al. (2012) reported that adipose tissue and 3T3-L1 adipocyte cell line express GPR84. Bouchard et al. (2007) reported that microglia express GPR84 in a strong and sustained manner.

5.3.1 Immune System

The functional study of GPR84 showed that CD3 antibody-induced IL-4, but not IFN-γ and IL-2 production was increased in GPR84 deficient mice compared to WT mice. However, the stimulation effects of various mitogen on the proliferation of T and B cells were not changed between GPR84-deficient and WT mice. Further, the levels of IL-4, but not IFN-γ mRNA was also increased in response to antibody stimulations of CD28 together with CD3. Also, the expression level of GPR84 mRNA was increased in monoyotes or differentiated into macrophages after stimulation of LPS. In addition, GPR84 expressed in microglia was induced by proinflammatory cytokines such as TNF-α and IL-1. The expression of GPR84 was potent observed in not only in mice suffering from endotoxemia, but also during experimental autoimmune encephalomyelitis (Bouchard et al. 2007). Hence, GPR84 might contribute to regulate neuroimmunological processes.

5.3.2 Adipose Tissue

GPR84 is reported to be expressed in adipose tissue and 3T3-L1 adipocytes. In HFD supplemented mice, GPR84 expression was detected in fat pads. Also, 3T3-L1 adipocytes co-cultured with a macrophage cell line RAW264, significantly induced GPR84 expression. On the other hand, medium-chain fatty acids reduced mRNA expression level of adiponectin in 3T3-L1 cell line through GPR84. This report suggested that macrophages that infiltrated into adipose tissue and secreted inflammatory cytokines such as TNF-α, contributed to enhance the expression of GPR84 mRNA (Nagasaki et al. 2012). Accordingly, GPR84 might play important physiological roles in the regulation of insulin sensitivity under inflammatory condition such as type 2 diabetes.

6 GPR120

6.1 Ligands

We previously isolated the GPR120 gene from genomic DNA. Medium to long-chain FFAs were identified as endogenous ligands of GPR120 using a receptor internalization assay (Fukunaga et al. 2006). GPR120 was activated by saturated

FFAs (C14–18) and unsaturated FFAs (C16–22). Various polyunsaturated fatty acids, regardless of ω-3 or ω-6 type, were found to act as agonists of GPR120 in the micromolar concentration range, with α-linolenic acid being the most potent (Hirasawa et al. 2005). The ligand profiles were similar to those for FFA1; however, the amino acid homology between GPR120 and FFA1 is only 10 %. The similarity in ligand specificity may be the result of convergent evolution. In addition, we synthesized a series of carboxylate group-containing compounds that were based on the structure of the thiazolidinedione (PPAR-γ) agonists. The relative ERK1/2 phosphorylation of these compounds examined in FFA1 and GPR120 expressing cells were correlated well with the calculated hydrogen bonding energy based on FFA1 and GPR120 homology model, respectively (Fig. 5a). The selective agonist NCG21 was developed (Suzuki et al. 2008) using a homology model and docking simulation for GPR120 (Fig. 5b) (Sun et al. 2010). To identify other natural ligands of GPR120, we screened for and identified a selective partial agonist from a series of natural compounds; grifolic acid, derived from the fruiting bodies of *Albatrellus ovinus* (Hara et al. 2009b). Grifolic acid activated GPR120 in GPR120 over-expressing cells and also in STC-1 cells, which express endogenous GPR120. Further Hashimoto et al. (2010) reported a synthetic compound (Compound 8) as a patented compound. More recently, Shimpukade et al. (2012) reported a potent and selective GPR120 agonist (Compound 9), which showed high potency on both human and murine GPR120. These compounds may be useful tools to monitor the physiological effects of GPR120 and may be useful for the development of novel drug candidates for the treatment of type 2 diabetes, obesity, and metabolic diseases.

