Role of the GDF15-GFRAL pathway under skeletal muscle mitochondrial stress

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Glossary of Terms

ACTH	Adenocorticotropic hormone
AgRP	Agouti-related peptide
AKT	Protein kinase B
AMH	Anti-Müllerian hormone
AN	Anorexia nervosa
AP	Area postrema
ARC	Arcuate nucleus of the hypothalamus
ATF	Activating transcription factor
ATP	Adenosine triphosphate
AUC	Area under curve
BMP	Bone morphogenetic protein
CCK	Cholecystokinin
cDNA	Complementary DNA
CeA	Central nucleus of the amygdala
CHOP	C/EBP homologous protein
CM	Conditioned media
CORT	Glucocorticoids
CREB	cAMP-response element binding protein
CRH	Corticotropin releasing hormone
CRHR1	CRH receptor 1
CRIF1	CR6-interacting factor 1
Cry	Cryptochrome circadian regulator
CSA	Cross-sectional area
Ct	Cycle threshold
CT	Circadian time
CTA	Conditioned taste aversion
CVD	Cardiovascular disease
CVO	Circumventricular organ
DEPC	Diethyl pyrocarbonate
DIO	Diet induced obesity

DMN	Dorsomedial nucleus of the hypothalamus
EDL	Extensor digitorium longus
eIF2α	Eukaryotic translation initiation factor 2α
EPM	Elevated plus maze
EPS	Electrical pulse stimulation
ER	Endoplasmic reticulum
ERG1	Early growth response protein 1
ERK1/2	Extracellular regulated kinase 1/2
FGF21	Fibroblast growth factor 21
Gastroc	Gastrocnemius
GCN2	General control nonderepressible 2
GDF15	Growth differentiation factor 15
GdKO	*Gdf15*-knockout
GDNF	Glial-cell-derived neurotropic factor family
GfKO	*Gfral*-knockout
GFRAL	GDNF family receptor α-like
GI	Gastrointestinal tract
GLP1	Glucagon-like peptide 1
GR	Glucocorticoid receptor
H&E	Hematoxylin-eosin
HFD	High fat diet
HPA	Hypothalamus-pituitary-adrenal
HTH	Hypothalamus
IP	Intraperitoneal
ISR	Integrated stress response
LHA	Lateral hypothalamic area
MC3R	Melanocortin receptor subtype 3
MC4R	Melanocortin receptor subtype 4
MD	Mitochondrial disease
MIC1	Macrophage inhibitory cytokine 1
MR	Mineralocorticoid receptor

MSH	Melanocyte stimulating hormone
mtDNA	Mitochondrial DNA
mtRNA	Mitochondrial RNA
NAG1	NSAID activated gene 1
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
OFT	Open field test
oGTT	Oral glucose tolerance test
ovBNST	Bed nucleus of the stria terminalis
OXPHOS	Oxidative phosphorylation
PBN	Parabrachial nucleus of the pons
PCSK	Proprotein convertase, subtilisin/kexin-type
PDF	Prostate derived factor
Per	Period
PFA	Paraformaldehyde
PLCγ	Phospholipase C γ
POMC	Proopiomelanocortin
PTGFB	Placental transformation growth factor-β
PVDF	Polyvinylidene difluoride
PVH	Paraventricular nucleus of the hypothalamus
PYY	Peptide YY
Quad	Quadriceps
rGDF15	Recombinant GDF15
RQ	Respiratory quotient
SAM	Sympathetic-adreno-medullar
SCN	Suprachiasmatic nucleus
SOL	Soleus
sWAT	Subcutaneous white adipose tissue
T2D	Type 2 diabetes

TA	Tibialis anterior
TG	HSA-*Ucp1*-transgenic
TGFβ	Transforming growth factor β
UCP1	Uncoupling protein 1
UESCs	Uterus endometrial stromal cells
UPR	Unfolded protein response
UPRmt	Mitochondrial unfolded protein response
VMN	Ventromedial nucleus of the hypothalamus
WT	Wildtype

Abstract

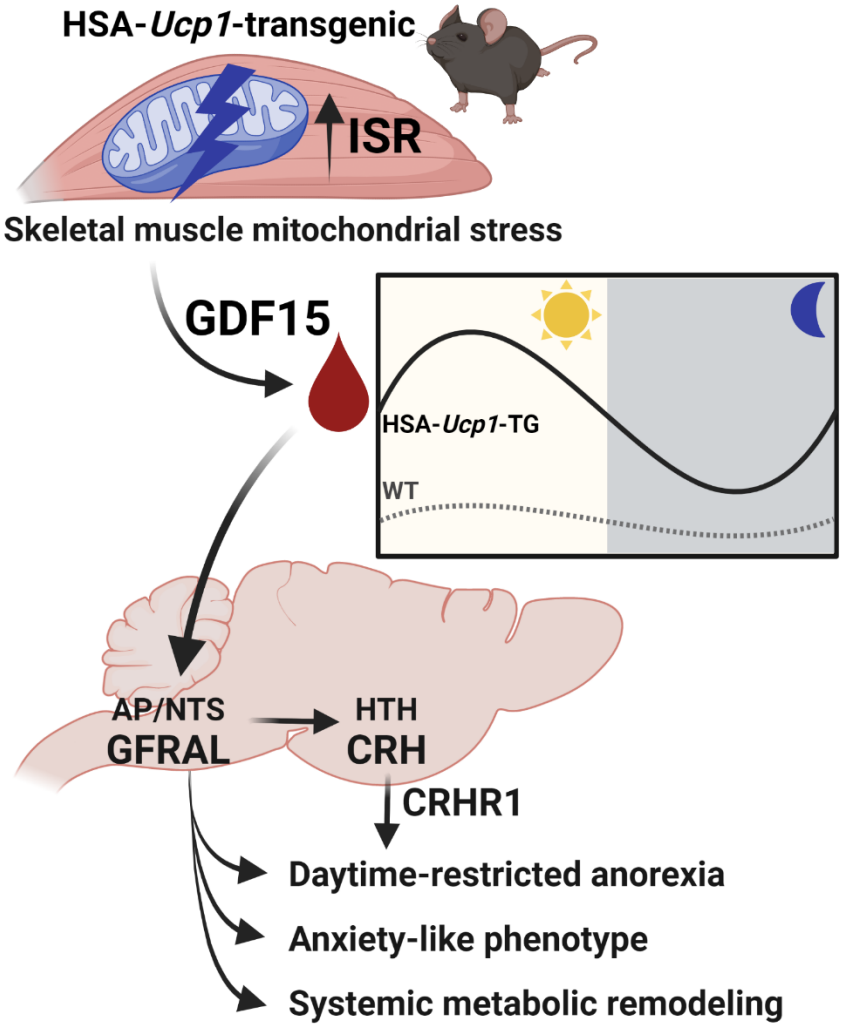
Growth differentiation factor 15 (GDF15) is a stress-induced cytokine secreted into the circulation by a number of tissues under different pathological conditions such as cardiovascular disease, cancer or mitochondrial dysfunction, among others. While GDF15 signaling through its recently identified hindbrain-specific receptor GDNF family receptor alpha-like (GFRAL) has been proposed to be involved in the metabolic stress response, its endocrine role under chronic stress conditions is still poorly understood. Mitochondrial dysfunction is characterized by the impairment of oxidative phosphorylation (OXPHOS), leading to inefficient functioning of mitochondria and consequently, to mitochondrial stress. Importantly, mitochondrial dysfunction is among the pathologies to most robustly induce GDF15 as a cytokine in the circulation.

The overall aim of this thesis was to elucidate the role of the GDF15-GFRAL pathway under mitochondrial stress conditions. For this purpose, a mouse model of skeletal muscle-specific mitochondrial stress achieved by ectopic expression of uncoupling protein 1 (UCP1), the HSA-*Ucp1*-transgenic (TG) mouse, was employed. As a consequence of mitochondrial stress, TG mice display a metabolic remodeling consisting of a lean phenotype, an improved glucose metabolism, an increased metabolic flexibility and a metabolic activation of white adipose tissue.

Making use of TG mice crossed with whole body *Gdf15*-knockout (GdKO) and *Gfral*-knockout (GfKO) mouse models, this thesis demonstrates that skeletal muscle mitochondrial stress induces the integrated stress response (ISR) and GDF15 in skeletal muscle, which is released into the circulation as a myokine (muscle-induced cytokine) in a circadian manner. Further, this work identifies GDF15-GFRAL signaling to be responsible for the systemic metabolic remodeling elicited by mitochondrial stress in TG mice. Moreover, this study reveals a daytime-restricted anorexia induced by the GDF15-GFRAL axis under muscle mitochondrial stress, which is, mechanistically, mediated through the induction of hypothalamic corticotropin releasing hormone (CRH). Finally, this work elucidates a so far unknown physiological outcome of the GDF15-GFRAL pathway: the induction of anxiety-like behavior.

In conclusion, this study uncovers a muscle-brain crosstalk under skeletal muscle mitochondrial stress conditions through the induction of GDF15 as a myokine that signals through the hindbrain-specific GFRAL receptor to elicit a stress response leading to metabolic remodeling and modulation of ingestive- and anxiety-like behavior.

Graphical Abstract



Graphical abstract: The role of the GDF15-GFRAL pathway under skeletal muscle mitochondrial stress. Skeletal muscle mitochondrial stress achieved by ectopic expression of uncoupling protein 1 (UCP1) in the mitochondria of skeletal muscle in HSA-*Ucp1*-transgenic mice triggers the activation of the integrated stress response (ISR) as well as the induction of the cytokine growth differentiation factor 15 (GDF15), which is released into the circulation in a diurnal manner. GDF15 signals through its hindbrain located receptor GDNF family receptor alpha-like (GFRAL) to induce a daytime-restricted anorexia, anxiety-like phenotype and systemic metabolic remodeling. Mechanistically, the GDF15-GFRAL pathway activates corticotropin releasing hormone (CRH) neurons in the hypothalamus (HTH) which signal through CRH receptor 1 (CRHR1) to control diurnal food intake. Figure created with BioRender.com

Zusammenfassung

Der Wachstum- und Differenzierungsfaktor 15 (GDF15) ist ein stressinduziertes Zytokin, dass bei u.a. Krebs, kardiovaskulären oder mitochondrialer Erkrankungen in den betroffenen Geweben stark induziert und ins Blut sekretiert werden kann. Die endokrine Funktion von GDF15 sowie die Bedeutung des kürzlich identifizierten und spezifisch im Hirnstamm exprimierten GDF15-Rezeptors GFRAL (GDNF family receptor alpha-like) unter chronischen Stressbedingungen ist jedoch noch unzureichend verstanden. Mitochondrialer Stress ist durch eine Fehlfunktion der oxidativen Phosphorylierung (OXPHOS) charakterisiert, was eine ineffiziente ATP-Synthese und eine gestörte zelluläre Energiehomöostase zur Folge hat. Ziel der Doktorarbeit war es, die biologische Funktion des GDF15-GFRAL-Signalwegs unter mitochondrialen Stressbedingungen aufzuklären.

Zu diesem Zweck wurde das etablierte transgene HSA-*Ucp1*-Mausmodel (TG) untersucht, welches durch eine chronisch verringerte OXPHOS-Effizienz spezifisch im Skelettmuskel sowie eine systemische Anpassung des Energiestoffwechsels charakterisiert ist. Dabei konnte in dieser Arbeit zunächst zeigt werden, dass mitochondrialer Stress im Skelettmuskel zell-autonom eine integrierte Stressantwort (ISR) induziert, wodurch die Expression und Sekretion von GDF15 in den Blutkreislauf als Myokin (muskelinduziertes Zytokin) stark erhöht wird. Zudem konnte erstmalig eine tageszeitliche Schwankung der muskulären *Gdf15* Genexpression und der im Blut zirkulierenden GDF15-Level bei TG Mäusen identifiziert werden. Durch weiterführende Zuchtkreuzungen der TG-Mäuse mit konstitutiven Knockout-Mäusen (KO) zur Inaktivierung der Gene *Gdf15* (GdKO) oder *Gfral* (GfKO), konnte zudem gezeigt werden, dass sowohl durch das zirkulierende GDF15 als auch die Aktivierung der GFRAL-Signalachse eine Tageszeit-abhängige Anorexie sowie die systemische Anpassung des Energiestoffwechsels im TG Mausmodell vermittelt werden. Mechanistisch konnte dabei erstmalig eine GFRAL-abhängige Induktion von Corticotropin-releasing Hormone (CRH) im Hypothalamus sowie ein erhöhtes, GFRAL-abhängiges Angstverhalten in TG Mäuse beschrieben werden.

Zusammenfassend unterstreichen die Ergebnisse die systemische Rolle von GDF15 als Myokin und die Bedeutung der endokrinen Kommunikation zwischen Skelettmuskel und Gehirn, vermittelt durch GDF15-GFRAL Signalachse, für die Energiehomöostase bei mitochondrialer Fehlfunktion. Die gewonnen Erkenntnisse dieser Doktorarbeit können somit zur Entwicklung neuer Therapieansätze für Patienten mit einer mitochondrialen bzw. Stoffwechselerkrankung beitragen.

1. Introduction

1.1 Regulation of energy homeostasis

Energy homeostasis or the control of energy balance is the biological mechanism by which organisms are able to maintain body weight. These mechanisms act by sensing the energy status of the organism and acting on the control of food intake and energy expenditure. Energy balance is maintained when energy intake equals energy expenditure and thus, a dysregulation of this system may lead to body weight gain or loss, which is the case of pathologies such as obesity or anorexia nervosa. The mechanisms by which mammals control energy balance include a variety of peripheral signals and complex neuronal circuits that aim to maintain body weight by sensing the energy status of the organism (for review see Morton, Meek et al. 2014).

1.1.1 Endocrine regulation of energy metabolism

Peripheral organs are able to sense energy surplus or deficit and send endocrine signals that aim to restore energy balance. These may be anorectic or satiety signals (aimed to decrease food intake) or orexigenic signals (aimed to stimulate food intake) which are sensed and integrated by the brain through specific receptors expressed in different brain areas. Anorectic or satiety signals include cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide 1 (GLP1) (secreted by the intestine), glucagon, insulin and amylin (secreted by the pancreas), as well as leptin (secreted by the adipose tissue). Orexigenic hormones include ghrelin (secreted by the stomach), or adiponectin (secreted from adipose tissue) (for review see Abdalla 2017).

