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The ghrelin system links dietary lipids with the endocrine control of energy homeostasis

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Summary

Ghrelin is a unique hunger-inducing stomach-borne hormone. It activates orexigenic circuits in the central nervous system (CNS) when acylated with a fatty acid residue by the Ghrelin *O*-acyltransferase (GOAT). Soon after the discovery of ghrelin a theoretical model emerged which suggests that the gastric peptide ghrelin is the first "meal initiation molecule". Ghrelin is also termed "hunger hormone" with a potentially important role as an endogenous regulator of energy balance. However, genetic deletion of ghrelin or its receptor, the growth hormone secretagogue receptor (GHSR), has only limited effects on appetite and obesity.

Here we introduce novel mouse models of altered ghrelin, GHSR and GOAT function to re-evaluate the role of the ghrelin system in regulating energy homeostasis. Simultaneous loss of ghrelin and GHSR function leads to decreased body weight and body fat, likely caused by increased energy expenditure and locomotor activity. Similarly, GOAT deficient mice are lighter and leaner than the wild-type controls. Mice overexpressing ghrelin and GOAT have increased body weight and fat mass along with decreased energy expenditure. Wild-type mouse studies show that fasting induces downregulation of the GOAT gene *Mboat4* and decreases acyl ghrelin concentration in blood. We therefore hypothesized that GOAT rather depends on dietary than endogenous derived lipids for ghrelin acylation. Feeding studies show that GOAT uses the unnatural fatty acid heptanoate (C7) to acylate ghrelin, which clearly supports our theory. Further, acylation of overproduced ghrelin in our transgenic mouse model requires dietary supplementation of medium-chain-triglycerides, the preferred GOAT substrate.

Our genetic models suggest that the ghrelin system plays an important physiological role in the control of energy metabolism. Thus, GOAT offers a novel peripheral drug target for the treatment of metabolic diseases. Moreover, our results suggest that ghrelin signaling may not be a result of absent nutrient intake, but indicate the availability of dietary lipids. We therefore propose that the ghrelin system functions as a novel lipid sensor, linking specific dietary lipids with the central-nervous control of energy metabolism.

Zusammenfassung

Ghrelin ist ein einzigartiges im Magen produziertes Hormon, da es von dem Enzym Ghrelin *O*-acyltransferase (GOAT) mit einer mittelkettigen Fettsäure acyliert werden muss, um biologische Aktivität zu erlangen. Kurz nach seiner Entdeckung entstand die Hypothese, dass Ghrelin das „Hungerhormon" sei und eine wichtige Rolle in der Regulation des Energiehaushalts spiele. Die genetische Manipulation von Ghrelin und seinem Rezeptor, dem GHSR, hat jedoch nur geringe Auswirkung auf Appetit und Körpergewicht. In der hier vorliegenden Studie stellen wir neuartige Mausmodelle mit abgewandelter Ghrelin-, GHSR- und GOAT-funktion vor, um den Einfluss des Ghrelinsystems auf die Regulation der Energiehomöostase zu reevaluieren. Weiterhin wird die endogene Regulation von GOAT erstmalig beschrieben.
Double-knockout Mäuse, die gleichzeitig defizitär für Ghrelin und GHSR sind, haben ein geringeres Körpergewicht, weniger Fettmasse und einen niedrigeren Energieverbrauch als Kontrolltiere. Knockout Mäuse für das GOAT Gen *Mboat4* sind leichter und schlanker als Kontrolltiere. Dementsprechend haben transgene Mäuse, die Ghrelin und GOAT überproduzieren, eine erhöhte Fettmasse und einen verminderten Energieverbrauch. Weiterhin können wir zeigen, dass GOAT, anders als auf Grund der allgemein bekannten Ghrelinfunktion angenommen, nicht durch Hungern aktiviert wird. Bei Mäusen, die gefastet haben, ist die Genexpression von *Mboat4* deutlich herunterreguliert, woraus ein geringer Blutspiegel von Acyl-Ghrelin resultiert. Daraus haben wir geschlossen, dass GOAT eventuell Nahrungsfette und nicht die durch Hungern freigesetzten endogen Fettsäuren zur Ghrelinacylierung benutzt. Fütterungsversuche bestätigen diese Hypothese, da GOAT die unnatürliche Fettsäure Heptan Säure (C7), die der Tiernahrung beigefügt wurde, zur Ghrelinacylierung verwendet. Ein weiteres Indiz für die Notwendigkeit von Nahrungsfetten für die Ghrelinacylierung ist, dass die transgenen Ghrelin/GOAT Mäuse nur massiv Acyl-Ghrelin produzieren, wenn sie mit einer Diät gefüttert werden, die mit mittelkettigen Fettsäuren angereichert ist.
Zusammenfassend zeigt die Studie, dass das Ghrelinsystem maßgeblich an der Regulation der Energiehomöostase beteiligt ist und dass die Ghrelinaktivierung direkt von Nahrungsfetten beeinflusst wird. Daraus könnte geschlossen werden, dass Ghrelin wohlmöglich nicht das Hungerhormon ist, wie bisher generell angenommen wurde. Ghrelin könnte vielmehr ein potentieller "Fettsensor" sein, der dem Gehirn die Verfügbarkeit von fettreicher Nahrung signalisiert und somit den Metabolismus zur optimalen Verwertung und Speicherung der aufgenommenen Energie beeinflusst.

1 Introduction

1.1 Ghrelin

Ghrelin is a gastrointestinal peptide mainly produced by the stomach. It was discovered in 1999 by the group of Kojima et al., which also identified its growth hormone (GH) secretagogue action (Kojima et al., 1999). Kojima et al. created the name "ghrelin" in order to refer to its function. The word ghrelin originates from *ghre*, the Proto-Indo-European root of the word grow and *-lin* indicating its secretagogue function. Only one year later Tschöp et al. discovered that ghrelin induces food intake and increases adiposity in rodents and humans, a major break through for the field of metabolic research (Tschop et al., 2000). Intriguingly, until now ghrelin remains the only circulating peptide identified to increase food intake and fat mass. Therefore, the endogenous ghrelin system today is less of a target for GH-related therapies, but rather an important basis for development of potential drugs that regulate energy metabolism and body mass. Genetic models support this pharmacological data since it has been described that both ghrelin and growth hormone secretagogue receptor (GHSR) knockout mice are resistant to high-fat diet (Wortley et al., 2005; Zigman et al., 2005; Pfluger et al., 2008).

1.1.1 Chemical structure of ghrelin and ghrelin synthesis

Ghrelin is mainly produced in the stomach from a distinct group of endocrine cells, called X/A like cells, which are located within the gastric oxyntic mucosa (Date et al., 2000). A certain amount of ghrelin is further produced along the gastrointestinal tract and pancreas (Date et al., 2000). Additionally, ghrelin is expressed to a smaller amount in numerous other tissues including the brain (Cowley et al., 2003; Mondal et al., 2005), testis (Tena-Sempere, 2008) pituitary (Korbonits et al., 2001), kidney (Mori et al., 2000), thyroid gland (Raghay et al., 2006), and placenta (Gualillo et al., 2001).

The human ghrelin gene is located on chromosome 3 (3p25–26) and embraces 5 exons (Kojima et al., 1999). It has two transcriptional starting sites resulting in two ghrelin transcripts, transcript-A and –B. Nevertheless, transcript-A is the predominant ghrelin messenger ribonucleic acid (mRNA). Exons 1 and 2 encode the 28 amino acids of the functional ghrelin peptide (Fig. 1). The human ghrelin gene contains predicted binding sites for several transcription factors including AP2, NF-IL6, NF-B and half sites for estrogen and glucocorticoid rezones elements (Kanamoto et al., 2004; Kishimoto et al., 2003; Tanaka et al., 2001).

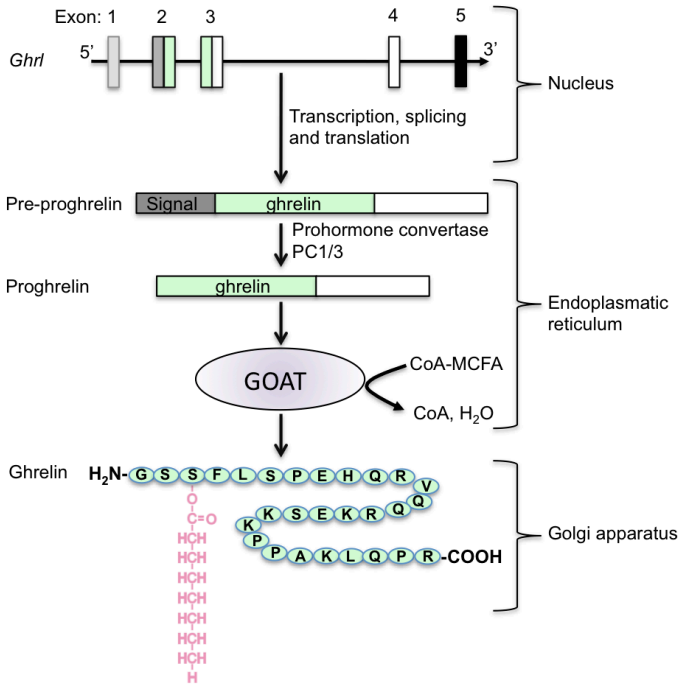

Figure 1 Ghrelin biosynthesis and acylation
The 28 amino acid peptide ghrelin is encoded by exons 2 and 3 of the ghrelin gene *Ghrl*. In a posttranslational step pre-proghrelin is cleaved to proghrelin by the prohormone convertase PC1/3. Located in the endoplasmatic reticulum GOAT couples a CoA activated medium-chain fatty acid (MCFA) to the third serine molecule of proghrelin. Acylated proghrelin is finally cleaved to acyl-ghrelin and packed into vesicles for secretion in the Golgi apparatus. Modified from (Kojima and Kangawa, 2005).

The amino acid sequence of ghrelin precursors is well conserved among mammals. The ghrelin precursor, pre-proghrelin, contains a signal peptide, which is directly followed by the 28 amino acid ghrelin sequence (Fig. 1). In the first step of processing pre-proghrelin to ghrelin, the signal sequence is removed to produce proghrelin. Next, proghrelin is cleaved between arginine and alanine of the C-terminal by the prohormone convertase PC1/3 (Zhu et al., 2006). The cleavage at this proline-arginine recognition site is rather uncommon for propeptide processing, however it is the same for all mammalian ghrelins (Steiner, 1998; Seidah and Chretien, 1999). In another post-translational step the hydroxyl group of serine-3 is acylated with n-octanoic acid or another medium-chain fatty acid (MCFA) (Fig. 1). This ghrelin modification is unique among proteins and necessary to activate ghrelin's receptor, the growth hormone secretagogue receptor (GHSR) (Kojima et al., 1999). However, it is not entirely clear yet when *n*-octanoylation of serine-3 takes place. In vitro presence of not only ghrelin *O*-acyltransferase (GOAT) but also prohormone convertase PC1/3 are necessary to produce acyl ghrelin. This finding suggests that proghrelin gets acylated before final cleavage to ghrelin (Takahashi et al., 2009).

1.1.2 Biological functions and clinical pharmacology of ghrelin

Ghrelin stimulates growth hormone (GH) release from primary pituitary cells (Kojima et al., 1999) and acts synergistically with growth hormone releasing hormone (GHRH) to stimulate GH secretion (Arvat et al., 2001). Under physiological conditions, ghrelin oscillates in a rhythmic expression pattern with circadian light-dark cycles (LeSauter et al., 2009) and reveals a

unique secretory pattern with surges immediately before a meal (Liu et al., 2008). It was therefore proposed that ghrelin triggers food anticipatory activity (LeSauter et al., 2009) and meal initiation (Drazen et al., 2006) possibly through activation of the sympathetic nervous system (Mundinger et al., 2006). Incoming nutrients suppress ghrelin levels after a meal (Tschop et al., 2001a). The magnitude of this ghrelin reduction seems to be in proportion to the caloric load and macronutrient content with ingested lipids being the least effective ghrelin suppressor (Foster-Schubert et al., 2008; Monteleone et al., 2003). However, the recovery of ghrelin levels in plasma does not seem to be an important determinant of intermeal intervals (Callahan et al., 2004). Ghrelin increases feeding frequency without affecting the meal size. It also increases gastric motility and decreases insulin secretion (Cummings et al., 2007; Cummings et al., 2001; Tschop et al., 2000). Recent evidence further suggests that ghrelin may function as a meal preparation factor: central infusion of ghrelin was shown to induce lipogenesis in white adipose tissue through inhibition of the hypothalamic melanocortin system (Nogueiras et al., 2007; Sangiao-Alvarellos et al., 2009).

Peripheral ghrelin administration reliably induces the sensation of hunger and increases food intake in both lean and obese, and both healthy and malnourished individuals (Wren et al., 2001). Interestingly, intravenous administration of ghrelin in healthy volunteers increases neural activity in response to food pictures in specific brain regions. Activation of these 'reward' centers by ghrelin suggests a novel mechanism by which food consumption is enhanced (Malik et al., 2008). Beyond a role in mediating meal-to-meal food intake, ghrelin plays a role in regulation of long-term energy balance, thereby defending energy deficiency. In humans, circulating ghrelin levels are inversely associated with adiposity, insulin resistance and weight gain (McLaughlin et al., 2004; Tschop et al., 2001b) while they are positively correlated with exercise-induced weight loss, low-caloric diet, mixed life-style modification or pathologic conditions such as anorexia nervosa and cachexia (Cummings et al., 2002; Nagaya et al., 2001). In contrast, patients with Prader-Willi syndrome and hyperphagia have very high circulation ghrelin levels (DelParigi et al., 2002).

Since the discovery of ghrelin, many investigators have used single injection or continuous infusion of ghrelin to study its roles in regulating GH secretion, food intake, body weight, energy homeostasis, glucose homeostasis and gastrointestinal motility. In humans, a single-dose injection of ghrelin *intra venous* (*i.v.*) as well as a continuous 24-h ghrelin infusion induces GH release acutely (Arvat et al., 2000) and increases 24-h pulsatile GH secretion (Veldhuis et al., 2008). The importance of ghrelin in GH regulation is supported by the observation that abnormalities of growth hormone secretagogue receptor function are associated with familial short stature (Pantel et al., 2006). Given at high doses, ghrelin also increases adrenocorticotropin, prolactin, and cortisol levels (Arvat et al., 2001). Consequently, ghrelin mimetics have been investigated with some promising effects as a therapeutic option for age-dependent GH decline (Nass et al., 2008). Nevertheless, lacking functional benefits, quality of life improvement and long-term safety data, the clinical use of ghrelin mimetics in the elderly deserves further investigation and is far from use in common practice.

1.1.3 Neuroendocrinology of ghrelin action

The biological actions of ghrelin are predominantly mediated via pathways in the central nervous system controlling food intake, energy expenditure, and nutrient partitioning. Ghrelin is the only blood-derived hormone capable of activating hypothalamic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons (Cowley et al., 2003; Dickson et al., 1993), which are currently regarded as very, if not the most, important players in regulating feeding and energy metabolism (Gropp et al., 2005; Luquet et al., 2005). The orexigenic effect of ghrelin is specifically modulated through the growth hormone secretagogue receptor 1a (GHS-R1a), also called the ghrelin receptor.

Peripheral and central ghrelin administration in rodents increases the number of stimulatory synapses on NPY/AgRP neurons (Nakazato et al., 2001) while it increases the number of inhibitory synapses on pro-opiomelanocortin (POMC) neurons (Cowley et al., 2003). NPY and AgRP are necessary for ghrelin's actions on feeding behavior, since ghrelin fails to increase food intake in mice lacking both NPY and AgRP (Chen et al., 2004). A crucial regulator for the expression of NPY and AgRP is the hypothalamic homeobox domain transcription factor Bsx (Sakkou et al., 2007), which is also essential for the control of the spontaneous physical activity and the hyperphagic response of ghrelin (Sakkou et al., 2007). Chronic ghrelin administration promotes weight gain and adiposity in rodents (Tschop et al., 2000) in the absence of overfeeding (Theander-Carrillo et al., 2006). The body weight gain induced by ghrelin is specifically based on accumulation of fat mass without changes in longitudinal skeletal growth or in lean mass (Tschop et al., 2000). This effect can be explained by direct actions on energy partitioning, which indirectly influences the other factors in the energy balance equation.

1.1.4 Ghrelin and insulin

Both ghrelin and its receptor are expressed in human pancreatic islets (Dezaki et al., 2004) and ghrelin inhibits insulin secretion in most animal studies (Dezaki et al., 2004; Reimer et al., 2003). Nevertheless, the effect of ghrelin on insulin secretion in humans is controversial. Plasma ghrelin and insulin levels seem to be negatively related since the two hormones exhibit reciprocal changes during the day and during euglycemic hyperinsulinemic clamp studies (Cummings et al., 2001; Flanagan et al., 2003). This relationship is consistent with epidemiologic studies as they show an inverse relationship between circulating ghrelin levels and indexes of insulin resistance (Tschop et al., 2001b). A single-dose injection of ghrelin *i.v.* increases plasma glucose levels significantly followed by a reduction in fasting insulin levels in lean (Broglio et al., 2001) and obese (Fusco et al., 2007) subjects which leads to the suggestion that ghrelin inhibits insulin secretion. This finding however has not been confirmed in more comprehensive studies. Conversely, studies showing that ghrelin has β-cell promoting effects which ameliorates type 1 diabetes, exist (Adeghate and Ponery, 2002; Irako et al., 2006) leaving the question open whether ghrelin has inhibiting or stimulating effects on insulin.

1.1.5 Degradation of ghrelin and functions of des-acyl ghrelin

Generally, serum C-esterases are the predominant enzymes responsible for ghrelin des-acylation while peptidases complete the ghrelin degradation. However, ghrelin des-acylation and

degradation differ highly among species. The half-life of acyl ghrelin in human serum is about 240 min whereas it is only 30 min in rat serum (De Vriese et al., 2004). Further, the mechanism of ghrelin cleavage and the kind of responsible enzymes for ghrelin des-acylation and degradation are crucially different between rodents and humans. In human serum butyrylcholinesterase is one of the major enzymes responsible for ghrelin des-acylation. Desoctanoylation in rodents is nine times faster and predominantly catalyzed by carboxylesterases (De Vriese et al., 2004). The ghrelin backbone has five cleavage sites, which differ between species and tissue within one species. Mostly, the cleavage sites are between the serine-2 and glutamic acid-8 residues, which leave relatively long C-terminal ghrelin fragments (De Vriese et al., 2004). These fragments are probably inactive because the minimal structural requirement to activate the ghrelin receptor is defined in the N-terminal region and requires *n*-acylation of serine-3 (Matsumoto et al., 2001).

Although octanoylation at serine-3 position is essential for ghrelin-bioactivity, i.e. the ghrelin receptor activation, the vast majority (80–90%) of circulating ghrelin is non-acylated. Since no possibly existing specific receptor for des-acyl ghrelin is known (Kojima et al., 1999) the general consensus appears to be that without this acyl-modification, ghrelin does not elicit relevant effects on energy balance. However, some controversial studies exist showing that des-acyl ghrelin has orexin-mediated effects on food intake (Toshinai et al., 2006), specific binding sites in cardiomyocytes (Lear et al., 2010) and GHSR independent effects on energy and glucose homeostasis (Thompson et al., 2004; Toshinai et al., 2006; Zhang et al., 2008).

1.2 Ghrelin *O*-acyltransferase

Only one enzyme exists that is able to activate ghrelin by acyl-modification, which is ghrelin *O*-acyltransferase (GOAT) (Gutierrez et al., 2008). An extensive search for this enzyme has been ongoing in numerous laboratories since ghrelin was discovered in 1999. Finally, in 2008 two independent teams of scientists identified membrane-bound *O*-acyltransferase 4 (MBOAT4) as being this specific acyltransferase and renamed it GOAT (Gutierrez et al., 2008; Yang et al., 2008a).

1.2.1 Discovery of GOAT

In the search for the enzyme that activates ghrelin Yang et al. (Yang et al., 2008a) generated several cell cultures that produce only des-acyl ghrelin by using cDNA transfection techniques. These cells were co-transfected with cDNA of 16 members of the family of membrane-bound *O*-acyltransferases (MBOATs), an enzyme family that is known to catalyze *O*-acylation reactions (Hofmann, 2000). The only candidate gene able to produce acyl-ghrelin after being transfected into the cell was *MBOAT4* (Yang et al., 2008a).

Simultaneously, Gutierrez et al. (Gutierrez et al., 2008) used gene silencing technology to identify GOAT. This group used TT cells derived from human medullary carcinoma cells, which are able to produce acyl-ghrelin when octanoic acid is added to the culture medium. Taking advantage of this property of the cells, the group designed silencing RNAs for 12

possible candidate genes. Silencing one candidate at a time, it was found that TT cells treated with small interfering RNA (siRNA) of *mboat4*, but not other candidate siRNAs, significantly decreased ghrelin acylation. This finding clearly indicated that MBOAT4 is crucial to produce acyl ghrelin. Further, the generation of GOAT deficient mice verified that GOAT is the only enzyme capable to acylate ghrelin since acyl ghrelin is completely absent in GOAT deficient mice (Gutierrez et al., 2008).

1.2.2 GOAT physiology

GOAT, like ghrelin, is conserved across vertebrates since functional GOAT activity has been shown in humans, rats, mice, and zebra fish (Gutierrez et al., 2008; Yang et al., 2008a). Further, sequences with clear amino acid similarities to GOAT are readily present in other vertebrates, consistent with the identification of octanoylated forms of ghrelin across vertebrates (Gutierrez et al., 2008). GOAT has eight predicted membrane-bound regions and is possibly located in the membrane of the endoplasmatic reticulum (Yang et al., 2008a). GOAT gene (*MBOAT4*) expression is highest in the stomach and pancreas in humans and is mostly co-localized with ghrelin expressing cells (Sakata et al., 2009). *MBOAT4* is also expressed to smaller amounts in various other tissues including heart, liver and colon (Gutierrez et al., 2008). Biochemically, GOAT has two critical substrates, des-acyl ghrelin and short- to medium-chain fatty acids conjugated as Coenzyme A (CoA) thioesters. In vitro data from cells expressing both ghrelin and GOAT show that GOAT can acyl modify ghrelin at its critical serine-3 residue with fatty acids ranging from acetate (C2) to tetradecanoic acid (C14) (Gutierrez et al., 2008). Nevertheless, most ghrelin in circulation is octanoyl and to a lesser degree decanoyl modified, although medium-chain triglycerides (MCT) are not abundant in circulation. Importantly, octanoyl- and decanoyl-modified ghrelin forms are the optimal ligands for activation of the GHSR-1a (Yang et al. 2008). The biochemical mechanisms involved in the generation of these medium-chain fatty acid substrates for GOAT in the ghrelin producing cells however remain to be described.

Ghrelin acylation studies show that GOAT acylates proghrelin before it is cleaved to the final ghrelin peptide (see 1.1.1 and (Yang et al., 2008b; Takahashi et al., 2009)). However, it also has been shown that acyl-modification of des-acyl ghrelin peptides is possible in vitro when fatty acid CoA-esters and GOAT containing microsomes are added to the medium (Ohgusu et al., 2009; Takahashi et al., 2009). These studies conclusively demonstrate that GOAT requires fatty acid substrates as high energy fatty acid CoA-thioesters, and that the short amino acid sequence GXSFX, where G, X, S, and F correspond to unblocked amino terminal glycine (G), any amino acid (X), serine (S) and phenylalanine (F) respectively, is sufficient as a substrate for GOAT acylation (Yang et al., 2008b; Ohgusu et al., 2009). The structural constraints defined by this amino acid motif appear specific only for ghrelin and suggest that ghrelin may be GOAT's only peptide substrate. Most recent studies comparing the in vitro selectivity of hexanoyl- and octanoyl-CoA substrates indicate that GOAT might actually prefer hexanoyl-CoA to octanoyl-CoA substrates. These studies highlight again the importance to study origin of these fatty acids and their metabolism in acyl ghrelin producing cells (Ohgusu et al., 2009).

1.3 Growth hormone secretagogue receptor

The GHSR is a 7 trans-membrane or G protein coupled receptor and, based on amino acid homology, belongs to a sub-family, which includes the motilin receptor, the two neurotensin receptors, the two neuromedin U receptors, the thyrotropin releasing hormone (TRH) receptor as well as the orphan receptor GPR39 (Holst et al., 2004). Importantly, the functions of this entire family of receptors are related to the control of food intake, gastrointestinal function and energy homeostasis.

1.3.1 Chemical structure and signaling pathways of the ghrelin receptor

Like ghrelin, GHSR is located on chromosome 3 in humans (Howard et al., 1996). All members of the ghrelin receptor family have an intron in the coding region between the transmembrane segment 5 and 6. Two different splice variants have been identified for the ghrelin receptor: the full-length functional ghrelin receptor 1a and the ghrelin receptor 1b that is truncated after the fifth transmembrane region. The truncated receptor 1b does not bind ghrelin but has been described to modulate the function of the full-length ghrelin receptor (Leung et al., 2007), potentially through interference with a homodimeric form of GHS-R1a, which possibly constitutes the functional unit at the plasma membrane. Furthermore, cross talk between the dopamine D1 and the ghrelin receptors has been described, which is possibly realized through heterodimerisation (Jiang et al., 2006).

Upon ligand binding, the ghrelin receptor couples to the $G\alpha_{q/11}$ subunit leading to activation of phospholipase C with release of inositol phosphate and diacylglycerol (Holst et al., 2004). Additionally, signaling via $G\alpha_{i}$ and $G\alpha_{s}$ coupling has been reported (Dezaki et al., 2007; Carreira et al., 2004). Calcium release, a read-out commonly used for screening of potential ligands for the ghrelin receptor, is mediated by the inositol phosphates or possibly through the $\beta\gamma$-subunit of the G-protein (Holst et al., 2005). Several kinases and transcription factors are then activated downstream of the $G\alpha_{q/11}$ protein-coupling and arrestin mobilization. The "energy sensor", AMP-activated kinase (AMPK), is one of the best-characterized kinases activated by ghrelin. In the hypothalamus it has been shown that ghrelin induced increase in NPY/AgRP expression. Increase in food intake is mediated through AMPK activation, which further increases the expression of uncoupling protein 2 (UCP-2) and upregulates mitochondrial respiration (Andrews et al., 2008). However, the pathway whereby ghrelin modulates AMPK is yet not fully understood. Release of calcium contributes to activation of calcium/calmodulin kinase kinase CaMKK2, which has recently been demonstrated to activate AMPK in the hypothalamus (Anderson et al., 2008). Figure 2 describes the currently accepted signal transduction pathways of ghrelin and the ghrelin receptor. However the redundancy in kinase activation complicates the description of the signaling pathways from ghrelin to induction of food intake especially since involvement of other intracellular mediators such as ERK, phosphatidylinositol 3-kinase, phosphodiesterase 3 and cAMP response element-binding protein (CREB) have been suggested (Kohno et al., 2007).

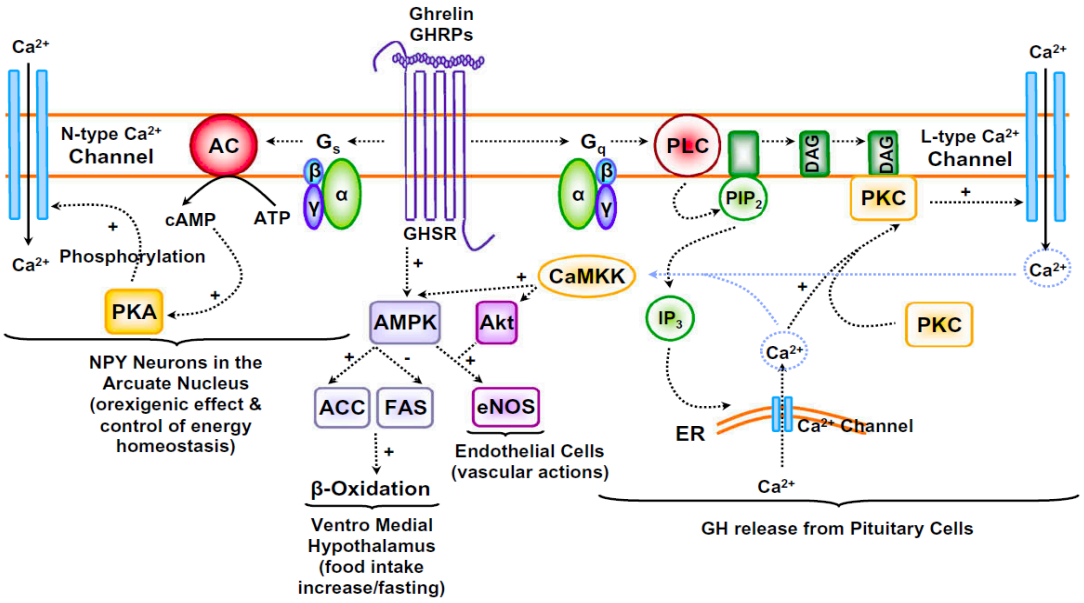

Figure 2 Mechanisms of ghrelin action
Acting at GHS-1a receptors in the hypothalamus and pituitary, ghrelin activates two signal transduction pathways. Ghrelin can increase calcium through the adenylate cyclase–protein kinase A (AC–PKA) pathway in *NPY*-expressing cells of the arcuate nucleus or through the phospholipase C–protein kinase C (PLC–PKC) pathway in the pituitary. Ghrelin can also inhibit vascular inflammation through the activation of the calmodulin-dependent kinase kinase (CaMKK), AMPactivated protein kinase (AMPK)/Akt and endothelial nitric oxide synthase (eNOS). AMPK together with acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) also mediate ghrelin induced increase in food intake in relation to fasting. From (Castaneda et al., 2010)

1.3.2 Constitutive signaling

An intriguing characteristic of the ghrelin receptor is its high level of agonist independent signaling as demonstrated in vitro in a number of signal transduction pathways, including inositol phosphate turnover, GTPγS binding and various transcriptional elements (Bennett et al., 2009; Holst et al., 2003). In general, it is very difficult to determine the physiological importance of such high constitutive signaling activity in the in vivo setting, mainly because most inverse agonists also act as antagonists, i.e. they also block the action of the endogenous agonist. However, for the ghrelin receptor a peptide has been described that is 100 fold more potent as inverse agonist than as antagonist (Holst et al., 2003). Continuous infusion of this compound in low concentration, which is still sufficient to obtain only inverse agonism, decreases food intake and body weight gain and decreases expression of *Ucp2* and *Npy* in rats (Petersen et al., 2009).

Rare genetic variants of the ghrelin receptor exist in humans, which selectively eliminate its constitutive activity without affecting the function of ghrelin. These mutations predominantly result in development of short statue indicating that the high constitutive activity of the ghrelin receptor is of physiological importance (Pantel et al., 2006).