6.2 Signal Transduction

Polyunsaturated fatty acids and synthetic ligands induced a rise in cytosolic free Ca^{2+} in GPR120 over-expressing HEK293 cells through GPR120, but they did not promote cAMP production. This suggested that GPR120 is coupled to the G_q protein family, similarly to FFA1, but not to the G_s or $G_{i/o}$ families (Hirasawa et al. 2005). GPR120 can also induce the activation of ERK1/2 under certain conditions, and the activation of PI3-kinase and the serine/threonine protein kinase Akt in GPR120-expressing cells (Katsuma et al. 2005). Oh et al. showed that the ω-3 FFAs, DHA and eicosapentaenoic acid (EPA) exert anti-inflammatory effects through GPR120. The underlying mechanism involved inhibition of TGF-β-activated kinase 1 (TAK1) phosphorylation related to the toll-like receptor (TLR), and tumor necrosis factor-α (TNF-α) inflammatory pathways through β-arrestin 2 signaling in monocytic RAW264.7 cells and primary intraperitoneal macrophages (Oh et al. 2010). Recently, Shah et al. showed that linoleic acid leads to activation of monovalent cation-specific transient receptor potential channel type M5 (TRPM5) in STC-1 cells (Shah et al. 2012). Polyunsaturated fatty acid-induced depolarization is significantly reduced by blockade of G proteins and PLC, and

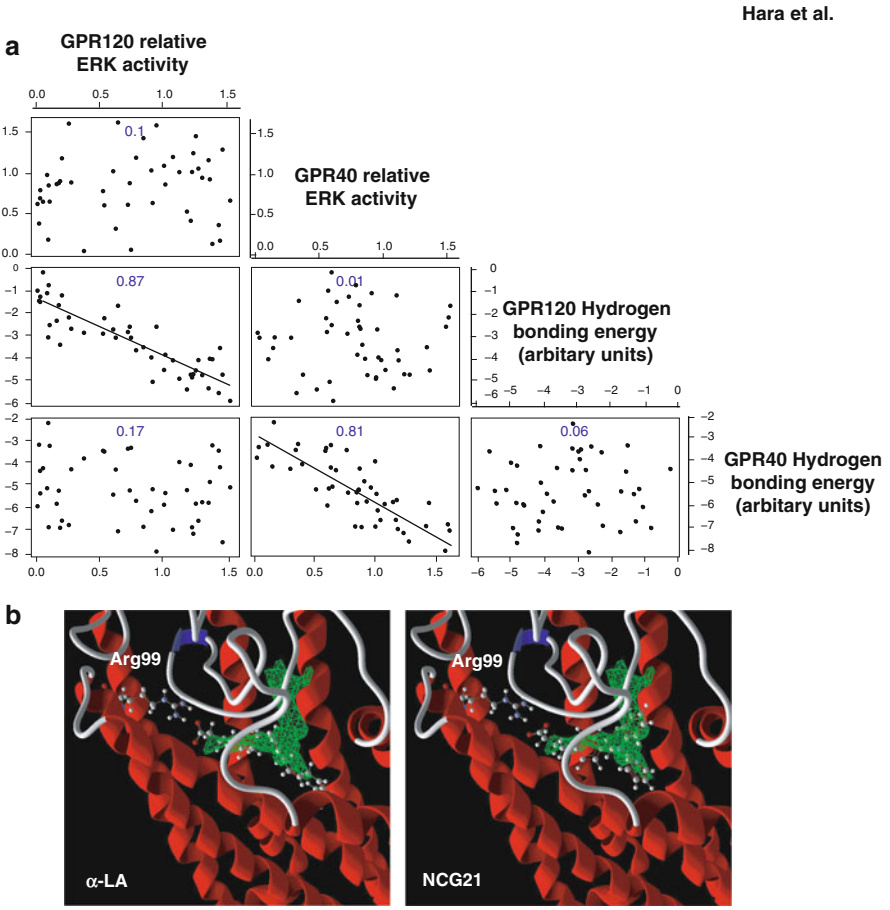

Fig. 5 Docking simulation of each compound in homology models. (**a**) The relative ERK activity versus the calculated energy of interaction based on each modeling was plotted. The coefficient of determination ($R^2 = 0.81$, FFA1 and 0.87, GPR120) reflects a high correlation between the hydrogen bonding energy and relative ERK activity. (**b**) GPR120 homology model docked with α-LA and NCG21. α-LA and NCG21 were docked into the binding pocket of GPR120. Red balls: oxygen atoms of carboxylate group; green: the predicted binding pocket by Molegro cavity detection algorithm

siRNA transfection against TRPM5 resulted in a significant reduction of α-LA-induced intracellular calcium rise as well as CCK secretion from STC-1 cells, suggesting that TRPM5 plays a crucial role in GPR120 signaling.