1.1.2 Neuronal circuits involved in energy homeostasis

Among the neuronal circuits controlling energy homeostasis, the melanocortin system has been the best studied and seems to be the most prominent in the regulation of energy balance. In this system, two sets of neurons located in the arcuate nucleus of the hypothalamus (ARC) have antagonizing actions to control food intake. On one hand, proopiomelanocortin (POMC) neurons lead to anorectic responses through the cleavage of POMC into α- and β-melanocyte stimulating hormone (α- and β-MSH), which bind to melanocortin receptor subtypes 3 and 4 (MC3R and MC4R) to reduce food intake and increase energy expenditure, thereby reducing body weight. On the other hand, neurons that produce neuropeptide Y (NPY) and agouti-related peptide (AgRP) are orexigenic. While NPY stimulates food intake and reduces energy expenditure, AgRP is an antagonist of

MC3R and MC4R, thereby preventing α/β-MSH from eliciting its anorectic effects (for review see Gao and Horvath 2008). Nevertheless, the ARC is not the only brain center involved in the regulation of food intake. Other important structures are the paraventricular nucleus of the hypothalamus (PVH), the lateral hypothalamic area (LHA), the ventromedial nucleus of the hypothalamus (VMN), the dorsomedial nucleus of the hypothalamus (DMN), the brainstem and structures involved in the reward system such as the amygdala, hippocampus, pre-frontal cortex and midbrain (for review see Gao and Horvath 2008, Abdalla 2017).

1.2 The stress response

Also known as “fight or flight” response, the stress response consists of a series of physical and emotional reactions to a potential stressor or threat. These physical reactions are aimed to acutely face the potential stressor and include an increased heart rate and breathing, decreased digestive activity and glucose release for its rapid utilization as fuel. Physiologically, the stress response comprises a wide range of brain structures that interconnect with each other and that can be divided into two main axes that work in a coordinated fashion: the sympathetic-adreno-medullar (SAM) axis and the hypothalamus-pituitary-adrenal (HPA) axis. The SAM constitutes the first phase of the stress response and ultimately secretes noradrenaline and norepinephrine to provide a rapid physiological response that raises awareness and alertness. On the other hand, the HPA axis involves the second phase of the stress response and is aimed to secrete glucocorticoids, resulting in a long lasting response (for review see Godoy, Rossignoli et al. 2018).

1.2.1 The HPA axis: structure and function

Anatomically, as its name indicates, the HPA axis consists of three main structures: the hypothalamus, more specifically the PVH, the pituitary gland and the adrenal glands (**Fig. 1**). The activation of the HPA axis starts in the PVH with the production of corticotropin releasing hormone (CRH). CRH is released into the circulation and stimulates the production of POMC in the pituitary gland, which is cleaved into adenocorticotropic hormone (ACTH) and again released into the circulation to stimulate the production of glucocorticoids (CORT) (cortisone in humans, corticosterone in rodents) by the adrenal glands. Glucocorticoids exert their effects by binding to the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), nuclear receptors that translocate to the nucleus to regulate gene expression after glucocorticoid binding. As a self-regulatory mechanism, glucocorticoids can act on the PVH and the pituitary gland to terminate the activation of the HPA-axis by blocking CRH and ACTH release and synthesis (for review see Gjerstad,

Lightman et al. 2018). Glucocorticoid secretion is regulated in a circadian manner in mammals with peak levels at start of the activity phase. Rhythmic glucocorticoid secretion is controlled by the central hypothalamic clock, the suprachiasmatic nucleus (SCN) and the peripheral clock of the adrenal glands (Dickmeis 2009).

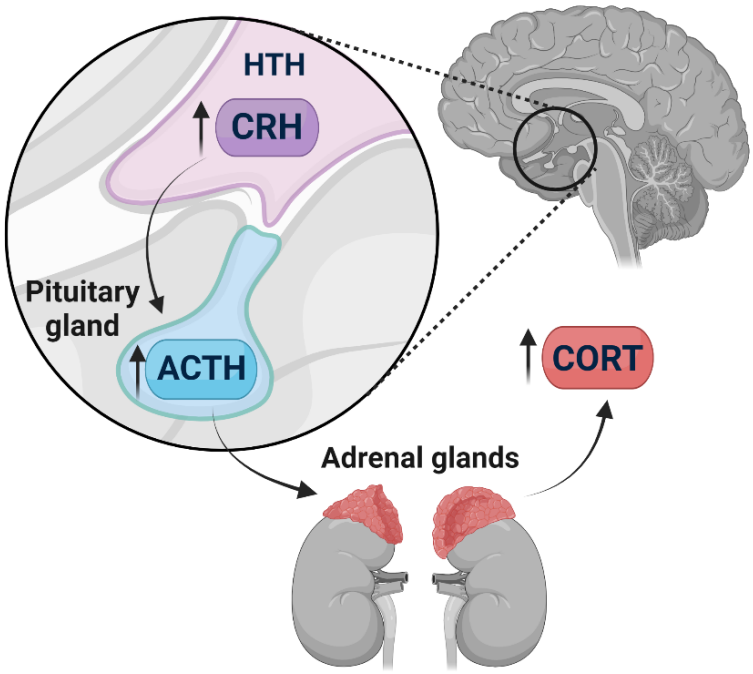


Figure 1. HPA axis signaling. Upon activation of the HPA axis, corticotropin releasing hormone (CRH) is produced and secreted from the hypothalamus (HTH) into the blood stream to reach the pituitary gland, where it stimulates the production of adenocorticotropic hormone (ACTH) which further reaches the circulation and stimulates the production of glucocorticoids (CORT) by the adrenal glands. Figure created with BioRender.com

1.2.2 Role of the HPA axis in energy metabolism

The HPA axis has an important regulatory function on energy balance, in particular under chronic stress conditions (Rabasa and Dickson 2016). There is a general consensus that glucocorticoids have orexigenic effects (Tataranni, Larson et al. 1996), and disturbed plasma cortisol levels represent different human pathologies. Addison's disease is characterized by hypocortisolism and leads to symptoms such as muscle weakness, fatigue and weight loss (Lovas and Husebye 2007). The other extreme is represented by Cushing's syndrome, which is characterized by hypercortisolism and leads to symptoms such as hypertension, insulin resistance, hyperglycemia and weight gain (Hankin, Theile et al. 1977). On the other hand, the anorectic effects of CRH have been well described. Intraventricular CRH infusion inhibits food intake in rats (Benoit, Thiele et al. 2000,

Richardson, Omachi et al. 2002). Furthermore, the anorectic effects of leptin have been proposed to occur through a CRH-dependent mechanism (Uehara, Shimizu et al. 1998). Mechanistically, CRH signaling has been suggested to act by inhibiting the orexigenic pathways involving NPY (Heinrichs, Menzaghi et al. 1993), but a broader mechanistic understanding of its anorectic effects is still lacking.

1.3 Growth differentiation factor 15

Growth differentiation factor 15 (GDF15), also named macrophage inhibitory cytokine 1 (MIC1) (Bootcov, Bauskin et al. 1997), placental transformation growth factor-β (PTGFB) (Li, Wong et al. 2000), NSAID activated gene 1 (NAG1) (Baek, Kim et al. 2001) and prostate derived factor (PDF) (Paralkar, Vail et al. 1998), was first identified in 1997 in activated macrophages and described as a divergent member of the transforming growth factor β (TGFβ) superfamily (Bootcov, Bauskin et al. 1997, Hsiao, Koniaris et al. 2000).

1.3.1 Physiological and molecular regulation of GDF15

Extensive literature has described GDF15 as a biomarker for a number of very diverse pathologies or conditions such as diabetes, cardiovascular disease (CVD) (Adela and Banerjee 2015), obesity (Vila, Riedl et al. 2011, Kempf, Guba-Quint et al. 2012), cancer (Wallentin, Zethelius et al. 2013, Wang, Yang et al. 2017) or mitochondrial disease (Fujita, Ito et al. 2015), among others. This, together with the fact that under normal circumstances GDF15 has a low expression level in most organs as well as low circulating levels, has contributed to the fact that GDF15 is regarded as a stress-induced cytokine. GDF15 is regulated by a number of factors both at the transcriptional and at the translational level. Since GDF15 is mostly recognized as a stress-induced factor, most of the known regulators, especially at the transcription level, are associated to cellular stress. Among the transcription factors that have been proposed to regulate *Gdf15* expression and that have binding sites in the *Gdf15* human promoter are p53 (Kelly, Lucia et al. 2009), SP1 and SP3 (Baek, Horowitz et al. 2001), early growth response protein 1 (ERG1) (Baek, Kim et al. 2004), activating transcription factor 3 (ATF3) (Lee, Kim et al. 2005) and cAMP-response element binding protein (CREB) (Impey, McCorkle et al. 2004). The proinflammatory transcription factor nuclear factor kappa light chain enhancer of activated B cells (NF-κB) also induces *Gdf15* expression (Ratnam, Peterson et al. 2017), which is in line with the roles in inflammation attributed to GDF15 as it was first discovered (Bootcov, Bauskin et al. 1997). Lately, the induction of *Gdf15* by the integrated stress response (ISR) via an activating transcription factor 4 (ATF4)/ C/EBP homologous protein (CHOP)-dependent mechanism through phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) has

been postulated as the main regulatory pathway for *Gdf15* induction under cellular stress, especially under conditions of mitochondrial- or endoplasmic reticulum (ER) stress (Chung, Ryu et al. 2017, Patel, Alvarez-Guaita et al. 2019) (**Fig. 2**). After transcription, GDF15 is synthesized in the cytoplasm as a pro-GDF15 monomer, which dimerizes to form a pro-GDF15 dimer which in turn can be cleaved at its RXXR motif to give rise to a mature GDF15 dimer. It has been demonstrated that cleavage of pro-GDF15 can be performed by the members of the proprotein convertase, subtilisin/kexin-type (PCSK) family PCSK3, PCSK5 and PCSK6 (Li, Liu et al. 2018). After cleavage, mature GDF15 is most likely secreted and released into the circulation (**Fig. 2**) while pro-GDF15 is kept locally and attaches to the extracellular matrix through its pro-peptide sequences (Bauskin, Brown et al. 2005).

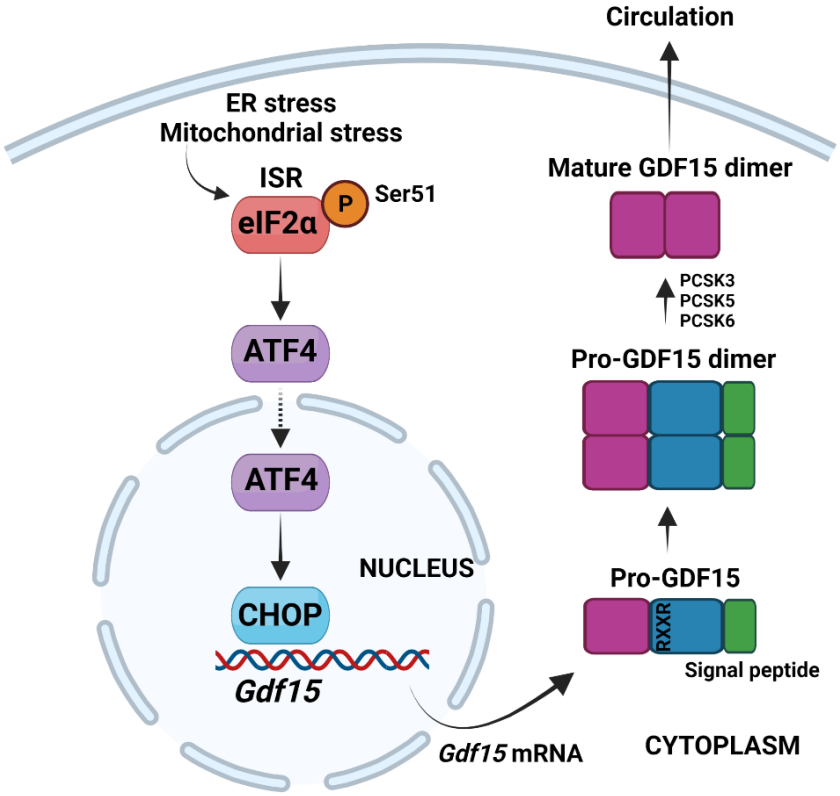


Figure 2. Induction of GDF15 by the integrated stress response. Cellular stress (endoplasmic reticulum (ER)- and mitochondrial stress) lead to the induction of the integrated stress response (ISR) involving the phosphorylation of eIF2α and the activation of the transcription factor ATF4, which translocates into the nucleus and subsequently activates CHOP, which in turn induces *Gdf15* gene expression. After transcription, *Gdf15* mRNA translocates to the cytoplasm where it is translated into the pro-GDF15 protein, which dimerizes to form a pro-GDF15 dimer and can be cleaved by PCSK3, 5 and 6 proteases at its RXXR motif, leading to the mature GDF15 dimer which is secreted into the circulation. Figure created with BioRender.com.

1.3.2 The GDF15 receptor: GFRAL

Four independent studies in 2017 identified the only so far known GDF15 receptor: GDNF family receptor alpha-like (GFRAL) (Emmerson, Wang et al. 2017, Hsu, Crawley et al. 2017, Mullican, Lin-Schmidt et al. 2017, Yang, Chang et al. 2017). As its name indicates, GFRAL belongs to the GDNF family of receptors, and, as such, signaling depends on the interaction with the tyrosine kinase coreceptor RET. GFRAL therefore associates with RET, whose phosphorylation is induced upon GDF15 binding to the complex. Phosphorylation of RET consequently induces the phosphorylation of the intracellular signaling mediators phospholipase C γ (PLCγ), protein kinase B (AKT) and extracellular regulated kinase 1/2 (ERK1/2) (Mullican, Lin-Schmidt et al. 2017, Yang, Chang et al. 2017) (**Fig. 3**).

Interestingly, these studies confirmed the exclusive location of GFRAL in the hindbrain, more specifically in two adjacent areas called the Area Postrema (AP) and the nucleus of the solitary tract, Nucleus Tractus Solitarius (NTS). A crucial function of the AP, one of the so-called circumventricular organs (CVOs), is the control of nausea and vomiting. Due to its location, it acts as a chemoreceptor for circulating toxins and emetic agents to induce vomiting (Miller and Leslie 1994). Furthermore, the AP has been demonstrated to be implicated in the regulation of food intake and energy homeostasis. Its privileged location outside the blood brain barrier makes it a perfect site for integration of appetite and satiety regulating signals coming from the periphery such as amylin, CCK, GLP1, PYY, adiponectin or ghrelin, hormones of which receptors have been found in this area (Price, Hoyda et al. 2008) (**Fig. 3**). Definite proof of the implication of this area in food intake behavior is that its ablation leads to highly increased food intake in rodents (Edwards and Ritter 1981). Furthermore, this area has been linked to anxiety-related behavior, since lesions in the AP lead to a decreased anxiety in rats as well as to elevated levels of NPY in the arcuate nucleus, the amygdala and the hippocampus. This elevation of NPY has been previously associated with diminished anxiety related behaviors (Miller, Holmes et al. 2002).