1.3.3 Ghrelin receptor agonism and antagonism

Long before the receptor was cloned and ghrelin was discovered, agonists were developed based on the finding that a synthetic hexa-peptide from a series of opioid-like peptides was able to release GH from isolated pituitary cells independent of the GHRH receptor (Smith et al., 1997). Consequently, several series of potent and efficient peptide and non-peptide agonist were developed as GH secretagogues for the then orphan receptor, which later became the

ghrelin receptor. However, none of these made it to the market officially due to lack of sufficient efficacy. In recent years the focus for agonists has changed from effects on GH secretion to treatment of cachexia, gastrointestinal disorders and heart failure based on ghrelin's effect on energy homeostasis and other functions. With the discovery of ghrelin as meal initiation factor, suddenly antagonists for the ghrelin receptor became pharmacologically interesting for the treatment of obesity and type-2 diabetes. An obstacle in designing ghrelin receptor antagonists is that they need to pass the blood-brain-barrier to efficiently decrease food intake and body weight in rodent models (Rudolph et al., 2007; Xin et al., 2006). It has been suggested that a combined antagonist and inverse agonist could be the preferred anti-obesity drug as it would decrease the activity of the ghrelin system also between meals when the ghrelin drive is low (Holst and Schwartz, 2004). However, although small oligopeptide based, potent inverse agonists have been described (Holst et al., 2006), very little is known of the inverse agonist properties of the various ghrelin receptor non-peptide antagonists (Holst et al., 2009). The ghrelin antagonists are still only in preclinical development or early phases of clinical development, and it is not known yet whether they will work as well in man as the physiology of the ghrelin hormone suggests.

1.4 Mouse models for altered ghrelin, GHSR and GOAT function

Gain- and loss-of-function mouse models offer an excellent tool to study physiological functions of peptides and proteins. In recent years, several laboratories generated global loss-of-function mouse models for the ghrelin and GHSR gene (De Smet et al., 2006; Sun et al., 2003; Wortley et al., 2004; Zigman et al., 2005). These studies describe ghrelin-deficient and GHS-R1a-deficient mice and contributed largely to our knowledge of physiological ghrelin and GHSR functions.

The generation of ghrelin mutant mouse models has revealed a surprising variety of other physiological functions of ghrelin in such distinct areas as learning and memory (Diano et al., 2006), thymopoiesis (Dixit et al., 2007), wake/sleep rhythms (Szentirmai et al., 2007), or aging (Smith et al., 2007). Recent pharmacological intervention trials further point to a neuroprotective role of ghrelin in neurodegenerative diseases such as Parkinson (Moon et al., 2009). Nevertheless, to date it remains unclear whether such neuroprotective effects of ghrelin, as well as other effects obtained through pharmacological intervention, are also observed in a more physiological setting. Ghrelin mutant mouse models will contribute to elucidate such novel aspects of ghrelin, and will specifically help to translate ghrelin pharmacology into organismal physiology.

1.4.1 Ghrelin-deficient mice

Interestingly, when fed a regular chow diet, ghrelin-deficient mice do not reveal any overt changes in body weight, body fat, or food intake compared to wild-type (Wt) littermates (Sun et al., 2003; Wortley et al., 2004). However, when ghrelin-deficient (ghrelin$^{-/-}$) mice are chronically exposed to high-fat diet (HFD) directly after weaning, a clear metabolic benefit from ghrelin-deficiency emerges: despite similar food intake, ghrelin$^{-/-}$ mice gain less body weight and body fat than Wt mice, which might be due to the observed increase in energy expendi-

ture and locomotor activity (Wortley et al., 2005). Moreover, respiratory quotient (RQ) is decreased in ghrelin-deficient mice fed HFD, which is indivcative for a change in metabolic fuel preference to favor fat oxidation. When fed chow diet, ghrelin$^{-/-}$ mice show normal glucose utilization. This observation leads to the suggestion that ghrelin is of limited relevance for the regulation of glucose homeostasis. However, when challenged with chronic exposure to HFD, ghrelin deficient mice show improved glucose disposal and insulin sensitivity compared to wild-type littermates (Wortley et al., 2005). Accordingly, mice with neuron-specific overexpression of ghrelin develop glucose intolerance although the body weight of these mice is lower compared to Wt mice (Reed et al., 2008).

When crossing ghrelin deficient mice with leptin deficient ob/ob mice, double mutants retain the marked body adiposity phenotype of ob/ob mice (Sun et al., 2006). However, the double mutants show a significant decrease in basal glucose and increase in basal insulin levels, as well as a better glucose tolerance and insulin sensitivity when stimulated with a glucose or insulin challenge, compared to the leptin deficient ob/ob mice. In addition, fasting glucose levels are normalized compared to ob/ob mice (Sun et al., 2006).

1.4.2 GHSR-deficient mice

Similar to ghrelin-deficient mice on HFD, mice deficient for GHSR are protected from diet-induced obesity, which at least in part could be explained by mild hypophagia (Longo et al., 2008; Zigman et al., 2005). Expression of a *Ghsr* antisense RNA in the arcuate nucleus of rats results in hypophagia, decreased body weight and body fat (Shuto et al., 2002) further pointing to a specific role of the hypothalamus in mediating the effects of ghrelin on energy metabolism. GHSR deficiency improves glucose disposal and insulin sensitivity in HFD fed mice compared to wild-type controls. However, GHSR deficiency does not affect body weight or fat mass when mice are exposed to standard chow diet (Zigman et al., 2005). Taken together, ghrelin or GHSR deficiency ameliorates the development of obesity and possibly glucose intolerance after chronic exposure to HFD but overall the phenotype of ghrelin$^{-/-}$ and GHSR$^{-/-}$ mice is rather mild.

1.5. Aim of the study

Ghrelin is the only circulating orexigenic hormone that potently increases food intake and fat mass (Tschop et al., 2000) and since its discovery over 10 years ago, ghrelin and its many functions have been extensively studied. Nevertheless, most of ghrelin's actions are still controversial, including its well-known function as being the "hunger hormone". Ghrelin levels in blood rise shortly before a meal but genetic and pharmacologic loss of ghrelin function studies indicate that ghrelin possibly has no major role in regulating appetite and long-term food intake (Sun et al., 2003; Wortley et al., 2004; Zorrilla et al., 2006). The recent discovery of GOAT (Gutierrez et al., 2008; Yang et al., 2008a) marked a major breakthrough in ghrelin research offering the possibility to better understand ghrelin physiology. However, at the time of this study very little was known about the physiological regulation and endogenous function of GOAT.

The aim of this work was to characterize the role of the GOAT/ghrelin/GHSR system in regulating energy and glucose homeostasis. For this purpose (1) a novel mouse model that is simultaneously deficient for ghrelin and the ghrelin receptor GHSR had to be generated and metabolically phenotyped. (2) GOAT physiology during different feeding and fasting paradigms had to be studied for the first time to reveal how *Mboat4* expression and activity may be regulated under physiological conditions. (3) Mouse models with loss of GOAT and gain of ghrelin and GOAT function had to be generated and metabolically characterized for the first time.

(1) Generation of a new Ghr-GHSR knockout mouse model to reevaluate the role of ghrelin in regulating energy homeostasis
Breeding strategies had to be developed to obtain mouse colonies of reasonable study size of age matched littermate wild-type, ghrelin and GHSR single knockout as well as double knockout mice for ghrelin and GHSR, respectively. Information about the role of ghrelin in regulating energy and glucose homeostasis was gained by comparing metabolic parameters including body weight, food intake, body composition, energy expenditure, locomotor activity and glucose tolerance of double knockout mice to single knockout and wild-type mice during different feeding regimes.

(2) Characterization of GOAT function and physiology
GOAT activity during several physiological states had to be characterized. Therefore, a novel mass spectrometry based ghrelin assay was used to study GOAT activity indirectly ex vivo by measuring blood concentrations of acyl and des-acyl ghrelin in fasted and fed mice in addition to measurement of mRNA levels of *Ghrl* and *Mboat4*.

(3) Mouse models with gain or loss of GOAT function to characterize the role of GOAT in regulating energy and glucose homeostasis
Novel mouse models with either systemic deletion of GOAT or the transgenic overproduction of ghrelin and GOAT had to be produced. In order to achieve successful overproduction of acyl ghrelin in the transgenic mice, a new diet containing medium-chain-triglycerides had to be developed. Another new mouse model simultaneously deficient for GOAT and leptin had to be generated to study the specific function of GOAT in regulation of glucose homeostasis. Finally, to characterize the putative physiological role for GOAT in metabolic regulation, parameters of energy and glucose homeostasis were studied in all mouse models of altered GOAT function.

2 Material and Methods

2.1 Materials

2.1.1 Mouse strains

Table 1 Mouse strains

Name	Abbreviation	Background	Supplier
Wild-type	Wt	C57BL/6J	Jackson Laboratories (Bar Harbor, ME)
Ghrelin knockout	$Ghr^{-/-}$	C57BL/6J	Mark Sleeman, Regeneron Inc. NY
GHSR knockout	$GHSR^{-/-}$	C57BL/6J	Mark Sleeman, Regeneron Inc. NY
Ghrelin-GHSR knockout	$Ghr^{-/-}GHSR^{-/-}$	C57BL/6J	Own breeding
GOAT knockout	$Mboat4^{-/-}$	C57BL/6	Eli Lilly Company, Indianapolis, IN
Human ghrelin human GOAT transgenic	Tg	C57BL/6	Taconic Farms, Germantown NY
ob/ob	ob/ob	C57BL/6	Taconic Farms, Germantown NY
GOAT-leptin knockout	$Mboat4^{-/-}$ob/ob	C57BL/6	Own breeding

2.1.2 Rodent diets

All rodent diets were purchased from Research Diets (New Brunswick, NJ) or from Harlan Teklad (Madiosn, WI).

Table 2 Rodent diets and their macronutrient composition

	Chow	MCT diet	45 % HFD	60% HFD
Manufacturer and product #	Harlan Teklad LM-485	Harlan Teklad TD.08622	Research Diets D12451	Research Diets D12331
Protein [kcal]	19.92	7.2	20	16.4
Carbohydrate [kcal]	67.93	66.3	35	25.5
Fat [kcal]	5.67	22.6	45	58
Kcal/g	3.75	4.0	4.73	5.56

MCT, Medium-chain-triglycerides; HFD, high-fat diet; kcal, kilocalorie

2.1.3 PCR primers

Primers for genotyping were synthesized, HPLC purified and lyophilized by Integrated DNA Technologies (Coralville, IA). All primers were diluted in DEPC-treated water to a stock solution of 100µM/µl.

Table 3 PCR primers and conditions for genotyping

Allele	Primer	PCR conditions	Product
Ghrl $^{+/+}$	F:5'-TAAAGGGGTTGGGGTATGGAGG 3' R:5'-ACCAGAGAGGAAGGTAGAAGGAGTG-3'	3min 94C, (30sec 94C, 45sec 64C, 45sec 72sec) 36 cycles, 3min 72C	800 bp
Ghrl $^{-/-}$	F: 5'-AGCTCCCACCCTTGAAAGAT-3' R:5'-ACCATTTTCAATCCGCACCTC-3'	3min initial 94C, 30sec 94C, 60sec 60C, 90sec 72sec, 36 cycles, final extension 3min 72C	1200 bp
Ghsr $^{+/+}$	F: 5'- TCCTTTCATCGCTAATGTTCG -3' R: 5'- TCAGAGAGTGAGTCGTTGCCG-3'	3min initial 94C, 30sec 94C, 45sec 60C, 45sec 72sec, 36 cycles, final extension 3min 72C	600bp
Ghsr $^{-/-}$	F: 5'- TCCTTTCATCGCTAATGTTCG -3' R: 5'-GTCTGTCCTAGCTTCCTCACTG-3'	3min initial 94C, 30sec 94C, 45sec 60C, 45sec 72sec, 36 cycles, final extension 3min 72C	550 bp
Mboat4$^{+/+}$	F: 5'-GGATGGATAAACCTGATGGC-3' R: 5'-GCTAAGAGTTCTATATCCAGATCG-3'	5min initial 95C, 30sec 95C, 30sec 60C, 60sec 72sec, 35 cycles, final extension 10min 72C	238 bp
Mboat4$^{-/-}$	F: 5'-GCTTAGGGACTCTAGGAAGG-3' R: 5'- GCTAAGAGTTCTATATCCAGATCG-3'	5min initial 95C, 30sec 95C, 30sec 60C, 60sec 72sec, 35 cycles, final extension 10min 72C	277 bp
Tg $^{+/+}$	F: 5'-AGGCTCAGAGGCACACAGGAGT-3' R: 5'-CCCTCTCACACTACCTAAACAC-3'	3min initial 94C, 30sec 94C, 30sec 60C, 60sec 72sec, 36 cycles, final extension 2min 72C	300 bp
OB/OB and ob/ob	F: 5'- TGTCCAAGATGGGACCAGACTC-3' R: 5'-ACTGGTCTGAGGCAGGGAGCA-3'	3min initial 94C, 30sec 94C, 60sec 62C, 45sec 72sec, 36 cycles, final extension 2min 72C, Digest product with DdeI at 37C for 7 hrs	OB/OB: 155 bp ob/ob: 55 and 100 bp

F, forward; R, reward; bp, base pairs; Ghr, ghrelin; Ghsr, Growth Hormone Secretagogue Receptor; Mboat4, Membranebound *O*-acyl transferase 4; ob, obese

Primers for qPCR were either ordered from Integrated DNA Technologies (Coralville, IA) or from Applied Biosystems (Foster City, CA).

Table 4 Real-Time PCR primers, Taqman gene expression IDs and Taqman probes

Gene	Primer/ Taqman gene expression assay ID	Taqman probe
Mboat4	F: 5'-ACCGGGCCAGGTACCT-3'	6FAM-CTGGACCCTTGAACACGAGCCTGAAA
	R: 5'-ACCCATGGCAGCAAAAGC -3'	
Ghrl	Mm00445450_m1	N/A
Ghsr	F: 5'-TCCGATCTGCTCATCTTCCT -3'	N/A
	R: 5'- GGAAGCAGATGGCGAAGTAG-3'	
Ucp1	F: 5'-GGGCCCTTGTAAACAACAAA-3'	N/A
	R: 5'-GTCGGTCCTTCCTTGGTGTA-3'	
Ucp3	F: 5'-GTCTGCCTCATCAGGGTGTT-3'	N/A
	R: 5'-CCTGGTCCTTACCATGCAGT-3'	
Cox2	F: 5'-ACGAAATCAACAACCCCGTA-3'	N/A
	R: 5'-GGCAGAACGACTCGGTTATC-3'	
Cox4(b)	F: 5'-AGATGAACCATCGCTCCAAC-3'	N/A
	R: 5'-ATGGGGTTGCTCTTCATGTC-3'	
Cycs	F: 5'-GTCTGCCCTTTCTCCCTTCT-3'	N/A
	R: 5'-CCAAATCTCCACGGTCTGTT-3'	
Atp5b	F: 5'-GAGGGATTACCACCCATCCT-3'	N/A
	R: 5'-CATGATTCTGCCCAAGGTCT-3'	
Pgc1α	F: 5'-ATGTGTCGCCTTCTTGCTCT-3'	N/A
	R: 5'-ATCTACTGCCTGGGGACCTT-3'	
Pepck	F: 5'-TCAACACCGACCTCCCTTAC-3'	N/A
	R: 5'-CCCTAGCCTGTTCTCTGTGC-3'	
G6pc	F: 5'-CCTCCTCAGCCTATGTCTGC-3'	N/A
	R: 5'-AACATCGGAGTGACCTTTGG-3'	
Mdh2	F: 5'-GCTTTGTCTTCTCCCTCGTG-3'	N/A
	R: 5'-CAAAGTCCTCGCCTTTCTTG-3'	
Pdk4	F: 5'-GCCTTGGGAGAAATGTGTGT-3'	N/A
	R: 5'-GAAGGCACTGGCTTTTTGAG-3'	
Arbp	F: 5'-GGCCCGAGAAGACCTCCTT-3'	6FAM-CCAGGCTTTGGGCATCACCACG
	R: 5'-TCAATGGTGCCTCTGGAGATT-3'	
L32	F: 5'-GCCAGGAGACGACAAAAAT-3'	N/A
	R: 5'-AATCCTCTTGCCCTGATCC-3'	

N/A, not applicable; *Mboat4*, Membranebound-*O*-acyltransferase 4; *Ghrl*, Ghrelin; *Ghsr*, Growth hormone secretagogue receptor; *Ucp1* and *Ucp 3*, Uncoupling protein 1 and 3; *Cox2*, Cytochrome c oxidase subunit II; *Cox4(b)*, Cytochrome C oxidase subunit IV isoform 2; *Cycs*, Cytochrome C somatic; *Atp5b*, ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit; *Pgc1*α, Peroxisome proliferator activated receptor gamma coactivator 1 alpha; *Pepck*, Phosphoenolpyruvate carboxykinase, *G6pc*, Glucose-6-phosphatase catalytic, *Mdh2*, Malate dehydrogenase 2 NAD (mitochondrial); *Pdk4*, Pyruvate dehydrogenase kinase, isoenzyme 4; *Arbp*, Acidic ribosomal phosphoprotein P0; *L32*, Ribosomal protein L32.

2.1.4 Antibodies

Table 5 Antibodies for Immunohistochemistry

Primary antibodies:	Dilution	Company and order ID
Gp anti-insulin	1:1000	Linco 4011-10F
Rb anti-glucagon	1:1000	Phoenix H-028-02
Rb anti-somatostatin	1:200	Phoenix H-060-03
Gp anti-pancreatic polypeptide	1:500	Linco 4041-01
Rb anti-ghrelin	1:250	Gift from Lori Sussel, Columbia University
Secondary antibodies:		
Cy3 donkey anti-gp	1:200	Jackson 706-165-148
Cy2 donkey anti-rb	1:200	Jackson 711-225-152

Gp, guinea pig; Rb, rabbit

2.1.5 Enzymes, PCR supplies and reaction kits

Table 6 PCR compound and reaction kits

Compund/reation kit	Supplier
Autokit Glucose and NEFA C	Wako (Neuss, Germany)
Betaine	Sigma-Aldrich (Saint Louis, MO)
DdeI	New England Biolabs (Beverly, MA)
DEPC-treated water	Fisher Scientific (USA)
Infinity Cholesterol Reagent	Thermo Electron (Pittsburgh, PA)
Infinity Triglyceride Reagent	Thermo Electron (Pittsburgh, PA)
dNTP mix	Invitrogen (Carlsbad, CA)
Mouse Leptin ELISA Kit	Diagnostic Systems Laboratories (Webster, TX)
Oligo(dT20) primers	Invitrogen (Carlsbad, CA)
RNeasy® Lipid Tissue Mini Kit	Quiagen (Hercules, CA)
Sensitive Rat Insulin RIA Kit	Linco Research (St. Charles, MO)
SuperScript® III First-Strand Synthesis System	Invitrogen, Carlsbad, CA)
Sybr Green® Supermix	Bio-Rad (Hercules, CA)
TaqMan® Universal PCR Master Mix	Applied Biosystems (Foster City, CA)
TaqPolymerase recombinant	Invitrogen (Carlsbad, CA)

2.1.6 Chemicals

Table 7 Chemicals

Chemical	Supplier
α-cyano-4-hydroxycinnamic acid	Sigma-Aldrich (Saint Louis, MO)
Chloroform	Sigma-Aldrich (Saint Louis, MO)
Complete® EDTA-free Protease Inhibitor Cocktail Tablets	Roche (USA)
D-glucose	Sigma-Aldrich (Saint Louis, MO)
Donkey and Goat Serum	Calbiochem (USA)
EDTA	Invitrogen (Carlsbad, CA)
Ethanol	Sigma-Aldrich (Saint Louis, MO)
Exendin-4	Kindly provided by Prof. Richard DiMarchi, University of Bloomington, IN
HEPES	Sigma-Aldrich (Saint Louis, MO)
Humolog Insulin Pen (100U/ml)	Eli Lilly Company (Indianapolis, IN)
NaCl	Sigma-Aldrich (Saint Louis, MO)
N-octyl glucopyranoside	Sigma-Aldrich (Saint Louis, MO)
OCT compound	Fisher Scientific (USA)
PBS-Triton X	Roche (USA)
Sterile saline	Fisher Scientific (USA)
Sucrose	Sigma-Aldrich (Saint Louis, MO)
Trizol	Quiagen (Hercules, CA)

2.1.7 Buffers and solutions

All used buffers and solutions were prepared with Millipore® filtered water. The pH was adjusted with 1N HCl or 1N NaCl.

Table 8 Buffers and Solutions

Buffer/Solution	Ingredients
Anti-proteolytic Cocktail (for ghrelin)	25 mM EDTA; 1 mg/ml Complete EDTA-free Protease Inhibitor Cocktail Tablets; 1 M NaCl; 200 mM HCl; pH 3
Donkey Serum (2%)	2 ml donkey serum in 98 ml PBS
Exendin-4 (0.07µl/µl)	stock solution (3.5µg/µl) diluted 50-fold in sterile saline
Glucose Solution (25%)	2.5 g D-glucose in 10 ml sterile saline
Goat Serum (5%)	5 ml goat serum in 95 ml PBS
Insulin (0.1 U/ml)	10µl insulin in 10 ml sterile saline
IPMS Buffer I	140 mM Tris-HCl; 50 mM HEPES; 150 mM NaCl; 0.1% N-octyl glucopyranoside; pH 7.5
IPMS Elution Buffer	60% acetonitrile in 0.1% trifluoroacetic acid
IPMS Wash Buffer	50mM Tris-HCl, 50 mM HEPES, and 150 mM NaCl; pH 7.5
PBS (1X)	114 mM NaCl; 2.7 mM KCl; 10 mM $Na_2HPO_4(H2O)_2$; 1.8 mM KH_2PO_4; pH 7.4
PBS-Triton X	Purchased as PBST from Roche (USA)
TAE (1X)	40 mM Tris-acetate; 1 mM EDTA; pH 8

2.2 Methods

2.2.1 Animals

All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati and the Eli Lilly Company, Indianapolis. All mice were group housed in Positive Individual Ventilation cages in dedicated animal housing rooms with a 12-h light, 12-h dark cycle and 22°C, with free access to food and water unless fasted. Unless otherwise indicated, all here represented results are predominantly from male mice. However, some data from female mice are included to compare loss of ghrelin and GOAT function between genders. For all studies mice were fed with standard rodent chow (chow), medium-chain-triglyceride diet (MCT diet) or high-fat diet (HDF). Information about the diet composition can be found in table 1.

2.2.1.1 Wild-type mouse studies

Male C57BL/6J wild-type mice purchased from Jackson Laboratories were used for all *Mboat4* expression studies and triheptanoate feeding studies. All mice were 2 to 3 months old, had a body weight of 23-33g at the time of study and were fed regular chow diet or chow enriched with triheptanoate, unless fasted or otherwise indicated. For *Mboat4* expression studies mice were fasted for 12 hrs (n=7), 24 hrs (n=7) or 36 hrs (n=8) and euthanized for tissue collection by decapitation. Another group of 8 mice served as control and was fed *ad libitum* until decapitation. To avoid confounding factors such as circadian rhythm at the time of sacrifice, all mice were sacrificed successively at the same day starting at 9:30 AM (see table 9 for the experimental time-schedule).

Table 9 Experimental timetable for Mboat4 expression studies after long-term fasting

Group	n	Start fasting	End fasting	Time sacrifice
ad lib	8	-	-	Friday, 11:15
12 h	7	Thursday, 21:30	Friday, 9:30	Friday, 9:30
24 h	7	Thursday, 10:45	Friday, 10:45	Friday, 10:45
36 h	8	Wednesday, 22:00	Friday, 10:00	Friday, 10:00

2.2.1.2 Generation of Ghr-Ghsr mice

Ghrelin and GHS-R1 deficient mice were generated using the high-throughput homologous recombination Velocigene technology (Valenzuela et al., 2003). Both knock-out mouse strains including wild-type littermates were a kind gift by Mark Sleeman from Regeneron Pharmaceuticals who generated the mice as previously described on a C57BL/6 and C57BL/J6 background (Wortley et al., 2004; Abizaid et al., 2006; Zigman et al., 2005). Briefly, ghrelin knockout mice ($Ghr^{-/-}$ mice) were generated using bacterial artificial chromosome (BAC)-based targeting vectors, in which the coding region of the *ghrl* locus was precisely deleted starting from the from ATG initiation codon to the termination codon. The *ghrl* locus was then replaced with an in-frame lacZ reporter gene and neomycin selectable marker, which were electroporated into embryonic stem (ES) cells. Heterozygote and homozygous mice derived from correctly targeted ES cells were identified by a real-time PCR-based "loss-of-native-allele" assay as described by Valenzuela et al. (Valenzuela et al., 2003).

GHS-R1 knockout mice ($Ghsr^{-/-}$ mice) were created by inserting a *loxP*-flanked transcriptional blocking cassette (TBC) into a putative intron located downstream of the transcriptional start site and upstream of the translational start site of the murine *Ghsr* gene (Fig. 3).

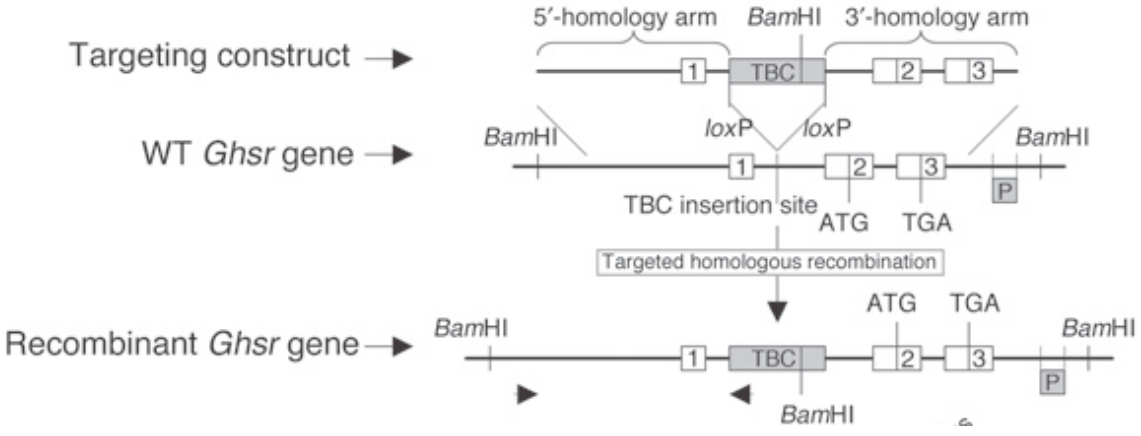

Figure 3 Schematic diagram of the derivation of GHSR-null mice by homologous recombination
The *lox-P* flanked transcriptional blocking cassette (TBC) was inserted into a mouse GHSR containing bacterial artificial chromosome 256 bp upstream of the *Ghsr* start codon (within a 196-bp putative intron) and downstream of the proposed *Ghsr* transcriptional start site. The final targeting construct consisted of the *loxP*-flanked TBC, which was flanked by 4.5-kb *Ghsr* homology arms. From (Zigman et al., 2005)

The TBC were derived from 4 different plasmids and included the following elements (in the order listed): a splice acceptor site from the mouse engrailed 2 gene (*En-2*) followed by an SV40 poly(A) signal, a SV40 enhancer followed by a neomycin resistance gene and 2 HSV-TK poly(A) signals, a synthetic poly(A) signal/transcriptional pause signal, and another synthetic poly(A) signal followed by a Myc-associated zinc finger protein-binding site (Yonaha and Proudfoot, 1999; Ashfield et al., 1994; Joyner et al., 1989). The final targeting construct, which consisted of the *loxP*-flanked TBC flanked by 4.5-kb *Ghsr* homology arms, was electroporated into ES cells, and correct targeting was confirmed by Southern blot and PCR analyses. After germline transmission was established, the chimera carrying the recombinant allele was crossed onto a C57BL/6J background to yield N2F1 animals. This gene modification approach, in which the *Ghsr* locus was modified by the addition of a *loxP*-flanked TBC rather than by the removal of a critical exon or exons, was used in order to enable Cre recombinase–mediated reactivation of GHSR expression in future experiments.

$Ghr^{-/-}$ mice of both sexes were intercrossed with $Ghsr^{-/-}$ mice of both sexes to generate a double-heterozygous F1 generation. The double-heterozygous F1 mice were inbred to produce a F2 generation of double knockout mice deficient for both, ghrelin and GHSR, respectively. F2 mice with both functional alleles for *Ghrl* and *Ghsr* were used as wild-type controls (Wt). Further, F2 mice with one functional allele ($Ghr^{-/-}$ or $Ghsr^{-/-}$ mice) were used as single knockout controls. All results were derived from male F2 littermates after inbreeding of the double heterozygous F1 generation. Mice from the F2 generation were studied on standard chow and after six-week exposure to 60% HFD. Confirmatory data on body weights and body lengths were derived from two additional F3 populations of male and female animals at the date of weaning (age 21 days) and after 13 weeks on either chow or 45% HFD. These mice were produced by homozygous inbreeding of F2 Wt, $Ghr^{-/-}$, $Ghsr^{-/-}$, or $Ghr^{-/-}$-$Ghsr^{-/-}$ mice. A group of mice consisting from all four genotypes (Wt, $Ghr^{-/-}$, $Ghsr^{-/-}$ and $Ghr^{-/-}$-$Ghsr^{-/-}$ mice) is here referred to as Ghr-GHSR mice.

2.2.1.3 Generation of $Mboat4^{-/-}$ mice

GOAT-deficient mice ($Mboat4^{-/-}$) were generated on a C57BL/6 background by Taconic/Artemis using C57BL/6N cells from C57/TacN mice. Homologous recombination using the PGK-Neo-pA targeting vector carrying *Mboat4* surrounding sequences flanked by Flippase Recognition Targeting cassettes was used to create embryonic stem cells. As a result these cells carried a 9838 bp disruption in the *Mboat4* genomic region beginning at the predicted start codon (ATG) and ending 208 bp into the 3' untranslated region (Fig 4). Flippase mediated recombination removed the Neomycin selectable marker, which resulted in entire disruption of the genomic loci except for the short FLR sequences.

$Mboat4^{-/-}$ mice were fed with standard chow, 58% HFD or MCT diet containing 10% of calories from trioctanoate and tridecanoate.

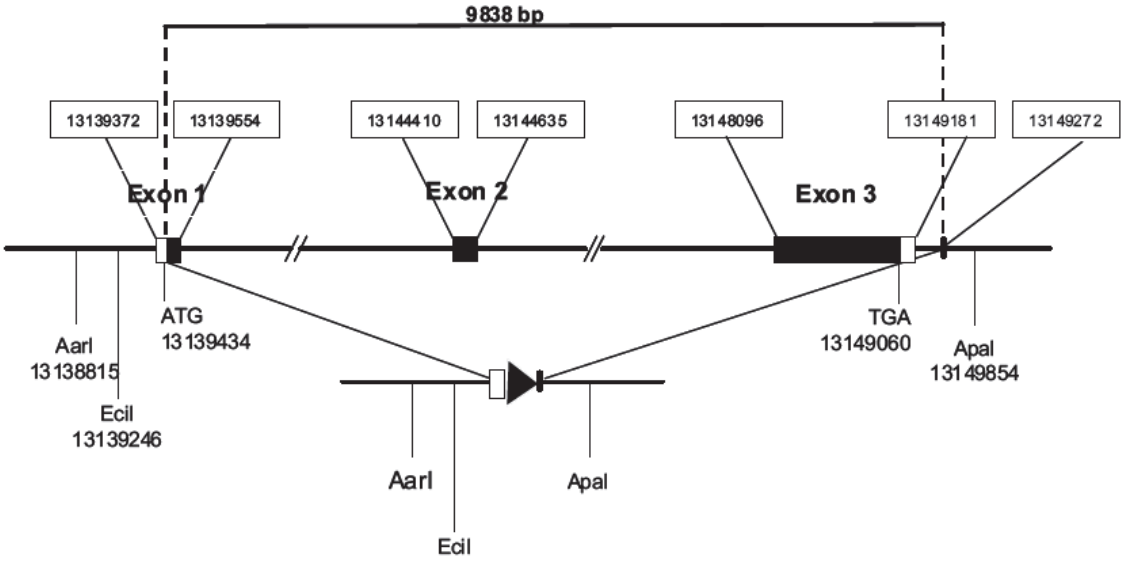

Figure 4 Deletion map of the *Mboat4* gene
Genetic map of mouse C57BL-6 chromosome 8 containing the *Mboat4* gene. Black bars indicate coding regions of exons, white bars indicate non-coding regions. The triangle indicates the FLP recombinase target of 34 bp that is retained after generation of the knockout allele.