6.3 Expression and Physiological Functions

6.3.1 Intestine

Endogenous expression of GPR120 was demonstrated in the intestines of humans and mice. Furthermore, the enteroendocrine cell line STC-1 expressed endogenous GPR120. Our previous study showed that GPR120-expressing cells are located in the GLP-1-expressing enteroendocrine cells in the large intestine (Hirasawa et al. 2005; Tanaka et al. 2008; Miyauchi et al. 2009). Stimulation by FFAs induced GLP-1 and CCK secretion in murine enteroendocrine STC-1 cells (Sidhu et al. 2000), and siRNA directed against GPR120 inhibited the FFA-induced effect on incretin secretion and $[Ca^{2+}]_i$ response. The effect of FFAs on plasma levels of GLP-1 and insulin were examined by the administration of FFAs into murine colon (Hirasawa et al. 2005). These reports tempt us to speculate about the physiological function of GPR120 on GLP-1 secretion in vivo. In addition, K cells that express GPR120 and also synthesize GIP were located in the intestinal tract (Parker et al. 2009). Moreover, recent reports suggest that GPR120 may play a role in the lipid-sensing cascade in ghrelin cells (Lu et al. 2012).

6.3.2 Macrophages

Oh et al. (2010) demonstrated endogenous expression of GPR120 in monocytic RAW 264.7 cells and in primary proinflammatory M1-like macrophages. Stimulation of GPR120 with ω-3 FFAs caused broad anti-inflammatory effects in these cells, all of which were abrogated by siRNA against GPR120. In vitro experiments revealed the molecular mechanism underlying ω-3 FFA-mediated anti-inflammatory effects. Stimulation of GPR120 specifically inhibited TAK1 phosphorylation and activation, providing a common mechanism for the inhibition of both TLR and TNF-α signaling. In vivo experiments showed that ω-3 FFA treatment inhibited inflammation and enhanced systemic insulin sensitivity in WT mice; however, these effects by ω-3 FFA were not observed in GPR120-deficient mice. These results showed that GPR120 is a functional ω-3 FFA receptor and that it mediates potent insulin sensitizing and antidiabetic effects in vivo by repressing macrophage-induced tissue inflammation.

6.3.3 Adipocytes

Gotoh et al. (2007) reported that adipose tissue expressed GPR120 and that the mRNA expression level in adipocytes was higher than in stromal-vascular cells. GPR120 expression was increased during adipocyte differentiation of 3T3-L1 cells. Small interfering RNA against GPR120 inhibited this effect on adipocyte differentiation (Gotoh et al. 2007). These findings suggested that GPR120 may play important