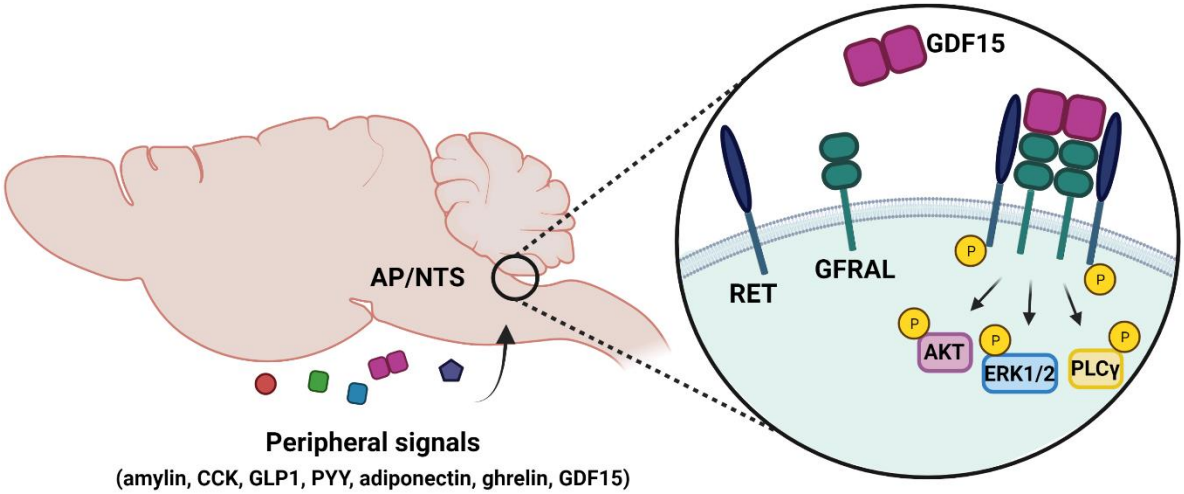


Figure 3. Localization and signaling of the GFRAL receptor in the mouse brain. The GFRAL receptor is located in the area postrema (AP) and nucleus of the solitary tract (NTS) in the hindbrain, a highly vascularized area that receives and integrates peripheral signals such as amylin, CCK, GLP1, PYY, adiponectin, ghrelin and GDF15. Upon GDF15 binding, the GFRAL receptor dimerizes with RET, which gets phosphorylated to allow for the signal transduction to take place through the phosphorylation of AKT, ERK1/2 and PLCγ. Figure adapted from Cimino, Coll et al. 2017 and created with BioRender.com.

The neuronal connections of GFRAL neurons to other brain areas have just started to emerge. Recent data showed that recombinant GDF15 injection leads to neuronal activation of the lateral parabrachial nucleus of the pons (PBN), the paraventricular nucleus of the hypothalamus (PVH), the central nucleus of the amygdala (CeA) and the oval sub-nucleus of the bed nucleus of the stria terminalis (ovBNST) (Worth, Shoop et al. 2020), but a broader understanding of downstream targets of the GDF15-GFRAL pathway is still lacking.

1.3.3 Role of GDF15 in appetite regulation and energy metabolism

So far, the most studied metabolic role of GDF15 is the control of food intake. First published in 2007 by Johnen and colleagues, tumor-induced GDF15 was shown to reduce food intake and body weight, a phenotype that was observed as well in transgenic GDF15-overexpressing mice (Johnen, Lin et al. 2007). With the discovery of the GDF15 receptor GFRAL, the mechanism of food intake suppression by GDF15 was partially elucidated. Food intake reduction, and in turn body weight loss induced by pharmacologically administered GDF15 was abolished in *Gfral*-knockout mice (Mullican, Lin-Schmidt et al. 2017), indicating a GFRAL-dependent effect of GDF15-induced anorexia.

GDF15 has not only been associated to body weight loss but also to other metabolic improvements in glucose metabolism. Macia and colleagues showed that GDF15-overexpressing transgenic mice display an improved glucose tolerance and response to

insulin (Macia, Tsai et al. 2012). Further, *ob/ob* mice (obese, leptin deficient mice) injected with GDF15 showed an increased insulin sensitivity and lipid catabolism (Chung, Ryu et al. 2017). Recently, metformin, the gold standard drug to treat type II diabetic patients, was shown to exert its beneficial effects on body weight and energy balance through GDF15 (Coll, Chen et al. 2019, Day, Ford et al. 2019). Interestingly, these studies also showed a GDF15-dependent improvement of glucose tolerance, most likely dependent on the associated body weight loss. Furthermore, the fact that GDF15 is induced by different nutritional stress conditions such as long-term high fat diet feeding (Xiong, Walker et al. 2017), fasting or lysine deficiency has promoted that it is regarded as an endocrine signal of nutritional stress (Patel, Alvarez-Guaita et al. 2019).

Up until now, the main suggested mechanism for a GDF15-dependent anorexia is the induction of nausea and emesis. In rats and mice, which are unable to vomit, recombinant GDF15 (rGDF15) injection or cisplastin-induced hepatic GDF15, respectively, lead to weight loss and pica behavior (intake of non-caloric substances such as kaolin). In shrews, rGDF15 injection induces vomiting and weight loss (Borner, Shaulson et al. 2020, Borner, Wald et al. 2020). In this regard, GDF15 has been shown to be positively associated with nausea and emesis in pregnant women (Fejzo, Arzy et al. 2018, Fejzo, Sazonova et al. 2018, Petry, Ong et al. 2018). Furthermore, rGDF15 infusion has proven to also promote conditioned taste aversion (CTA) (Patel, Alvarez-Guaita et al. 2019, Borner, Wald et al. 2020, Sabatini, Frikke-Schmidt et al. 2021), also in line with a role in promoting sickness-related behavior. Of note, all studies to date have been performed using high doses of rGDF15 or inducing it acutely via toxins, but there is as yet no evidence that pathological and pathophysiological states of chronically induced endogenous GDF15 induce similar physiological outcomes.

Altogether, it can be assumed that GDF15 is a crucial metabolic mediator that governs energy homeostasis under different physiological stress conditions.

1.3.4 Auto-/paracrine roles for GDF15

Although a role for GDF15 as a mediator of energy metabolism through its brainstem receptor GFRAL has been established, a large body of literature presents evidence that speaks for a role of GDF15 in an auto-/paracrine fashion.

The association between GDF15 and macrophages has been largely studied. In tumor cells, GDF15 is expressed in a NF-kB dependent manner to suppress macrophage killing activity (Ratnam, Peterson et al. 2017). Interestingly, GDF15 expression in macrophages itself is thought to improve oxidative function thereby improving insulin resistance in *ob/ob* mice and high fat diet (HFD)-fed mice (Jung, Choi et al. 2018). Furthermore, a recent study

reported a role of growth factor-expressing macrophages in orchestrating regenerative inflammation in skeletal muscle through GDF15 (Patsalos, Halasz et al. 2022).

In whole body *Gdf15*-knockout mice, the skeletal muscle gene expression of known members of the unfolded protein response (UPR), a cellular mechanism aimed to alleviate stress and restore cellular homeostasis, was exacerbated during the recovery phase after strenuous exercise, suggesting a possible role for GDF15 in reducing skeletal muscle damage after exercise (Gil, Ost et al. 2019). In liver, diet-induced GDF15 expression was shown to protect from non-alcoholic steatohepatitis (NASH) phenotypes such as hepatic inflammation and fibrosis (Kim, Kim et al. 2018). Furthermore, GDF15 has also been reported to protect from cardiovascular complications. In GDF15 deficient mice, isquemia/reperfusion injury led to greater infarct sizes and displayed an increased cardiomyocyte apoptosis at the injury site (Kempf, Eden et al. 2006). Mechanistically, GDF15 was shown to play a role in repressing the recruitment of polymorphonuclear leukocytes by interfering with chemokine signaling and integrin activation, thereby playing a crucial anti-inflammatory role (Kempf, Zarbock et al. 2011).

Taken together, it seems that GDF15 might play an auto-/paracrine role in injured tissues to alleviate stress and cellular damage, but further investigations are required to better understand its mechanisms of action.

1.4 Mitochondrial dysfunction

Mitochondria are crucial organelles for organism survival (Nunnari and Suomalainen 2012). They are often addressed as the powerhouse of the cell because of their ability to generate adenosine triphosphate (ATP), the energy currency of the cell, through a process called oxidative phosphorylation (OXPHOS). Five protein complexes and two electron carriers that are located in the inner membrane of mitochondria take part in OXPHOS. Complexes I-IV have the overall aim to generate an electrochemical gradient which ultimately drives ATP synthesis by ATP synthase (also called complex V). While ATP synthesis is the best-known function of mitochondria, these organelles are involved in other important cellular processes such as cell death, cell proliferation or storage of calcium ions. Therefore, any disturbances leading to mitochondrial dysfunction may have severe consequences for the organism. In humans, mitochondrial dysfunction can lead to a pathology called mitochondrial disease (MD), which is defined as a clinically heterogeneous disorder ultimately caused by a variety of mutations in genes that encode for proteins involved in mitochondrial function, predominantly in OXPHOS. Patients with MD suffer from a variety of symptoms that can affect different organs, among which are respiratory failure, liver failure, diabetes mellitus or pancreatitis. Furthermore, MD may lead to neurological-related symptoms such as

epilepsy, migraine, parkinsonism, psychiatric disorder or myopathy, among others (Russell, Gorman et al. 2020).

1.4.1 GDF15 and mitochondrial dysfunction

Mitochondrial dysfunction is one of the best-described physiological conditions that induces GDF15 (Yatsuga, Fujita et al. 2015, Montero, Yubero et al. 2016, Dominguez-Gonzalez, Badosa et al. 2020, Maresca, Del Dotto et al. 2020, Lehtonen, Auranen et al. 2021). One of the main mechanisms that mitochondria use to fight stress is the activation of the mitochondrial unfolded protein response (UPRmt), a signaling pathway that aims to activate communication between the mitochondria and the nucleus in order to restore mitochondrial function. The UPRmt ultimately converges with the ISR (**Fig. 2**), therefore leading to the phosphorylation of eIF2α and subsequent ATF4 and CHOP activation.

The ultimate role of the UPRmt is to promote the transcription of genes involved in restoring mitochondrial function, and, interestingly, among these genes is *Gdf15*, which in this context is regarded as a mitokine (mitochondrial stress-induced cytokine) (Kim and Lee 2021). Nevertheless, it is still unclear whether GDF15 induction plays a protective or a detrimental role during the mitochondrial stress response, both in an auto-/paracrine and in an endocrine fashion.

1.4.2 Mouse models of mitochondrial dysfunction

There is a large scientific community working on the field of mitochondrial dysfunction and making use of different mouse models of tissue-specific mitochondrial stress. These mouse models target different mitochondrial proteins that are involved in crucial functions for the normal functioning of mitochondria such as mitochondrial import, dynamics and quality control, OXPHOS efficiency, UPRmt, as well as mitochondrial RNA or DNA (mtRNA/mtDNA) dynamics (for review see Keipert and Ost 2021). Of note, most of these mouse models share what has been regarded as a healthy metabolic phenotype, including resistance to diet induced obesity (DIO), protection from hepatic steatosis, improved glucose homeostasis and/or insulin sensitivity. Furthermore, some of these models have reported an increased expression of GDF15 mRNA at the target organ of the mitochondrial mutation (Chung, Ryu et al. 2017, Choi, Jung et al. 2020, Keipert, Lutter et al. 2020, Pereyra, Rajan et al. 2020, Kang, Choi et al. 2021) and consequently, high levels of circulating GDF15 (Chung, Ryu et al. 2017, Choi, Jung et al. 2020, Pereyra, Rajan et al. 2020, Kang, Choi et al. 2021).

1.4.3 The HSA-*Ucp1*-transgenic mouse model

Skeletal muscle is the main organ in the body involved in the control of energy homeostasis due to its contribution to resting metabolic rate and total energy expenditure, playing as well a crucial role glucose homeostasis (for review see Frontera and Ochala 2015). This makes it, therefore, a promising target to study physiological outcomes of tissue-specific mitochondrial dysfunction.

The HSA-*Ucp1*-transgenic (TG) mouse is a model of skeletal muscle targeted mild mitochondrial dysfunction (Klaus, Rudolph et al. 2005, Keipert, Klaus et al. 2010). In this model, skeletal muscle mitochondrial dysfunction is achieved by the ectopic expression of low levels of uncoupling protein 1 (UCP1) in the inner membrane of the mitochondria that ultimately lead to the uncoupling of OXPHOS and thus, to a diminished respiratory capacity (Ost, Werner et al. 2014), thereby resulting in mitochondrial stress. As a consequence, these mice display a muscle atrophy with reduced muscle mass and myofiber size (Couplan, Gelly et al. 2002, Ost, Werner et al. 2014, Ost, Keipert et al. 2015). Furthermore, they present important metabolic adaptations such as resistance to DIO, improved glucose homeostasis, and an increased browning of subcutaneous white adipose tissue (sWAT) (Neschen, Katterle et al. 2008, Keipert, Voigt et al. 2011, Keipert, Ost et al. 2014), a hallmark of metabolically active fat regarded as beneficial to increase energy expenditure and counteract body weight gain (Herz and Kiefer 2019). Interestingly, the first human MD documented, Luft's disease, was shown to display clinical presentation of skeletal muscle atrophy due to increased muscle-specific mitochondrial respiratory uncoupling, energy depletion, and systemic hypermetabolism (Luft, Ikkos et al. 1962), phenotypical traits that seem to be similar to those of TG mice.

2. Aims of the study

The overall aim of this PhD thesis was to elucidate the role and mode of action of GDF15 and its receptor GFRAL under chronic skeletal muscle mitochondrial stress conditions using the established HSA-*Ucp1*-transgenic (TG) mouse as a model.

The first chapter of this thesis aimed to investigate the auto/paracrine and endocrine role of GDF15 on mitochondrial stress-induced metabolic adaptations in TG mice. For that, TG mice were crossed with whole body *Gdf15*-knockout (GdKO) mice in order to obtain TGx*Gdf15*-knockout (TGxGdKO) mice, on which *in vivo* metabolic and molecular analyses were carried out in comparison to TG mice using wildtype (WT) and GdKO as controls.

The second chapter of this thesis focused on identifying physiological responses and outcomes associated to an activation of the GDF15-GFRAL axis under muscle mitochondrial stress with an aim to further elucidate the molecular mechanisms elicited by this pathway. For this, TG mice in comparison to WT mice were subjected to different behavioral tests and molecular tissue analyses to narrow down physiological- and molecular pathways that may be involved in downstream effects of GDF15-GFRAL signaling.

The third chapter of this thesis focused on understanding the involvement of the GDF15 receptor GFRAL on the metabolic, behavioral and molecular outcomes identified in chapters 1 and 2. To achieve this, TG mice were crossed with whole body *Gfral*-knockout (GfKO) mice to generate TGx*Gfral*-knockout (TGxGfKO) mice on which an *in vivo* metabolic, behavioral and molecular characterization was performed in comparison to TG mice. WT and GfKO mice were used as controls.