2.2.1.4 Human ghrelin/human GOAT transgenic mice

The human ghrelin and GOAT transgenic (Tg) mice were generated on a C57BL/6 background at the Model Animal Research Centre in Nanjing China. The open reading frames for the *Mboat4* gene were independently cloned with the addition of a 5' Kozak sequence into multiple cloning site of a plivhHL1-derived vector. The plivhHL1 vector 2 has the constitutive human apolipoprotein-E gene promoter and carries the hepatic control region for liver specific tissue expression. Both transgenes were microinjected at equimolar amounts into fertilized eggs to generate transgenic mice. DNA PCR analyses specific for the transgenic sequences were performed to identify founder mice harboring both transgenes. Functional expression of both genes was determined by blood ghrelin immunoprecipitation mass spectrometry analysis. All here presented results are derived from male Tg mice, which were fed chow or MCT diet.

2.2.1.5 Mboat4$^{-/-}$-ob/ob mice

To generate mice that are deficient for both, GOAT and leptin (Mboat4$^{-/-}$-ob/ob mice), male Mboat4$^{-/-}$ mice were intercrossed with female OB/ob mice purchased form Taconic. From the resulting F1 generation double heterozygous Mboat4$^{+/-}$-OB/ob mice were chosen and inbred to generate a F2 generation of Wt, Mboat4$^{-/-}$, ob/ob and Mboat4$^{-/-}$-ob/ob mice. Male and female Mboat4$^{-/-}$-ob/ob mice and ob/ob littermate controls were studied on chow and MCT diet.

2.2.2 Genotyping

To establish genotypes, polymerase chain reactions (PCR) were performed to amplify defined DNA fragments that are characteristic for the knockout and wild-type alleles of each mouse line. DNA was extracted from tail snips on the day of weaning (21 days after birth) by the Genetic Variation and Gene Discovery Core Facility of the Cincinnati Children's Hospital. PCRs were performed using the primers and thermal conditions described in table 3. All

primers were purchased from Integrated DNA Technologies, Inc in lyophilized form. Primers were diluted with DEPC-treated water to a stock concentration of 100mM. All PCRs were performed on an Eppendorf Mastercycler in a total reaction-volume of 25 µl using 50-100 ng DNA, 2.5 µl 10x polymerase buffer, 2.5 µl Betaine, 10-50 pmol each forward and reverse primer, 0.25 mM dNTP-mix, 2 units polymerase and DEPC-treated water ad 25 µl. Depending on the production size PCR products were separated on 1-3% agarose gels containing ethidium-bromide. More detailed PCR protocols can be found in the supplementary information. In addition, all genotypes were confirmed by either performing radioimmuno-assays to measure total ghrelin in plasma of $Ghrl^{-/-}$ mice, MALDI-Tof mass spectrometry to measure acyl-ghrelin in blood of $Mboat4^{-/-}$ and Tg mice or by measuring pituitary *Ghsr* gene expression in $Ghsr^{-/-}$ mice, respectively. The genotype of the ob/ob mice could be confirmed phenotypically by the development of an obese statue 6 weeks after birth.

2.2.3 RNA extraction and gene profiling

After finishing all in vivo studies, mice were decapitated. Tissues were collected, immediately frozen on dry ice and stored at -80°C until the day of analysis. For RNA extraction 50-100 mg tissue was disrupted with a sterile stainless steel bead in 1ml Trizol using a TissueLyser (Quiagen, USA) at 27 Hz for 3 min. RNA was extracted using the RNeasy Mini Kit (Qiagen USA), according to the manufacturer's instructions. Specifically, 200 µl chloroform (Sigma-Aldrich, Saint Louis, MO) was added after tissue disruption. After shaking for ca. 30 sec the mixture was centrifuged for 15 min at 8000g at room temperature. After centrifugation only 350 µl of supernatant was collected and mixed with 350 µl 70% ethanol (Sigma, Saint Louis, MO). After washing steps according to the manufacturer's protocol RNA was eluted in 50 µl warm (60°C) RNase-free water. RNA concentration was measured using a NanoDrop 2000c spectrometer (Thermo Scientific, USA).

After subsequent DNase treatment, reverse transcriptions were performed with 4µg RNA using SuperScript III and oligo(dT20) primers. Quantitative real-time PCRs (qPCR) were performed to either validate the genotype (*Ghsr* expression in $Ghsr^{-/-}$ mice) or to study candidate gene expression. qPCRs were performed on a Bio-Rad iCycler by using iQ SybrGreen Supermix or on a ABI Prism 7900HT Sequence Detection System using Taqman probes. Gene expression was normalized to the housekeeping genes Acidic ribosomal phosphoprotein P0 (*Arbp*) or ribosomal protein L32 (*L32*) and evaluated using the delta-delta CT method (Pfaffl, 2001). A list of all primers and probes used for qPCR analysis can be found in Table 4 of the Materials section.

2.2.4 Energy balance physiology measurements

Weekly body weight (BW) and food intake (FI) were measured with a laboratory balance (Metler Toledo, USA) with the accuracy of 0.1 g. Body length was defined as the distance between nose and rectum and was measured by using a digital high-precision sliding caliper (Victor Machinery Exchange, USA). Body core temperature was measured in the beginning of the light phase in nonanesthetized mice by using a rectal thermometer (Physitemp, Clifton, NJ). At the same time peritoneal body surface temperature was measured using an infrared thermometer (Fluke,

Grossostheim, Germany). In order to measure the surface temperature accurately mice were shaved at the area between the shoulder blades and neck. Fat mass (FM) and lean mass (LM) was measured using NMR technology (EchoMRI, Houston, TX). Fat fee mass (FFM) was calculated by subtracting the FM from the BW using the following equation 1:

$$\text{Equation 1 FFM [g]} = \text{BW [g]} - \text{FM [g]}$$

Energy intake and energy expenditure (EE), as well as home-cage activity, were studied by using a combined indirect calorimetry system (TSE Systems, Bad Homburg, Germany). The calorimetry system was located in a designated room within the animal housing facility with a 12-h light, 12-h dark cycle and 22°C room temperature. Mice had free access to food and water in the system. After adaptation to the cages for >12 h, oxygen consumption and carbon dioxide production were measured every 45 min for a total of 76 h to determine the respiratory quotient (RQ, see Eq. 2) and energy expenditure. To ensure a true effect of genotype on EE, the EE was always calculated per whole animal (EE_{BW}), per fat free mass (EE_{FFM}) and per $BW^{0.75}$ ($EE_{BW0.75}$) using equations 3, 4 and 5. Only changes that reached significant difference in all three forms of normalization were considered as true effect.

$$\text{Equation 2 RQ} = CO_2\text{exiting} / (O_2\text{entering} \times (100 - O_2\text{exiting} - CO_2\text{exiting}) / (100 - O_2\text{entering}) - O_2\text{exiting})$$

$$\text{Equation 3 } EE_{BW} = \text{Volume } O_2 = (O_2\text{entering} - O_2\text{exiting}) \times \text{airflow/BW}$$

$$\text{Equation 4 } EE_{FFM} = \text{Volume } O_2 = (O_2\text{entering} - O_2\text{exiting}) \times \text{airflow/FFM}$$

$$\text{Equation 5 } EE_{BW0.75} = \text{Volume } O_2 = (O_2\text{entering} - O_2\text{exiting}) \times \text{airflow/ } BW^{0.75}$$

Simultaneous to measurement of EE, food and water intake as well as meal patterns were determined continuously for 76 h by integration of scales into the sealed cage environment. Meals were defined as food intake events with a minimum duration of 60 s, and a break of 300 s between food intake events. Food intake was normalized per g consumed in 24 h. In parallel, home-cage locomotor activity was determined using a multidimensional infrared light beam system with beams installed on cage bottom and cage top levels and activity being expressed as beam breaks. Stationary motor activity (fidgeting) was defined as consecutive breaks of one single light beam at cage bottom level, ambulatory movement as breaks of any two different light beams at cage bottom level, and rearing as simultaneous breaks of light beams on both cage bottom and top level.

2.2.5 Glucose tolerance test and insulin tolerance test

For the measurements of glucose tolerance and insulin sensitivity, at 8:00 AM mice were single housed in fresh cages, weighed and the food was removed. After 6 hrs of fasting at 2:00 PM mice were injected *intra peritoneal* (*i.p.*) with 2 g glucose/kg body weight for the glucose tolerance test (GTT), and 1 U insulin/kg body weight for the insulin tolerance test (ITT). Tail

blood glucose levels (mg/dl) were measured using a handheld glucometer (TheraSense Freestyle, USA) before (0 min) and at 15, 30, 60, and 120 min after injection.

2.2.6 Exendin-4 test

Ghr-GHSR mice, which have been fed with HFD since weaning, were single housed in fresh cages and fasted on the morning of the test. A fresh solution of Exendin-4 was prepared by diluting the stock solution (3.5 μg/μl) 50 fold in sterile saline to a final concentration of 0.07 μg/μl. After 6 hrs of fasting (0 min) blood glucose from tail blood was tested with a handheld glucometer (TheraSense Freestyle, USA). The mice were injected immediately after measuring the 0 min glucose level with 50 nmol/kgBW Exendin-4 i.p. Blood glucose was then measured 30 min, 60 min and 90 min after injection.

2.2.7 Blood analysis

For measurement of all blood parameters except IPMS analysis of ghrelin (see below) blood was collected after an overnight fast from tail veins using EDTA-coated Microvette tubes (Sarstedt, Nuremberg, Germany) and immediately chilled on ice. After 15 min of centrifugation at 3,000 g and 4°C, plasma was collected and stored at -80°C.

Plasma insulin levels were quantified with a radioimmunoassay from Linco. Plasma leptin levels were measured using an ELISA kit. Plasma glucose and nonesterified fatty acid (NEFA) were measured by using commercially available enzymatic assay kits from Wako. Plasma cholesterol levels were determined with the cholesterol oxidase method by using Infinity Cholesterol reagent, and plasma triglycerides were quantified by using the Infinity Triglyceride reagent. All assays were performed according to the assay manufacturer's instructions. Plasma parameters of the Mboat4$^{-/-}$ mice (except ghrelin) were analyzed by the Rules Based Medicine Company, Austin Texas. The lipoprotein profile of Mboat4$^{-/-}$ mice was measured with Mass Spectrometry at the Eli Lilly Company in Indianapolis. Lipoproteins were captured using an affinity resin beads (Liposorb, Calbiochem, EMD Chemicals Inc. North America). After capture and washing, proteins were digested with trypsin directly on the beads. Resulting tryptic peptides were quantified on an ion trap mass spectrometer in MRM mode. Multiple peptides (two or more) were measured for each protein and average values were reported for each protein. Peak intensity for each peptide was calibrated against normal mouse serum serially diluted in horse serum. In this manner, all reported concentrations are % of normal mouse serum.

2.2.8 Ghrelin IPMS assay - Immunoprecipitation Reaction (IP) and Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-ToF MS)

Quantification of ghrelin is normally achieved with single-site immuno-assays such as RIAs and ELISAs using a single recognition site for the primary antibody. Due to interference from peptide fragments or cross-reactivity at a single epitope, single-site assays can be less specific than extracted or deproteinized samples used for mass spectrometry analysis (Nussbaum et

al., 1987; Rauh et al., 2007). Further commercial available primary ghrelin antibodies are generally specific towards either the acylated N-terminus for acyl ghrelin or the C-terminus for total ghrelin (acyl + des-acyl ghrelin), requiring two specific assays to measure acyl and total ghrelin (des-acyl levels are calculated from the difference). To overcome those limitations, an immunoprecipitation (IP) matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-ToF/MS) assay for ghrelin was established in collaboration with the Eli Lilly Company at the Corporate Center's Research Laboratories in Indianapolis. Using antibodies toward the C-terminus of ghrelin and stable isotope labeled ghrelin standards, both acyl and des-acyl ghrelin were measured simultaneously.

2.2.8.1 Blood collection

Ghrelin was extracted from blood and quantified using the ghrelin IPMS assay (Gutierrez et al., 2008), a technique that combines immunoprecipitation (IP) and mass spectrometry (MS). For ghrelin IPMS analysis 2 ml collection tubes with an imprinted volume scale (Eppendorf, USA) were marked with a black horizontal line at the 1ml volume notch. Tubes were then prefilled with freshly made 500 µl anti-proteolytic cocktail (Table 7) and kept on wet ice or 4°C until blood collection. The pH of the anti-proteolytic cocktail was measured with an electronic pH meter and adjusted with 1N HCl or 1N NaCl to a pH of 3. Due to the cocktail's acidity EDTA eventually crystallized. Therefore the prefilled collection tubes were mixed by vortexing for 30 sec immediately before the blood collection. Mice were decapitated and using a clean plastic funnel for each mouse exactly 500 µl trunk blood (until fill-volume reached the pre-marked black line) was collected into the chilled collection tubes pre-filled with 500 µl anti-proteolytic cocktail. The blood-preservative mixture was carefully mixed by rotation (ca. 30 sec) until the EDTA was soluble and the mixture reached a dark red color. The blood-preservative mixture was then immediately frozen on dry ice and stored at -80°C until time of IPMS analysis.

2.2.8.2 Ghrelin immunoprecipitation

For ghrelin IP monoclonal ghrelin antibodies (D4-7.1) derived at the Eli Lilly Company, Indianapolis were covalently coupled to Invitrogen/Dynal (Invitrogen, Carlsbad, CA) magnetic beads following the manufacturer's instructions. Blood was carefully thawed at 4°C, acidified with 50 µl 1N HCl and extracted on tC18 Sep Pak cartridges (Millipore, USA, catalog no. WAT036805). Peptides were eluted in IPMS elution buffer (Table 7) and lyophilized to pellets. The pellets were re-suspended in 275 µl of IPMS buffer I. If necessary, the buffer was re-adjusted to pH 7.5 and the pellet-buffer mixture was exposed to ~1 µg of anti-ghrelin antibody. The mixture was then bound to the coupled magnetic beads and incubated either overnight at 4°C or for 2 h at room temperature. Antibody–antigen complexes were washed twice in 500 µl IPMS wash buffer (Table 7) and twice in distilled water. Immunocomplexes were acidified with 10 µl of a 0.1% trifluoroacetic acid solution, removed from magnetic beads, and processed with C18 ZipTips (Millipore USA, catalog no. ZTC18S). Ghrelin peptides were eluted in 3 µl of a solution containing 50% acetonitrile, 0.1% TFA saturated with α-cyano-4-hydroxy-cinammic acid matrix (Sigma-Aldrich, Saint Louis, MO).

2.2.8.3 Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry

MALDI-ToF target plates were thoroughly cleaned and coated with "Seed Crystals" (Gutierrez et al., 2005). To layer "Seed Crystals", the plates were processed on the plate spreader with a 2% solution of α-cyano-4-hydroxycinnamic acid in methanol. A 1 µl-volume from each ghrelin eluate (see above) was then spotted on the target plates and analyzed by MALDI-ToF/MS using an Applied Biosciences 4700 MALDI-ToF mass spectrometer under optimized conditions for ghrelin peptide detection. The laser was operated at a fixed fluence just above the threshold value and the spectra were automatically collected for each spot by a random, center-biased pattern. Data were analyzed with the Maldi_Quant Software, which was specifically written for the MALDI-ToF analysis using the R Statistical Computing Environment. Data processing included mass calibration, spectrum smoothing, local baseline subtraction, and Lorentzian peak fitting for peak quantification.

2.2.9 Immunohistochemistry

For immunohistochemistry fresh pancreas was collected into cold 4% paraformaldehyde in PBS and incubated overnight at 4°C. The next day the pancreas was removed, rinsed twice in cold phosphate buffered saline and incubated overnight in 30% sucrose dissolved in PBS at 4°C. On the next day the pancreas was transferred to a solution prepared from 50% OCT compound and 50% sucrose (30%) and mixed at periodically at room temperature (RT) for 15 –30 min followed by 30 min incubation in 100% OCT compound at RT. Finally, the pancreas was embedded with 100% OCT compound in plastic cassettes, frozen on dry ice and stored at –80°C until analysis. On the day of analysis the pancreas was sectioned on a Cryostat (Leica, Wetzlar Germany) into 8 µm thick slices and transferred to consecutively numbered superfrost plus slides (Fisher-Scientific USA). Slides were numbered into 8 series (A to H) with 6 slides per series (A1-6; B1-6,…). A total of 140-150 sections were collected from each pancreas and 3 consecutive sections were grouped on each slide starting with slide A1 and ending with slide H6. Each slide was immediately stored on dry ice after tissue collection. The first slide of each series was dyed with Harris Hematoxylin and Eosin to identify the series with the greatest number of islet cells. The remaining 5 slides of the chosen series were used for immunohistochemistry. Slides were rinsed in 1x PBS and blocked for 30 min with 5% goat serum in PBS-TritonX. Primary antibodies were diluted in 2% donkey serum in PBST and two antibodies that were each rose in different animals were applied to each slide and incubated over night at 4°C. On the next day slides were rinsed 3 times for 5 min in PBST and incubated with both secondary antibodies that were diluted in 2% donkey serum in PBST for 2 hrs at RT. After incubation the slides were washed twice for 5 min in PBST and covered with a few drops of DAKO Anti-fade. Slides were coversliped and stored dark and moist at 4°C until visualization. A list of primary and secondary antibodies, their dilution and manufacturers references can be found in table 5.

2.2.10 Statistical analysis

Unless indicated otherwise, all statistical analyses were performed on the basis of planned comparisons and/or using Fishers least significant difference (LSD) test. These analyses of

statistical validity were selected because in each case simple comparisons were made with strong a-priori predictions on the basis of the genetic deletion or overexpression of the here described target genes. Differences in gene expression during fasting between groups, as well as in glucose levels during GTT or ITT were examined by two-way ANOVAs, and Dunnett's Multiple Comparison post-hoc tests. All statistical analyses were performed with Statistica 6.0 (StatSoft, Tulsa, OK) and GraphPad Prism version 5.01 for Windows (GraphPad Software, SanDiego, CA). Experiment-wise α-error was set at $P < 0.05$. All results are presented as means $\pm$ standard error of the mean (SEM).

3 Results

Ghrelin has been thought of as the hunger hormone, a molecule that initiates food intake and potentially prepares the body for incoming nutrients. Even though ghrelin administration significantly increases food intake and adipogenesis in humans and rodents, ghrelin or GHSR deficient mice are not hypophagic and lean. The lack of a metabolic phenotype, at least on a standard chow diet, could be explained with the existence of additional GHSR ligands or possible other ghrelin receptors.

3.1 Ghr-GHSR mice

To completely shut down the ghrelin system and to test for the potential existence of other ligands or ghrelin receptors we generated and metabolically phenotyped mice deficient for ghrelin ($Ghr^{-/-}$), GHS-R1 ($Ghsr^{-/-}$) and ghrelin and GHS-R1a simultaneously ($Ghr^{-/-}Ghsr^{-/-}$). $Ghr^{-/-}$ and $Ghr^{-/-}Ghsr^{-/-}$ mice had no detectable levels of total ghrelin in plasma whereas $Ghsr^{-/-}$ and Wt mice showed normal levels of total ghrelin in plasma (Fig 5). Pituitary gene expression of *Ghsr* was normal in Wt and $Ghr^{-/-}$ mice but undetectable in $Ghr^{-/-}Ghsr^{-/-}$ and $Ghsr^{-/-}$ mice (data not shown). Fertility was normal in all mice, and no differences in mortality rate or overall health could be observed between groups.

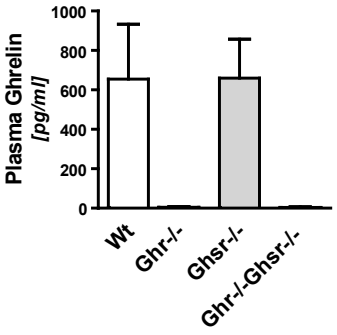

Figure 5 Ghrelin levels of Ghr-GHSR mice
Plasma concentration of total ghrelin in 13.4 month old male mice was measured with an Radio Immuno Assay to confirm the validity of the genotypes. $Ghr^{-/-}$ and $Ghr^{-/-}Ghsr^{-/-}$ mice had undetectable levels of ghrelin in plasma. Values are means ± SEM.

3.1.1 $Ghr^{-/-}Ghsr^{-/-}$ mice were leaner than single knock-out and Wt littermates

At the age of weaning (21 days after birth) BW and BL was not significantly different between the four studied genotypes in male and female mice (Fig. 6). $Ghr^{-/-}Ghsr^{-/-}$ mice grew more slowly than their Wt and single knockout littermates. At the age of 2 month chow fed $Ghr^{-/-}Ghsr^{-/-}$ mice showed already a significant reduction in BW together with a slight but significant decrease in LM. However, when normalized to BW the percentage of FM and LM was comparable between all groups (Table 10).

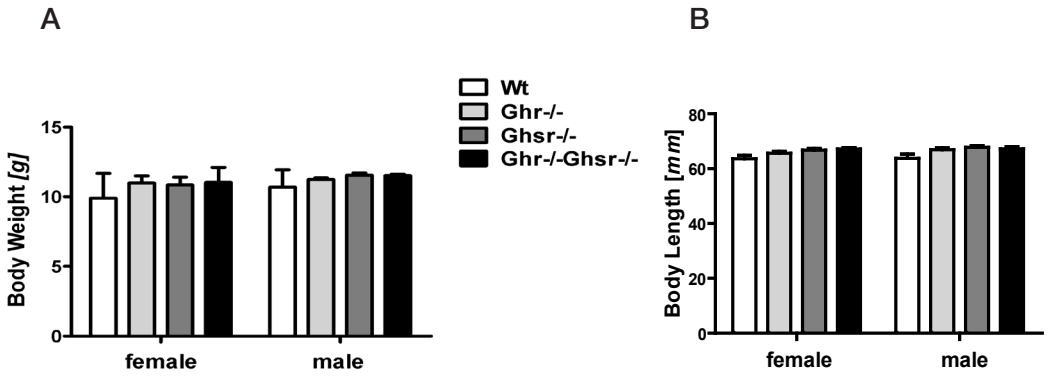

Figure 6 Body Weight and Length of Ghr-GHSR mice
(A) Body weight and (B) body length of male and female mice was measured at the day of weaning (21 days after birth; n=9-20 per group). (A) Neither body weight nor (B) body length differed between the groups. Values are means ± SEM.

The decrease in BW was sustained throughout aging. At an age of 13 month $Ghr^{-/-}Ghsr^{-/-}$ were still significantly lighter than Wt controls (Fig. 7A) and the difference in BW was predominantly due to a decrease in FM (Fig. 7C) but not to changes in LM (Fig. 7D). Body length of the 13 month old $Ghr^{-/-}Ghsr^{-/-}$ mice was reduced (P= 0.013, Fig. 6B), whereas young $Ghr^{-/-}Ghsr^{-/-}$ mice did not yet differ from Wt controls (P= 0.78, Table 10) that might indicate an age related phenotype of body length.

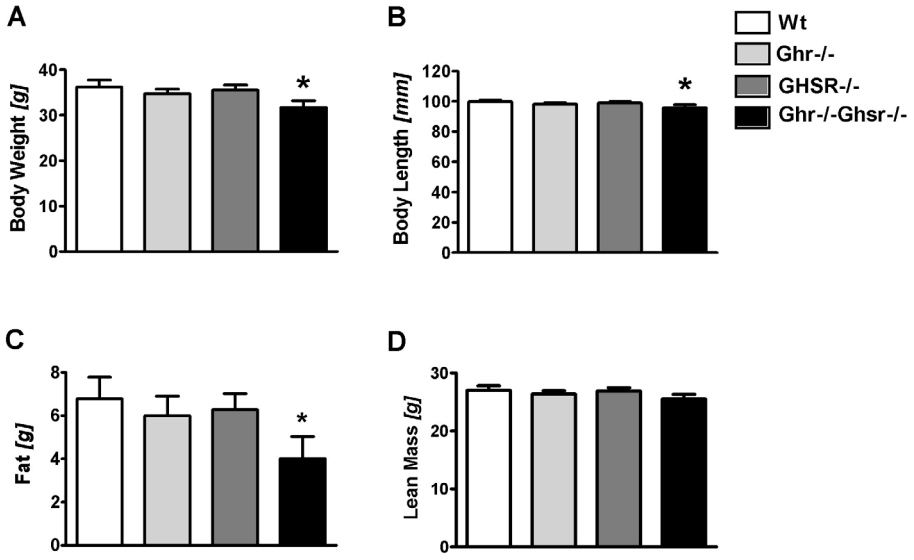

Figure 7 Body weight and body composition of chow fed Ghr-GHSR mice
(A) Body weight, (B) body length, (C) fat mass and (D) lean mass were measured in 13 Wt, 12 $Ghr^{-/-}$, 13 $Ghsr^{-/-}$, and 6 $Ghr^{-/-}Ghsr^{-/-}$ mice on standard chow diet. Both body weight (A) and body length (B) were decreased in $Ghr^{-/-}Ghsr^{-/-}$ mice compared to Wt controls. Fat mass (C) was also decreased in $Ghr^{-/-}Ghsr^{-/-}$ mice compared with Wt mice, whereas lean mass did not differ (D). Values are means ± SEM. *$P < 0.05$.

Table 10 Anthropometric measures of Ghr-GHSR mice

	Wt (n=13)	$Ghr^{-/-}$ (n=14)	$Ghsr^{-/-}$ (n=9)	$Ghr^{-/-}Ghsr^{-/-}$ (n=11)
Body Weight [g]	27.22±0.69	26.09±0.47	26.20±0.38	24.98±0.54*
Body Length [mm]	89.2±0.9	90.8±0.8	88.2±1.2	88.8±0.7
Fat Mass [g]	1.91±0.29	2.38±0.33	1.52±0.20	1.87±0.29
Lean Mass [g]	23.15±0.59	21.77±0.46*	21.93±0.41	21.29±0.35*
Fat Mass [%]	6.94±0.95	8.79±1.06	5.93±0.73	7.34±0.91
Lean Mass [%]	85.6±0.98	83.34±1.18	86.47±0.99	85.37±0.8
IGF-1 [ng/mL][1)]	548±32	510±25	492±28	610±43

*$P < 0.05$.

To challenge the metabolic system and to investigate if $Ghr^{-/-}Ghsr^{-/-}$ mice are protected against diet-induced obesity we exposed adult and young $Ghr^{-/-}$, $Ghsr^{-/-}$, $Ghr^{-/-}Ghsr^{-/-}$ and Wt mice chronically to HFD in two different experiments. Thirteen month old mice that have been exclusively fed with standard chow were exposed to HFD containing 58% of calories from fat for six weeks. As described above these $Ghr^{-/-}Ghsr^{-/-}$ mice were lighter and leaner than the Wt and single knockout littermates before starting HFD feeding. During exposure to HFD, $Ghr^{-/-}Ghsr^{-/-}$ sustained the decreased BW and did not reach the level of obesity of the remainder genotypes (Fig. 8A). Nevertheless, on HFD mice of all four genotypes gained the same amount of weight, FM and LM (Fig. 8B), respectively.

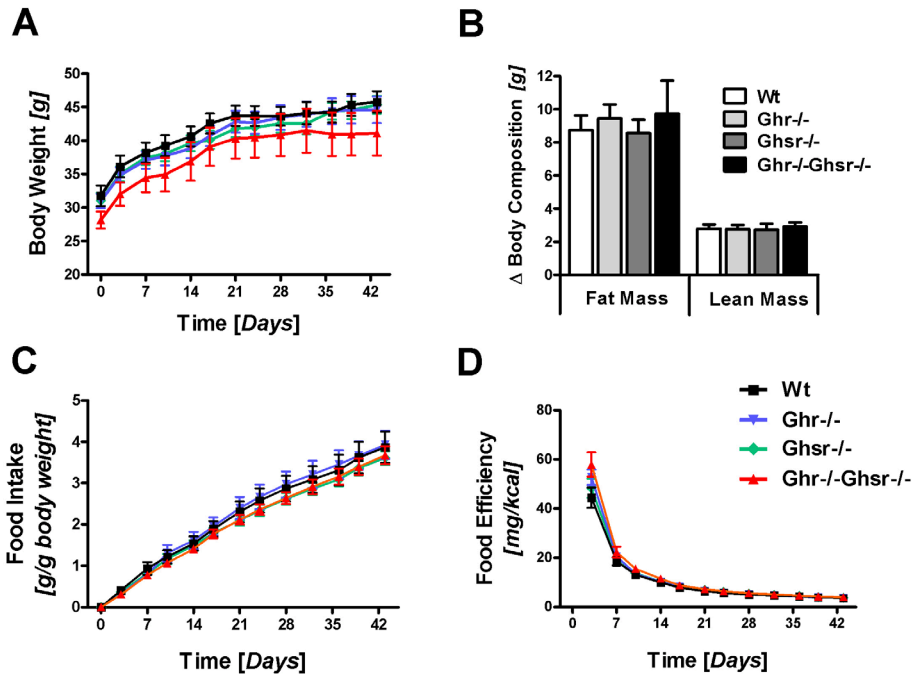

Figure 8 Body weight, body composition and food intake of Ghr-GHSR mice after HFD feeding
(A) Body weight and (C) food intake were measured weekly from the time when high-fat diet (HFD) feeding was started (time point 0) in 12 Wt, 12 $Ghr^{-/-}$, 13 $Ghsr^{-/-}$ and 6 $Ghr^{-/-}Ghsr^{-/-}$ 13 month old male mice. (B) Body composition was measured with NMR after exposure to HFD for 42 days. (D) Food efficiency was calculated by dividing body weight gain by ingested kilocalories. Body weight gain (A) strongly tended to be smaller in $Ghr^{-/-}Ghsr^{-/-}$ mice whereas body composition (B), food intake (C) and food efficiency (D) remained unchanged. Values are means ± SEM.

3.1.2 Ghr$^{-/-}$Ghsr$^{-/-}$ mice were not hypophagic

Meal patterns were analyzed in chow and HFD fed Ghr-GHSR mice to reveal effects of absolute ghrelin deficiency. This analysis was done not only to measure gross food intake but also food intake behavior such as meals patterns and meal seize.