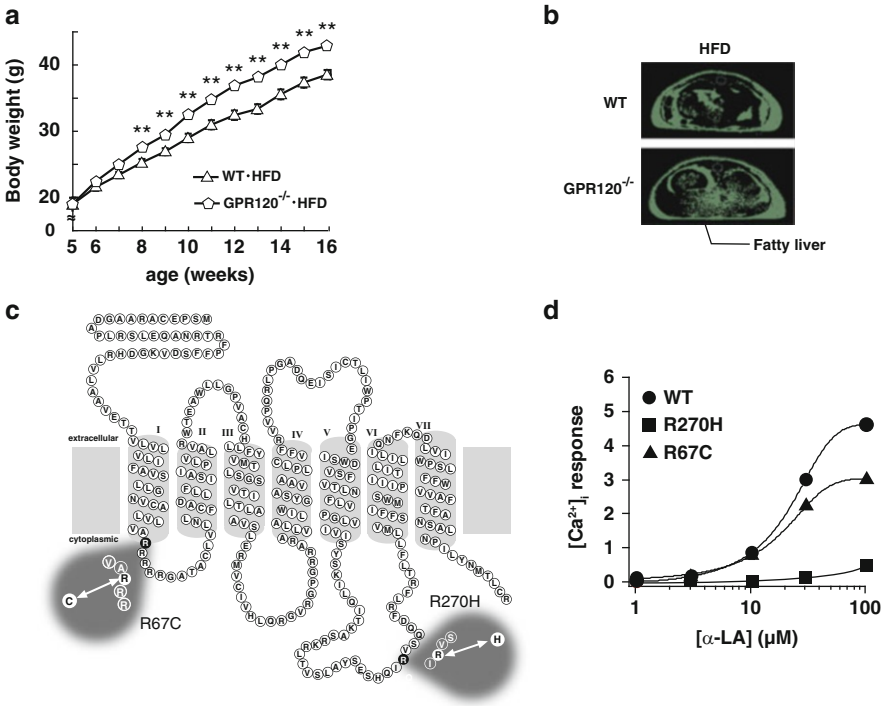

Fig. 6 Obesity and hepatic steatosis in HFD-fed GPR120-deficient mice and pharmacological characterization of two non-synonymous variants of GPR120. (**a**) Body weight changes of WT and GPR120-deficient mice fed HFD. Data represent mean ± s.e.m. $^{**}p < 0.01$ versus the corresponding WT data. (**b**) Representative cross-sectional images of WT and GPR120-deficient mice subjected to microcomputed tomography analysis of the in situ accumulation of fat. Fat depots are demarcated (green) for illustration. The fatty liver in HFD-fed GPR120-deficient mouse was indicated as black line. (**c**) Schematic diagram of two-dimensional topology of GPR120 receptor. Two non-synonymous variants p.R67C and p.R270H were shown. (**d**) α-LA-induced $[Ca^{2+}]_i$ responses in cells expressing WT GPR120 or a p.R67C or p.R270H variant

roles in differentiation, and also in the maturation processes of adipocytes. Moreover, we recently reported that dysfunctional GPR120 led to obesity in both mice and humans (Ichimura et al. 2012). We found that GPR120-deficient mice fed HFD developed obesity and fatty liver with decreased adipocyte differentiation and lipogenesis, and enhanced hepatic lipogenesis (Fig. 6a and b). Insulin resistance in such mice was associated with reduced insulin signaling and enhanced inflammation in adipose tissue. We showed that GPR120 expression in human adipose tissue was significantly higher in obese individuals than in lean controls. GPR120 exon

sequencing in obese subjects revealed two non-synonymous mutation p. R270H and p. R67C (Fig. 6c). The p. R270H variant that inhibited GPR120 signaling activity (Fig. 6d) might be significantly associated with obesity.

Further, since HFD-fed GPR120-deficient mice showed fat liver and obesity, the molecular basis of the metabolic changes in adipose tissues and livers of HFD-fed GPR120-deficient mice and WT mice were examined by using gene expression analysis (Fig. 7a and b). Approximately 1,600 and 600 differentially expressed genes were identified in adipose tissues and livers, respectively. Notably, adipocyte differentiation (*Fabp4*), lipogenesis (*Scd1*) and insulin signal (*Irs2* and *Insr*) related genes were depressed in adipose tissues, whereas these genes together with a fatty acid transporter gene (*Cd36*) were upregulated in livers from GPR120-deficient mice. Therefore, Overall, this study demonstrated that the lipid sensor GPR120 had a key role in sensing dietary fat and thus, in the control of energy balance in both humans and rodents.

6.3.4 Taste Buds

Matsumura et al. reported the expression of GPR120 in taste bud type II cells, as determined by double immunostaining for GPR120 and markers of type II taste cells (phospholipase-Cb2 and ⟨-gustducin) (Matsumura et al. 2009). Cartoni et al. (2010) also reported the expression of GPR120 in CV sections. Short-access test using a lick meter showed that preference for fatty acids but not for other tastes was inhibited in GPR120 KO mice (Cartoni et al. 2010).

6.3.5 Lung

Endogenous expression of GPR120 is also found in other cells and tissues. Furthermore, a GPR120 antibody that recognizes the extracellular domain of murine GPR120 has been developed. This antibody was used to detect GPR120 protein expression in lung and adipose tissues, in which GPR120 mRNA expression was already known (Miyauchi et al. 2009). Pulmonary Clara cells that expressed the Clara cell 10-kDa protein as a marker, stained positively for GPR120 with this antibody (Miyauchi et al. 2009). Further studies are needed to reveal the physiological function of GPR120 in the lung.