3. Materials and methods

3.1 Animal experiments

All animal experiments presented in this thesis were approved by the ethics committee of the Ministry of Agriculture and Environment (State Brandenburg, Germany) (Permission number 2347-9-2016 for mouse study 1 and 2347-16-2020 for mouse studies 2, 3 and 4). Mice with a C57BL/6 background were used to generate the experimental genotypes. Unless otherwise indicated, mice were group-housed and random-caged with *ad libitum* access to standard chow diet (Sniff, Soest, Germany) and acidified, autoclaved water at 23ºC under a 12:12-h dark-light cycle. Mice were sacrificed with isoflurane narcosis followed by heart puncture, blood was collected in lithium-heparin tubes (#41.1503.005., Sarstedt, Germany) and tissues were snap-frozen in liquid nitrogen and stored at -80ºC.

3.1.1 Mouse study 1

For mouse study 1 (results presented in Results Chapter 1), HSA-*Ucp1*-transgenic mice were crossed with whole body *Gdf15*-knockout mice kindly provided by Dr. Se-Jin Lee (University of Connecticut School of Medicine, Department of Genetics and Genome Sciences) to generate the four experimental genotypes: wildtype (WT), *Gdf15*-knockout (GdKO), HSA-*Ucp1*-transgenic (TG) and TGx*Gdf15*-knockout (TGxGdKO). Mice were sacrificed at different ages after 3 hrs of food withdrawal.

3.1.1.1 Body weight and body composition measurement

Body weight and body composition were monitored biweekly. Quantitative magnetic resonance (QMR, EchoMRI 2012 Body Composition Analyzer, Houston, USA) was used for the measurement of body composition.

3.1.1.2 Oral glucose tolerance test

An oral glucose tolerance test (oGTT) was performed in male mice at 17 weeks of age. A glucose bolus of 2 mg per gram body weight was applied via oral gavage after 2 hrs of food withdrawal. Blood glucose levels were measured before (baseline) and 15, 30, 60 and 120 min after glucose treatment using a Contour Next glucose sensor (Bayer, Germany). Plasma was collected from the tail vein with microvettes (CB 300, Sarstedt, Germany) before (baseline) and 15, 30 and 60 min after application and insulin was measured with an ELISA assay (DRG Instruments GmbH, Germany). oGTT was performed in male mice at 17 weeks of age.

3.1.1.3 Indirect calorimetry

Using indirect calorimetry, energy expenditure, food intake, physical activity, and respiratory quotient (RQ = CO_2 produced/O_2 consumed) were assessed. The system used consists of an open respiratory system with the simultaneous measurement of cage activity, food, and water intake (TSE PhenoMaster, TSE Systems, Germany). Indirect calorimetry measurements were performed in male mice at 17-18 weeks of age in 10 min intervals over a period of 72 hrs. The last 24 hrs were used for analysis of energy metabolism parameters.

3.1.2 Mouse study 2

For mouse study 2 (results presented in Results Chapter 1, Fig. 14), WT and TG male mice were used. Mice were single-caged for one week prior to sacrifice. Mice were sacrificed at 16-18 wks of age along the 24 hrs light-dark cycle in 4 hrs intervals (6, 10, 14, 18, 22 and 2 hrs). Care was taken to minimize disturbances of sleep-awake cycles and stress levels upon time of sacrifice.

3.1.3 Mouse study 3 and 4

Mouse study 3 (results presented in Results Chapter 2) was conducted in WT and TG male and female mice. For mouse study 4 (results presented in Results Chapter 3), TG mice were crossed with *Gfral*-knockout (GfKO) mice to generate TGx*Gfral*-knockout (TGxGfKO) mice in order to obtain the four experimental genotypes: WT, GfKO, TG and TGxGfKO. Experiments were performed on male and female mice.

3.1.3.1 Open Field Test

At 10 weeks of age an Open Field Test (OFT) was conducted. Tests were performed for a total duration of 10 minutes between 9:00 and 11:00 hrs. The open field apparatus consisted of a 50x50cm enclosure. The mouse was placed in the center of the field and recorded with a camera using the software ANY-maze 5.2. Data were analyzed using the same software.

3.1.3.2 Elevated Plus Maze test

At 12 weeks of age an Elevated Plus Maze (EPM) test was performed. The test had a duration of 10 minutes and was performed between 9:00 and 11:00 hrs. The EPM apparatus consisted of two open (30x5x0.5cm) and two closed (30x5x15cm) arms, which cross each other in a middle platform (5x5x0.5cm) designated as the center. To start the test mice were placed in one of the open arms and were recorded using ANY-maze 5.2. Data were analyzed using the same software. Blood was collected from the tail vein one day before and right after the test with lithium-heparin microvettes (CB 300, Sarstedt, Germany) for corticosterone measurements.

3.1.3.3 Kaolin preference test and indirect calorimetry

A kaolin preference test was performed at 18-19 weeks of age in mouse study 3. The test was performed in indirect calorimetry cages (described previously in section 3.1.1.3). Mice were presented with two food hoppers, one with standard chow diet and the other with kaolin pellets (#K50001, Research Diets, USA). After an adaptation period of 5 days, food intake of both food hoppers was recorded during 48 hrs, with a change of the position of the food hoppers after the first 24 hrs.

Indirect calorimetry was performed in mouse study 4 as previously described for mouse study 1 (section 3.1.1.3).

3.1.4 Antalarmin treatment with simultaneous recording of food intake

To perform the antalarmin treatment experiment with simultaneous recording of food intake a “Food and Drink Measurement” system (Phenomaster, TSE Systems GmbH) was used. This system allows for automatic recording of food intake. Mice were single-caged in the “Food and Drink Measurment” system cages for 72 hrs prior to start of the measurements. The measurements took place during the next three consecutive days. On day one, no intervention was performed in order to obtain basal food intake measurements. On day two, an intraperitoneal (IP) injection of vehicle (0,9% saline and 10% Cremophore® EL (Merk, #238470)) was conducted. On day three, mice received an IP injection of the CRHR1 antagonist antalarmin (30 mg/kg) (Sigma-Aldrich, #A8727) dissolved in vehicle. IP injections were conducted at 8:00 hrs. Food intake was recorded until 24 hrs after antalarmin injection.

3.2 Tissue analyses

3.2.1 Plasma analyses

Whole blood was centrifuged at 9,000 g for 10 min at 4ºC and plasma was stored at -80ºC. Plasma GDF15 was quantified using the Mouse/Rat GDF-15 Quantikine ELISA Kit (#MGD150, Bio-techne, USA). Plasma corticosterone was measured with a corticosterone ELISA kit (#ADI-900-097, Enzo, USA). Plasma ghrelin and plasma leptin were measured with a Meso-Scale Discovery (MSD) multiplex assay (MSD instruments, USA).

3.2.2 Gene expression analyses

3.2.2.1 RNA isolation

Quadriceps and sWAT tissues were ground prior to start of RNA isolation protocol while whole hypothalamus, pituitary gland and AP/NTS were used for the isolation. 40-70 mg of

tissue or whole organs were homogenized for 3 min at 50 Hz using peqGOLD Trifast (#732-3314, VWR International GmbH, Germany), zirconium beads and a TissueLyser LT (#85600, Qiagen, USA). After lysis, 200-400 µL chloroform were added to the sample, which was vortexed and incubated for 10 min at room temperature. Afterwards, samples were centrifuged at 18,400 g for 20 min at 4ºC and the resulting upper aqueous phase was mixed with 500 µL of isopropanol in order to start the precipitation of RNA. Tubes were inverted until a homogeneous, clear mix was observed and incubated on ice for 10 min. RNA was precipitated at 18,400 g for 60-120 min according to pellet size. After the pellet was washed once with 75% EtOH and once again with 100% EtOH, it was air dried and resuspended in diethyl pyrocarbonate (DEPC) water (MP Biomedicals LLC, USA) in a volume adjusted to the pellet size (20-40 µL). Samples were incubated at 60ºC for 5 min and immediately transferred to ice prior to measurement of RNA concentration using a Take3 plate (Gene5 2.05, BIOTEK).

3.2.2.2 DNAse digestion

A DNAse digestion was performed in order to avoid contamination of DNA traces in the purified RNA. Following manufacturer instructions (#EN0521, Fisher Scientific, USA), 4 to 8 µg were mixed with DEPC water to a final volume of 24 µL. 6 µL of a master mix containing 3µL of 10X reaction buffer (with $MgCl_2$), 2 µL of DNAse (1 U/µL), 0,75 µL of Ribolock (RNAse, 1 U/µL) and 0,25 µL of DEPC water was added to each reaction. The mix was incubated for 30 min at 37ºC and after the incubation, 1 µL of 50mM EDTA was added to each reaction and further incubated for 10 more min at 65ºC. After incubation samples were immediately placed on ice.

3.2.2.3 Reverse transcription

Complementary DNA (cDNA) was synthesized from 1 µg RNA. Reverse transcription was performed according to manufacturer instructions (RT SuperMix Kit E3010, LunaScript ®, NEB, USA). 1 µg RNA was diluted to a total volume of 16 µL in DEPC water. 4 µL of LunaScript RT SuperMix (5X) were added to each sample and the mix was incubated in the thermocycler with the following steps (**Table 1**):

Table 1. Reverse transcription temperature protocol

Cycle step	Temperature	Time
Primer annealing	25°C	2 minutes
cDNA synthesis	55°C	10 minutes
Heat inactivation	95°C	1 minute

After incubation samples were diluted with 180 µL DEPC water to a final volume of 200 µL and a final cDNA concentration of 5 ng/µL. Until usage, samples were stored at -20ºC to -80ºC.

3.2.2.4 qPCR

For performing qPCR analyses, 384 well plates (#731-0193, VWR International GmbH, Germany) were used and samples were pipetted in triplicates. Per reaction, 1 µL of cDNA (5 ng/µL) and 4 µL of a master mix containing 2,5 µL of Luna Universal Master Mix (#M3003E, NEB, USA), 0,5 µL of 3 µM forward and reverse primers (for primer sequences see **Table 2**) and 0,5 µL of DEPC water was used. Plates were centrifuged, sealed with an ultra-clear foil (#G060/UC-RT, Kisker, Germany) and inserted into the qPCR device (Quantstudio 7, Fisher Scientific, USA), which was programmed to accomplish the PCR protocol presented in **Table 3**.

Table 2. qPCR primer sequences

Gene ID	Gene name	Primers
Actb	*Actin Beta*	5′ GCCAACCGTGAAAAGAGAC 3′ (F)
		5′ TACGACCAGAGGCATACAG 3′ (R)
Agrp	*Agouti Related*	5’ TTGGCGGAGGTGCTAGAT 3’ (F)
	Neuropeptide	5’ ACTCGTGCAGCCTTACACAG 3’ (R)
Aspg	*Asparaginase*	5′ AGGCATCAGAGTGTCATT 3′ (F)
		5′ GGCACAGTGTCCATCATA 3′ (R)
Atf4	*Activating Transcription*	5′ GGAATGGCCGGCTATGG 3′ (F)
	Factor 4	5′ TCCCGGAAAAGGCATCCT 3′ (R)
Atf5	*Activating Transcription*	5′ CTACCCCTCCATTCCACTTTCC 3′ (F)
	Factor 5	5′ TTCTTGACTGGCTTCTCACTTGTG 3′ (R)
Atf6	*Activating Transcription*	5′ CTTCCTCCAGTTGCTCCATC 3′ (F)
	Factor 6	5′ CAACTCCTCAGGAACGTGCT 3′ (R)
B2m	*Beta-2-Microglobulin*	5′ CCCCACTGAGACTGATACATACGC 3′ (F)
		5′ AGAAACTGGATTTGTAATTAAGCAGGTTC 3′ (R)
Chop	*C/EBP Homologous Protein*	5′ AGAGTGGTCAGTGCGCAGC 3′ (F)
		5′ CTCATTCTCCTGCTCCTTCTCC 3′ (R)
Cpt1b	*Carnitine*	5′ GAAGAGATCAAGCCGGTCAT 3′ (F)
	Palmitoyltransferase 1B	5′ CTCCATCTGGTAGGAGCACA 3′ (R)
Crh	*Corticotropin Releasing*	5’ CAACCTCAGCCGGTTCTGAT 3’ (F)
	Hormone	5’ CAGCGGGACTTCTGTTGAGA 3’ (R)
Dio2	*Iodothyronine Deiodinase 2*	5′ TGCCACCTTCTTGACTTTGC 3′ (F)
		5′ GGTTCCGGTGCTTCTTAACC 3′ (R)
Gapdh	*Glyceraldehyde-3-*	5′ AACTTTGGCATTGTGGAAGG 3′ (F)
	Phosphate Dehydrogenase	5′ ACACATTGGGGGTAGGAACA 3′ (R)
Gdf15	*Growth Differentiation*	5′ GAGCTACGGGGTCGCTTC 3′ (F)
	Factor 15	5′ GGGACCCCAATCTCACCT 3′ (R)
Gfral	*GDNF Family Receptor*	5’ CGAAATGATGAATTATGCAGGA 3’ (F)
	Alpha Like	5’ TGCAGGTCTCATCTTCATGG 3’ (R)
Pomc	*Proopiomelanocortin*	5’ AACCTGCTGGCTTGCATC 3’ (F)
		5’ GACCCATGACGTACTTCCG 3’ (R)
Ppara	*Peroxisome Proliferator*	5’ TGGCAAAGTCTTAGTGCCAGA 3’ (F)
	Activated Receptor Alpha	5’ TCACTAGGTCACACAGCCTCT 3’ (R)
Ucp1	*Uncoupling Protein 1*	5′ TGGAGGTGTGGCAGTATTC 3′ (F)
		5′ AGCTCTGTACAGTTGATGATGAC 3′ (R)

Cycle threshold (Ct) values were obtained by adjusting a threshold for each gene at the exponential phase of the logarithmic amplification curve with the Quantstudio 12K Flex Software. Relative differences in gene expression were calculated by the delta Ct (dCt) and delta delta Ct (ddCt) methods, in which at first Ct values of the gene of interest are related to the reference or house keeper gene (dCt) and second, dCt values are normalized to a control group (ddCt).

Table 3. qPCR temperature protocol

Step	Temperature	Time	Number of cycles
Initial denaturation	95ºC	60 sec	1
Denaturation	95ºC	15 sec	40
Extension	60ºC	30 sec	
Melt curve	60-95ºC	various	1

3.2.3 Protein expression analyses

3.2.3.1 Protein isolation for GDF15 ELISA analysis

For the measurement of GDF15 in muscle tissue, 40 mg of ground quadriceps tissue were aliquoted. Tissue was lysed using 5 sterile zirconium beads and 120 µL of lysis buffer (10 mM Tris pH 7.4, 0,025% Triton-X-100, Sigma-Aldrich) for 3 min at 50 Hz in a TissueLyser LT (#85600, Qiagen, USA). Lysed samples were incubated for 10 min on ice and centrifuged for 10 min at 23,400 g and 4°C. After centrifugation the clear supernatant was transferred into low binding Eppendorf tubes (#72.706.600, Sarstedt, Germany). GDF15 was measured with the Mouse/Rat GDF-15 Quantikine ELISA Kit (#MGD150, Bio-techne, USA) using 50 µL of undiluted lysate. GDF15 content measured by the ELISA was normalized to total protein content, which was measured by the Lowry method (detailed in section 3.2.3.3).