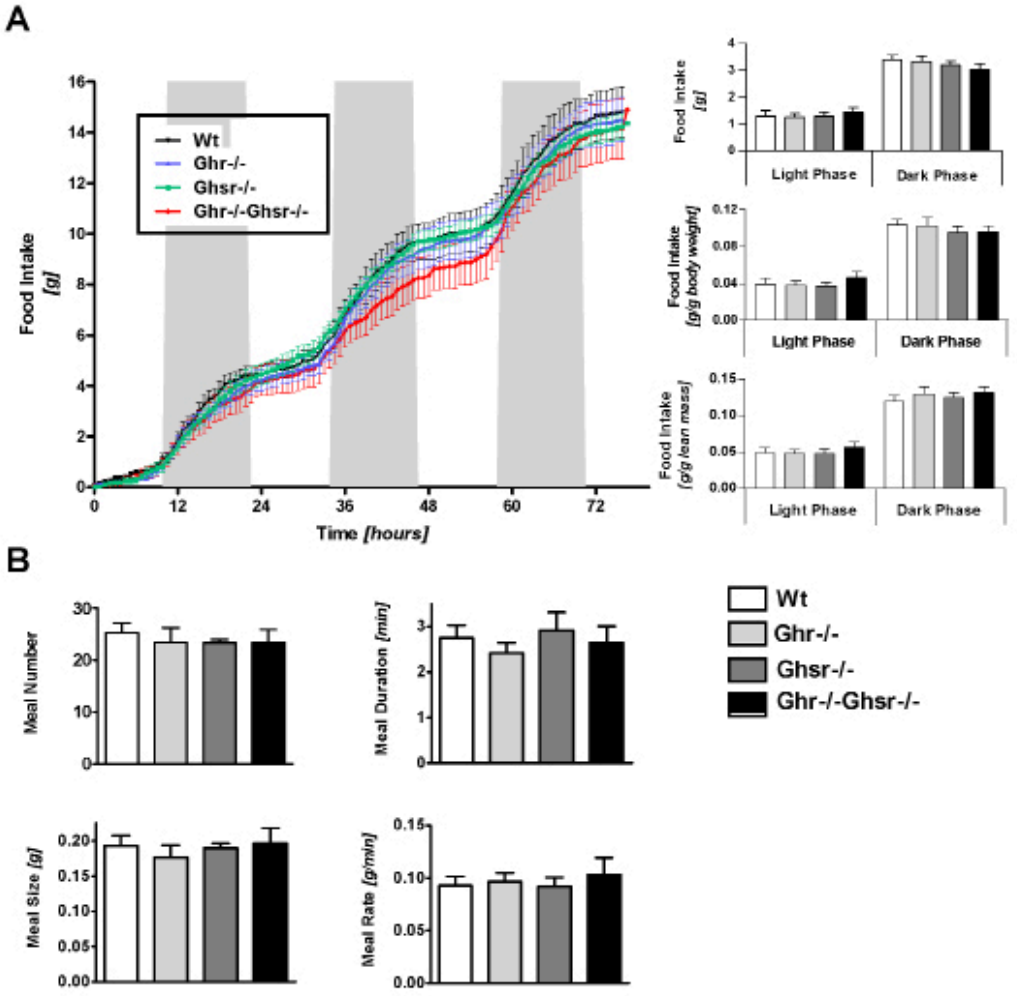

Figure 9 Food intake and meal pattern analysis of chow fed Ghr-GHSR mice
Ingestive behavior was studied in 7 Wt, 8 Ghr$^{-/-}$, 8 Ghsr$^{-/-}$, and 6 Ghr$^{-/-}$Ghsr$^{-/-}$ male mice that had access to standard chow diet *ad libitum*. (A, left) No differences in cumulative food intake were observed between groups during a representative 76-h study period. (A, right, top) Average 24-h food consumption was comparable between groups during both dark and light phases. Normalization of average cumulative 24-h food consumption to body weight (A, right, middle) or lean mass (A, right, bottom) also did not reveal differences between genotypes. (B) Meal pattern analysis showed no differences regarding average meal number (B, left, top), meal duration (B, right, top), meal size (B, left, bottom), or ingestion rate (B, right, bottom). Values are means ± SEM.

No differences in cumulative food and water intake (data not shown), which could have explained the lower BW and FM of Ghr$^{-/-}$Ghsr$^{-/-}$ mice, were found between Ghr$^{-/-}$, Ghsr$^{-/-}$, Ghr$^{-/-}$Ghsr$^{-/-}$ and Wt mice at all ages on both, chow (Fig. 9A, left) and HFD (Fig. 8C). More specifically, on chow and HFD twentyfour-hour FI did not differ between genotypes during the light or dark phase, respectively (Fig. 9A, right, top for chow). In addition, normalization of 24-h chow intake per BW (Fig. 9A, right, middle) or LM (Fig. 8A, right, bottom) to control for the changes in FM and body length between genotypes did not reveal any differences in

relative FI between the groups. Meal patterns were also not affected by deletion of Ghr, Ghsr, or both genes on either diet. The number of meals per 24 h (Fig. 9B, left, top for chow), the average duration of the meals (Fig. 8B, right, top for chow), the average size of a meal (Fig. 9B, left, bottom for chow), and the average meal rate (Fig. 9B, right, bottom for chow) did not differ between the four genotypes. Further food efficiency, a parameter that indicates how much weight is gained per ingested calorie, was not changed between groups (Fig. 8D) when feeding HFD.

3.1.3 Normal fasting induced hyperphagia in Ghr$^{-/-}$Ghsr$^{-/-}$ mice

To study if absence of ghrelin influences fasting induced hyperphagia 13 month old mice of all four genotypes were fasted for 20 h and then given free access to standard chow. EE, FI and meal patterns were analyzed using indirect calorimetry and an automated food intake monitoring system during the 20 hrs fasting and 24 hrs re-feeding process. The significant increase in EE that could be found in Ghr$^{-/-}$Ghsr$^{-/-}$ mice during regular chow feeding (see below) was maintained throughout the 20 hrs period of fasting. Respectively, no changes in overall food consumption were observed between genotypes during the re-feeding period even when FI was normalized to BW or LM. Further, analysis of the re-feeding meal patterns revealed no differences between the genotypes. Neither number (P= 0.60 Wt vs. Ghr$^{-/-}$Ghsr$^{-/-}$; Fig. 10B, left, top), duration (P= 0.29 Wt vs. Ghr$^{-/-}$Ghsr$^{-/-}$; Fig. 10B, left, bottom), nor sizes of meals (P= 0.29 Wt vs. Ghr-/-Ghsr-/-; Fig. 10B, left, bottom) were significantly different between groups. However, there was a trend towards lower food ingestion rate in the Ghr$^{-/-}$Ghsr$^{-/-}$ compared to Wt mice (P= 0.11; Fig. 10B, right, bottom) during the first 4 hrs of re-feeding.

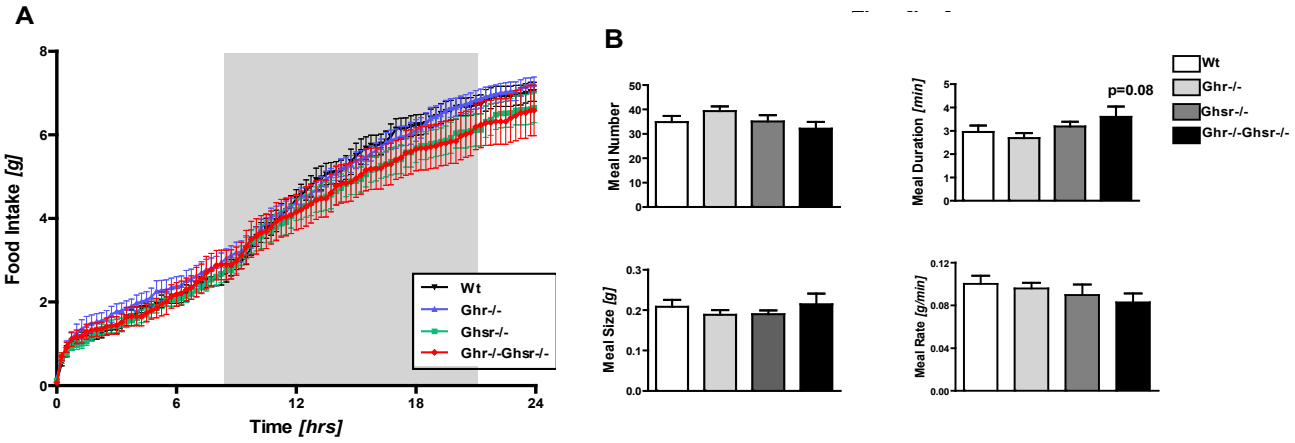

Figure 10 Fasting induced hyper-phagia in Ghr-GHSR mice
Six to 9 previously chow fed male mice per group were fasted for 20 h and subsequently given free access to standard chow in an automated food-monitoring system. (A) Cumulative food intake was unchanged in all groups throughout the 24-hr study period. (B) Meal pattern analysis revealed no significant changes in the meal rate (B right, bottom), the meal duration (B right, top), or the number and size of meals (B left, top and bottom, respectively) during the fisrt 4 hours of re-feeding. Values are means ± SEM.

3.1.4 Ghr$^{-/-}$Ghsr$^{-/-}$ mice had increased energy expenditure and body core temperature

Ghr$^{-/-}$Ghsr$^{-/-}$ mice had a leaner phenotype in spite of unchanged FI. In search of an possible explanation, we measured EE on both diets with our indirect calorimetry system.

Only Ghr$^{-/-}$Ghsr$^{-/-}$ mice but none of the single knockout mice showed significantly higher EE (Fig. 11A, left) compared to Wt mice during chow feeding throughout the light (P = 0.0006) and dark phase (P= 0.0007; Fig. 11A, right, top) of the photoperiod. Normalization of the average EE to LM, which is considered to be the "metabolically active" tissue mass, supported this finding (light phase P= 0.0027, dark phase P= 0.006 vs. Wt, respectively; Fig. 11A, right, bottom). This increase in EE was consistent with higher body core temperature of Ghr$^{-/-}$ Ghsr$^{-/-}$ mice (P= 0.043; Fig. 11C, top, +0.5°C), whereas peritoneal surface temperature (Fig. 11C, bottom) was not found to be significantly higher (P = 0.73). Further, we were unable to find any changes in the respiratory quotient between groups, which could have indicated a change in fuel preference. Ghr$^{-/-}$Ghsr$^{-/-}$ that had a higher EE on chow diet continued to show a strong trend towards increased EE after HFD exposure for 6 weeks. However, this increased did not reach statistical significance when compared to Wt controls (data not shown).

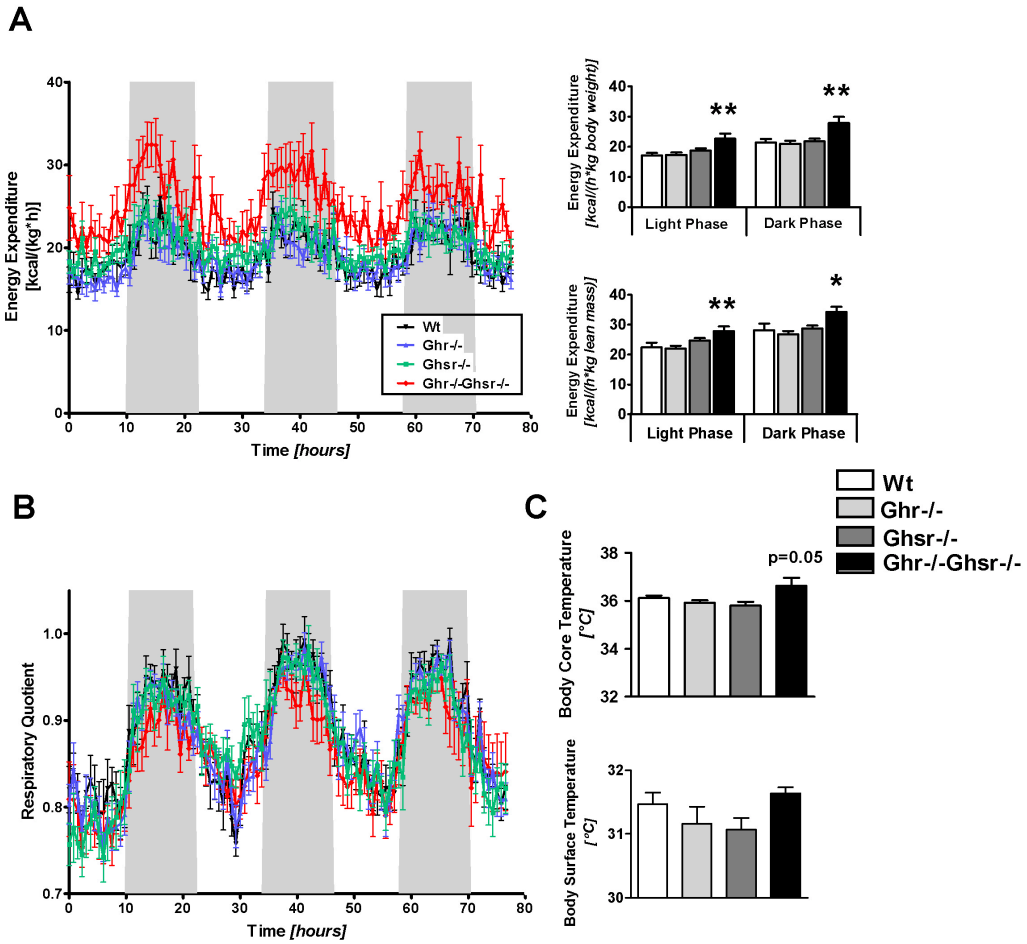

Figure 11 Energy expenditure, respiratory quotient, body core temperature, and peritoneal body surface temperature of chow fed Ghr-GHSR mice

(A) Average energy expenditure and (B) respiratory quotient were measured in 7 Wt, 9 Ghr$^{-/-}$, 8 Ghsr$^{-/-}$, and 6 Ghr$^{-/-}$Ghsr$^{-/-}$ mice for a period of 76 h. Ghr$^{-/-}$Ghsr$^{-/-}$ mice had significantly higher energy expenditure than Wt mice in both the dark phase and light phase (A, right, top). Normalization to the metabolically active lean tissue mass (measured by NMR) verified the increase in energy expenditure (A, right, bottom). (C, top) Body core temperature was higher in Ghr$^{-/-}$Ghsr$^{-/-}$ mice (C, bottom) in contrast with a similar body surface temperature. Values are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

3.1.5 Exposure of Ghr$^{-/-}$Ghsr$^{-/-}$ mice to HFD had a strong effect on locomotor activity

In parallel to measuring EE as described above, we monitored locomotor activity by using an infrared beam break systems to study changes in mouse activity induced by ghrelin and GHSR ablation. Total locomotor activity (Fig. 12A) was further dissected into ambulatory movements (Fig. 12B), stationary movements (Fig. 12C), and vertical movements (rearing, Fig. 12D) to differentiate between movements such as grooming, rearing, running and so forth.

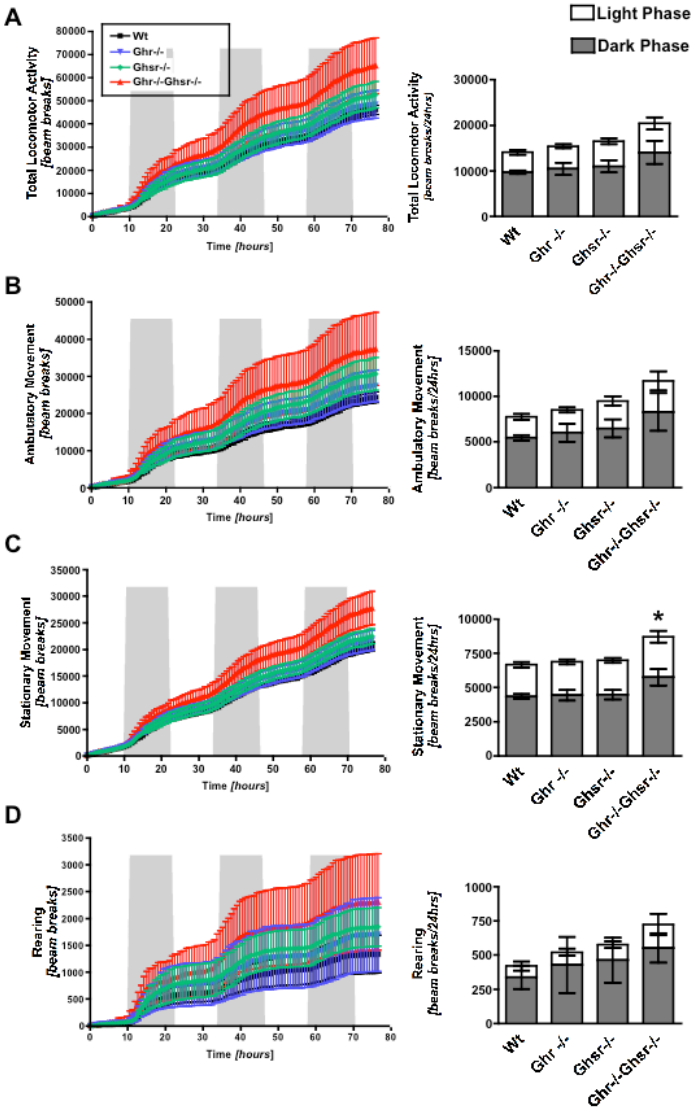

Figure 12 Spontaneous locomotor activity of chow fed Ghr-GHSR mice

Home-cage physical activity was monitored in 7 Wt, 9 Ghr$^{-/-}$, 8 Ghsr$^{-/-}$, and 6 Ghr$^{-/-}$Ghsr$^{-/-}$ male mice by using an infrared beam break system. (A) Total locomotor activity was further dissected into (B) ambulatory movement, (C) stationary movement and (D) rearing. Left: cumulative locomotion for the 76-h monitoring period. Right: 24-hr mean values for the light phase activity (white bars) and the dark phase activity (grey bars). Ghr$^{-/-}$Ghsr$^{-/-}$ mice (red lines) in general appeared to be more active than Wt mice, but significance was only reached for dark-phase stationary activity (C, right). Values are means ± SEM. *P < 0.05.

Ghr$^{-/-}$Ghsr$^{-/-}$ mice showed significantly more stationary movements (P= 0.033 dark phase; P= 0.036 light phase; Fig. 12C) and exhibited strong tendencies toward higher ambulatory and rearing activity when chow fed. Interestingly, Ghsr$^{-/-}$ mice showed no significant differ-

ences in locomotor activity, and Ghr$^{-/-}$ mice only showed a minor increase in total locomotor activity during the light phase, compared to Wt control mice (P = 0.004; Fig. 12 A, B, and D, right). Since a strong trend towards increased physical activity was apparent in the chow fed Ghr$^{-/-}$ Ghsr$^{-/-}$ mice the analysis of locomotor activity and fine movements was repeated during HFD feeding conditions. Surprisingly, six weeks of feeding HFD had a dramatic effect on physical activity of Ghr$^{-/-}$Ghsr$^{-/-}$ mice (Fig. 13). Total locomotor activity (Fig. 13A) was highly increased in Ghr$^{-/-}$Ghsr$^{-/-}$ mice compared to Wt littermates (*P*= 0.0019).

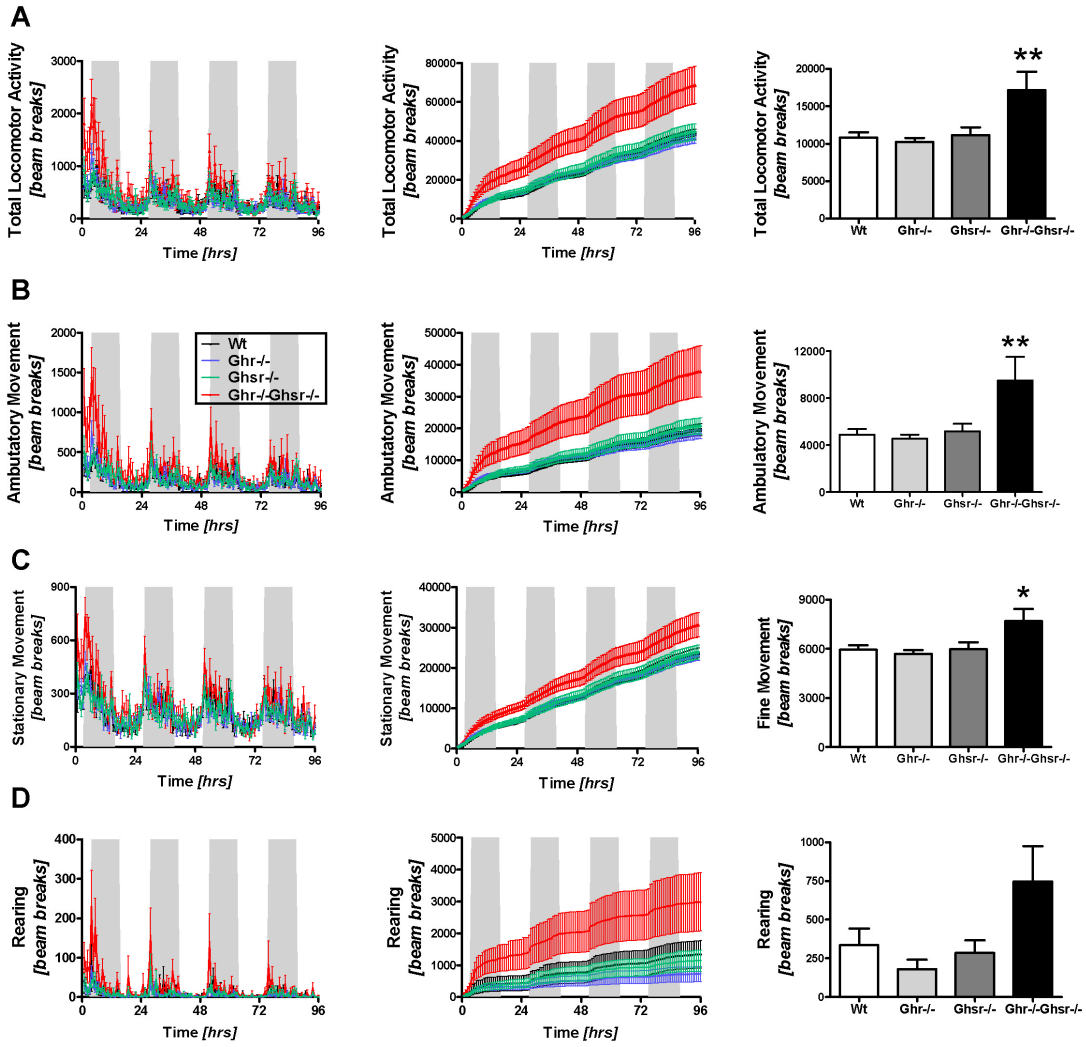

Figure 13 Analyzes of fine movement in Ghr$^{-/-}$Ghsr$^{-/-}$ mice after HFD exposure
Physical activity was measured by using an infrared beam break system in 9 Wt, 8 Ghr$^{-/-}$, 8 Ghsr$^{-/-}$, and 6 Ghr$^{-/-}$Ghsr$^{-/-}$ male mice after feeding with HFD for 6 weeks. (A) Total activity, which already tended to be increased in chow fed Ghr$^{-/-}$Ghsr$^{-/-}$ mice, was strongly increased in Ghr$^{-/-}$Ghsr$^{-/-}$ mice after the HFD exposure. Further, (B) ambulatory movement, (C) stationary movement and (D) rearing were strongly increased in Ghr$^{-/-}$Ghsr$^{-/-}$ mice compared to Wt controls. Values are means ± SEM. *$P < 0.05$; **$P < 0.01$

Further dissection of locomotor activity into fine movement elements revealed that all analyzed elements of activity, ambulatory movement (Fig. 13B; *P*= 0.004), fine movement (Fig. 13C; *P*= 0.018) and rearing (Fig. 13D; *P*= 0.03), were significantly increased after HFD exposure in Ghr$^{-/-}$Ghsr$^{-/-}$ compared to Wt mice.

3.1.6 Glucose homeostasis was impaired in Ghr$^{-/-}$Ghsr$^{-/-}$ mice after early exposure to HFD

The role of ghrelin on glucose homeostasis is highly controversial. On the one hand ghrelin clearly inhibits glucose induced insulin release. On the other hand it is suggested that ghrelin has a beta-cell protective effect and that ghrelin administration ameliorates insulin depended diabetes. We studied glucose homeostasis in Ghr$^{-/-}$Ghsr$^{-/-}$ mice by measuring glucose and insulin tolerance. After an overnight fast in chow fed mice, plasma glucose (P= 0.86, Fig. 14A) and plasma insulin levels (P= 0.12, Fig. 14B) were unchanged in old and young male (data not shown) Wt versus Ghr$^{-/-}$Ghsr$^{-/-}$ mice.

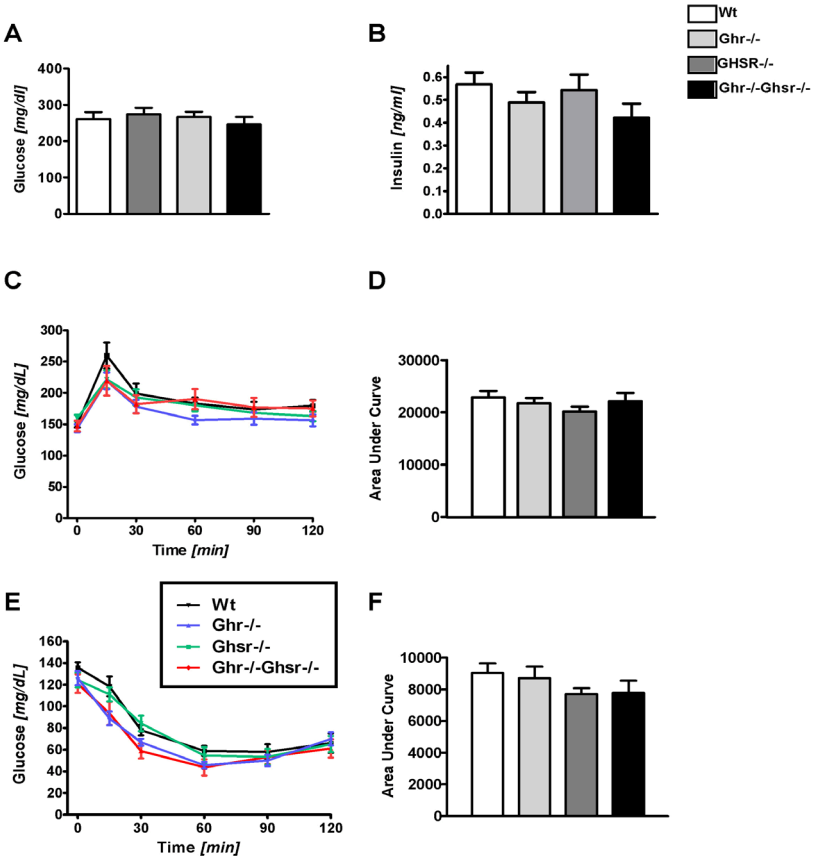

Figure 14 Glucose homeostasis and insulin sensitivity of chow fed Ghr-GHSR mice
(A) Fasting glucose levels and (B) fasting insulin levels in 12 or 13 Wt, 12 Ghr$^{-/-}$, 12 or 13 Ghsr$^{-/-}$, and 5 or 6 Ghr$^{-/-}$Ghsr$^{-/-}$ male mice did not differ between groups. (C) A glucose tolerance test (GTT) using a dose of 2 g glucose/kg body weight *intra peritoneal* did not reveal any differences of glucose tolerance between groups in the 120-min study period. (D) Area under the curve values for the GTT were also unchanged. (E) In an insulin tolerance test (ITT) using 1 U/kg insulin, no significant changes in glucose excursions or (F) the respective area under the curve values could be observed between the groups. GTTs and ITTs were performed in 13 Wt, 12 Ghr$^{-/-}$, 13 Ghsr$^{-/-}$, and 6 Ghr$^{-/-}$Ghsr$^{-/-}$ male mice, respectively. Values are means ± SEM.

Additionally, under chow feeding conditions glucose tolerance between the four genotypes at various ages was similar during oral and an *intra peritoneal* (*i.p*) glucose tolerance tests (GTTs) in males (Fig. 14C and D) and females (data not shown). Older male mice deficient in Ghr, GHSR, or both showed lower glucose peak levels 15 min after glucose injection, possibly indicating that lack of ghrelin signaling results in faster release of insulin (Fig. 14C).

To further investigate the endogenous role of the ghrelin system in the control of glucose metabolism, male mice were subjected to an insulin tolerance test (ITT) (1 U insulin/kg BW) (Fig. 14E and F). In $Ghr^{-/-}$ mice, glucose levels were similar to Wt mice. In $Ghr^{-/-}Ghsr^{-/-}$ and $Ghsr^{-/-}$ mice, glucose levels, however, dropped more rapidly. After 15 min, glucose values in $Ghr^{-/-}Ghsr^{-/-}$ mice were 25 mg/dl (P= 0.08), or 11% lower than in Wt mice. In $Ghsr^{-/-}$ mice, glucose levels were 30 mg/dl (P= 0.0061), or 15.7% lower than glucose levels of WT mice after 15 min. In general, glucose levels of $Ghr^{-/-}Ghsr^{-/-}$ and $Ghsr^{-/-}$ mice tended to remain lower compared with Wt mice throughout the 120 min period of the ITT (Fig. 14E). However, although integrated (area under the curve) glucose levels in both $Ghsr^{-/-}$ and $Ghr^{-/-}Ghsr^{-/-}$ mice tended to be lower compared with Wt control mice ($Ghr^{-/-}Ghsr^{-/-}$: P= 0.105, and $Ghsr^{-/-}$: P= 0.065; Fig. 14F) the deficiency of ghrelin, its receptor, or both did not seem to have a major impact on overall insulin sensitivity or the overall regulation of glucose homeostasis in chow fed mice.