6.4 Genetic Contribution to Type 2 Diabetes

Recently, Taneera et al. performed a systems genomics approach to indentify genes for type 2 diabetes, and GPR120 was in the top 20 ranked list (Taneera et al. 2012). In this report, GPR120 expression in human islets was positively correlated with

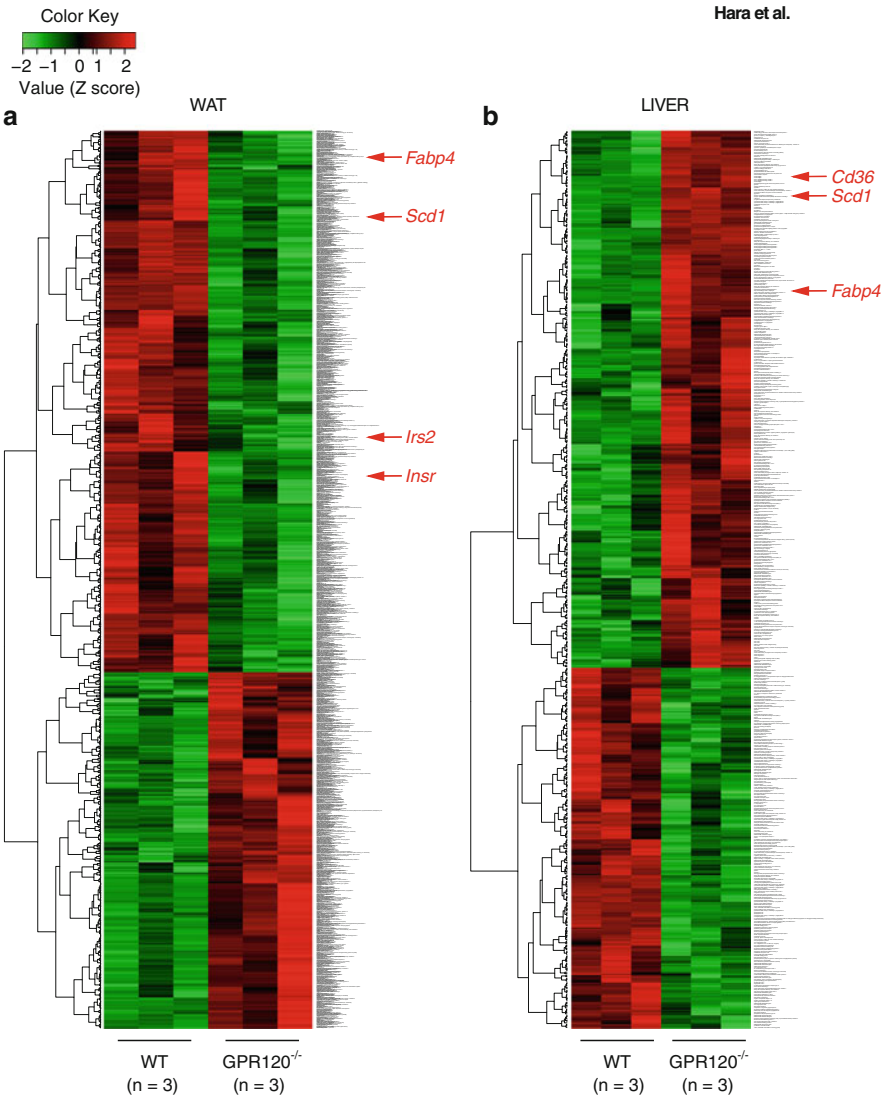

Fig. 7 Changes in gene expression and connectivity map of differentially expressed genes in 16-week-old HFD-fed GPR120-deficient mice. Heatmap comparison of (**a**) epididymal white adipose tissue (WAT) and (**b**) liver of HFD-fed WT and GPR120-deficient mice using gene expression microarray analysis. Gene changes were considered significant if $P < 0.05$ and fold-change > 1.5. The heat map was generated using z-scores across all samples

insulin secretion and insulin content, and with lower HbA1c. Although the physiological function still remains to be cleared, GPR120 expression in pancreas was detected by RT-PCR analysis with the low expression level (Gotoh et al. 2007). Second, activation of GPR120 with EPA prevented lipid-induced apoptosis, and increased cell viability. Although this is not consistent with previous reports (Nagasumi et al. 2009) that FFA1 is predominantly detected in murine pancreatic islets, these data suggest that GPR120 can protect pancreatic islets from lipotoxicity in humans.