3.2.3.2 RIPA protein isolation

To generate protein lysates of quadriceps muscle for Western Blot analyses, muscles were ground and 25 mg were weighted and aliquoted. Ground muscle was homogenized with 450 µL of a lysis buffer consisting of RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 0,25% Na-Desoxycholate and 1% Triton X-100) and Halt Protease and Phosphatase Inhibitor Cocktail (100x) (#10085973, Thermo Scientific, USA). After adding lysis buffer to the sample, 10 zirconium beads were added and samples were homogenized for 3 min at

50 Hz in a TissueLyser LT (#85600, Qiagen, USA). After homogenization, samples were transferred into protein low binding Eppendorf tubes (#72.706.600, Sarstedt, Germany) and centrifuged at 21,000 g at 4ºC for 30 min. After centrifugation, the clear supernatant was transferred into a fresh low binding tube and the lysates were stored at -20ºC until further usage.

3.2.3.3 Protein determination by Lowry

For protein determination, a protein assay kit (#500-0116, Bio-Rad, USA) based on the Lowry detection method was used. For the assay, protein lysates were diluted 1:20 in autoclaved Milli-Q (Merck Millipore, USA) water in low binding protein tubes. A standard curve with nine ascending concentrations (0,1 µg/µL to 1 µg/µL) was made from a 2 mg/ml BSA stock (#224B-B, Bioscience, Germany). 15 µL of samples (in triplicates), 15 µL of standards (in duplicates) and 15 µL of blank (1:20 lysis buffer in water) were added to a 96-well plate (#82.1581, Sarstedt). A working solution of reagents A and S was made according to manufacturer instructions, 25 µL were added to each well and the plate was mixed gently. 200 µL of reagent B were added to each well, the plate was again gently mixed and incubated at room temperature for a minimum of 15 minutes. After incubation, absorbance was measured at a wavelength of 750 nm. Protein concentrations were determined by extrapolation to the standard curve through linear regression.

3.2.3.4 Western Blot

For Western Blot, samples were diluted in 4x laemmli solution (0,25 M Tris-HCl, 8% SDS, 40% glycerol, 0,04% bromphenol blue (pH 6.8)) with 10% 1 M dithiothreitol (DTT) that was freshly prepared. 50 µL of protein lysates were mixed with 16,67 µL of laemmli/DTT solution, vortexed and incubated for 5 min in a thermoshaker at 95ºC.

For electrophoresis, running and stacking gels were prepared as described in **Table 4**. After polymerization of the gels, the electrophoresis system was mounted (#1658002, Mini-Protean®Tetra Cell) 1x running buffer (#1610732, 10x Tris/Glycine/SDS Buffer, BioRad, Germany) was added into the chamber and a total of 15 µg of protein was loaded for every sample. Electrophoresis was carried out at 60 V for the first 30 min or until the samples collected at the beginning of the running gel and at 120 V for up to 120 min until desired separation of proteins was achieved.

Table 4. Running and stacking gel composition

Reagents	Running gel (10%)	Stacking gel (4%)
Milli Q water	2,9 mL	1,6 mL
10% SDS	60 µL	25 µL
4x Running gel buffer[1]	1,5 mL	-
4x Stacking gel buffer[2]	-	0,625 mL
Acrylamide/Bis 30[3]	1,5 mL	0,25 mL
APS 10%[4]	40 µL	15 µL
TEMED[5]	6 µL	2,5 µL

[1]1,5M Tris Base (pH 8.8); [2]0,5M Tris Base (pH 6.8); [3]#3029.1, Carl Roth; [4]#9592.3, Carl Roth; [5]#2367.3, Carl Roth

Western blotting was performed in a semi-dry fashion. All components were equilibrated in transfer buffer (48 mM Tris, 1,3 mM SDS, 20% methanol, pH 9.2) prior to blotting. A sandwich was built using (from bottom to top) 6 filter papers, a polyvinylidene difluoride (PVDF) membrane, the gel and 6 more filter papers. The PVDF membrane was activated in methanol for one minute, washed in water and equilibrated in transfer buffer prior to use. Transfer of two sandwiches was performed simultaneously using the Trans Blot Turbo™ system (BioRad, USA) for 30 min at 25 V. After transfer, membranes were incubated in ponceau solution (0,4% Ponceau-S, 40% ethanol, 15% acetic acid) for confirmation of successful protein transfer. Membranes were then washed three times for 5 min in TBST (1x TBS Buffer (10x TBS buffer: 200 mM Tris 1,37 M sodium chloride), 1% Tween-20) and further incubated for 1 h in 5% milk in TBST solution for blocking of free binding sites. After the blocking step, membranes were again washed three times for 5 min in TBST and incubated overnight at 4ºC on a shaker in the primary antibody solution (1:1000 p-eIF2α^{Ser51} (#3597, Cell Signaling Technology, USA) in 5% BSA/TBST solution). Membranes were then washed three times for 5 min in TBST and incubated for one hour at room temperature in anti-rabbit secondary antibody (#7074, Cell Signaling Technology, USA) diluted 1:5000 in 5% milk/TBST. Membranes were again washed and proteins were detected using a commercially available kit for generating a chemiluminescent reaction (Advansta, Biozym, Germany) and a Fusion SL Vilber Lourmat (Peqlab, Erlangen, Germany) device. After detection, membranes were washed and stripped with stripping buffer (200mM glycerin,

0,1% SDS, 0,1% Tween20, pH 2.2) in order to detach antibodies from the membrane. Membranes were again blocked and the same procedure was repeated to detect total eIF2α (#5324, Cell Signaling Technology, USA; dilution 1:2000 in 5% BSA/TBST).

Protein expression was analyzed with the software ImageJ. Raw intensity values were normalized to the control group.

3.2.4 Histology

For histological analyses, tissues were fixed in 4% paraformaldehyde (PFA) immediately after dissection. Tissues were then embedded in paraffin and cut into 2 µm sections that were placed in a microscope slide and stained with the hematoxylin-eosin (H&E) method. Stained tissues were observed and imaged under the microscope (Eclipse E800, Nikon GmbH, Germany). For the calculation of myofiber cross-sectional area (CSA), images were analyzed using the software Image J.

3.3 Statistical analysis

For statistical analysis the software GraphPad Prism 9 (GraphPad Software Inc.) was used. Outliers were identified with the ROUT method and excluded for further analysis of data. Data were tested for normality using the Kolmogorov-Smirnov test. Normally distributed data were analyzed using one-way ANOVA with Tukey's multiple comparison test (for analysis of differences between more than two groups) or a two tailed unpaired student's t-test (for analysis of differences between two groups). Statistically significant data were defined when the *P*-value was below 0.05 (**P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001).

4. Results

4.1 Chapter 1: Role of GDF15 as a myokine under muscle mitochondrial stress

As previously described, GDF15 is a cytokine secreted by different tissues under various stress conditions. The overall aim of the study presented in this chapter was to understand its role under skeletal muscle-specific mitochondrial stress or dysfunction making use of the TG mouse model.

4.1.1 GDF15 is secreted as a myokine under skeletal muscle mitochondrial stress

First, the aim was to characterize whether skeletal muscle mitochondrial stress in TG mice leads to the induction of GDF15 as a myokine (muscle-induced cytokine). Therefore, a multi-tissue transcriptomic profiling of *Gdf15* gene expression was conducted in TG mice using WT as a control, confirming the induction of *Gdf15* gene expression specifically in skeletal muscle tissues (**Fig. 4A**) Furthermore, muscle mGDF15 protein content was confirmed to be increased in TG mice (**Fig. 4B**). In order to understand whether GDF15 is secreted by the muscle in TG mice, soleus (SOL) and extensor digitorium longus (EDL) muscles were dissected and GDF15 was measured in the supernatant of conditioned medium after 2 hrs of incubation. Indeed, an increased secretion of GDF15 in both SOL and EDL muscles of TG mice in comparison to WT was evident (**Fig. 4C**). This result was in line with plasma GDF15 concentrations, which were highly increased in TG male and female mice compared to their WT littermates (**Fig. 4D**).

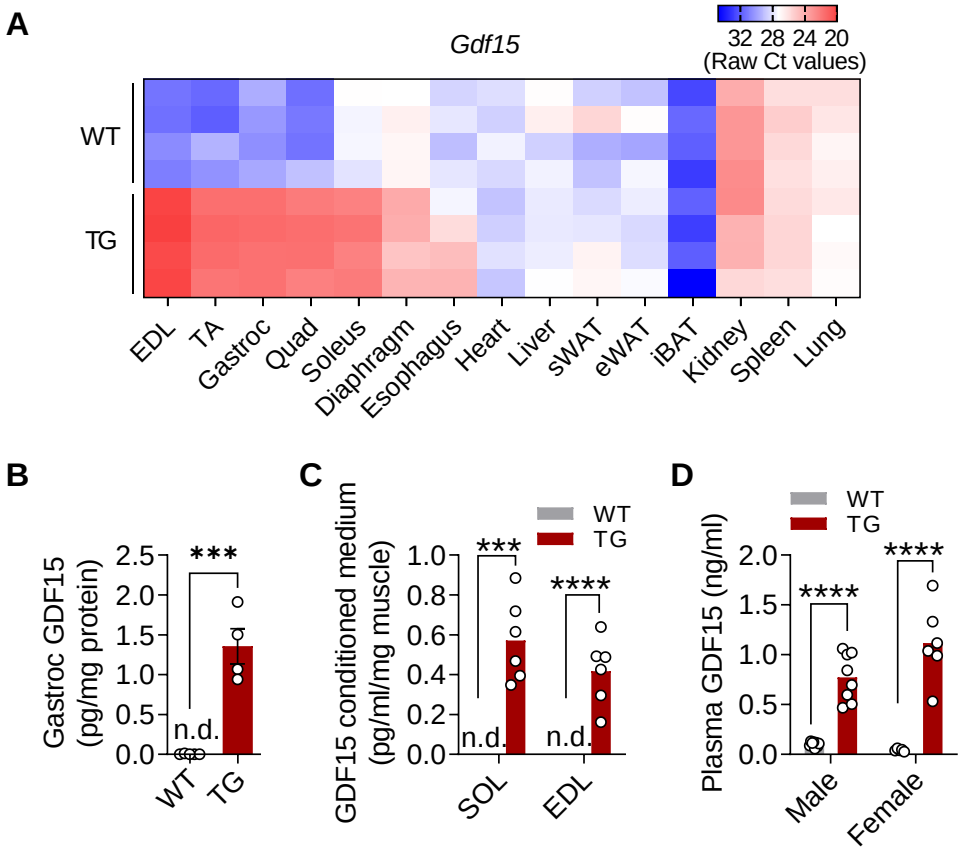


Figure 4. Muscle mitochondrial stress promotes GDF15 as a myokine. (**A**) Multi-tissue transcriptomic profiling of *Gdf15* gene expression. Heatmap is shown as raw Ct expression values (n=4). (**B**) Gastrocnemius (gastroc) GDF15 protein content normalized to total protein content (n=4-5). (**C**) *Ex vivo* secretion of GDF15 in soleus (SOL) and extensor digitorum longus (EDL) after 2 hrs incubation normalized to muscle wet weight (mg) (n=6) (**D**) GDF15 plasma levels in male and female mice (n=5-9). Data correspond to 20 wks old male mice and are expressed as means ± SEM; *P*-value calculated by unpaired t-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; TG, HSA-*Ucp1*-transgenic.

The ISR has been suggested as a mechanism of GDF15 induction under mitochondrial stress (Chung, Ryu et al. 2017). Therefore, the induction of the ISR in the muscles of TG mice was investigated in order to evaluate its involvement in the induction of GDF15. Indeed, gene expression of the well-established ISR members *Atf4*, *Atf5*, *Atf6* and *Chop* was significantly increased in the muscles of TG mice (**Fig. 5A**). Furthermore, phosphorylation of eIF2α was induced in the skeletal muscle of TG mice (**Fig. 5B, C**), confirming an activation of the ISR in skeletal muscle of TG mice possibly leading to the induction of GDF15.

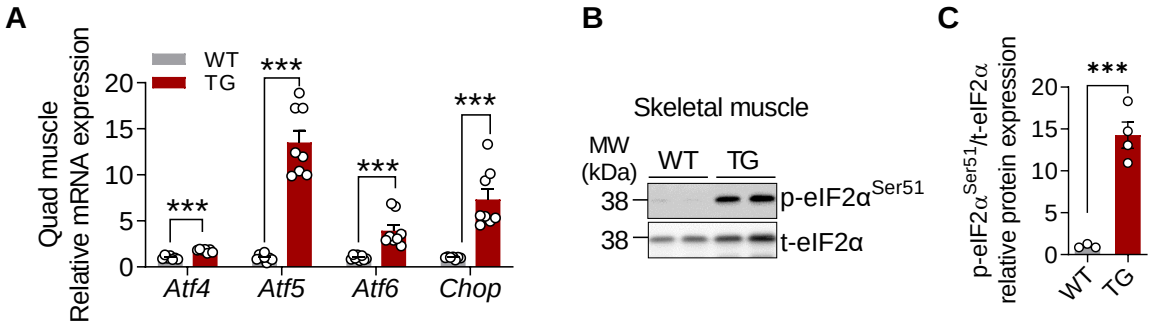


Figure 5. Muscle mitochondrial stress activates the integrated stress response in TG mice. (**A**) Quadriceps (quad) relative mRNA expression of the integrated stress response (ISR) markers *Atf4*, *Atf5*, *Atf6* and *Chop* (n=7-8). (**B**) Representative immunoblots of p-eIF2α^{Ser51} and t-eIF2α in skeletal muscle (quadriceps) and (**C**) quantification of p-eIF2α^{Ser51} relative to total protein expression in quadriceps muscle (n=3-4). Data correspond to 20 wks old male mice and are expressed as means ± SEM; *P*-value calculated by unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. WT, wildtype; TG, HSA-*Ucp1*-transgenic.

4.1.2 GDF15 does not contribute to muscle wasting and mitochondrial stress response in TG mice

In order to elucidate the role of GDF15 under muscle mitochondrial stress in TG mice, *Gdf15*-knockout TG mice (TGxGdKO) were generated and subjected to a molecular and metabolic phenotyping. To generate TGxGdKO mice, TG mice were crossed with whole body *Gdf15*-knockout mice (GdKO), since these mice do not present metabolic alterations under normal conditions and *Gdf15* mRNA induction is specific to skeletal muscle in TG mice (**Fig. 4A**). Both muscle mRNA expression of *Gdf15* (**Fig. 6A**) and circulating GDF15 (**Fig. 6B**) were undetectable in TGxGdKO mice, validating the mouse model for further investigations.