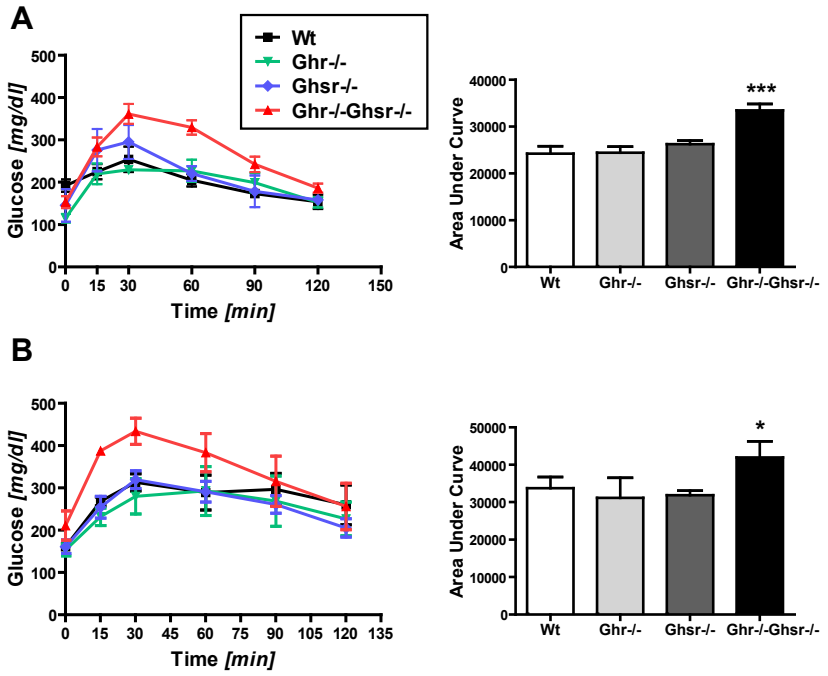

Figure 15 Glucose tolerance in GhrGHSR mice after early exposure to HFD
Glucose tolerance tests using an intraperitoneal administered dose of 2 g glucose/kg body weight were performed in (A) 3 month old and (B) 6 month old male Ghr-GHSR mice, which have been fed with HFD since weaning. (A) Three month old $Ghr^{-/-}Ghsr^{-/-}$ mice had a highly impaired glucose tolerance compared to the other genotypes as shown by the glucose excursion curve (A left) and the area under the curve (A right), respectively. Additionally, blood glucose after glucose challenge was significantly higher in 6 month old $Ghr^{-/-}Ghsr^{-/-}$ mice (B left) compared to Wt littermates. GTTs were performed in 7 Wt, 6 $Ghr^{-/-}$, 10 $Ghsr^{-/-}$ and 5 $Ghr^{-/-}Ghsr^{-/-}$ 3 month old male mice and in 7 Wt, 5 $Ghr^{-/-}$, 2 $Ghsr^{-/-}$ and 7 $Ghr^{-/-}Ghsr^{-/-}$ 6 month old male mice. Values are means ± SEM.
*P < 0.05; ***P < 0.0001

Next we studied glucose homeostasis in mice, which were fed with HFD from the day of weaning to test if absence of ghrelin signaling protects from diet induced glucose intolerance. Glucose tolerance evaluated with an i.p GTT was significantly impaired in three (Fig 15A; P= 0.006) and six mo old (Fig 15B; P= 0.05) male $Ghr^{-/-}Ghsr^{-/-}$ mice compared to Wt mice and unchanged in 6 mo old female mice (data not shown).

To test if the impaired glucose tolerance in male mice is due to a decrease in insulin sensitivity we subjected the males to an ITT. Early-onset HFD feeding did not seem to affect insulin sensitivity in 3 mo old (Fig 16A; *P*= 0.92) and 6 mo old (Fig 16B; *P*= 0.054) $Ghr^{-/-}Ghsr^{-/-}$ mice since there were no differences in glucose levels after insulin injection between the four studied genotypes.

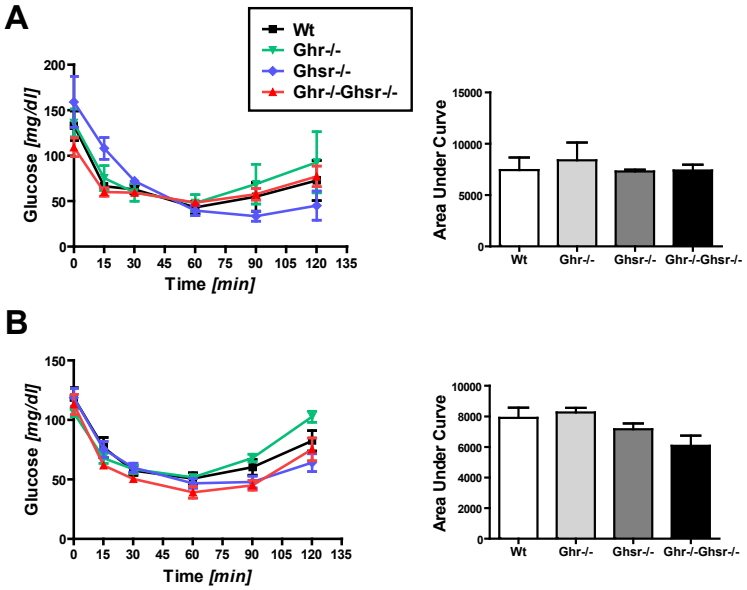

Figure 16 Insulin tolerance in $Ghr^{-/-}Ghsr^{-/-}$ mice after early exposure to HFD
The same 3 and 6 month old Ghr-GHSR mice that had undergone glucose tolerance testing were subjected to insulin tolerance tests using 1U insulin/kg body weight after a one-week recovery phase. Despite impaired glucose tolerance no difference between insulin sensitivity represented by blood glucose after insulin injection could be found in (A) 3 month old and (B) 6 month old Wt and $Ghr^{-/-}Ghsr^{-/-}$ mice. ITTs were performed in 7 Wt, 6 $Ghr^{-/-}$, 10 $Ghsr^{-/-}$ and 5 $Ghr^{-/-}Ghsr^{-/-}$ 3 month old male mice and in 7 Wt, 5 $Ghr^{-/-}$, 2 $Ghsr^{-/-}$ and 7 $Ghr^{-/-}Ghsr^{-/-}$ 6 month old male mice, respectively. Values are means ± SEM.

We suggested therefore that the impaired glucose tolerance of the $Ghr^{-/-}Ghsr^{-/-}$ mice is potentially caused by impaired insulin production. To test this hypothesis we injected 3 and 6 mo old mice with Exendin-4 (50 nmol/kgBW) and measured blood glucose before and 30 min, 60 min and 90 min after the injection. Exendin-4 is a glucagon-like peptide agonist that stimulates insulin secretion from the beta-cells of the pancreas. According to our hypothesis we expected to find impaired insulin secretion in $Ghr^{-/-}Ghsr^{-/-}$ mice after Exendin-4 injection, which would be indicated by higher blood glucose levels of $Ghr^{-/-}Ghsr^{-/-}$ mice.

As expected, blood glucose fell in all four genotypes after Exendin-4 injection. During the entire observation period, young and older $Ghr^{-/-}Ghsr^{-/-}$ mice tended to have slightly higher glucose concentrations after Exendin-4 injection, however the difference did not reach statistical significance (3 month old *P*= 0.23; 6 month old *P*= 0.3; Fig. 17). Since this Exendin-4 test could not answer the question why $Ghr^{-/-}Ghsr^{-/-}$ mice have impaired glucose tolerance in a diet-induced obese state, we measured plasma insulin levels of ad libitum HFD fed male mice and plasma insulin concentrations after glucose challenge.

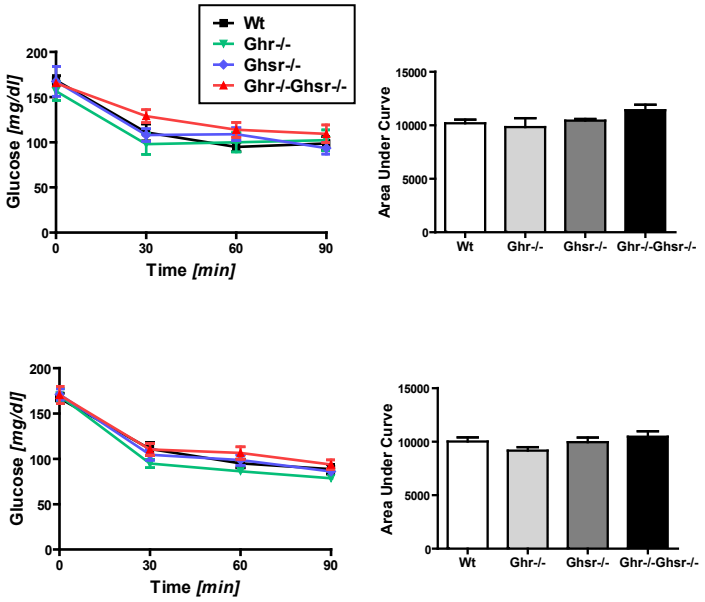

Figure 17 Exendin-4 response of HFD fed Ghr-GHSR mice
Three and six month old male Ghr-GHSR mice that were fed with HFD from the day of weaning were injected with 50 nmol/kgBW Exendin-4 *i.p.*, a GLP-1 receptor agonist. As expected blood glucose fell in all four studied genotypes after Exendin-4 injection. However, there were no differences between the genotypes. The test was performed in (top) 7 Wt, 6 $Ghr^{-/-}$, 10 $Ghsr^{-/-}$ and 5 $Ghr^{-/-}Ghsr^{-/-}$ three month old male mice and (bottom) in 7 Wt, 5 $Ghr^{-/-}$, 2 $Ghsr^{-/-}$ and 7 $Ghr^{-/-}Ghsr^{-/-}$ six month old male mice. Values are means ± SEM.

There were no changes between insulin levels of the four genotypes when fed HFD ad libitum (Fig. 18A). Correspondingly, glucose injection (2g/kgBW) *i.p.* resulted in comparable insulin secretion in Wt, $Ghr^{-/-}$, $Ghsr^{-/-}$ and $Ghr^{-/-}Ghsr^{-/-}$ mice (Fig. 18B). Only $Ghsr^{-/-}$ mice seemed to have a trend toward increase insulin secretion after the glucose challenge. Since the insulin secretion test could not explain why $Ghr^{-/-}Ghsr^{-/-}$ mice have an impaired glucose tolerance after HFD feeding we studied pancreatic beta-cell architecture in the male mice. Pancreatic tissue of male mice that have been exclusively fed with HFD was stained with antibodies against insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin. Fluorescent visualization of the labeled beta-cells did not reveal any abnormalities in beta-cell seize or structure (supplemental data) of the $Ghr^{-/-}Ghsr^{-/-}$ mice.

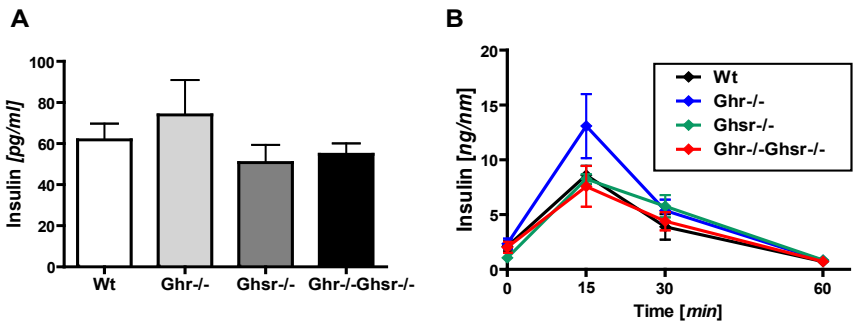

Figure 18 Insulin secretion test in Ghr-GHSR mice
(A) Plasma insulin levels were measured from tail blood using RIA in 5 month old Ghr-GHSR mice fed chronically with HDF. $Ghr^{-/-}$ mice seemed to have slightly higher plasma insulin levels compared to the other genotypes. (B) Six month old male Ghr-GHSR mice were injected with 2g glucose/kg body weight and blood was collected into EDTA coated tubes by tail bleeding before (0 min) and 15, 30 and 60 min after insulin injection. Total plasma insulin levels were measured in with an ELISA. $Ghr^{-/-}$ mice tended to have a higher insulin spike than the other genotypes after 15 min but overall glucose induced insulin production was comparable in all groups respectively. Values are means ± SEM.

3.1.7 $Ghr^{-/-}Ghsr^{-/-}$ mice had lower plasma cholesterol levels than Wt controls

Ghrelin stimulates the release of growth hormone (GH) and a shorter body statue of $Ghr^{-/-}Ghsr^{-/-}$ mice could possibly be explained by lack of ghrelin induced GH stimulation. However, GH secretion is pulsatile and varies substantial throughout the day. Limited by the amount and volume of blood collections that are allowed in mice per day we decided to measure only plasma concentrations of Insulin-like growth factor 1 (IGF-1) to study the length related phenotype of $Ghr^{-/-}Ghsr^{-/-}$ mice. IGF-1 is a polypeptide released by the liver that plays an important role in pre-pubertal growth. GH stimulates IGF-1 secretion and therefore the shorter body length of $Ghr^{-/-}Ghsr^{-/-}$ mice could be possibly explained by a lack of GH-stimulated IGF-1 signaling. Nevertheless, plasma IGF-1 levels of 2 and 13 mo old mice did not differ between genotypes and did not accompany the observed changes in fat mass or body length, respectively (Table 11). Further, free fatty acid and triglyceride levels were not changed. Interestingly, cholesterol levels were significantly lower in old $Ghr^{-/-}Ghsr^{-/-}$ mice compared to Wt controls ($P = 0.015$, Table 11) after an overnight fast.

Table 11 Selected blood parameters of Ghr-GHSR mice

	Wt	$Ghr^{-/-}$	$Ghsr^{-/-}$	$Ghr^{-/-}Ghsr^{-/-}$
Leptin [ng/ml]	7.31+/-1.46	5.76+/-1.52; ns	4.71+/-1.14; ns	4.61+/-1.61; ns
Cholesterol [mg/dl]	89.5+/-3.6	81.4+/-2.8; ns	80.1+/-2.7; ns	70.7+/-6.9, p<0.01
Triglycerides [mg/dl]	58.3+/-4.1	60.7+/-2.6; ns	58.2+/-2.7; ns	61.2+/-7.3; ns
FFA [mM]	0.54+/-0.06	0.57+/-0.03; ns	0.54+/-0.03; ns	0.50+/-0.06; ns`
IGF-1 [ng/ml]	440.7+/-18.0	405.6+/-20.5; ns	419.0+/-22.6; ns	391.3+/-44.4; ns
Insulin [ng/ml]	0.57+/-0.18	0.49+/-1.6	0.54+/-2.5	0.42+/-1.5

3.2. Ghrelin-O-acyl transferase physiology studies

While studies with the Ghr-GHSR mice were still ongoing, ghrelin-O acyl- transferase (GOAT) was discovered as the only enzyme to acylate ghrelin (Gutierrez et al., 2008; Yang et al., 2008a). The discovery of GOAT offered the opportunity to better understand ghrelin biology, especially since experiments with the Ghr-GHSR mice still left many questions unanswered. In order to study endogenous regulation of GOAT several studies in wild-type mice were conducted.

3.2.1 *Mboat4* expression was downregulated during fasting

First, GOAT gene (*Mboat4*) expression was measured in wild type mice that were fed *ad libitum* or fasted. Further GOAT activity was indirectly measured in these mice by quantifying blood concentrations of acyl and des-acyl ghrelin with mass spectrometry. Due to ghrelin's nature being the hunger hormone it was hypothesized that acyl ghrelin blood concentrations and GOAT expression are highest after long term fasting. Interestingly, only blood concentrations of des-acyl ghrelin (Fig. 19B) were increased after fasting whereas acyl-ghrelin in blood (Fig. 19A) remained unchanged during food deprivation. Further, GOAT gene expression

was downregulated after fasting (Fig. 19D), which could possibly explain the lack of ghrelin acylation.

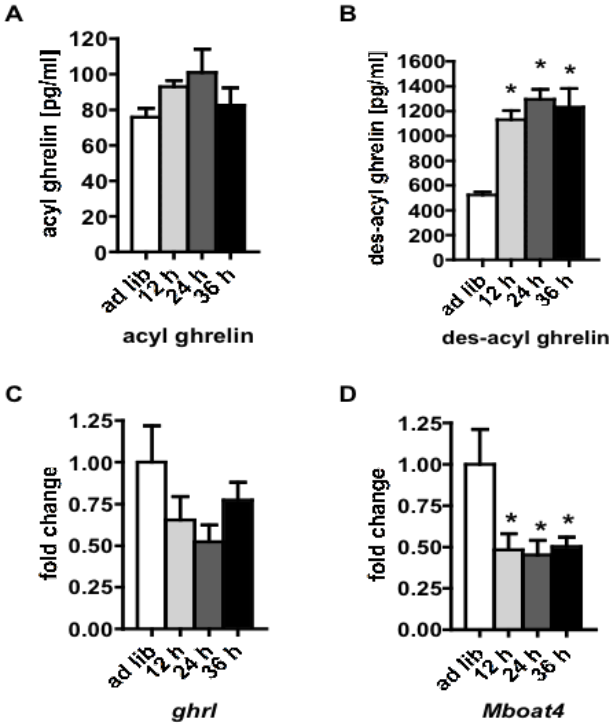

Figure 19 Ghrelin and GOAT response to long term fasting
Seven to eight 3 month old male wild-type mice were fasted for 12, 24 or 36 hours. Another group was fed chow *ad libitum*. After each time point trunk blood and stomach was collected and immediately frozen on dry ice. (A) Blood concentrations of acyl and (B) des-acyl ghrelin were analyzed using Mass Spectrometry. (C) Gastric ghrelin and (D) GOAT mRNA levels were measured using real-time PCR. (B) Only concentrations of des-acyl ghrelin but not (A) acyl ghrelin increased significantly upon long term fasting. (C) Ghrelin (*ghrl*) mRNA levels remained stable whereas (D) GOAT gene (*Mboat4*) expression was significantly downregulated, suggesting that ghrelin production and secretion might be two timely independent processes. Values are means ± SEM. **P*< 0.05

3.2.2 GOAT used dietary fatty acids for ghrelin activation

Concluding from these findings it was hypothesized that GOAT eventually relies on dietary fatty acids to acylate ghrelin. In order to test this hypothesis, mice were fed with a diet enriched in triheptanoate. Triheptanoate is a triglyceride that contains the unnatural fatty acid heptanoate (C7:0). The appearance of C7 acylated ghrelin in blood and stomach tissue would prove that GOAT uses dietary lipids as a substrate for ghrelin acylation. Des-acylated and acylated ghrelin of the triheptanoate and control fed mice was analyzed using mass spectrometry. Mice of both diet had normal levels of des-acyl ghrelin in blood (Fig. 20A) and stomach (Fig. 20B). Also, normal levels of octanoyl-ghrelin could be found in both groups. However, the C7 fed mice had a relatively high amount of ghrelin that was acylated with heptanoyl. The heptanoyl acylation of ghrelin seemed to be to the cost of octanoyl acylation since both groups had the same amount of total ghrelin but the C7 fed mice had significantly less octanoylated ghrelin compared to the control group. This finding confirms that dietary lipids are not only principally used but are also preferred to acylate ghrelin.

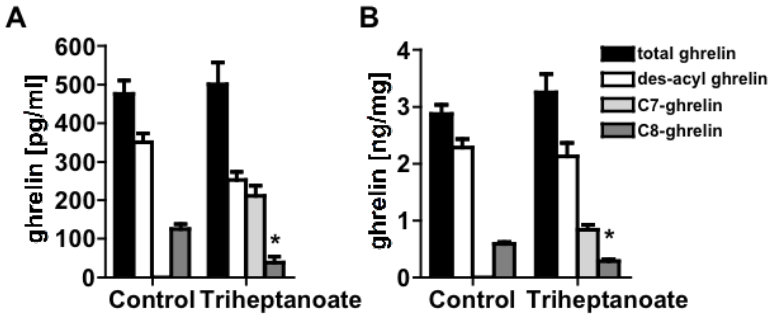

Figure 20 Triheptanoate feeding results in heptanoyl-ghrelin
Eight wild-type mice per group were fed for three days with either chow enriched with triheptanoate (C7:0) or standard chow *ad libitum*. Blood (A) and stomach (B) ghrelin was extracted and ghrelin acyl-side chains analyzed with Mass Spectrometry. A relatively high amount of C7-acylted ghrelin could be found in blood (A) and stomach (B) of the triheptanoate fed mice. Total ghrelin remained unchanged but octanoylated ghrelin was significantly reduced in blood and stomach of the triheptanoate fed mice. Values are means ± SEM. *P< 0.05

3.3 GOAT deficient Mboat4$^{-/-}$ mice

To study if GOAT has a role in regulating energy or glucose homeostasis GOAT knockout mice (Mboat4$^{-/-}$) were developed as previously described (Gutierrez et al., 2008). Mboat4$^{-/-}$ mice have zero levels of acylated ghrelin proving that GOAT is the only enzyme that acylated ghrelin (Fig. 21). Total ghrelin in blood, which is exclusively des-acyl ghrelin in these mice, was higher compared to wild-type littermates.

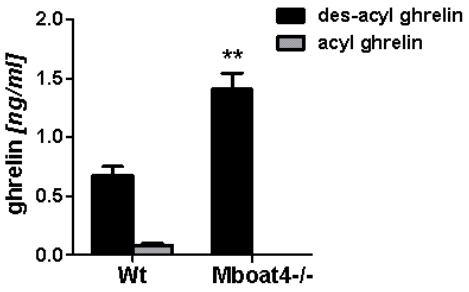

Figure 21 Blood ghrelin profile of Mboat4$^{-/-}$ mice
Mboat4$^{-/-}$ mice had a zero level of acyl ghrelin in blood proving that GOAT is the only enzyme acylating ghrelin. Compared to age-matched Wt littermates Mboat4$^{-/-}$ mice had significantly higher des-acyl ghrelin. Values are means ± SEM. **P< 0.01

Currently, there is no consensus about biological effects of des-acyl ghrelin. Some studies postulate that des-acyl ghrelin has orexigenic effects similar to those of acyl ghrelin and that it has anti-diabetic functions.

Phenotypical differences between the Mboat4$^{-/-}$ and Wt mice are likely caused by the lack of acyl ghrelin, therefore making Mboat4$^{-/-}$ mice an excellent tool to study the effects between non-active des-acyl and active acyl ghrelin. To compare absolute ghrelin deficiency (Ghr$^{-/-}$ mice), lack of ghrelin signaling (Ghsr$^{-/-}$ mice) and the absence of GOAT (Mboat4$^{-/-}$ mice) under the same conditions, Mboat4$^{-/-}$ and Wt littermate mice were studied on chow and HFD. Additionally, a third cohort of Mboat4$^{-/-}$ mice was exposed to medium-chain-triglyceride (MCT) diet.

3.3.1 Mboat4$^{-/-}$ mice showed no changes in body weight on standard chow but on HFD

On chow diet male Mboat4$^{-/-}$ mice had slightly but non-significant lower BW (Fig. 22A left *P*= 0.584) and FM (Fig. 22A middle *P*= 0.513) compared to age matched littermate wild-types. Chow intake was not changed between Mboat4$^{-/-}$ and Wt mice. Similarly, female Mboat4$^{-/-}$ mice had the same BW as Wt mice (Fig 22B left). However, they seemed to have a trend towards higher FM (Fig. 22B middle *P*= 0.301).

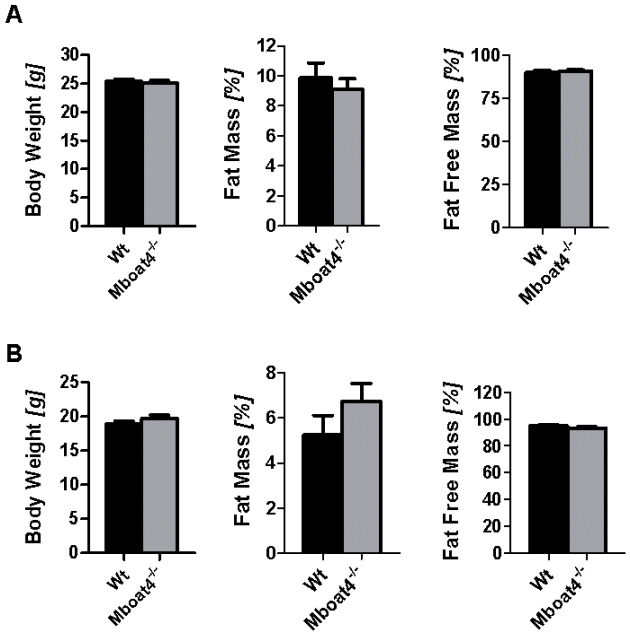

Figure 22 Body composition of chow fed Mboat4$^{-/-}$ mice
Body weight, fat mass and fat free mass of 2 month old (A) male and (B) female Mboat4$^{-/-}$ mice measured by NMR. (A) Chow fed male Mboat4$^{-/-}$ mice showed a trend towards decreased fat mass whereas (B) the females seemed to have a slightly increased fat mass. Values are means ± SEM.

To study if GOAT deficiency ameliorates diet-induced obesity, Mboat4$^{-/-}$ and Wt mice were fed with 58% HFD directly after weaning. After 4 months BW was significantly decreased in male Mboat4$^{-/-}$ mice (*P*= 0.047) but FM and FFM were not changed. To rule out that the decreased BW is due to differences in body seize, body length was measured but did not reveal any differences between groups (Fig. 23A right). Similar to chow feeding, female Mboat4$^{-/-}$ mice had a trend towards increased BW (Fig. 23B left) and FM (Fig. 23B middle-left) after exposure to HFD for four month. Again, there were no changes in body length (Fig. 23B right).

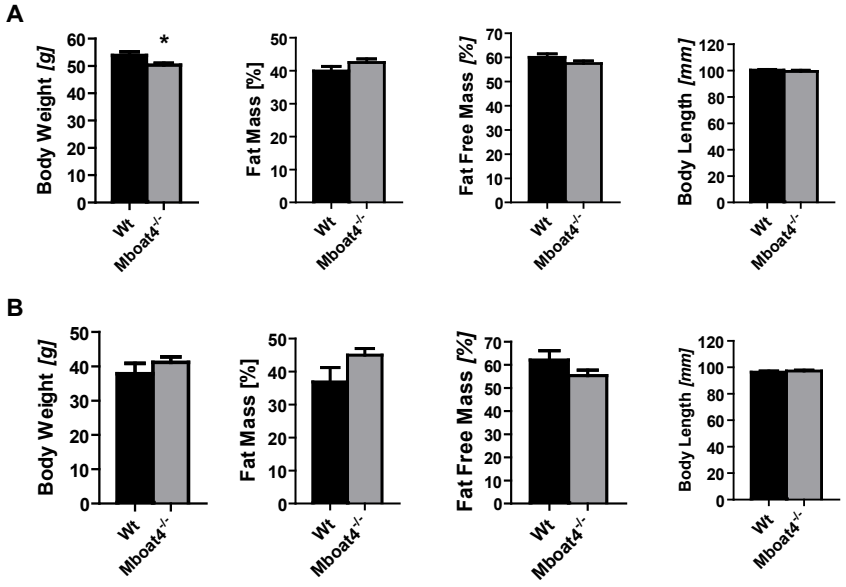

Figure 23 Body weight and body composition of diet-induced obese Mboat4$^{-/-}$ mice
Adult (A) male and (B) female Wt and Mboat4$^{-/-}$ mice fed with 58% HFD for 20 weeks. (A) After 20 weeks male Mboat4$^{-/-}$ mice had a significantly lower body weight than the Wt controls. (B) Female Mboat4$^{-/-}$ mice still showed a trend towards higher BW and FM. Values are means ± SEM. *P< 0.05

3.3.2 Mboat4$^{-/-}$mice had decreased body weight and fat mass on medium-chain-triglyceride diet

Since GOAT directly uses dietary lipids to acylate ghrelin (see above) male Mboat4$^{-/-}$ and Wt mice were fed with a diet enriched with MCT. In theory this diet would have no effect on the Mboat4$^{-/-}$ mice. However, it should enhance ghrelin acylation in Wt mice maximizing the effects between the two genotypes. BW of male Mboat4$^{-/-}$ mice was significantly decreased compared to Wt mice after feeding MCT diet for four weeks (Fig. 24 left, P= 0.016). This difference was mainly due to a decrease in FM (Fig. 24 middle left, P= 0.040) but not in LM, which was significantly greater in Mboat4$^{-/-}$ mice (Fig. 24 middle right, P= 0.040). Interestingly, changes in FI could not explain the difference in BW and FM since FI was increased in Mboat4$^{-/-}$ mice (Fig. 24 right, P= 0.021).

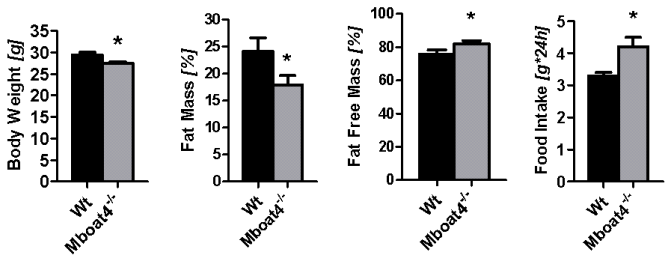

Figure 24 Body weight, body composition and food intake of Mboat4$^{-/-}$ mice on MCT diet
Three month old male Mboat4$^{-/-}$ mice that have been fed with a medium-chain triglyceride (MCT) enriched diet for 6 weeks had significantly lower body weight and fat mass than Wt littermates. The lean mass and food intake was significantly increased in Mboat4$^{-/-}$ mice. Values are means ± SEM. *P< 0.05

3.3.3 Energy expenditure was increased in Mboat4$^{-/-}$ mice on MCT diet

Mboat4$^{-/-}$ mice had lower BW on HFD and lower BW and FM on MCT diet. However, Mboat4$^{-/-}$ mice were not hypophagic, which could have explained the difference in BW and FM. As a matter of fact, FI was increased in Mboat4$^{-/-}$ mice on MCT. In order to find a possible explanation for the leaner phenotype of Mboat4$^{-/-}$ mice energy expenditure and locomotor activity was measured using indirect calorimetry. Energy expenditure normalized to BW tended to be higher in male Mboat4$^{-/-}$ mice on chow (P= 0.255) and HFD (P= 0.51) but this increase did not reach statistical significance. On MCT diet however, Mboat4$^{-/-}$ mice showed a significantly increase in EE during the light phase of the photoperiod (Fig. 25A; P= 0.022) and a strong trend towards increased total EE. Fuel preference, indicated by the respiratory quotient, and total locomotor activity was not changed between the genotypes on during MCT feeding (Fig. 25B and C).

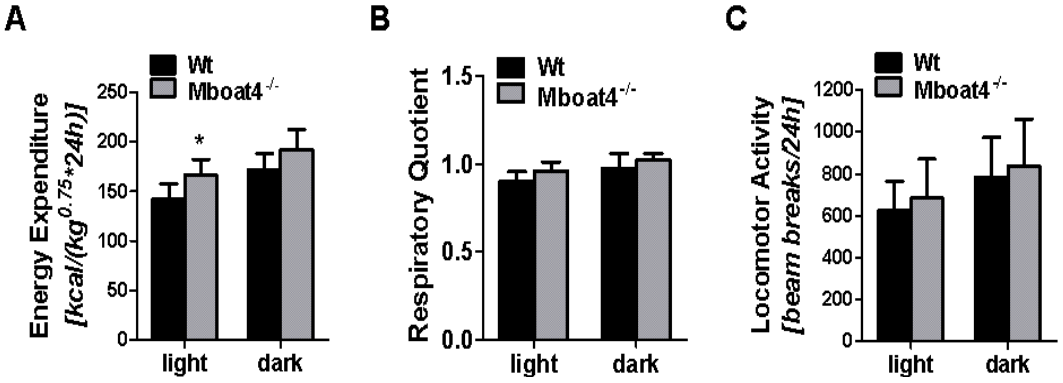

Figure 25 Energy expenditure, fuel preference and total activity of Mboat4$^{-/-}$ mice on MCT diet
Energy expenditure, respiratory quotient and locomotor activity of MCT diet fed male mice was measured simultaneously in a system that combines indirect calorimetry and infrared beam brake counts. Energy expenditure was normalized per body weight to the potency of 0.75 to control for differences in body weight and fat mass between the genotypes. (A) Energy expenditure of Mboat4$^{-/-}$ mice fed MCT diet is significantly lower during the light photoperiod. (B) Respiratory quotient was comparable between Mboat4$^{-/-}$ and Wt mice. (C) Total locomotor activity measured by infrared beam breaks tended to be increased in Mboat4$^{-/-}$ mice on MCT diet. All mice were 3 month old and n=5 per group. Values are means ± SEM. *P< 0.05

3.3.4 Absence of acyl ghrelin did not change glucose homeostasis

Male Mboat4$^{-/-}$ and Wt mice were subjected to GTTs *i.p.* on chow (Fig. 26A), HFD (Fig. 26B) and MCT (Fig. 26C) diet to study if selective deletion of acyl ghrelin has an effect on glucose tolerance. Mice were fasted for 6 hrs and subsequently challenged with 2g glucose/kgBW. Generally, glucose tolerance was comparable between genotypes on either diet. The HFD group had a slower glucose clearance than the chow and MCT diet group, which was expected and could be explained by the diabetogenic effects of the diet. However, absence of acyl ghrelin did not seem to rescue the diet induced glucose intolerance.