6.5 *The Future*

Since FFARs were originally identified as the receptor for FFAs, a remarkable amount of evidence has been gathered to understand the various physiological functions of FFARs. FFARs are activated by FFAs, which are mainly derived from food and the corresponding digested or fermented products in the gastrointestinal tract. Reports using in vitro and in vivo studies indicated that the physiological functions of FFARs conclusively contribute to regulation of metabolic energy (Fig. 8). However, a number of questions remain to be answered. The relative contributions of each of these FFARs to regulation of metabolic energy in the body are currently unclear. The precise signaling mechanisms involving the activation of $[Ca^{2+}]_i$, or ERK1/2 response via G-protein dependent or G-protein independent pathways that are responsible for the reported physiological functions remain to be explored. In addition to our report that genetic analyses of GPR120 could identify a loss-of-function GPR120 variant presented in obese patients, further genetic analysis of GPR120 and other FFARs should be performed to identify gene variants associated with its protein function. Additionally, since the evidence of rare gene variants of major effect on disease risk was reported (Cirulli and Goldstein 2010), we should focus not only on common variant but also on rare variant. Moreover, because the expression, but not the function, of FFARs has been reported in several tissues, functional analysis may provide further evidence for understanding the mechanisms underlying energy metabolism associated with FFARs. Furthermore, early clinical trial evaluation has yielded beneficial results for synthetic agonists of FFA1, thereby suggesting that future research will increase the therapeutic potential of FFARs. Taken together, additional analysis of FFARs may also be important to better understand the nutrient sensing process and to develop therapeutic compounds to treat metabolic energy disorders such as obesity and type 2 diabetes.

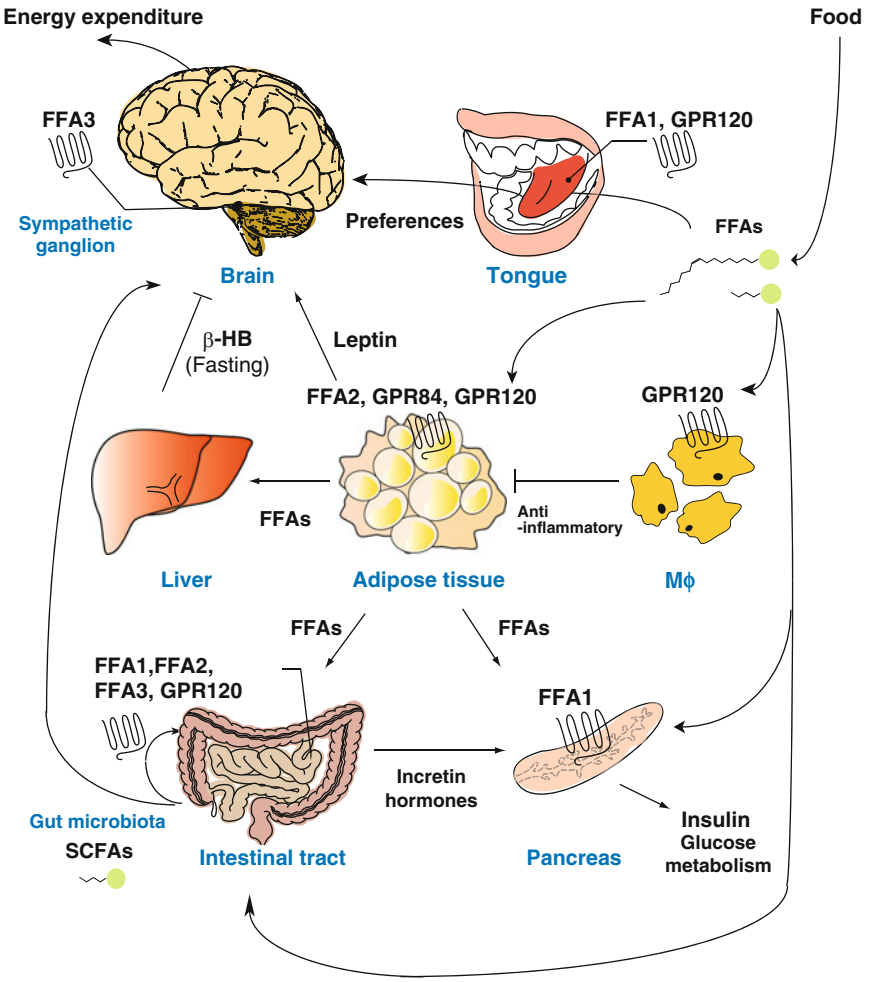

Fig. 8 Functional relationship between FFAs and FFARs. Nutritional and endogenous FFAs stimulate FFARs expressing in various tissues and thereby promote secretion of insulin and incretin hormones, regulate cell differentiations and modulate sympathetic nerve activity. FFARs play key roles in the regulation of FFAs-mediated energy homeostasis in the body

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