First, aiming to characterize possible auto-/paracrine roles of GDF15, the focus was put on understanding the potential effects of GDF15 on skeletal muscle structure and function. While TG mice present a significantly lower skeletal muscle tissue mass compared to WT mice (Ost, Keipert et al. 2015), this was not affected by the loss of GDF15 (**Fig. 6C**), which also did not have an effect on the reduced myofiber size in TG mice (**Fig. 6D**) as confirmed by the quantification of myofiber cross-sectional area (CSA) (**Fig. 6E**).

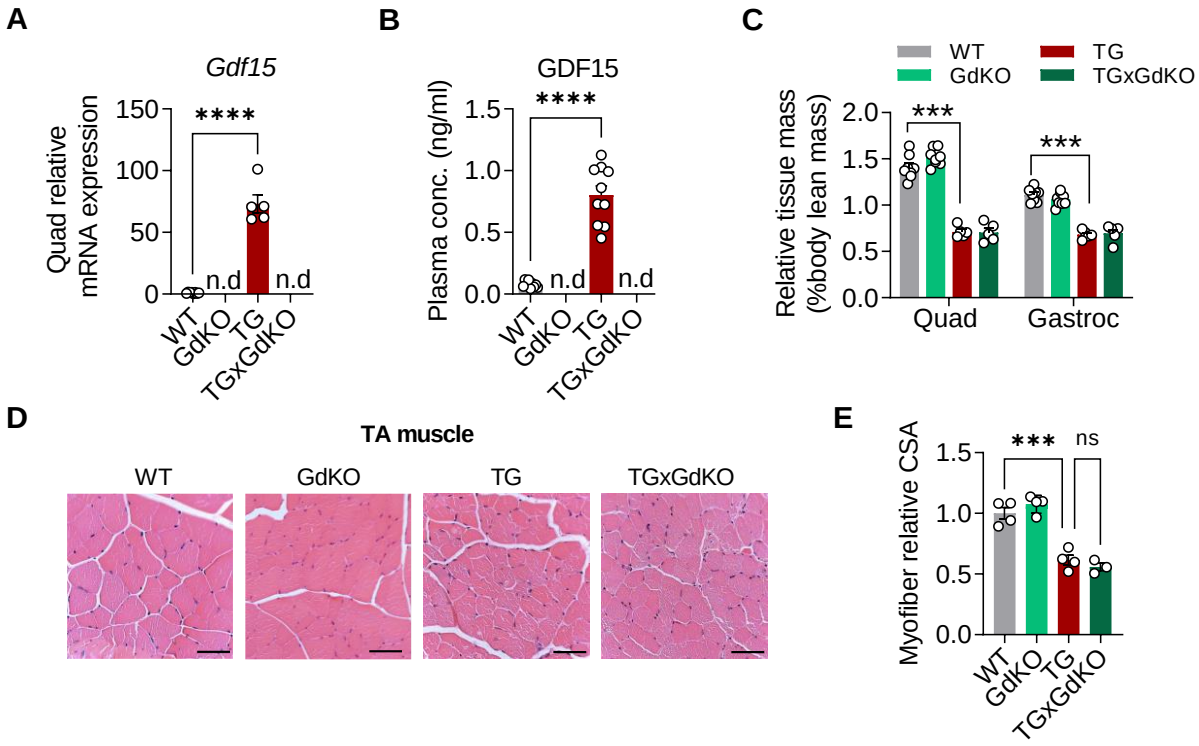


Figure 6. Muscle atrophy in TG mice is independent of GDF15. (**A**) Quadriceps (quad) relative mRNA expression of *Ucp1* and *Gdf15* (n=5-8); (**B**) Plasma circulating GDF15 (n=9-10); (**C**) Relative tissue mass to body lean mass of quadriceps (quad) and gastrocnemius (gastroc) muscles (n=5-8). (**D**) Representative H&E histological staining of tibialis anterior (TA) muscle (scale bar represents 50 µm) and (**E**) myofiber relative cross-sectional area (CSA) normalized to WT (n=3-4). Data correspond to 95 wks old male mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; GdKO, *Gdf15*-knockout; TG, HSA-*Ucp1*-transgenic; TGxGdKO, HSA-*Ucp1*-transgenic x *Gdf15*-KO.

Further, the effects of GDF15 loss on the induction of the ISR were investigated, confirming that gene expression of the ISR components *Atf4, Atf5, Atf6* and *Chop* (**Fig. 7A**) as well as phosphorylation of eIF2α (**Fig 7B, C**) were unaffected by the lack of GDF15. Altogether, these results indicate that GDF15 is neither involved in skeletal muscle atrophy nor in the modulation of the stress response in TG mice in an auto-/paracrine fashion.

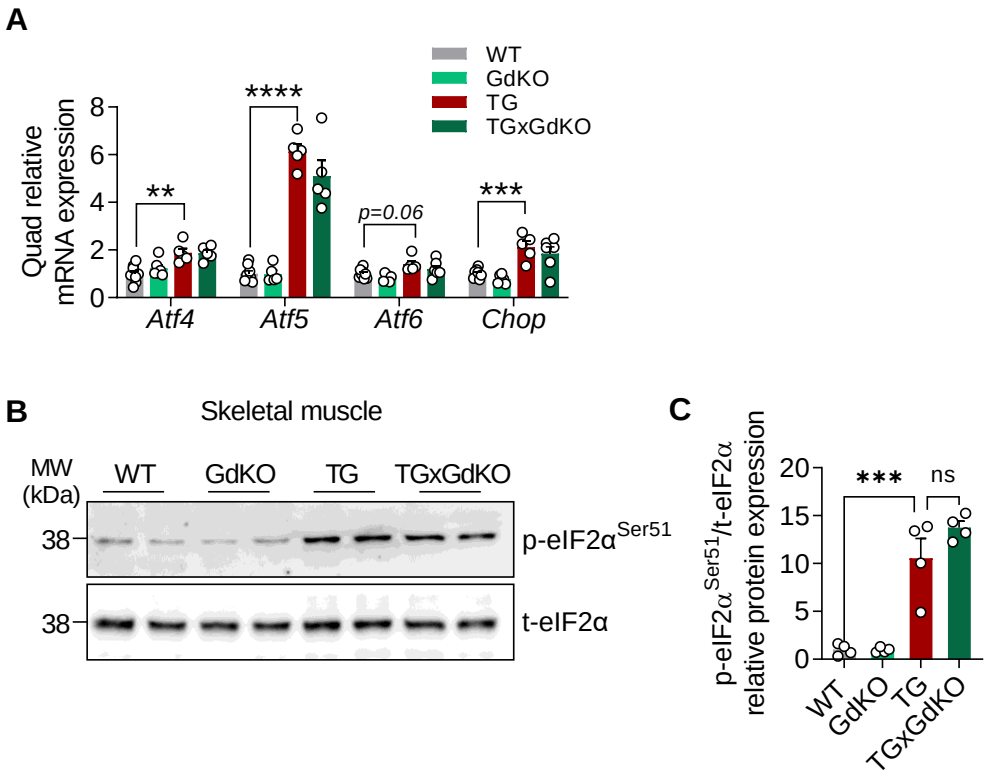


Figure 7. Muscle mitochondrial stress-induced integrated stress response works independently of GDF15. (**A**) Quadriceps (quad) relative mRNA expression of the integrated stress response (ISR) markers *Atf4*, *Atf5*, *Atf6* and *Chop* (n=5-8). (**B**) Representative immunoblots of p-eIF2α and t-eIF2α in quadriceps muscle and (**C**) quantification of p-eIF2α^{Ser51} relative to total protein expression in quadriceps muscle (n=4). Data correspond to 95 wks old male mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. WT, wildtype; GdKO, *Gdf15*-knockout; TG, HSA-*Ucp1*-transgenic; TGxGdKO, HSA-*Ucp1*-transgenic x *Gdf15*-KO.

4.1.3 GDF15 controls body composition and metabolic remodeling in TG mice

The next step was to evaluate potential endocrine effects of GDF15 under muscle mitochondrial stress. Interestingly, TGxGdKO mice gained body mass compared to TG mice (**Fig. 8A**), indicating an involvement of GDF15 in the control of body weight. This body mass increase was not due to an increase in body lean mass, which remained unchanged in TGxGdKO mice compared to TG mice (**Fig. 8B**), but to a significant increase in body fat mass (**Fig. 8C**).

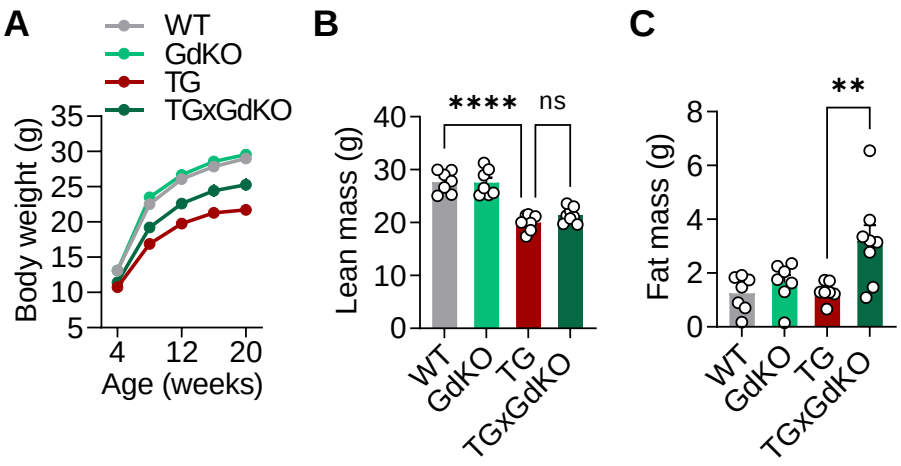


Figure 8. GDF15 controls body composition during muscle mitochondrial dysfunction. (**A**) Body mass, (**B**) lean mass and (**C**) fat mass at 20 wks of age. Data correspond to male mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. WT, wildtype; GdKO, *Gdf15*-knockout; TG, HSA-*Ucp1*-transgenic; TGxGdKO, HSA-*Ucp1*-transgenic x *Gdf15*-KO.

In line with these results, there was an increase of fat depot weight in TGxGdKO mice compared to TG mice (**Fig. 9A**). It has previously been shown that muscle mitochondrial stress promotes the metabolic activation of sWAT depots ("browning") in TG mice and other mouse models (Tyynismaa, Carroll et al. 2010, Kim, Jeong et al. 2013, Keipert, Ost et al. 2014, Chung, Ryu et al. 2017, Becker, Kukat et al. 2018). Interestingly, loss of GDF15 in TG mice abolished the increased gene expression of the browning markers *Ucp1*, *Cpt1b*, *Dio2*, *Aspg* and *Ppara* in sWAT tissue (**Fig. 9B**). In line with these results, H&E staining of sWAT revealed a browning-like multilocular morphology in TG mice that was absent in TGxGdKO mice (**Fig. 9C**). These results, therefore, indicate that GDF15 is involved in the browning phenotype of TG mice.

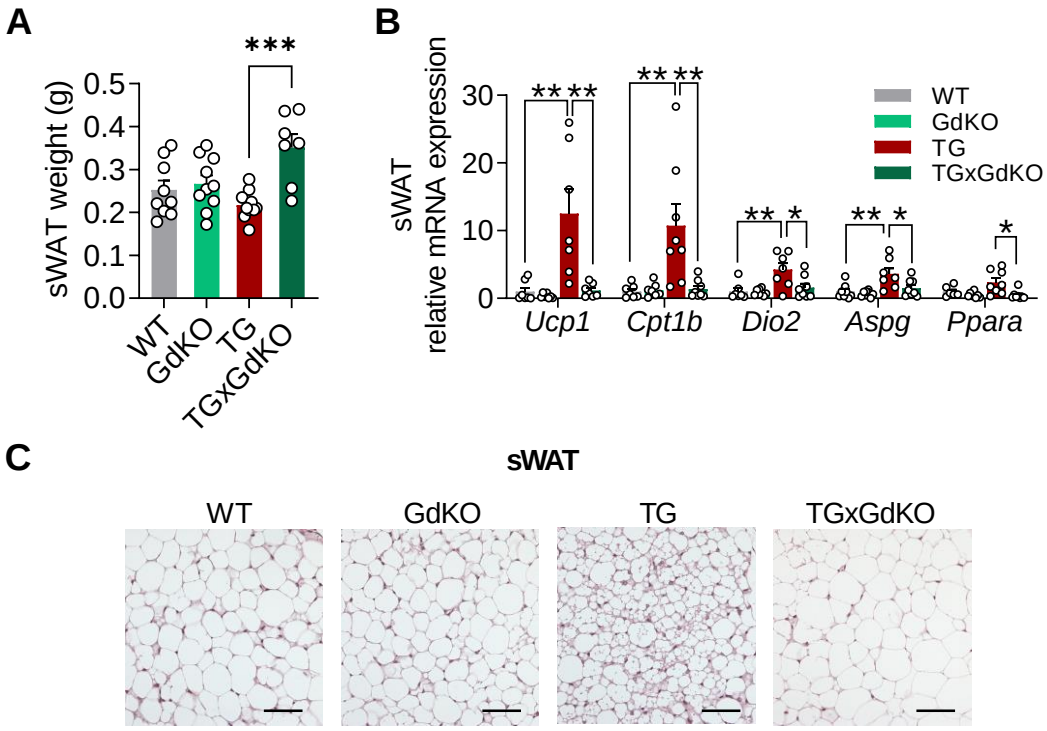


Figure 9. GDF15 controls browning of subcutaneous white adipose tissue during muscle mitochondrial dysfunction. (A) Subcutaneous white adipose tissue (sWAT) weight (n=7-10). (**B**) sWAT relative mRNA expression profile of browning markers (n=7-8). (**C**) Representative H&E histological staining of sWAT (scale bars represent 50 µm). Data correspond to 20 wks old male mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; GdKO, *Gdf15*-knockout; TG, HSA-*Ucp1*-transgenic; TGxGdKO, HSA-*Ucp1*-transgenic x *Gdf15*-KO.

In order to investigate the effects of GDF15 on glucose homeostasis under muscle mitochondrial stress, an oral glucose tolerance test (oGTT) was conducted (**Fig. 10A-C**). Interestingly, while glucose levels remained unchanged between genotypes throughout the oGTT (**Fig. 10A**), post-absorptive hypoinsulinemia in TG mice was lost in TGxGdKO mice (**Fig. 10B, C**), indicating that GDF15 is involved in mediating insulin sensitivity in TG mice.