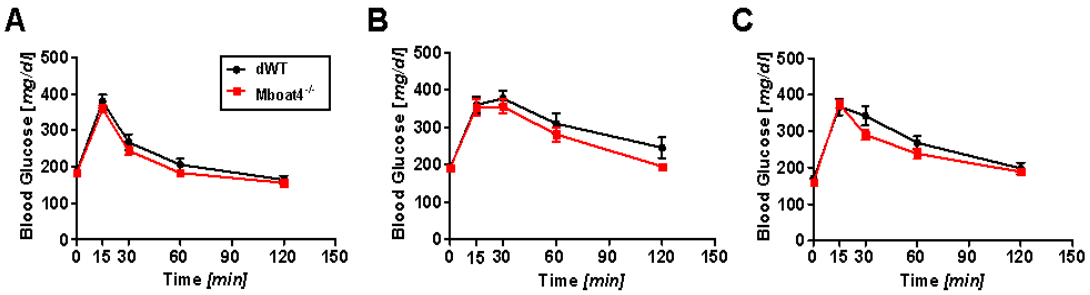

Figure 26 Glucose tolerance test in male Mboat4$^{-/-}$ mice on different diets
Glucose tolerance tests with intra peritoneal doses of 2g glucose/kg body weight were conducted in Wt and Mboat4$^{-/-}$ mice under different diet conditions. (A) Standard chow fed 2 month old Mboat4$^{-/-}$ mice (n= 13) had a comparable glucose tolerance to Wt littermates (n= 10). (B) Glucose tolerance was impaired to a similar level in both, 6 month old Mboat4$^{-/-}$ (n= 7) and Wt (n= 8) mice after exposure to HFD for 2 month. (C) During MCT feeding Mboat4$^{-/-}$ mice (n= 13) developed a trend towards slightly improved glucose tolerance compared to Wt controls (n= 10). Values are means ± SEM

3.3.5 Mboat4$^{-/-}$ mice had decreased markers of inflammation

Tail blood from 3 month old Mboat4$^{-/-}$ and Wt mice fed MCT diet ad lib was drawn to measure blood parameters that are known to be associated with energy and glucose homeostasis. Further, the complete spectrum of lipoproteins was analyzed using Mass Spectrometry (Fig. 27). Obesity is associated with increased production of inflammatory cytokines. These cytokines can be measured in blood as marker of inflammation. The cytokines Interleukin-6 (IL-6) and Interleukin-10 (IL-10) were significantly decreased in Mboat4$^{-/-}$ mice (Table 12) compared to Wt littermates on MCT diet. Further, peptide tyrosine tyrosine (PYY), a polypeptide produced in the intestines, was decreased in the Mboat4$^{-/-}$ mice.

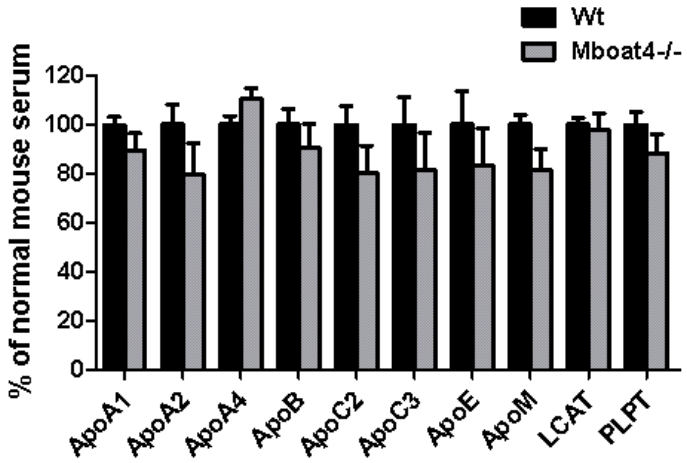

Figure 27 Lipoprotein profile of MCT diet fed Mboat4$^{-/-}$ mice
Mice were fed MCT diet ad lib and tail blood was collected in to EDTA coated tubes on ice. Lipoproteins were extracted from plasma and analyzed with mass spectrometry. Generally, lipoproteins tended to be lower in Mboat4$^{-/-}$ mice compared to Wt controls.

Table 12 Blood profile of 3 month old Mboat4$^{-/-}$ and Wt mice on MCT diet

Variable	Unit	Mboat4$^{-/-}$		SEM	Wt		SEM	p-value
ACE (Angiotensin-converting enzyme)	ng/ml	330.33	+/-	6.66	339.4	+/-	8.14	0.393
ACTH (Adrenocorticotropic Hormone)	pg/ml	102.23	+/-	5.30	121.3	+/-	11.46	0.115
Adiponectin	ng/ml	61.28	+/-	8.75	51.8	+/-	9.30	0.472
C3a des Arg	**ng/ml**	**486.67**	**+/-**	**22.50**	**389.9**	**+/-**	**22.75**	**0.006**
Cortisol	ng/ml	195.47	+/-	12.44	203.0	+/-	12.85	0.680
Galanin	pg/ml	161.57	+/-	14.88	258.9	+/-	51.71	0.059
GH (Growth Hormone)	pg/ml	3070.06	+/-	418.23	3744.8	+/-	642.28	0.365
GLP-1 total (Glucagon-like peptide 1)	pg/ml	127.75	+/-	12.04	152.7	+/-	13.59	0.180
Glucagon	pg/ml	86.96	+/-	8.57	85.5	+/-	10.48	0.912
GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor)	pg/ml	1.99	+/-	0.12	2.5	+/-	0.28	0.082
IGF-1 (Insulin-like Growth Factor)	ng/ml	67.36	+/-	9.92	59.6	+/-	11.34	0.608
IL-10 (Interleukin-10)	**pg/ml**	**1024.00**	**+/-**	**62.78**	**1213.3**	**+/-**	**65.13**	**0.047**
IL-11 (Interleukin-11)	pg/ml	507.67	+/-	57.52	632.5	+/-	74.77	0.188
IL-1b (Interleukin-1 beta)	**pg/ml**	**1935.33**	**+/-**	**165.57**	**2550.0**	**+/-**	**198.08**	**0.023**
IL-6 (Interleukin-6)	**pg/ml**	**5.09**	**+/-**	**0.35**	**7.9**	**+/-**	**0.78**	**0.001**
Insulin	u I U / ml	19.55	+/-	4.19	23.6	+/-	3.56	0.486
Leptin	ng/ml	8.61	+/-	1.16	10.3	+/-	1.30	0.327
LH (Luteinizing Hormone)	pg/ml	41.87	+/-	11.51	64.8	+/-	12.87	0.197
Lymphotactin	pg/ml	520.94	+/-	34.58	622.4	+/-	44.55	0.077
MCP-1 (Monocyte Chemoattractant Protein-1)	**pg/ml**	**116.32**	**+/-**	**5.79**	**145.8**	**+/-**	**7.33**	**0.003**
M-CSF (Macrophage-Colony Stimulating Factor)	ng/ml	13.13	+/-	1.41	16.3	+/-	1.52	0.141
Progesterone	ng/ml	31.62	+/-	11.54	67.1	+/-	29.05	0.267
Prolactin	ng/ml	33.76	+/-	0.96	37.0	+/-	2.14	0.144
PYY (Peptide tyrosine tyrosine)	**pg/ml**	**101.62**	**+/-**	**6.53**	**122.9**	**+/-**	**6.20**	**0.028**
RANTES	pg/ml	1.23	+/-	0.09	1.5	+/-	0.09	0.064
Resistin	ng/ml	0.06	+/-	0.05	0.0	+/-	0.03	0.694
Secretin	pg/ml	418.12	+/-	36.97	660.1	+/-	130.61	0.063
Testosterone	ng/ml	5.15	+/-	1.54	5.9	+/-	1.90	0.758
TNF-alpha	ng/ml	1.02	+/-	0.07	1.2	+/-	0.08	0.154

Bolded parameters are $P< 0.05$

3.4 Ghrelin and GOAT overexpressing transgenic mice

In another approach to uncover physiological GOAT function, transgenic (Tg) mice were created to express the human genes for ghrelin and GOAT in liver under control of the ApoE promoter. Multiple genetic lines were generated that stably carried and expressed both genes (data not shown) and one line was selected for additional studies.

3.4.1 The transgenic model was diet dependable

As predicted the Tg mice had high circulating levels of the 1-28 and 1-30 splice variants of human des-acyl and acetyl (C2) ghrelin. Despite this high amount of human des-acyl ghrelin production, Tg mice did not produce human octanoyl-ghrelin. Concluding from the earlier findings that ghrelin acylation is highly influenced by dietary lipids (see above), it was suggested that under normal dietary conditions there are not sufficient MCFAs in liver to acylate ghrelin. When fed with MCT diet, Tg mice finally started to produce high amounts of octanoyl-ghrelin. This shows clearly that the transgenic model is dependent on dietary availability of MCFA (Fig. 28).

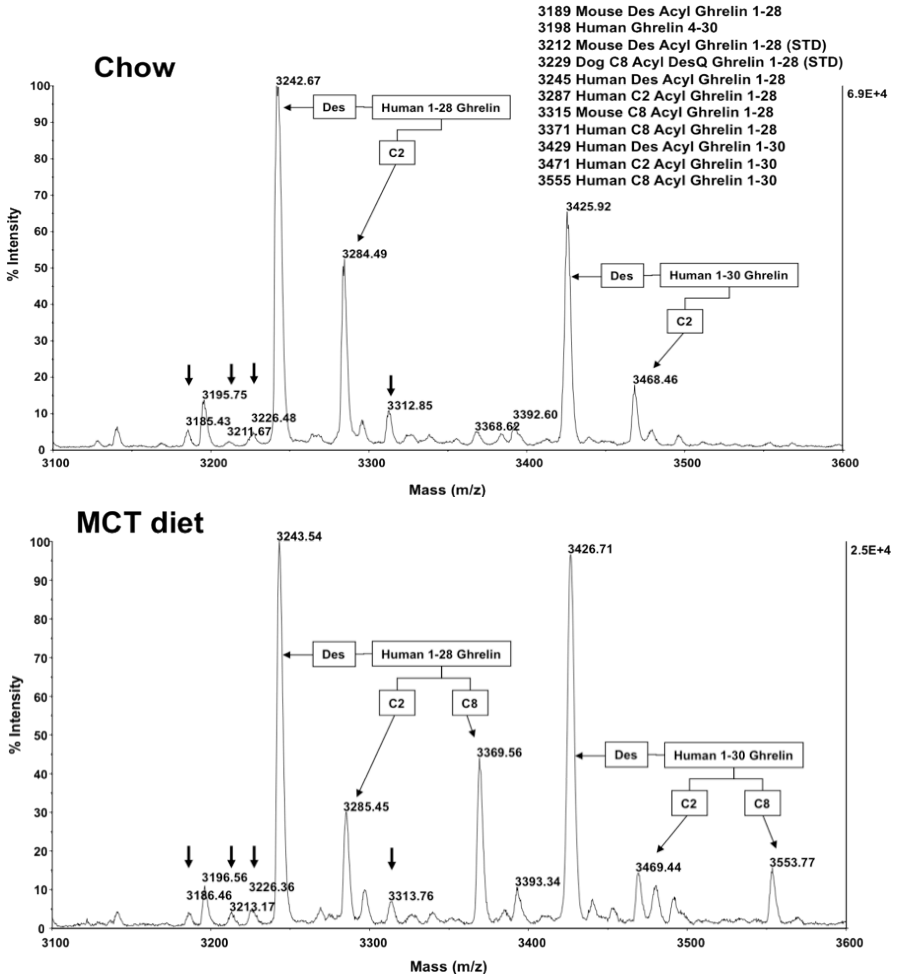

Figure 28 Ghrelin isoforms in blood of Tg mice on different diets

Ghrelin immunoprecipitation MALDI-ToF MS analyses of blood from Tg mice fed either (A) regular chow or (B) MCT diet. Downward arrows denote endogenous mouse des acyl (m/z3189) or octanoyl modified (m/z 3315) ghrelin forms or the standard peptides (m/z 3212, 3229) used in these studies. Peaks at m/z 3245, 3287, and 33711 represent human des acyl-, acetyl-, and octanoyl-modified forms of ghrelin 1-28. Peaks at m/z 3429, 3471, and 3555 correspond to human des acyl-, acetyl-, and octanoyl-modified forms of ghrelin 1-30. (B) Only Tg mice fed MCT diet produce high concentrations of octanoyl (C8) ghrelin whereas the same mice fed chow diet (A) have only des-acyl and acetyl (C2) modified ghrelin forms in circulation.

3.4.2 Transgenic mice had increased adiposity on MCT diet

Under chow feeding conditions male Tg mice had normal blood concentrations of acyl ghrelin (Fig. 28A) and showed no differences in body weight and body composition compared to Wt littermates (data not shown). When fed MCT diet Tg mice started producing human octanoyl ghrelin (Fig. 28B and 29B) leading to 40 fold increased blood concentrations of human acyl ghrelin compared to MCT diet fed Wt mice (Fig. 29B right).

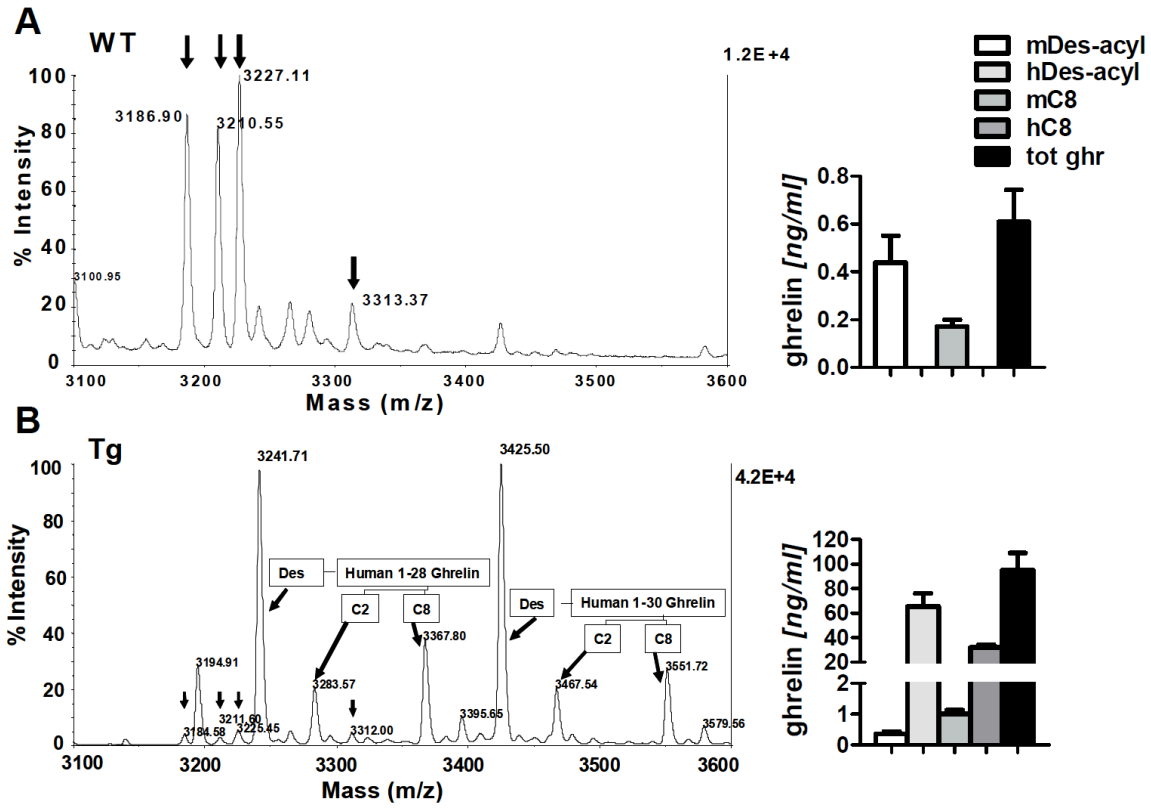

Figure 29 Ghrelin derivates in blood of Tg and Wt mice fed MCT diet
Ghrelin modification was measured with MALDI-ToF MS in blood of (A) 8 Wt and (B) 8 Tg mice that were fed with MCT diet for at least 4 weeks. Downward arrows denote endogenous mouse des acyl (m/z 3,189) or octanoyl-modified (m/z 3,315) ghrelin forms or the standard peptides (m/z 3,212, 3,229) used in these studies. Peaks at m/z 3,245, 3,287 and 3,371 represent human des-acyl–, acetyl- and octanoyl-modified forms of ghrelin 1–28. Peaks at m/z 3,429, 3,471 and 3,555 correspond to human des–acyl–, acetyl- and octanoyl-modified forms of ghrelin 1–30. Bar graphs represent mean ghrelin concentrations of (A) Wt and (B) transgenic mice fed MCT diet. m, mouse; h, human; Des-acyl, des-acyl ghrelin; C8, octanoyl ghrelin; tot ghr, total ghrelin. Values are means ± SEM.

After four weeks on MCT diet, male Tg mice developed higher BW than Wt littermates (P= 0.0158). Analysis of body composition revealed that this increase in BW was most likely due to an increase in FM, which was significantly increased in the Tg mice compared to littermate controls (P= 0.0478) (Fig. 30).

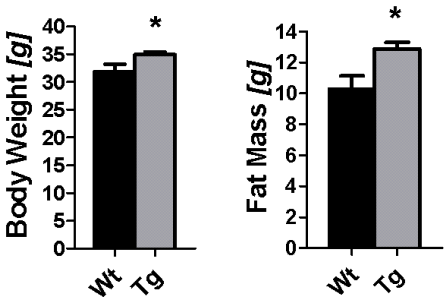

Figure 30 Body weight and fat mass of ghrelin and GOAT overproducing transgenic mice
Body weight and fat mass of three month old Tg mice (n=8) is significantly reduced compared to Wt littermates (n=8) when fed MCT diet. Values are means ± SEM.

3.4.3 Transgenic mice had decreased energy expenditure

GOAT deficiency led to increased EE in Mboat4$^{-/-}$ mice. To study whether simultaneous ghrelin and GOAT overproduction influences EE as well, Tg mice were placed in the indirect calorimetric system where EE and automated FI were measured simultaneously. As expected and opposite to the phenotype of Mboat4$^{-/-}$ mice, Tg mice exhibited decreased EE during the light and dark phase (Fig. 31A; P= 0.0068). Respiratory quotient tended to be slightly increased pointing towards lesser fat oxidation in Tg mice during the light phase. However, the difference was not statistically significant. FI was not changed between Tg and Wt mice (Fig. 31C). Locomotor activity (data not shown) was further not different from Wt mice.

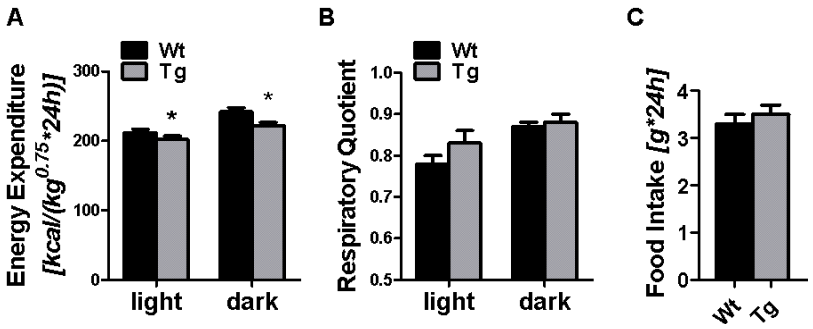

Figure 31 Energy expenditure and food intake of transgenic mice
Energy expenditure, respiratory quotient and automated food intake were measured simultaneously during the light and dark phase in 8 Tg and 8 Wt mice that were fed with MCT diet. (A) Energy expenditure was significantly increased in Tg mice compared to Wt littermates during the light and dark phases. (B) Respiratory quotient and (C) food intake were similar to Wt mice. Values are means ± SEM. *P< 0.05

3.4.4 Genes involved in respiratory function were downregulated in Tg mice

To identify possible downstream mechanisms responsible for the decreased EE observed in Tg mice, gene expression of candidate genes was measured with quantitative real-time PCR in muscle (quadriceps), liver and brown adipose tissue of MCT diet fed male Tg and Wt mice.

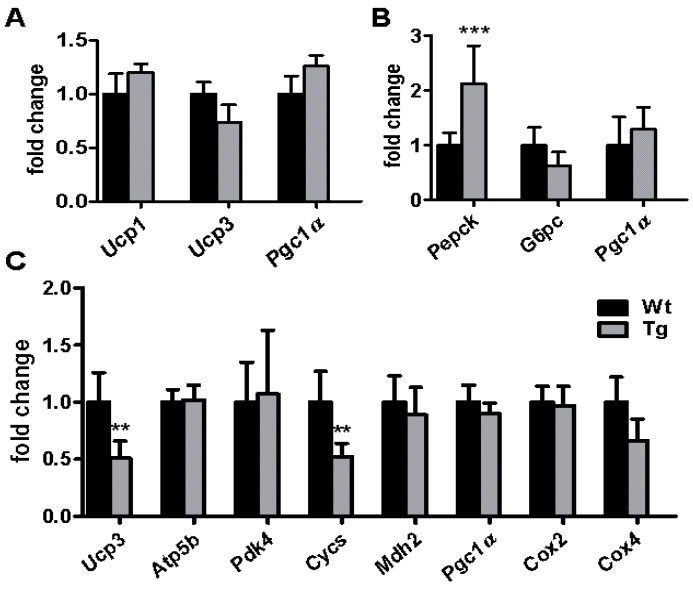

Figure 32 Gene expression profiling in Tg mice fed MCT diet
Gene expression of candidate genes was measured with real-time PCR in brown adipose tissue, liver and quadriceps of 8 Tg and 8 Wt mice after exposure to MCT diet for 6 weeks. (A) Gene expression of *Ucp1* (uncoupling protein 1), *Ucp3* (uncoupling protein 3) and *Pgc1*α (peroxisome proliferator-activated receptor-g coactivator 1 α) in brown adipose tissue was not changed between Wt and Tg mice. (B) *Pepck* mRNA was two fold upregulated in liver tissue of Tg mice. (C) Gene expression of genes involved in regulation of energy homeostasis in quadriceps of Wt and Tg mice. *Ucp3* and *Cycs* expression was significantly decreased in Tg mice.Values are means ± SEM. ***P*< 0.01; ****P*< 0.001

In brown adipose tissue mRNA levels of the genes encoding uncoupling protein 1 and 3 (*Ucp1, Ucp3*) and peroxisome proliferative activated receptor gamma coactivator 1 (*Pgc1*α) were not changed (Fig. 32A). Phosphoenolpyruvate carboxykinase (*Pepck*), a key gene involved in gluconeogenesis, was upregulated 2-fold in liver tissue of Tg mice (Fig. 32B; *P*= 0.0006) compared to Wt mice. Gene expression of *G6pc* and *Pgc1*α however was unchanged in liver. Expression of genes encoding the proteins UCP-3 and Cytochrome C, which are both important for mitochondrial respiratory chain function, was significantly reduced (both *P*= 0.001) in quadriceps of Tg mice compared to Wt mice (Fig. 32C).

3.5 GOAT and leptin deficient Mboat4$^{-/-}$-ob/ob mice

Ghrelin's influence on glucose homeostasis is still highly controversial. Recent studies suggest that des-acyl ghrelin has beneficial effects in type 1 and 2 diabetes (Granata et al., 2007). Taking into consideration the results from the Ghr-GHSR double knockout mouse studies (see above) it is possible that ghrelin has a beta-cell protective effect. The GOAT deficient mouse offers an elegant tool to differentiate between the functions of bioactive acyl ghrelin and the non-active des-acyl ghrelin since Mboat4$^{-/-}$ mice produce relatively high amounts of des-acyl but zero acyl ghrelin. To better understand the association between the ghrelin/GOAT system and glucose homeostasis GOAT deficiency was studied on a type 2 diabetic background using leptin deficient ob/ob mice.

3.5.1 Mboat4$^{-/-}$-ob/ob mice tended to have decreased body weight on MCT diet

GOAT function influences energy homeostasis and body fat mass as shown above. In order to

test whether loss of GOAT function has the same effect in an obese and diabetic background, GOAT was deleted in leptin deficient ob/ob mice to create the double-knockout Mboat4$^{-/-}$-ob/ob mouse. When fed standard chow there were no differences in BW, FI or body composition between 2 month old male or female Mboat4$^{-/-}$-ob/ob and ob/ob littermate mice (supplemental data). To compare results from Mboat4$^{-/-}$-ob/ob mice to the other mouse models of altered GOAT function (see above), a new generation of Mboat4$^{-/-}$-ob/ob and ob/ob mice was generated and fed with MCT diet early after weaning. At this time point Mboat4$^{-/-}$-ob/ob and ob/ob mice had the same body weight. However, over time Mboat4$^{-/-}$-ob/ob mice seemed to gain BW more slowly than the ob/ob littermates. Although the difference did not reach the level of statistical significance at the end of the observation period, Mboat4$^{-/-}$-ob/ob mice developed a persistent trend towards decreased body weight (Fig. 33A).

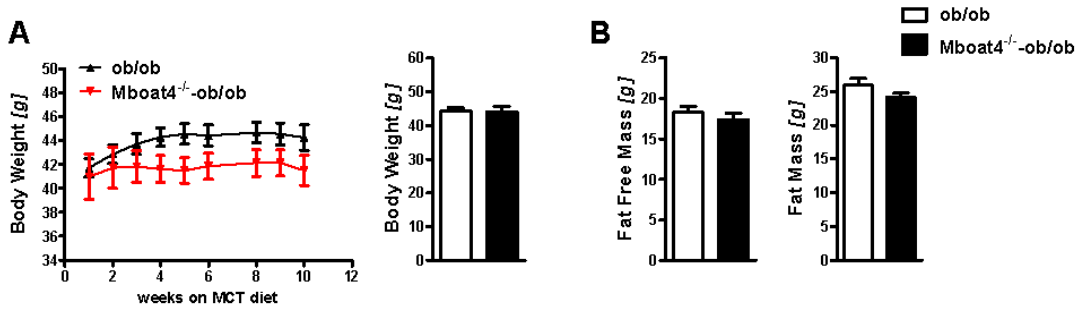

Figure 33 Body weight and body composition of Mboat4$^{-/-}$-ob/ob mice
Seven ob/ob and 8 Mboat4$^{-/-}$-ob/ob mice were fed with MCT diet starting at an age of 4 weeks. Body weight and body composition was monitored over a period of 10 weeks. (A) Mboat4$^{-/-}$-ob/ob mice seem to grow more slowly than ob/ob mice resulting in a trend towards decreased BW, which was nevertheless not significant after feeding with MCT diet for 10 weeks (A right). (B) Fat free and fat mass tended to be decreased in Mboat4$^{-/-}$-ob/ob mice after 10 weeks. Values are means ± SEM.

ob/ob mice are hyperphagic and are therefore known to gain body weight very quickly. In our experiments neither ob/ob nor Mboat4$^{-/-}$-ob/ob mice gained weight substantially over the observation period of 10 weeks. This effect was most likely due to the chronic exposure to the MCT diet. In earlier Wt mouse studies we experienced that mice gain only little weight while they are exposed to MCT diet.

3.5.2 Mboat4$^{-/-}$-ob/ob mice showed tendency towards increased locomotor activity

Six weeks after begin of MCT feeding, male Mboat4$^{-/-}$-ob/ob and ob/ob mice were placed in the indirect calorimetric system. Automated food intake was not changed between genotypes (Fig. 34A). EE followed the expected circadian rhythm and was similar in both groups (Fig. 34B). RQ tended to be decreased in Mboat4$^{-/-}$-ob/ob mice during the light and dark phase indicating the possibility of higher fat oxidation in Mboat4$^{-/-}$-ob/ob mice (Fig. 34C). Interestingly, locomotor activity showed a strong trend towards being increased in Mboat4$^{-/-}$-ob/ob mice (Fig. 34D). Nevertheless, this difference was not significant possibly due to small groups size and high variance between the animals.

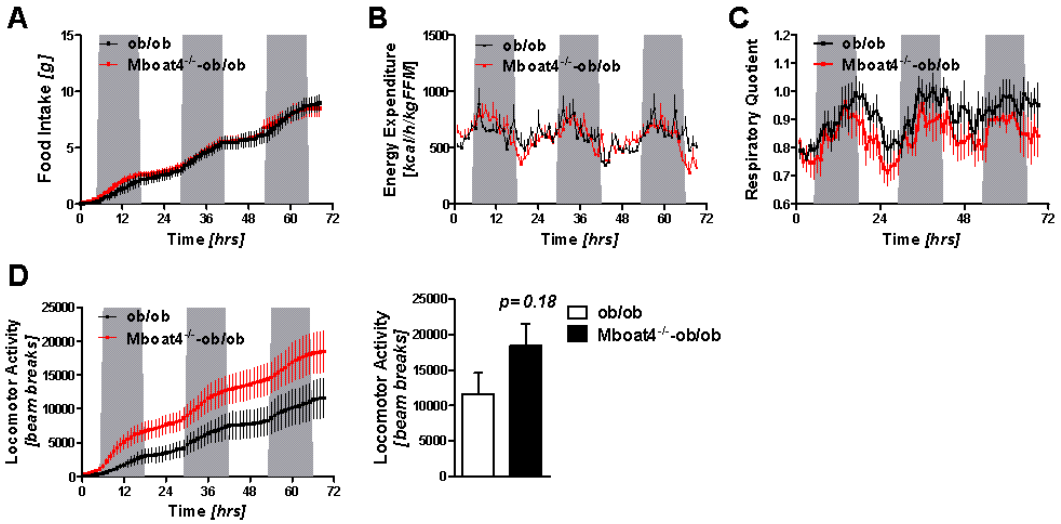

Figure 34 Indirect calorimetry and locomotor activity of Mboat4$^{-/-}$-ob/ob mice
Automated food intake, EE, RQ and homecage activity was measured in 4 ob/ob and 6 Mboat4$^{-/-}$-ob/ob male 7 weeks old mice. (A) Food intake and (B) EE was not changed between groups. (C) RQ tended to be decreased in Mboat4$^{-/-}$-ob/ob mice. (D) Mboat4$^{-/-}$-ob/ob mice tended a strong trend towards increased locomotor activity. Values are means ± SEM.

3.5.3 Deletion of GOAT did not rescue the diabetic phenotype of ob/ob mice

Two month old Mboat4$^{-/-}$-ob/ob and ob/ob mice of both genders were subjected to an ITT using 1U insulin/kgBW *i.p.* to study insulin sensitivity (Fig. 35). Fasting blood glucose was normal in both groups of male and female mice before insulin injection. However, female Mboat4$^{-/-}$-ob/ob seemed to have slightly higher fasting glucose levels compared to female ob/ob littermates (Fig. 35B). Despite insulin injection, blood glucose continued increasing in male and female mice of both genotypes reaching its highest point 15 min after insulin injection. This phenomenon was most likely due to the high stress level of the animals. After 15 min blood glucose dropped in male and female Mboat4$^{-/-}$-ob/ob and ob/ob mice to a similar extend. Overall, insulin sensitivity was comparable between genotypes and genders throughout the entire study period of 120 min (Fig. 35).

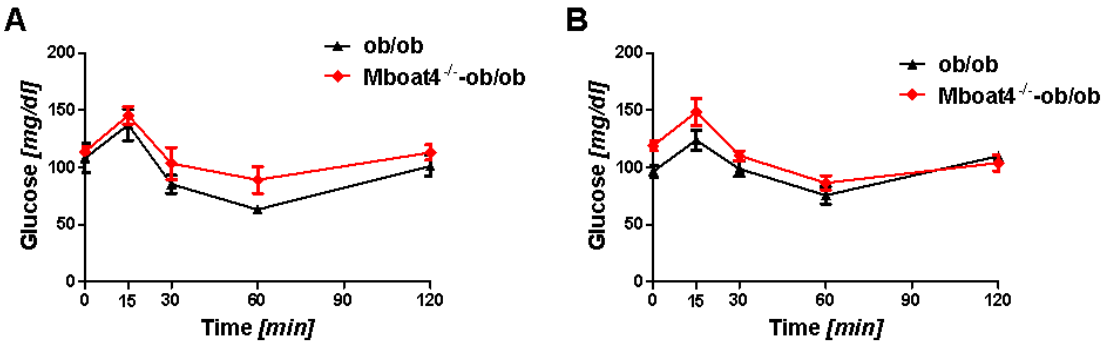

Figure 35 Insulin tolerance test in Mboat4$^{-/-}$-ob/ob mice
Male and female Mboat4$^{-/-}$-ob/ob mice were subjected to an intraperitoneal insulin tolerance test after feeding with MCT diet for almost 3 month. Mice were fasted for 6 hrs before the test. (A) Blood glucose of male ob/ob (n=6) and Mboat4$^{-/-}$-ob/ob (n=10) mice after insulin injection was similar in both genotypes throughout the observation period. (B) Blood glucose of female ob/ob (n=5) and Mboat4$^{-/-}$-ob/ob (n=9) mice was also comparable between genotypes after insulin injection. Values are means ± SEM.

4. Discussion

The development of mutant mouse models with gain- or loss-of-function provides a key tool to study the physiological roles of proteins such as ghrelin and GOAT. Simultaneous deficiency for ghrelin and the ghrelin receptor in mice leads to changed energy balance and glucose tolerance, which is not observed in mice deficient for either the ligand or the receptor alone. Importantly, many significant data presented here result from studies of $Ghr^{-/-}Ghsr^{-/-}$ mice maintained on standard chow diet, whereas previously reported energy balance or metabolism phenotypes from mouse ghrelin or GHSR deficient mice require chronic exposure to high-fat diet (Wortley et al., 2005; Zigman et al., 2005). Further, it could be shown that the GOAT/ghrelin system is inactive during fasting, since dietary lipids serve as GOAT substrate. Genetic modulation of GOAT function in mice demonstrates that the GOAT/ghrelin system is an endogenous regulator of body weight and body fat. Therefore, GOAT could be a valuable peripheral drug target.

4.1 $Ghr^{-/-}GHSR^{-/-}$ mice have a stronger phenotype than the single knockout $Ghr^{-/-}$ and $GHSR^{-/-}$ mice

We observed significant differences in energy expenditure, locomotor activity, body weight, body fat, and body length between double knockout $Ghr^{-/-}GHSR^{-/-}$ mice and Wt but not between single knockout and Wt mice. Further, most described differences between $Ghr^{-/-}$ $GHSR^{-/-}$ and Wt mice do not rely on metabolically challenging early-onset exposure to HFD and are statistically significant during standard chow feeding. For most parameters studied, ghrelin deficiency has less impact on energy metabolism components than GHSR deficiency. These findings are consistent with the functional relevance of constitutive GHSR activity (Holst et al., 2003). Deletion of ghrelin terminates the ligand-induced receptor signaling. Consequently ghrelin deficiency interrupts only direct ghrelin signaling but not the ghrelin-independent constitutive GHSR signaling (Fig. 36A). Indeed, deletion of GHSR silences GHSR signaling more efficiently as the $Ghr^{-/-}$ and $GHSR^{-/-}$ mouse data show (Fig. 36C). Nevertheless, simultaneous deletion of ghrelin and GHSR is even more efficient than GHSR ablation alone and only this simultaneous deficiency for both genes leads to significant differences compared to wild-type control mice independent of the diet. A possible explanation for these results is the putative existence of other ghrelin system components, such as an additional ghrelin receptor or other ghrelin-like GHSR ligands encoded by genes other than Ghr and Ghsr. Mice deficient for both, ghrelin and GHSR, are thus a useful tool to investigate this hypothesis.

The current understanding of the ghrelin-GHSR system, with only one ghrelin receptor and only one receptor ligand, is shown in the center of figure 36. Since ghrelin deficiency results only in a mild phenotype that cannot be purely explained by the effects of constitutive GHSR signaling, the existence of another GHSR ligand can be suggested (Fig. 36B). Similarly, GHSR deletion does not lead to the complete absence of ghrelin-induced metabolic effects (Fig. 36C), which suggests the existence of another ghrelin receptor (Fig. 36D). We therefore pro-

pose a new Ghrelin-GHSR model that includes a putative other ghrelin receptor and GHSR ligand (Fig. 36E). As a result, deletion of ghrelin (Fig. 36F) or GHSR (Fig. 36G) has no metabolic consequences since ghrelin and GHSR signaling would still exist. Only simultaneous deletion of ghrelin and GHSR are sufficient to interrupt the metabolic effects of ghrelin-GHSR signaling (Fig. 36H). However, it should be noted that the proposed model only works, if the putative other ligand is not able to bind to the putative other receptor.

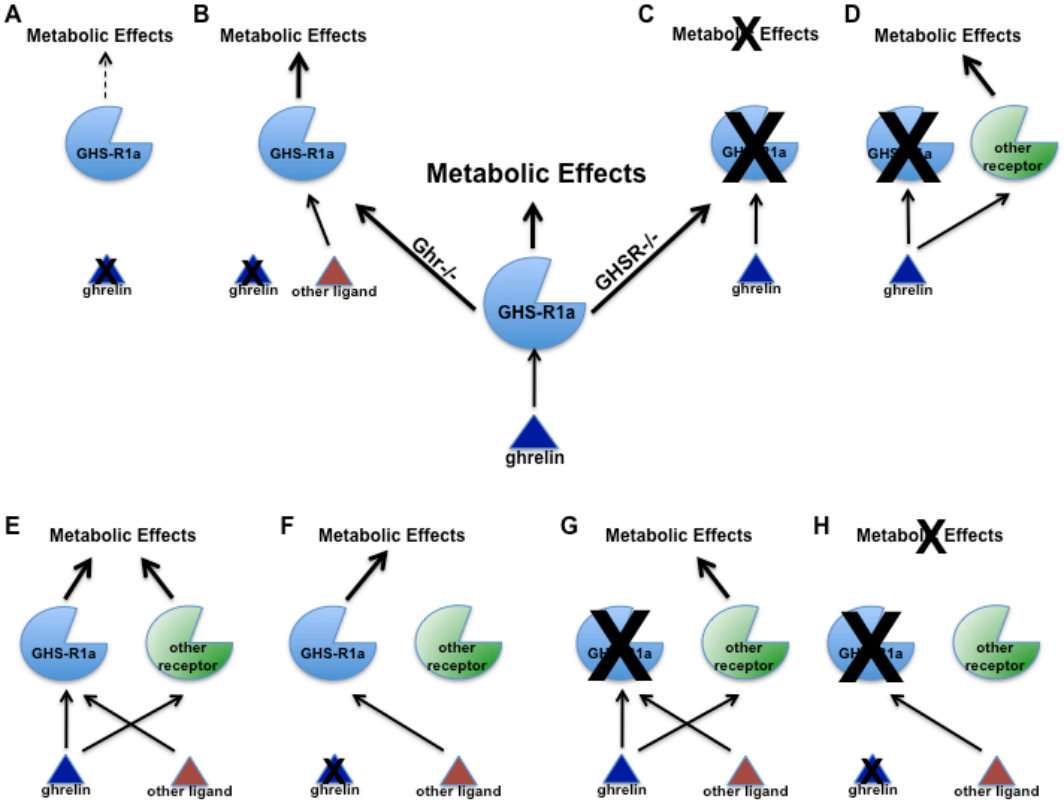

Figure 36 Scheme of the ghrelin-GHSR system and possible other ligands and receptors
The currently accepted ghrelin-GHSR system is represented in the center. (A) Deletion of ghrelin or (C) GHSR would therefore significantly interfere with the metabolic effects of ghrelin. Since this assumption cannot be completely verified in knockout mouse models a putative other ligand (B) and receptor (D) might exist. This assumption leads to a new ligand-receptor model (E) that includes another ligand as well another receptor. The new model could explain the mild phenotype of (F) ghrelin and (G) GHSR deficient mice. (H) Only simultaneous deletion of ghrelin and GHSR leads to interruption of ghrelin/GHSR induced metabolic effects.

Additionally to the new ligand and receptor theory an additive "gene-dosage"-like effect, potentiating the outcome of single gene knockout to a more powerful genotype of the double knockout, might exist. An example for such an effect is loss of NPY in ob/ob mice (Erickson et al., 1996). In normal mice loss of NPY is compensated and does not lead to hypophagia or starvation. These compensatory mechanisms however are insufficient in ob/ob mice, which results in a leaner and less diabetic phenotype of $NPY^{-/-}$ob/ob compared to ob/ob mice (Erickson et al., 1996). The findings of the $NPY^{-/-}$ob/ob mouse might indicate that deletion of a single gene can be compensated for whereas deletion of another related gene results in a more profound phenotype. Extrapolated to our double knockout model this interpretation could mean that loss of ghrelin or GHSR alone has only minor effects on metabolism whereas the simultaneous deletion of both adds up to a condition that cannot be easily compensated

for. Nevertheless, it should be noted that disruption of both genes results in a significant but rather mild phenotype compared to Wt mice. Like NPY$^{-/-}$ob/ob mice, which have amelioration but not complete reversal of the obese phenotype, Ghr$^{-/-}$GHSR$^{-/-}$ mice show an improved regulation of energy homeostasis but are not exceptionally lean or entirely protected against diet-induced obesity. Possible involvement of other neuromodulators in the response to ghrelin and GHSR deletion might be responsible for the mild phenotype of Ghr$^{-/-}$GHSR$^{-/-}$ mice.

4.2 Genetic modulation of the GOAT/ghrelin and the ghrelin/GHSR systems in mice changes energy homeostasis

In summary, we conclude that ghrelin has a physiological role in the regulation of energy expenditure and body weight under unchallenged environmental conditions. Mice simultaneously deficient for ghrelin and its receptor have significantly lower body weight and fat mass than single knockout or wild-type littermates. Consequently, mice lacking GOAT are lighter and leaner whereas transgenic mice overproducing ghrelin and GOAT have higher body weight and fat mass than Wt controls. The leaner phenotype of Ghr$^{-/-}$GHSR$^{-/-}$ mice is observed on standard chow and HFD whereas the phenotype of the Mboat4$^{-/-}$ mice only develops on HFD and MCT diet. Transgenic mice require MCT diet feeding to switch on the GOAT/ghrelin system and to develop adiposity.

The described changes in body weight and fat mass might be explained by the observed changes in energy expenditure, body core temperature and locomotor activity. These changes possibly lead to negative energy balance in Ghr$^{-/-}$GHSR$^{-/-}$ and Mboat4-/- mice and to positive energy balance in Tg mice. Especially during chronic exposure to HFD, Ghr$^{-/-}$GHSR$^{-/-}$ mice increase their homecage activity dramatically to defend their body weight. We are unable to verify the previously reported increase of energy expenditure in Ghr$^{-/-}$ mice on chow diet (De Smet et al., 2006) but rather find the additional absence of GHSR to be necessary to increase energy expenditure compared to Wt controls in Ghr$^{-/-}$GHSR$^{-/-}$ mice. Nevertheless, absence or overproduction of acyl-ghrelin without changes in GHSR seems to be sufficient to modulate body weight fat mass and energy expenditure in Mboat4$^{-/-}$ and Tg mice. Another possible explanation for the decreased body weight in Ghr$^{-/-}$GHSR$^{-/-}$ mice could be that older Ghr$^{-/-}$GHSR$^{-/-}$ mice exhibit a modestly, but significantly, shorter body length that possibly contributes to the decreased body weight. However, the similar body weights in three week old mice, as well as the comparable body lengths in two month old animals, do not suggest an impaired embryonic or pubertal development. Furthermore, IGF-I levels are neither changed in Ghr$^{-/-}$GHSR$^{-/-}$ nor Mboat4$^{-/-}$ mice. Therefore, the lower body weights in old and young Ghr$^{-/-}$GHSR$^{-/-}$ mice are unlikely to be a consequence of decreased body length alone.

The exact cause for the changes in body weight and fat mass of Ghr$^{-/-}$GHSR$^{-/-}$, Mboat4$^{-/-}$ and Tg mice cannot be easily identified on the basis of the present data. Increased locomotor activity in the absence of ghrelin signaling is consistent with earlier observations. It could be demonstrated that intracerebroventricular ghrelin administration decreases spontaneous physical activity in rats (Tang-Christensen et al., 2004). Furthermore, increased locomotor activity and energy expenditure was previously described in ghrelin-deficient (Wortley et al.,

2005) and GHSR-deficient mice (Zigman et al., 2005) when chronically challenged with a high caloric diet. Consequently, increased locomotor activity, energy expenditure and core temperature likely contribute to the leaner phenotype of $Ghr^{-/-}GHSR^{-/-}$ mice but more detailed molecular-biological experiments are necessary to fully explain the underlying mechanisms. It is known that central administration of ghrelin increases gene expression of fat storage-promoting enzymes while simultaneously decreasing expression of fat oxidizing enzymes (Theander-Carrillo et al., 2006). Consequently, the complete silencing of the ghrelin/GHSR system in $Ghr^{-/-}GHSR^{-/-}$ mice and absence of acyl-ghrelin in $Mboat4^{-/-}$ mice possibly resulted in promotion of fat oxidation and inhibition of fat deposition in white adipose tissue.

In Tg mice gene expression of candidate genes involved in the regulation of energy homeostasis was measured in BAT, liver and muscle to identify possible mechanisms responsible for the decreased energy expenditure. Uncoupling proteins are mitochondrial inner membrane proteins that can uncouple the proton gradient in the respiratory chain from energy-consuming systems such as adenosine triphosphate (ATP) synthase, which results in heat instead of ATP production. UCP-1 is mainly present in the BAT and is involved in non-shivering thermogenesis (Nicholls and Locke, 1984). *Ucp1* and *Ucp3* expression in BAT is not changed between Tg and Wt mice indicating that mechanisms other than thermogenesis might contribute to the decreased energy expenditure.

In muscle of Tg mice, expression of *Cycs* and *Ucp3* are significantly downregulated. It has been suggested that UCP-3 in muscle is responsible for the export of excess fatty acids out of mitochondria in a situation of elevated mitochondrial fatty acid oxidation (Kozak and Harper, 2000). Cytochrom C is, like uncoupling proteins, located in the inner mitochondrial membrane and an essential part of the electron transport chain. Therefore, decreased expression of *Ucp3* and *Cycs* could hamper mitochondrial function and possibly decrease energy expenditure. Nevertheless, decreased gene expression does not necessary translate into decreased protein production or enzyme activity. Therefore, protein levels of UCP-3 and Cytochrom C should be measured in muscle of Tg mice to confirm the theory of decreased mitochondrial function. Additionally, mitochondrial respiratory capacity could be measured in isolated muscle cells of Tg and Wt mice.

4.3 Modulation of the ghrelin-GHSR-axis does not alter feeding behavior in mice

Administration of ghrelin increases food intake in humans and rodents more potently than any other known molecule except NYP (Asakawa et al., 2001; Tschop et al., 2000; Tschop et al., 2001a; Wren et al., 2001) indicating an important role for ghrelin as a hunger hormone or meal initiation factor (Cummings et al., 2001). Therefore, mice deficient for total or acyl ghrelin are expected to be hypophagic or even anorectic. Surprisingly, neither $Ghr^{-/-}$ (Wortley et al., 2004; Sun et al., 2003), $GHSR^{-/-}$ (Zigman et al., 2005) nor the here introduced $Ghr^{-/-}$ $GHSR^{-/-}$ and $Mboat4^{-/-}$ mice show changes in food intake or fasting-refeeding behavior compared to Wt mice. Further, massive ghrelin over production and over acylation does not increase food intake in Tg mice. All here described mice with genetic manipulation of either ghrelin, GOAT

or GHSR have the same chow and HFD intake compared to Wt and single knockout littermates. Only Mboat4$^{-/-}$ mice show a slight but significant increase in food intake when fed MCT diet, which is in opposition to ghrelin's known function as meal initiator (Cummings et al., 2001). Meal pattern analysis, which precisely describes the duration and size of individual meals, does not reveal any changes between the genotypes. Even fasting induced hyperphagia is not influenced by disruption of the ghrelin-GHSR system in Ghr$^{-/-}$GHSR$^{-/-}$ mice.

It is possible that ghrelin only acutely influences food intake between short inter-meal intervals or when food is anticipated (Drazen et al., 2006) but that chronic ghrelin signaling rather modulates energy expenditure, fuel preference and adipogenesis without affecting food intake (Theander-Carrillo et al., 2006; Strassburg et al., 2008). Another explanation for the normal feeding behavior of the Mboat4$^{-/-}$ and Ghr$^{-/-}$GHSR$^{-/-}$ mice could be developmental compensation. The orexigenic drive is crucial for survival and therefore it can be assumed that during development other orexigenic systems possibly compensate the lack of ghrelin signaling. Eminent examples for such developmental compensation are mice deficient for NPY/AgRP neurons (Luquet et al., 2005). NPY and AgRP expressing neurons in the hypothalamus are thought to be essential regulators of feeding behavior and body weight. (Levine and Morley, 1984; Stanley and Leibowitz, 1984). Deletion of NPY neurons during the early postnatal period affects feeding behavior only minimally (Luquet et al., 2005). Nevertheless, temporally controlled ablation of NPY/AgRP neurons in adult mice results promptly in starvation (Luquet et al., 2005). This finding clearly indicates that during development functional defects resulting from degenerated neurons might be overcome possibly by reorganization of the neuronal circuits and synaptic plasticity but that they cannot be compensated when development of the central nervous system is completed. In order to test that hypothesis it would be crucial to develop inducible and eventually tissue-specific knockout models for GOAT, ghrelin and GHSR.

The ghrelin/GOAT Tg mice might be an already existing inducible tool to study food intake more intensely. Tg mice do not overproduce octanoyl ghrelin when fed standard chow, which leads to a non significant phenotype between Tg and Wt mice. In our model exposure to MCT diet is necessary to induce acylation of the transgenically overproduced human ghrelin, therefore making the mouse model diet-inducible. Switching MCT diet back to chow resulted in loss of the energy expenditure phenotype in Tg mice already after two weeks. However, at this time point (2 weeks after switch to chow feeding) no changes in food intake could be measured between MCT diet fed and chow fed Tg mice. Possibly, changes in food intake occur in the immediate hours after the diet switch when ghrelin acylation is switched on or off and therefore still acts acutely. To answer that question, close monitoring of food intake, preferentially in our indirect calorimetry system with automated measurement of food intake, is required in Tg mice during diet switch periods.

4.4 Deletion of ghrelin/GHSR signaling but not acyl ghrelin deficiency impairs glucose tolerance in diet-induced obese mice

The role of ghrelin as a glucose-regulating hormone remains controversial. It has been suggested that ghrelin may have paracrine or autocrine effects on the pancreas. Thereby ghrelin could contribute to the regulation of insulin secretion (Date et al., 2002). A logical piece of evidence supporting such a theory is that ghrelin and its receptor are expressed in pancreatic islet cells (Date et al., 2002; Dezaki et al., 2004; Volante et al., 2002). The controversy, however, originates from whether ghrelin inhibits insulin secretion (Broglio et al., 2001; Colombo et al., 2003; Reimer et al., 2003) or enhances insulin secretion (Adeghate and Ponery, 2002; Date et al., 2002; Granata et al., 2007). Ghrelin deficient mice exhibit significantly better glucose metabolism than Wt littermates after early-onset exposure to HFD by improving glucose tolerance and lowering plasma concentrations of insulin, glucose, leptin, triglycerides, and cholesterol (Wortley et al., 2005). These data suggest that ghrelin deficiency may protect rodents from HFD-induced hyperglycemia and hyperinsulinemia. Similarly, GHSR deficient mice demonstrate a strong trend toward improved insulin sensitivity and glucose tolerance on chow diet (Zigman et al., 2005).

Deducted from the existing ghrelin and GHSR mouse mutants, we expected $Ghr^{-/-}GHSR^{-/-}$ mice to have improved glucose and insulin tolerance. Under chow feeding conditions $Ghr^{-/-}$ $GHSR^{-/-}$ mice show no difference in glucose and insulin tolerance compared to Wt or single knockout mice. Similarly $Mboat4^{-/-}$ mice have normal glucose tolerance when fed chow, HFD or MCT diet. Surprisingly, in the diet induced obese state, achieved by early-onset exposure to HFD, three and six month old $Ghr^{-/-}GHSR^{-/-}$ mice exhibit decreased glucose tolerance despite being still insulin and GLP-1 receptor agonist sensitive. Ghrelin is highly expressed in the pancreas during neonatal development suggesting a crucial role for pancreatic function and development (Prado et al., 2004). Ghrelin and GHSR deficiency therefore might lead to dysfunctional beta-cell proliferation or beta-cell loss, which possibly results in impaired glucose tolerance. Immunohistochemical studies of β-cell seize and architecture in adult $Ghr^{-/-}$ $GHSR^{-/-}$ mice however show no abnormalities. Since lean and obese $Mboat4^{-/-}$ mice have normal glucose tolerance compared to Wt littermates a special role for des-acyl ghrelin in regulating glucose homeostasis can be assumed. Granata et al. previously suggested that des-acyl ghrelin improves insulin secretion and has anti-apoptotic effects (Granata et al., 2007).

Leptin is produced by adipocytes and signals the brain about the abundance of adipose tissue. Leptin signaling therefore results in inhibition of food intake (Halaas et al., 1995). Mice with a mutation in the leptin gene (ob/ob) develop severe obesity, hyperglycemia, hyperinsulinemia, glucose intolerance, and hypothermia (Arvaniti et al., 1998; Meinders et al., 1996; Muzzin et al., 1996). When crossing leptin deficient ob/ob mice with ghrelin-deficient mice, Sun et al. (Sun et al., 2006) found that double-knockout mice for leptin and ghrelin displayed lower basal glucose and higher insulin concentrations than ob/ob mice without having differences in body fat mass. Both glucose and insulin tolerance tests revealed lower glucose concentrations in ghrelin deficient ob/ob mice compared with ob/ob mice. In addition, plasma glucose levels

normalized during fasting conditions in ghrelin-deficient ob/ob mice. The authors therefore concluded that ghrelin deficiency improved glucose sensitivity and pancreatic beta-cell function (Sun et al., 2006). To differentiate whether acyl or des-acyl ghrelin might be responsible for this improvement in glucose tolerance we generated Mboat4$^{-/-}$-ob/ob mice, which are deficient for GOAT and leptin. Mboat4$^{-/-}$-ob/ob mice have the same body weight, fasting blood glucose and insulin sensitivity compared to ob/ob littermates on chow and MCT diet. Thus, the absence of acyl ghrelin likely does not rescue the obese or diabetic phenotype of ob/ob mice and it is possible that des-acyl ghrelin plays an important role in regulation of glucose homeostasis.

4.5 Acyl-Ghrelin deficiency decreases circulating markers of inflammation and cholesterol

Chow fed Ghr$^{-/-}$GHSR$^{-/-}$ mice have significant lower levels of cholesterol in plasma compared to Wt littermates. Mboat4$^{-/-}$ mice have decreased plasma concentrations of cytokines, including Il-6, Il-10, Monocyte Chemoattractant Protein-1 (MCP-1), and complement component 3 (C 3 des arg). A role for ghrelin in influencing the immune system has been suggested before. GHSR is potentially expressed in leukocytes and in vitro studies indicate that T cells produce ghrelin (Hattori et al., 2001; Dixit et al., 2004). However, most studies involving ghrelin and immune function show that ghrelin administration ameliorates pathologic inflammatory processes (Gonzalez-Rey et al., 2006; Dembinski et al., 2003; Li et al., 2004). Mechanisms that could be involved in the improved immune response could be the ghrelin-induced increased release of IL-10 (Gonzalez-Rey et al., 2006; Li et al., 2004; Ma et al., 2001).

In the present study, ghrelin seems to act opposite since markers of inflammation are reduced by the absence and not administration of acyl ghrelin. A possible explanation could be the decreased fat mass of Mboat4$^{-/-}$ compared to Wt mice. Adipose tissue is involved in the production of markers of inflammation and therefore circulating cytokines correlate with the fat mass (Hotamisligil, 2008). Another reason for the improved inflammatory state of Mboat4$^{-/-}$ mice might be that des-acyl and not acyl ghrelin is responsible for the modulation of the immune function. Mboat4$^{-/-}$ mice have increased blood concentrations of des-acyl ghrelin compared to Wt mice. Therefore it might be possible that the relative high levels of des-acyl ghrelin contributed to the decrease in markers of inflammation. More studies, possibly involving administration of acyl versus des-acyl ghrelin in vivo and in vitro, are required to clarify the role of ghrelin in immune modulation.

4.6 GOAT is inactive during fasting

Because ghrelin plasma concentrations surge shortly before a meal, Mboat4 mRNA levels are expected to follow the ghrelin secretion pattern, namely increasing in the fasted state and decreasing postprandially. To test this hypothesis, we fasted four groups of C57BL/6J mice over a time course of 36 h and examined the expression patterns of *Mboat4* and *Ghrl*. In addition, we measured plasma concentrations of both acyl and des-acyl ghrelin in these mice. To our surprise, gastric *Mboat4* expression is highest when mice are fed *ad libitum* and decreases

significantly with fasting for 12, 24 or 36 h. *Ghrl* mRNA remains constant during prolonged fasting, but blood concentrations of des-acyl ghrelin double. As the latter seems to be a stable increase, the data suggest translational control of the message, with potential involvement of microRNA. The twofold decrease of *Mboat4* mRNA after fasting does not result in lower acyl ghrelin concentrations in blood. Therefore, GOAT activity might not be a bottleneck for ghrelin acylation in ad libitum or fasting conditions. Owing to the fact that there are two substrates for GOAT, ghrelin and MCFA, and that Ghrl expression is relatively constant during fasting, these data suggest that *Mboat4* is possibly downregulated due to the lack of available MCFAs. Surprisingly, GOAT does not seem to use lipids derived from endogenous lipolysis, which should be extremely increased after 36 hours of fasting, to acylate ghrelin. The measurement of GOAT activity and development of GOAT antibodies capable of determining amounts of the mostly intramembrane GOAT protein are necessary to confirm this theory.

Our finding that acyl-ghrelin is downregulated during fasting, clearly contradicts existing literature and the current view of ghrelin as being the hunger hormone. The discrepancy to literature might origin from the generally used incorrect sample collection and lack of sophisticated commercially available assays specifically for acyl ghrelin. Although DeVriese et al. already showed in 2004 that EDTA could not prevent des-acylation of ghrelin (De Vriese et al., 2004) many researchers still solely add EDTA instead of specific serine esterase inhibitors to blood samples. Further, the fact that ghrelin degrading esterases are 9 times higher and of different nature in rodent compared to human blood (De Vriese et al., 2004) is often neglected. When analyzing acyl ghrelin from rodent samples protocols for human ghrelin protection are not suitable (De Vriese et al., 2004). Another major confounding factor is the lack of specific acyl ghrelin antibodies that reliably recognize only active acyl ghrelin and not inactive degradation fragments. In our studies we use a specifically developed sample collection protocol to optimally protect acyl ghrelin from des-acylation and degradation. Further, instead of commercially available ghrelin assays we used the IPMS assay to analyze all our samples, which precisely measures acyl ghrelin. The IPMS assay involves ghrelin extraction to work in a protease- and esterase-free environment, in-house developed specific anti-ghrelin antibodies and mass spectrometry. We therefore believe that our acyl ghrelin measurements are more reliable than previously published data and that acyl ghrelin is truly downregulated upon fasting. Moreover, a sophisticated in-house acyl ghrelin ELISA was recently developed in the laboratories of Prof. Thorner supporting our findings (Liu et al., 2008).

4.7 Dietary lipids are an important activator of the GOAT/ghrelin system

To further test our hypothesis, that GOAT does not rely on endogenous derived fatty acids, we fed C57BL/6 mice with a diet rich in glycerol triheptanoate, a medium-chain triglyceride constituted of heptanoic acid (C7:0), which is not synthesized de novo in mice. It has previously been shown that dietary lipids can directly influence ghrelin acylation (Nishi et al., 2005). Similar to those results, we find that heptanoic acid is used for ghrelin acylation. The fact that heptanoylated ghrelin is more abundant than octanoylated ghrelin in mice fed this special diet supports our theory that dietary lipids have a major role in ghrelin acylation and that regulation of acyl ghrelin production and secretion is dependent on the dietary MCFA substrate.

Furthermore supporting our theory is the fact that chow fed ghrelin/GOAT transgenic mice lack octanoyl-modified forms of human ghrelin in circulation. This is probably due to the lack of MCFAs in liver under normal dietary conditions. To test this notion, we fed transgenic mice with MCT diet resulting in production of large amounts of octanoylated ghrelin. To show the dependence of ghrelin acylation on dietary MCT lipid availability, we switched the transgenic mice back to regular chow diet. After 2 weeks, the body weight difference between transgenic and Wt mice disappears as well as differences in energy expenditure and respiratory quotient. Therefore, our model of octanoylation of overexpressed human ghrelin is diet inducible and reversible.