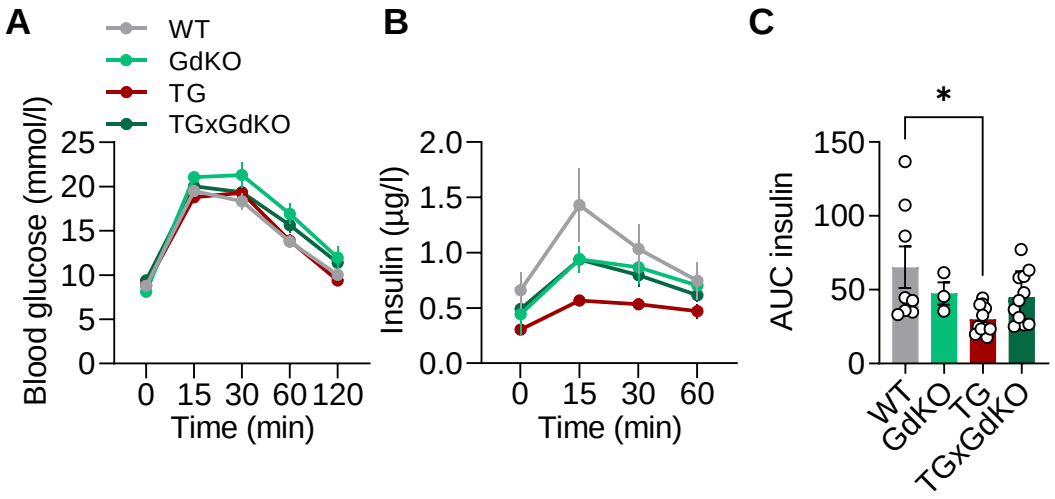


Figure 10. Loss of GDF15 abolishes hypoinsulinemia in TG mice. (**A**) Blood glucose and (**B**) insulin levels with (**C**) total area under the curve (AUC) of insulin during oral glucose tolerance test (oGTT) at 17 weeks of age (n=3-11). Data correspond to 17-18 wks old male mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; GdKO, *Gdf15*-knockout; TG, HSA-*Ucp1*-transgenic; TGxGdKO, HSA-*Ucp1*-transgenic x *Gdf15*-KO.

An increased metabolic flexibility, the ability to adapt fuel oxidation to fuel availability evidenced by measuring the respiratory quotient (RQ, VCO_2/VO_2), is a well described metabolic trait of TG mice (Klaus, Rudolph et al. 2005). In order to further investigate the involvement of GDF15 in the control of energy metabolism, specifically on metabolic flexibility, the RQ of TGxGdKO mice was analyzed. Strikingly, loss of GDF15 in TGxGdKO mice completely abolished the increased metabolic flexibility of TG mice as evidenced by an increased RQ amplitude (**Fig. 11A, B**), indicating an involvement of GDF15 in the control of energy metabolism.

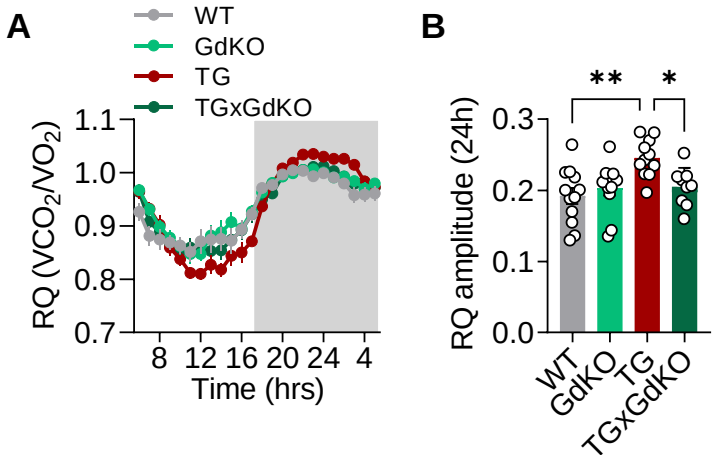


Figure 11. Loss of GDF15 abolishes metabolic flexibility in TG mice. (**A**) Respiratory quotient (RQ) over 24 hrs and (**B**) RQ amplitude (n=9-11). Data correspond to 17-18 wks old male mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; GdKO, *Gdf15*-knockout; TG, HSA-*Ucp1*-transgenic; TGxGdKO, HSA-*Ucp1*-transgenic x *Gdf15*-KO.

Altogether, these results indicate that, under muscle mitochondrial stress conditions, GDF15 is involved in the control of energy homeostasis leading to a metabolic remodeling in TG mice.

4.1.4 GDF15 leads to daytime-restricted anorexia under skeletal muscle mitochondrial stress

Aiming to further understand the metabolic actions of GDF15, a comprehensive *in vivo* metabolic phenotyping using indirect calorimetry was conducted (**Fig. 12**). While physical activity was not different among genotypes (**Fig. 12A, D**), GDF15 loss affected energy expenditure and food intake in a daytime specific manner (**Fig. 12B, C, E, F**). Interestingly, GDF15 proved to be responsible for a daytime-restricted anorexia in TG mice (**Fig. 12C, F**), likely affecting energy expenditure, which mirrored the food intake pattern (**Fig. 12B, E**).

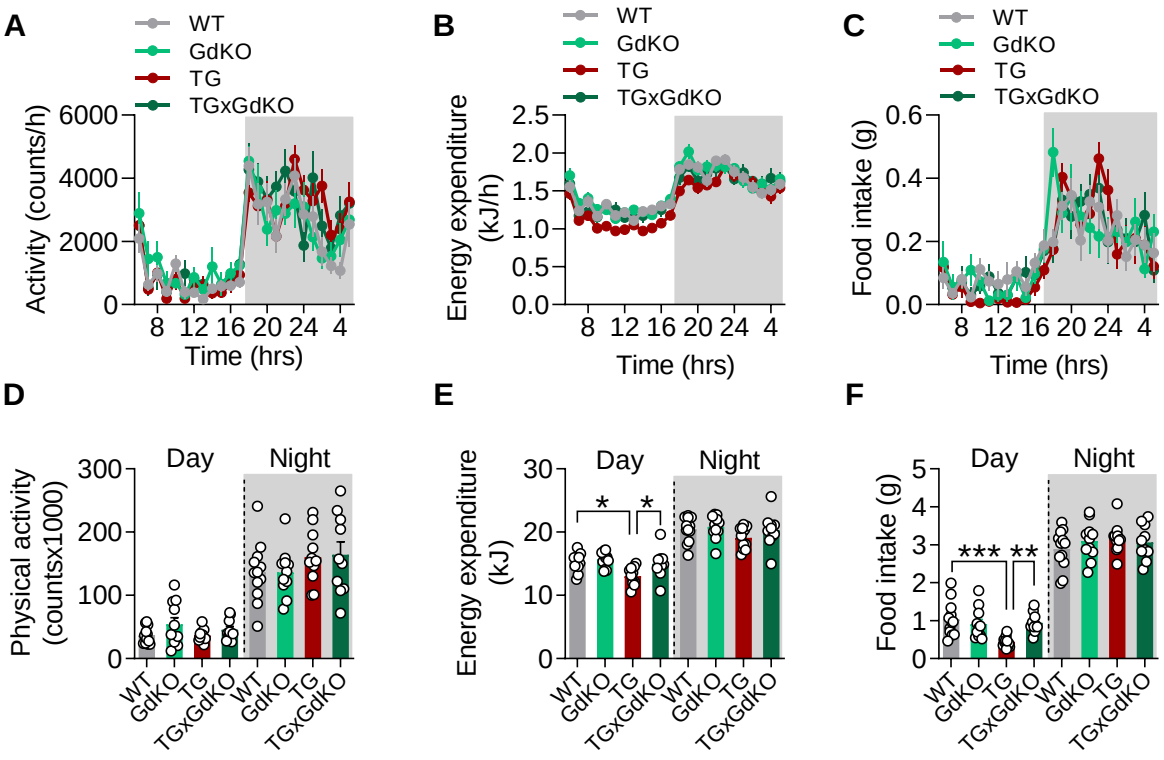


Figure 12. GDF15 leads to daytime-restricted anorexia during mitochondrial dysfunction in TG mice. (**A**) Longitudinal physical activity counts, (**B**) energy expenditure and (**C**) food intake over 24 hrs. (**D**) Total physical activity, (**E**) energy expenditure and (**F**) food intake during the light (day) and dark (night) phase (n=10-12). Data correspond to 17-18 wks old male mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; GdKO, *Gdf15*-knockout; TG, HSA-*Ucp1*-transgenic; TGxGdKO, HSA-*Ucp1*-transgenic x *Gdf15*-KO.

GDF15 has been shown to oscillate in humans in a diurnal pattern (Tsai, Macia et al. 2015). This raises the possibility that the diurnal regulation of food intake in TG mice could be related to diurnal oscillations of GDF15 levels. In order to test this hypothesis, WT and TG mice were sacrificed at day (10:00 hrs) and night (22:00 hrs) and variations in skeletal muscle *Gdf15* gene expression were examined. Indeed, skeletal muscle gene expression in TG mice between day and night presented a diurnal variation (**Fig. 13A**). Next, in order to determine a possible origin for these oscillations, the skeletal muscle ISR was investigated. Interestingly, skeletal muscle gene expression of *Atf4*, *Atf5*, *Atf6* and *Chop* (**Fig. 13B**) as well as phosphorylation of eIF2α (**Fig. 13C, D**) were similar at 10:00 and 22:00 hrs, likely indicating that the ISR does not play a role in *Gdf15* diurnal regulation in skeletal muscle of TG mice.

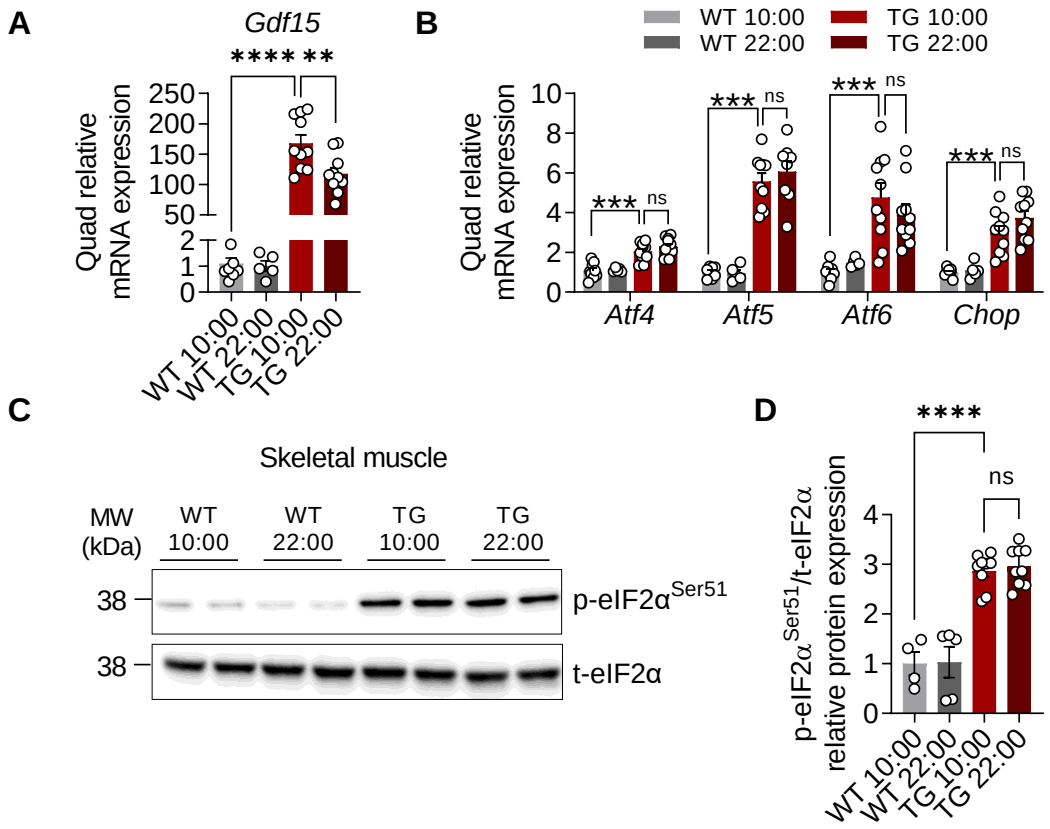


Figure 13. Diurnal variation in skeletal muscle *Gdf15* expression in TG mice is independent of the integrated stress response. (**A**) Relative quadriceps (quad) mRNA expression of *Gdf15* (n=5-10) (**B**) Relative quad mRNA expression of the integrated stress response (ISR) components *Atf4, Atf5, Atf6*, and *Chop* (n=5-10). (**C**) Representative immunoblots of ISR component eIF2α and (**D**) and quantification of phospho-eIF2α (p-eIF2α^{Ser51}) relative protein expression in gastrocnemius muscle (n=4-9). Data correspond to 34-40 wks old male mice sacrificed at 10:00 and 22:00 hrs and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001. WT, wildtype; TG, HSA-*Ucp1*-transgenic.

Finally, in order to further elucidate the circadian nature of GDF15 regulation in TG mice, WT and TG mice were dissected every 4 hrs along the 24 hrs day/night cycle and plasma circulating GDF15 levels were measured. Indeed, plasma GDF15 displayed a strong diurnal variation with peak at 10:00 hrs and a nadir at 22:00 hrs in TG mice (**Fig. 14**), confirming the diurnal regulation of GDF15 as a myokine under skeletal muscle stress conditions.

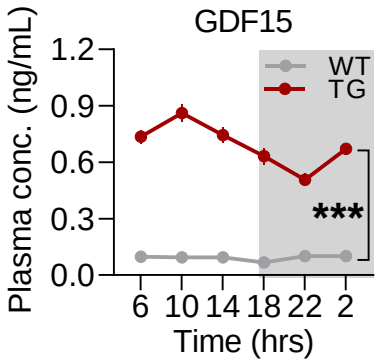


Figure 14. Plasma GDF15 oscillates in a circadian manner in TG mice. Circulating GDF15 plasma levels every 4 hrs (n=9-10). Data correspond to 16-18 wks old male mice and are expressed as means ± SEM; *P*-value calculated by unpaired t-test between individual timepoints. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P*< 0.0001. WT, wildtype; TG, HSA-*Ucp1*-transgenic.

4.2 Chapter 2: Physiological mechanisms of GDF15-induced anorexia under muscle mitochondrial stress

After establishing the role of GDF15 as a diurnal modulator of food intake and mediator of energy metabolism under skeletal muscle mitochondrial stress, the next aim was to further uncover the physiological and behavioral mechanisms behind GDF15's biology in a gender specific fashion, thus investigating male and female mice.