It remains unclear why endogenously derived fatty acids mobilized from adipose stores are apparently not used to massively acylate ghrelin during fasting. MCTs, the substrate for GOAT, can principally be produced during metabolism of long-chain fatty acids by beta oxidation, where the long-chain fatty acid is shortened by two carbons with each cycle in an markedly rapid process (Dole and Rizack, 1961). Only recently, more data on modulation of fatty acid profiles have become available, but such profiles were usually measured after a substantial fast rather than between meals when acyl ghrelin concentrations would be affected in a considerable manner (Liu et al., 2008). However, even after 12 h of fasting, MCFAs remain present in plasma (Dole and Rizack, 1961) and are not completely oxidized. Notably, ghrelin-secreting cells in the gastrointestinal tract are of the closed- and open-type, with the open-type cells being more abundant in the duodenum (Date et al., 2000) where fatty acids are absorbed. Thus, it is possible that the open-type ghrelin cells take up dietary lipids and directly use them for ghrelin acylation. We therefore conclude, on the basis of the existing body of published data in combination with our results, that ghrelin acylation and the secretion of acylated ghrelin probably represent two independent processes and that GOAT-ghrelin might act as a lipid sensor that is activated when certain fatty food is consumed. Follow-up studies providing improved insight into fatty acid uptake and destination could help to understand and more efficiently explore the mechanistic details of ghrelin acylation.

4.8 GOAT, ghrelin and GHSR as potential drug targets

The search for not only GH replacement therapy but also for potential treatment of obesity and type 2 diabetes mellitus has driven the development of GHSR agonist and antagonists (Smith et al., 1997). Further, a ghrelin antagonist has been developed (Zorrilla et al., 2006). Until now these pharmacological modulators of ghrelin and GHSR function are not on the market because of lack of significant efficacy (see Introduction). Data form our $Ghr^{-/-}$ $GHSR^{-/-}$ mice show that simultaneous targeting of ghrelin and GHSR is necessary to affect energy and glucose metabolism. This finding indicates that possibly other ghrelin receptors and GHSR ligands exist. Agonizing or antagonizing ghrelin function might be more fruitful in the future. However, until these putative other players in the ghrelin/GHSR system are not identified it will be difficult to effectively antagonize the effects of ghrelin signaling.

Gastric *MBOAT4* expression might offer an accessible and elegant drug target for the treatment of metabolic diseases. As our loss- and gain of GOAT- function models prove its role in regulation of diet-induced adiposity. Thus, GOAT modulators may be potential new anti-obesity drugs or anti-cachexia therapeutics. But even before the development of such pharmaceutical GOAT modulators, our data suggest that it might be possible to influence GOAT activity with the choice of dietary lipids. In nature, MCTs are found in milk along with long-chain fatty acids, and because MCTs are more efficiently digested, some infant formulae are enriched with MCTs. For the same reason, MCT therapy is indicated for individuals with malabsorption (Zurier et al., 1966). Our findings indicate that the orexigenic, positive energy balance–promoting GOAT-ghrelin system is triggered by dietary MCTs and offer a new perspective for the potential role of MCTs in milk and infant formula that deserves further study. Coconut is a natural source of large amounts of MCTs. Studies conducted in the Polynesian islands, where meals are composed of up to 60% from coconut, show high prevalence of obesity and dyslipidemia, which can be linked to the high intake of coconut products (Prior et al., 1981). Although ghrelin abundance was not measured in this population, it can be assumed that the extreme levels of MCT intake trigger ghrelin acylation, possibly leading to increased peripheral lipid storage and hypercholesterolemia (Prior et al., 1981), two phenomena that are associated with ghrelin signaling as shown by Lopez et al. (Lopez et al., 2008) and in our $Ghr^{-/-}$ $GHSR^{-/-}$ mice.

4.9 Ghrelin as a novel nutrikine

Pharmacological doses of ghrelin reliably increase food intake in humans and rodents (Tschop et al., 2000; Wren et al., 2001). However, the ghrelin induced hyperphagia seems to be most profound when ghrelin is delivered centrally, a very unnatural route of delivery, and the increased food intake lasts only for a couple of hours. Physiologically, in humans acylated ghrelin concentrations rise before a meal and drop afterwards, thus suggesting a role for ghrelin as a hunger signal (Cummings et al., 2001). However, our wild-type mouse studies and a recent study show that plasma concentrations of acyl ghrelin do not increase under conditions of prolonged fasting (Liu et al., 2008). Liu and colleagues corroborate the well-known ghrelin secretion pattern with surges before a meal and suppression after a meal for the inactive and the active form of ghrelin. Nevertheless, during prolonged fasting for 72 h or longer, in humans plasma concentrations of acyl ghrelin are consistently at the basal level (Liu et al., 2008). These results suggest that separate secretion and activation processes may regulate ghrelin. Further, ghrelin probably acts more as a meal preparation cue rather than a meal initiation factor or a hunger signal that responds to persistent starvation. The interpretation of ghrelin as a meal preparation factor preparing the organism to optimally metabolize and store energy could explain why we do not find increased food intake in the GOAT and ghrelin–transgenic mice. Additionally, gastric ghrelin might has more of a role in the regulation of energy metabolism in muscle and liver. Along the line of thought that ghrelin is acting rather as a meal preparation cue than as a hunger signal, prolonged food deprivation in our studies also does not lead to increased acyl ghrelin blood concentrations. In addition, neither gastric *Mboat4* expression nor *Ghrl* mRNA levels are upregulated during fasting, as we would have expected on the basis of the traditional model of ghrelin function. Taken together, our results do not point toward

a prominent role for both *Ghrl* and *Mboat4* mRNA expression as a key process for the generation of a hunger signal that indicates an empty stomach. However, ghrelin acylation and thereby activation seem to be influenced substantially by food intake and fatty acid composition of the ingested food. This finding leads to the suggestion that ghrelin is a gastric lipid sensor, or nutrikine, signaling the brain abundance of calories.

Our new model shows that ghrelin is a nutrikine, as represented schematically in Figure 37. In a physiological state, such as in between meals, basal ghrelin signaling can be found (Fig. 37A). The fatty acids used for ghrelin acylation could be either derived by endogenous lipolysis or origin from dietary lipids that remained in the stomach from previous meals. In the state of hunger or even long term starvation, dietary lipids are rare, resulting in a lack of GOAT substrate. Endogenous derived free fatty acids, which are abundant during hunger, are apparently not used to compensate the lack of dietary fatty acids (Fig. 37B). However, they are used to a small extend to maintain basal ghrelin signaling, since blood concentrations of acyl ghrelin never reach zero, even after long term starvation (Liu et al., 2008). Downregulation of *Mboat4* transcript and shortage of dietary fatty acids lead to a relative overproduction of des-acyl ghrelin, which cannot bind or activate GHSR.

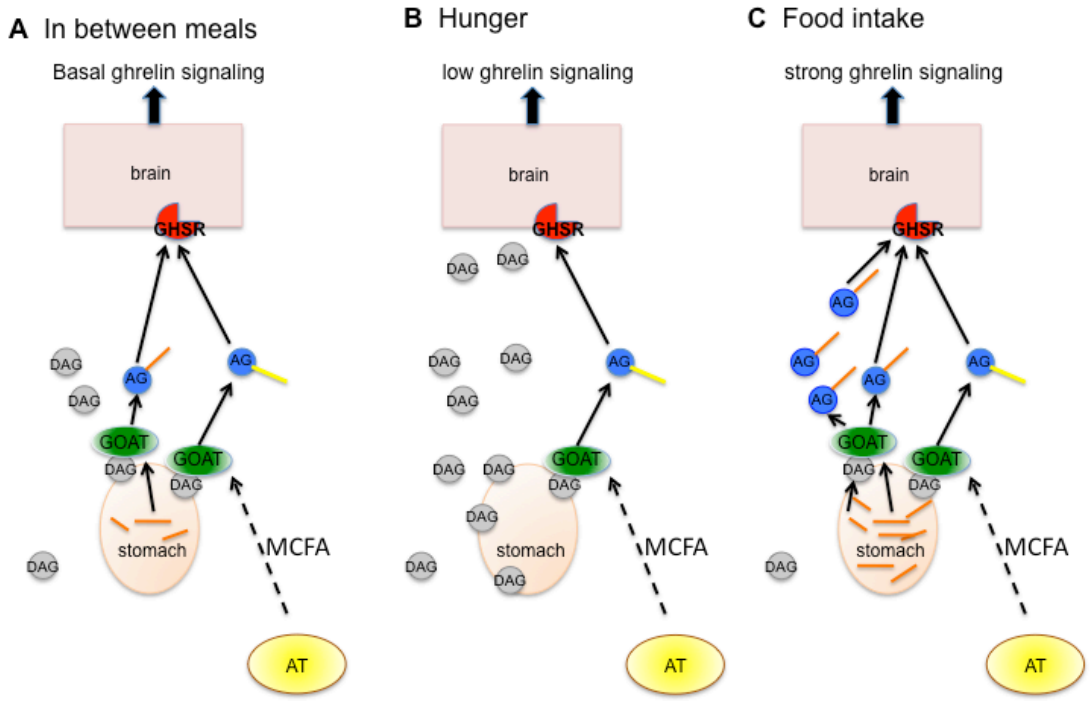

Figure 37 Ghrelin as a nutrikine that signals the brain abundance of dietary lipids
(A) Basal ghrelin signaling is comprised of dietary and endogenous derived medium-chain fatty acids (MCFA) that are coupled to ghrelin via GOAT to form the bioactive acyl ghrelin (AG). (B) During hunger or persisting starvation ghrelin acylation is reduced due to a lack of the GOAT substrate dietary lipids and downregulation of the GOAT gene *Mboat4*. Only a small amount of adipose-tissue (AT) derived fatty acids are used to maintain low ghrelin signaling. Gene expression of *Ghrl* is not downregulated by fasting, which results in relative overproduction of des-acyl ghrelin (DAG). (C) Ingested MCFA are directly used by GOAT to acylate ghrelin. The high blood concentration of AG leads to strong GHSR signaling possibly reporting the brain a high caloric environment.

During food intake MCFA are readily absorbed and used for ghrelin acylation (Fig. 37C). Coupled to ghrelin, the MCFAs enter into cells of the CNS via GHSR. The fate of the MCFA, once the acyl ghrelin-GHSR complex is internalized remains unknown but recent studies show that the orexigenic effects of ghrelin require hypothalamic fatty acid metabolism (Lopez et al., 2008).

Two recently published studies highlight the emerging importance of dietary fat as a substrate for endogenous modulators of energy balance (Gillum et al., 2008; Schwartz et al., 2008). The lipid mediator oleoylethanolamide and its precursor NAPE affect food intake and energy metabolism by using dietary fat as a substrate. Further, an acyltransferase is involved in oleoylethanolamide activation (Gillum et al., 2008; Schwartz et al., 2008). The new model we propose, with GOAT-ghrelin as a lipid sensor, seems logical when we review lipogenesis as the main function of ghrelin as well as induction of growth hormone release, two features that seem to be counterintuitive during hunger. It would make more sense for an organism to increase cell proliferation and support fat storage while nutrients are coming in. Therefore, although more studies are needed to completely dissect acyl from des-acyl ghrelin functions or ghrelin activation from ghrelin secretion processes, we propose that ghrelin's predominant physiological function may not necessarily, or at least not exclusively, be that of a hunger signal, that reflects an empty stomach as previously assumed. On the basis of the data presented here it seems more likely that the GOAT/ghrelin/GHSR system acts as a nutrient sensor by using readily absorbable MCFAs to signal the brain that high caloric food is available. This signal leads to optimization of nutrient partitioning and growth signals.

This concept, although largely hypothetical at this point, also fits well with some of the known effects of ghrelin in the brain: Shifting nutrient partitioning and substrate choice to achieve maximum efficiency not only in energy uptake but also metabolic energy storage. If the GOAT/ghrelin/GHSR system would in fact function as dietary lipid-sensing system, it may explain why treatment with acyl-ghrelin enhances preference for fat in rodents (Beck et al., 2002). The fact that ghrelin triggers brain centers of reward and addiction seems to make much more sense when we think of ghrelin as a signal, which signals the brain that MCT-rich energy sources are consumed and (or) stored fat is being utilized for energy. Taking this one step further, it could be speculated that ghrelin's promoting effects on learning and memory (Diano et al., 2006) may be evolutionarily shaped to remember where places with plenty of calories are found. Similarly, ghrelin's regulatory influence on reproductive processes could indicate that ghrelin signals sufficient nutrient availability as a prerequisite for appropriate control of reproduction (Nakahara et al., 2003; Tena-Sempere, 2008).

5 Literature

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6 Supplements

6.1 List of Tables

6.2 List of Figures

6. 3 Abbreviations

AgRP	agouti-related protein
AMPK	AMP-activated protein kinase
BAT	brown adipose tissue
BL	body length
BW	body weight
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
CREB	camp response element-binding protein
DEPC	diethylpyrocarbonate
dNTPs	deoxynucleotides
EDTA	Ethylenediaminetetraacetic acid
EE	energy expenditure
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal regulated kinase
ES	embryonic stem cell
F1	first filial generation
F2	second filial generation
F3	third filial generation
g	gravity
GH	growth hormone
GHRH	growth hormone releasing hormone
GHSR	growth hormone secretagogue receptor
GHS-R1a	growth hormone secretagogue receptor 1-a
GOAT	ghrelin O-acyltransferase
FFM	fat free mass
FI	food intake
FM	fat mass
HCl	hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	high-fat diet
i.p	intra peritoneal
IP	immunoprecipitation
i.v.	intra venous
Kcal	kilocalorie
LM	lean mass
MALDI-ToF	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
Mboat4	membrane-bound O-acyltransferase 4
MCFA	medium-chain fatty acids
MCT	medium-chain triglycerides

$MgCl_2$	magnesium chloride
mRNA	messenger ribonucleic acid
MS	mass spectrometry
n	amount
N2	second backcrossed generation
n/a	not applicable
NaCl	sodium chloride
NEFA	non-esterified fatty acids
NF	nuclear factor
NPY	neuropeptide Y
PBS	phosphate buffered saline
PBST	phosphate buffered saline Octylphenolpoly (ethylenglycolether)x
PCR	polymerase chain reaction
POMC	proopiomelanocortin
qPCR	quantitative polymerase chain reaction
RIA	radioimmunoassay
RNA	ribonucleic acid
RQ	respiratory quotient
RT	room temperature
SEM	standard error of the mean
siRNA	small interfering RNA
TAE	Tris-acetate-EDTA
TBC	transcriptional blocking cassette
Tg	transgenic
TFA	Trifluoroacetic acid
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris(hydroxymethyl)aminomethane in sodium chloride
TRH	thyrotropin releasing hormone
U	unit
UCP	uncoupling protein
Wt	wild type

6.4 Genotyping Protocols

6.4.1 Ghrl $^{+/+}$ PCR protocol

Master Mix	Concentration	1x	15x	30x	64x	80x	100x
Nuclease free H_20		14.5	217.5	435	928	1160	1450
10X Mango Buffer	10X	2.5	37.5	75	160	200	250
Betaine (Sigma)	5M	2.5	37.5	75	160	200	250
$MgCl_2$	50mM	1	15	30	64	80	100
dNTPs	10mM	0.5	7.5	15	32	40	50
Ghrl +/+ fwd.	20µM	0.5	7.5	15	32	40	50
Ghrl +/+ rev.	20µM	0.5	7.5	15	32	40	50
Mango Taq	1U/µl	1	15	30	64	80	100

Step 1: Work on wet ice! Prepare master mix. Add Taq as very last step.
Step 2: Add 2µl of each control and blank into corresponding well.
Add 2µl of each unknown into corresponding well.
Step 3: Dispense 23µl of master mixture into each well, mix with pipette.
There should be no visible air bubbles in wells.
Final volume = 25µl

Run on: Eppendorf Master Cycler
Program name: Ghr+_+,-_-,GHSR-Uni
Cycles: 94ºC, 3min x 1
94ºC, 30sec x 36
60ºC, 60sec x 36
72°C, 90sec x 36
72ºC, 3min x 1
4ºC, ∞
Run results on 1.0% agarose gel in 1X TAE.

Product Size: 800 bp
Primer Sequence:
Ghrl +/+ fwd. 5' TAA AGG GGT TGG GGT ATG GAG G 3'
Ghrl +/+ rev. 5' ACC AGA GAG GAA GGT AGA AGG AGT G 3'

6.4.2 Ghrl $^{-/-}$ PCR protocol

	Concentration	1x	15x	30x	64x	80x	100x
Nuclease free H_20		14.5	217.5	435	928	1160	1450
10X Mango Buffer	10X	2.5	37.5	75	160	200	250
Betaine (Sigma)	5M	2.5	37.5	75	160	200	250
$MgCl_2$	50mM	1	15	30	64	80	100
dNTPs	10mM	0.5	7.5	15	32	40	50
5J Ghrelin fwd.	20µM	0.5	7.5	15	32	40	50
Ghrl -/- rev.	20µM	0.5	7.5	15	32	40	50
Mango Taq	1U/µl	1	15	30	64	80	100

Step 1: Work on wet ice! Prepare master mix. Add Taq as very last step.
Step 2: Add 2µl of each control and blank into corresponding well.
Add 2µl of each unknown into corresponding well.
Step 3: Dispense 23µl of master mixture into each well, mix with pipette.
There should be no visible air bubbles in wells.
Final volume = 25µl

Run on: Eppendorf Master Cycler
Program name: Ghr+_+,-_-,GHSR-Uni
Cycles: 94ºC, 3min x 1
94ºC, 30sec x 36
60ºC, 60sec x 36
72°C, 90sec x 36
72ºC, 3min x 1
4ºC, ∞
Run results on 1.0% agarose gel in 1X TAE.

Product Size: 1200 bp
Primer Sequence:
5J Ghrelin fwd. 5' AGC TCC CAC CCT TGA AAG AT 3'
Ghrl -/- rev. 5' ACC ATT TTC AAT CCG CAC CTC 3'

6.4.3 GHSR del PCR protocol

Master Mix	Concentration	1x	15x	30x	64x	80x	100x
Nuclease free H_20		14.5	217.5	435	928	1160	1450
10X Mango Buffer	10X	2.5	37.5	75	160	200	250
Betaine (Sigma)	5M	2.5	37.5	75	160	200	250
$MgCl_2$	50mM	1	15	30	64	80	100
dNTPs	10mM	0.5	7.5	15	32	40	50
GHSR del fwd.	20µM	0.5	7.5	15	32	40	50
GHSR 3J rev.	20µM	0.5	7.5	15	32	40	50
Mango Taq	1U/µl	1	15	30	64	80	100

Step 1: Work on wet ice! Prepare master mix. Add Taq as very last step.
Step 2: Add 2µl of each control and blank into corresponding well.
Add 2µl of each unknown into corresponding well.
Step 3: Dispense 23µl of master mixture into each well, mix with pipette.
There should be no visible air bubbles in wells.
Final volume = 25µl

Run on: Eppendorf Master Cycler
Program name: GHSR-del
Cycles: 94ºC, 3min x 1
94ºC, 30sec x 36
60ºC, 45sec x 36
72°C, 45sec x 36
72ºC, 3min x 1
4ºC, ∞
Run results on 1.0% agarose gel in 1X TAE.

Product Size: 609 bp
Primer Sequence:
GHSR del fwd. 5' GTA TCG GCC CTG GAA CTT CG 3'
GHSR 3J rev. 5' AAC CAA GGA CAG AGA CAG AC 3'

6.4.4 GHSR Uni PCR protocol

Master Mix	Concentration	1x	15x	30x	64x	80x	100x
Nuclease free H_20		14.5	217.5	435	928	1160	1450
10X Mango Buffer	10X	2.5	37.5	75	160	200	250
Betaine (Sigma)	5M	2.5	37.5	75	160	200	250
$MgCl_2$	50mM	1	15	30	64	80	100
dNTPs	10mM	0.5	7.5	15	32	40	50
GHSR 5J fwd.	20µM	0.5	7.5	15	32	40	50
Universal 5J rev.	20µM	0.5	7.5	15	32	40	50
Mango Taq	1U/µl	1	15	30	64	80	100

Step 1: Work on wet ice! Prepare master mix. Add Taq as very last step.
Step 2: Add 2µl of each control and blank into corresponding well.
Add 2µl of each unknown into corresponding well.
Step 3: Dispense 23µl of master mixture into each well, mix with pipette.
There should be no visible air bubbles in wells.
Final volume = 25µl

Run on: Eppendorf Master Cycler
Program name: Ghr+_+,-_-,GHSR-Uni
Cycles: 94ºC, 3min x 1
94ºC, 30sec x 36
60ºC, 60sec x 36
72°C, 90sec x 36
72ºC, 3min x 1
4ºC, ∞
Run results on 1.0% agarose gel in 1X TAE.

Product Size: 550 bp
Primer Sequence:
GHSR 5J fwd. 5' TCC TTT CAT CGC TAA TGT TCG 3'
Universal 5J rev. 5' GTC TGT CCT AGC TTC CTC ACT G 3'

6.4.5 GOAT-KO PCR protocol

Genotyping PCR performed according to SOP 1452 detects heterozygous/homozygous conventional alleles. (i.e. conventional KO not conditional)
Primers:
1452_29: GCTTAGGGACTCTAGGAAGG
1452_30: GCTAAGAGTTCTATATCCAGATCG
1260_1: GAGACTCTGGCTACTCATCC
1260_2: CCTTCAGCAAGAGCTGGGGAC

Reaction		x1	
H2O (DECP)		13	µl
PCR Buffer 10x (Invitrogen)		2.5	µl
Betaine (5M, Sigma)		2.5	µl
MgCl2 (50mM)		1	µl
dNTPs (10mM)		0.5	µl
Primer 1452_29 (5µM)		1.25	µl
Primer 1452_30 (5µM)		1.25	µl
Primer 1260_1 (5µM)		0.25	µl
Primer 1260_2 (5µM)		0.25	µl
Taq Polym.(5U/µl, Invitrogen)		0.5	µl
	total volume:	23	µl
2µl DNA		2	µl

Program:

95°C	5 min	
95°C	30 sec	x 35
60°C	30 sec	x 35
72°C	1 min	x 35
72°C	10 min	
4°C	hold	

Expected Fragments [bp]: 277
PCR SOP 1537 allows in connection with LLY0004 LOC234155 KO conventional PCR = SOP1452 to distinguish between heterozygous and homozygous.
As you expect to detect the absence and presence of a fragment I recommend including the control primer of SOP 1260 as a positive control to ensure that the PCR performed well.

6.4.6 GOAT-Wt PCR protocol

Genotyping PCR performed according to SOP 1537 detects wild type alleles.
Primers:
1537_34: GGATGGATAAACCTGATGGC
1452_30: GCTAAGAGTTCTATATCCAGATCG
1260_1: GAGACTCTGGCTACTCATCC
1260_2: CCTTCAGCAAGAGCTGGGGAC

Reaction		x1	
H2O (DECP)		13	µl
PCR Buffer 10x (Invitrogen)		2.5	µl
Betaine (5M, Sigma)		2.5	µl
MgCl2 (50mM)		1	µl
dNTPs (10mM)		0.5	µl
Primer 1537_34 (5µM)		1.25	µl
Primer 1452_30 (5µM)		1.25	µl
Primer 1260_1 (5µM)		0.25	µl
Primer 1260_2 (5µM)		0.25	µl
Taq Polym.(5U/µl, Invitrogen)		0.5	µl
	total volume:	23	µl
2µl DNA		2	µl

Program:		
	95°C	5 min
	95°C	30 sec x 35
	60°C	30 sec x 35
	72°C	1 min x 35
	72°C	10 min
	4°C	hold

Expected Fragments [bp]: 238
PCR SOP 1537 allows in connection with SOP 1452 PCR to distinguish between heterozygous and homozygous mice.

6.4.7 ob/ob PCR protocol

Digestion of PCR product necessary before running the gel!

Reaction Components	Vol 1x	
H20	2.76	µl
10 X Taq PCR Buffer	1.2	µl
Betaine	1.2	µl
25 mM MgCl2	0.96	µl
2.5 mM dNTP	0.96	µl
20 µM oIMR 1151	0.6	µl
20 µM oIMR 1152	0.6	µl
5 mM DNA Loading Dye*	1.66	µl
5 U/µl Taq Pol. (Invirtogen)	0.06	µl
DNA*	3.5	

Notes: *Use 3.5 µl (5-20ng) DNA per reaction. DNA loading dye = 60% sucrose/5mM cresol red. Our genotyping laboratory purchases Cresol Red (Sodium Salt) from Sigma, catalog number C9877.
Cycling conditions:

Step	Temp	Time	Note
1	94 °C	3 min	
2	94 °C	30 sec	
3	62 °C	1 min	
4	72 °C	45 sec	repeat steps 2-4 for 35 cycles
5	72 °C	2 min	
6	10 °C		hold**

Primers:
oIMR1151 5'- TGT CCA AGA TGG ACC AGA CTC -3'
oIMR1152 5'- ACT GGT CTG AGG CAG GGA GCA -3'

after PCR: **Incubate 8 µl restriction digest with 13 µl PCR product for a minimum of 6 hours at 37C prior to running on gel. WT product uncut. MUT product cut.

Restriction Reaction	1x	
NEBuffer 3 (10 X)	1.32	µl
H20	6.18	µl
Dde1 (New England Biolabs)	0.5	µl

Separate digested products on a 2.0% agarose gel.

expected products: HET = 55 bp, 100 bp and 155 bp
MUT = 55 bp and 100 bp
WT = 155 bp

6.4.8 Human ghrelin human GOAT transgene PCR protocol

	# of Rxns:	100
Reaction Components	Vol/Rxn	Total Vol [µl]
H20	9.2	920
5 X Flexi PCR Buffer green	5	500
Betaine	2.5	250
25 mM MgCl2	2	200
2.5 mM dNTP	2	200
20 µM PJS300	1	100
21 µM PJS301	1	100
5 U/µl Bromega Flexi Taq	0.3	30
DNA	2	

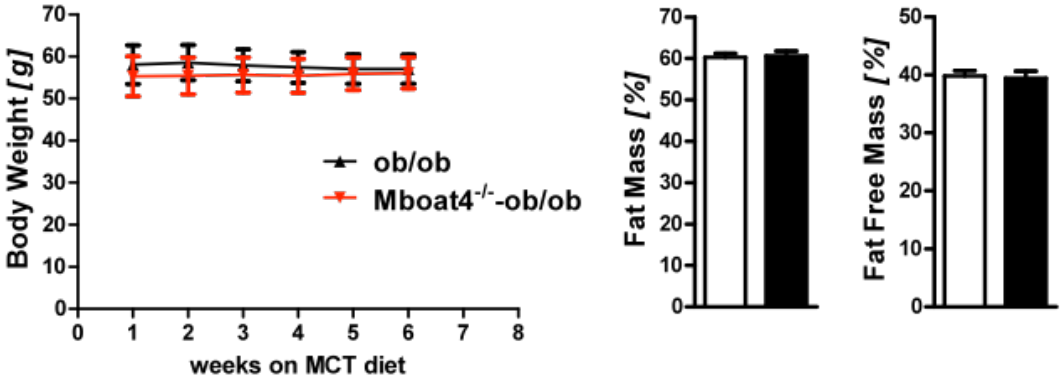
Cycling conditions:

Step	Temp	Time	Note
1	94 °C	3 min	
2	94 °C	30 sec	
3	60 °C	30sec	
4	72 °C	60 sec	repeat steps 2-4 for 35 cycles
5	72 °C	2 min	
6	6 °C		hold

Expected product seize: 300 bp

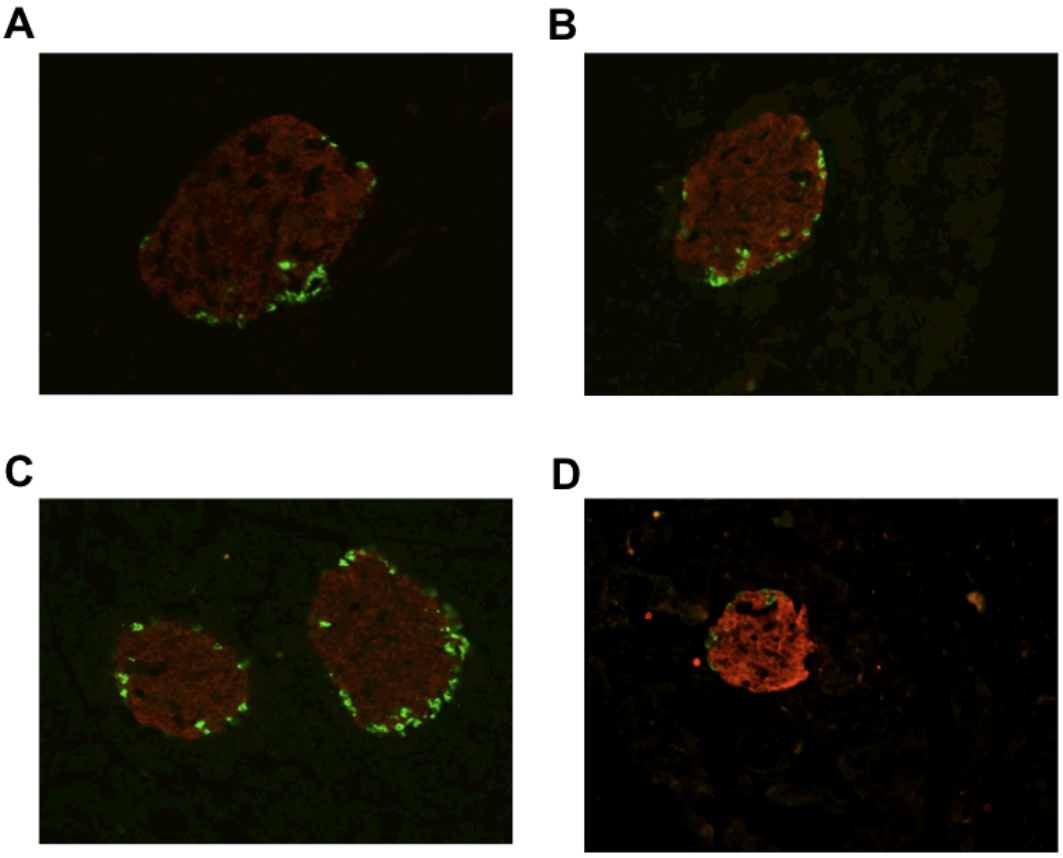
Primers:
PJS300 5'AGGCTCAGAGGCACACAGGAGT
PJS301 5''CCCTCTCACACTACCTAAACA

6.5 Female Mboat$^{-/-}$ob/ob mice



Body weight and body composition of female Mboat4$^{-/-}$ob/ob mice after 7 weeks of exposure to MCT diet.

6.6 Beta-cell structure of GhrGHSR mice



Immunohistochemistry of beta-cells from Ghr-GHSR mice
Pancreas of Ghr-GHSR mice, which have been chronically fed with HFD, was collected and stained for insulin (red) and glucagon (green). Representative pictures from (A) Wt, (B) Ghr$^{-/-}$GHSR$^{-/-}$, (C) Ghr$^{-/-}$ and (D) GHSR$^{-/-}$ mice.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die Arbeit wurde bisher an keiner anderen Hochschule eingereicht.

Henriette Kirchner Berlin, im Juni 2010