4.2.1 Elevated GDF15 under mitochondrial stress does not induce nausea

Very recently, GDF15 has been proposed to lead to nausea and emesis as a mechanism for its well-established anorectic action (Borner, Shaulson et al. 2020, Borner, Wald et al. 2020). Therefore, the first aim of this chapter was to characterize whether daytime anorexia in TG mice is associated to this physiological outcome. To test for visceral malaise in TG mice, a so-called kaolin preference test was conducted. Mice were offered a choice of standard chow diet and kaolin diet, a non-palatable substance that rodents, which are unable to vomit, tend to consume when feeling nauseous. While the already established daytime-restricted anorexia could be observed in male and female TG mice (**Fig 15A, B**), differences in kaolin consumption between WT and TG mice were not apparent (**Fig. 15C, D**). These results indicate that nausea is not associated with elevated GDF15 levels in TG mice and thus, likely not the physiological mechanism leading to daytime-restricted anorexia.

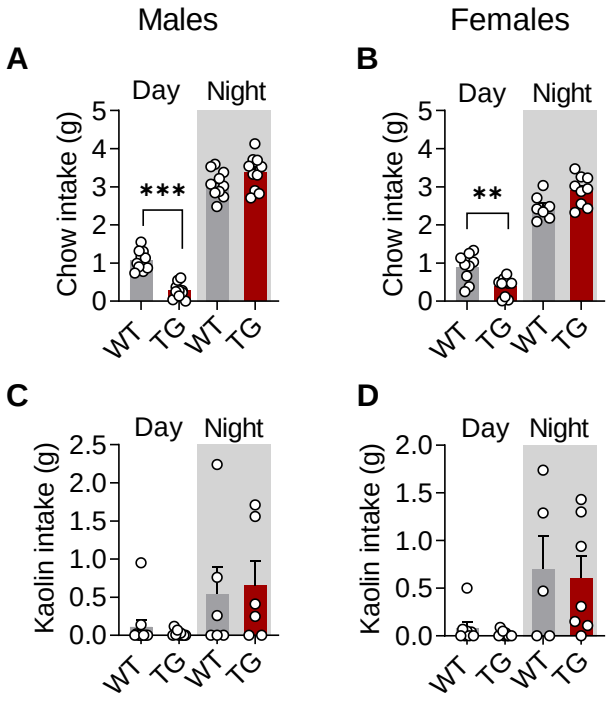


Figure 15. Muscle mitochondrial stress-induced daytime-restricted anorexia is not associated with nausea. (**A, B**) 24 hrs (day vs. night) chow intake during a kaolin preference test. (**C, D**) 24 hrs (day vs. night) kaolin intake during a kaolin preference test. Data correspond to 18-19 wks old male (left panel) and female (right panel) mice and are expressed as means ± SEM; *P*-value calculated by unpaired t-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; TG, HSA-*Ucp1*-transgenic.

4.2.2 Muscle mitochondrial stress-induced GDF15 associates with anxiety-like behavior

A behavioral characterization of whole body *Gdf15*-knockout mice showed a reduced anxiety-like behavior and an increased exploratory behavior compared to WT mice (Low, Ambikairajah et al. 2017), suggesting that GDF15 might be involved in controlling anxiety-like behavior. Stress-related behavior and ingestive behavior are known to be interlinked (for review see Yau and Potenza 2013). Therefore, the question arises whether increased GDF15 levels in TG mice might be associated to an increased anxiety-like behavior, potentially associated with their food intake phenotype. In order to address this question, Open Field Test (OFT) and Elevated Plus Maze test (EPM) were performed to assess anxiety and exploratory behavior in TG mice. In the OFT, TG mice displayed a reduced number of entries and time spent in the center as well as an increased time spent in the corners (**Fig. 16A, D**). Along with these results, mice spent an increased time freezing in the EPM (**Fig. 16B, E**). Of note, male but not female TG mice showed a higher induction of

circulating corticosterone after the EPM than WT littermates (**Fig. 16C, F**), indicating an increased stress susceptibility and, possibly, HPA axis sensitivity.

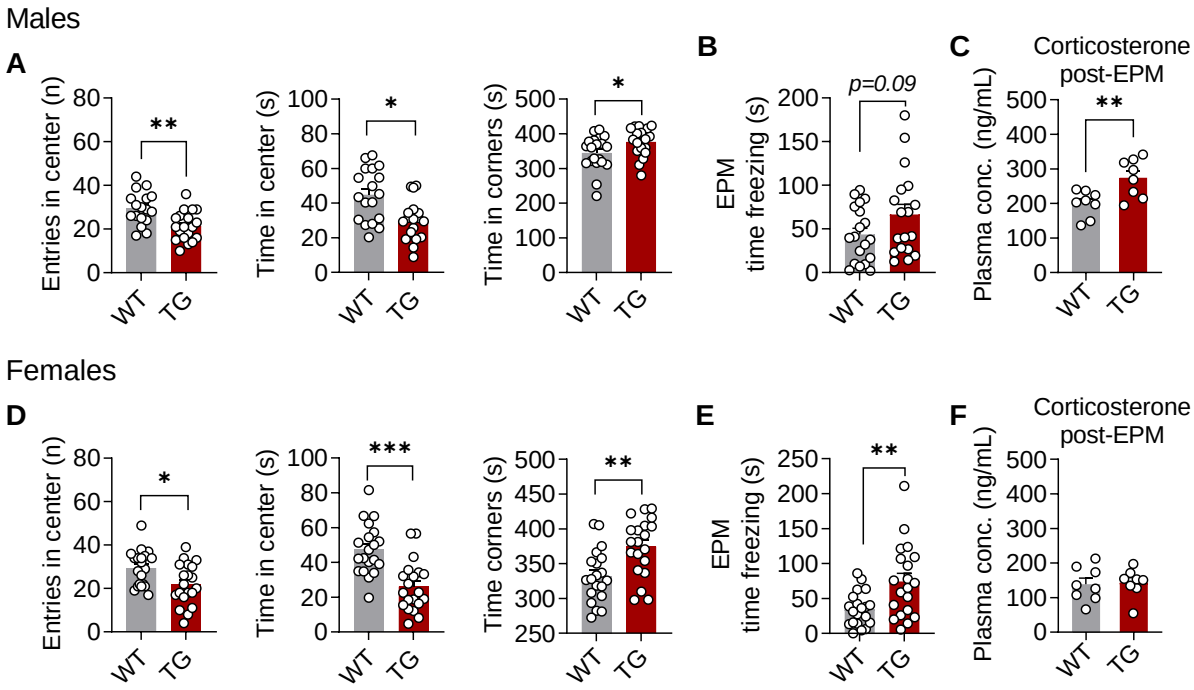


Figure 16. **Muscle mitochondrial stress is associated with increased anxiety-like behavior.** (**A, D**) Entries in the center, time spent in the center and time spent in the corners during an open field test (n=20). (**B, E**) Time spent freezing during an elevated plus maze (EPM) test (n=20). (**C, F**) post-EPM plasma corticosterone levels (n=8). Data correspond to 10-13 wks old male (upper panel) and female (lower panel) mice and are expressed as means ± SEM; *P*-value calculated by unpaired t-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P*< 0.0001. WT, wildtype; TG, HSA-*Ucp1*-transgenic.

4.2.3 Muscle mitochondrial stress-induced GDF15 associates with increased hypothalamic corticotropin releasing hormone

The HPA axis is the main system to control the stress response in mammals. Given the previously established anxiety-like phenotype in TG mice, a logical next step was to investigate the molecular regulation of the HPA axis in TG mice compared to WT mice. Interestingly, an induction of hypothalamic *Crh* mRNA expression was evident in both male and female TG mice (**Fig. 17A, D**), but was not accompanied by a further activation of the HPA axis as shown by the unchanged *Pomc* mRNA expression levels in the pituitary gland (**Fig. 17B, E**) and circulating corticosterone levels (**Fig. 17C, F**).

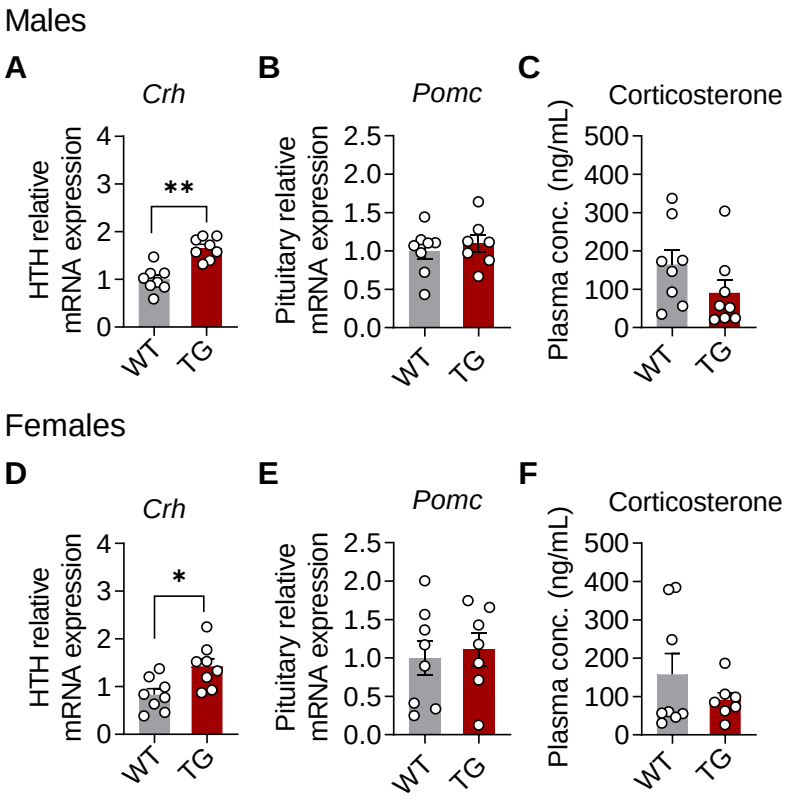


Figure 17. Muscle mitochondrial stress is associated with increased hypothalamic *Crh* independent of the corticosterone response. (**A, D**) Hypothalamus (HTH) *Crh* relative mRNA expression (n=8). (**B, E**) Pituitary *Pomc* relative mRNA expression (n=8). (**C, F**) Basal plasma corticosterone concentration (n=7-8). Data correspond to 16-20 wks old male (upper panel) and female (lower panel) mice and are expressed as means ± SEM; *P*-value calculated by unpaired t-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P*< 0.0001. WT, wildtype; TG, HSA-*Ucp1*-transgenic.

These results indicate that, under basal undisturbed conditions, hypothalamic CRH might play an alternative role other than the activation of the HPA axis in TG mice.

4.3 Chapter 3: Role of the GDF15-GFRAL axis in the control of food intake and anxiety-like behavior

Having established the association of skeletal muscle mitochondrial stress with an increased anxiety-like behavior and the hypothalamic induction of CRH in TG mice, the next goal was to uncover a possible link through the activation of the GDF15-GFRAL axis. For this, TG mice were crossed with *Gfral*-knockout (GfKO) mice in order to obtain GFRAL ablated TG mice (TGxGfKO) and a metabolic, behavioral and molecular phenotyping was conducted using WT and GfKO mice as a control. GFRAL ablation was confirmed by gene expression analysis of *Gfral* mRNA expression in the AP and NTS in both GfKO and TGxGfKO mice (**Fig. 18A, D**). Additionally, GFRAL ablation led to slightly increased skeletal

muscle *Gdf15* mRNA levels in female but not in male mice (**Fig. 18B, E**), whereas it slightly increased circulating GDF15 levels in male, but not in female mice (**Fig. 18C, F**).

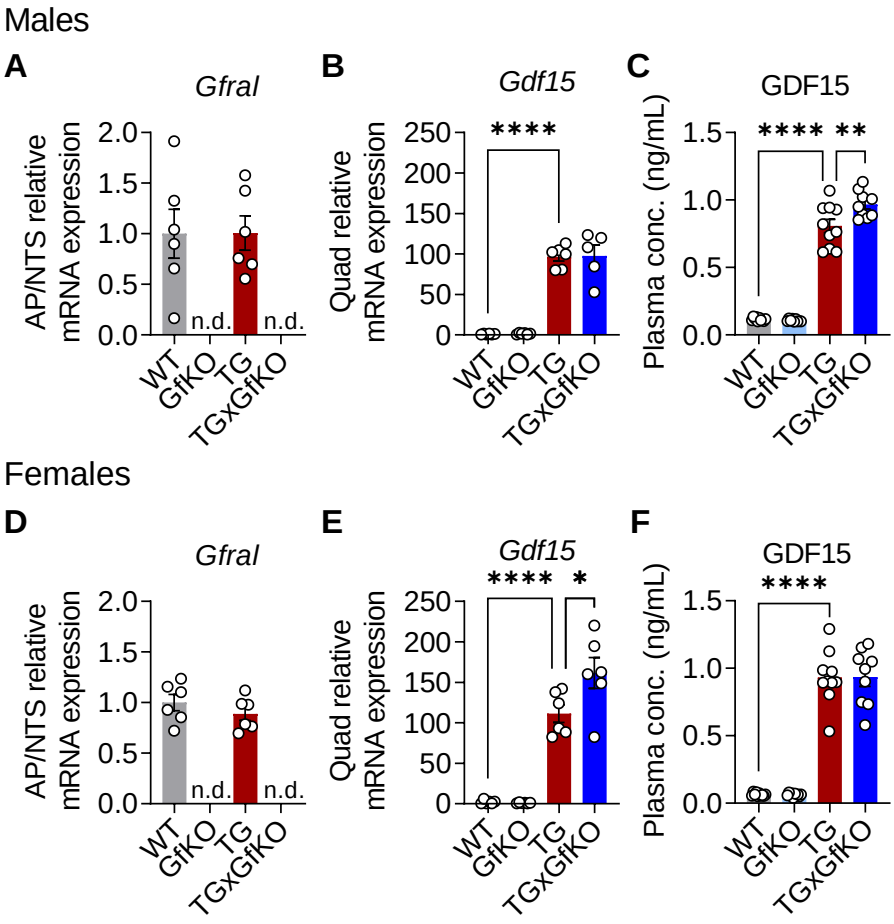


Figure 18. Loss of GFRAL affects circulating GDF15 levels in male but not female TG mice. (**A, D**) *Gfral* mRNA expression in the area postrema (AP) and nucleus of the solitary tract (NTS) (n=6). (**B, E**) Quadriceps (quad) *Gdf15* mRNA expression (n=6). (**C, F**) Circulating GDF15 plasma levels (n=10). Data correspond to 20 wks old male (upper panel) and female (lower panel) mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P*< 0.0001. WT, wildtype; GfKO, *Gfral*-knockout; TG, HSA-*Ucp1* -transgenic; TGxGfKO, HSA-*Ucp1* -transgenic x *Gfral*-KO.

Loss of GFRAL had a strong effect on body weight development of TG mice (**Fig. 19A, D**). Interestingly, body lean mass had the strongest contribution to the increase of body weight of TGxGfKO mice compared to TG mice (**Fig. 19B, E**), while body fat mass remained unaffected (**Fig. 19C, F**).

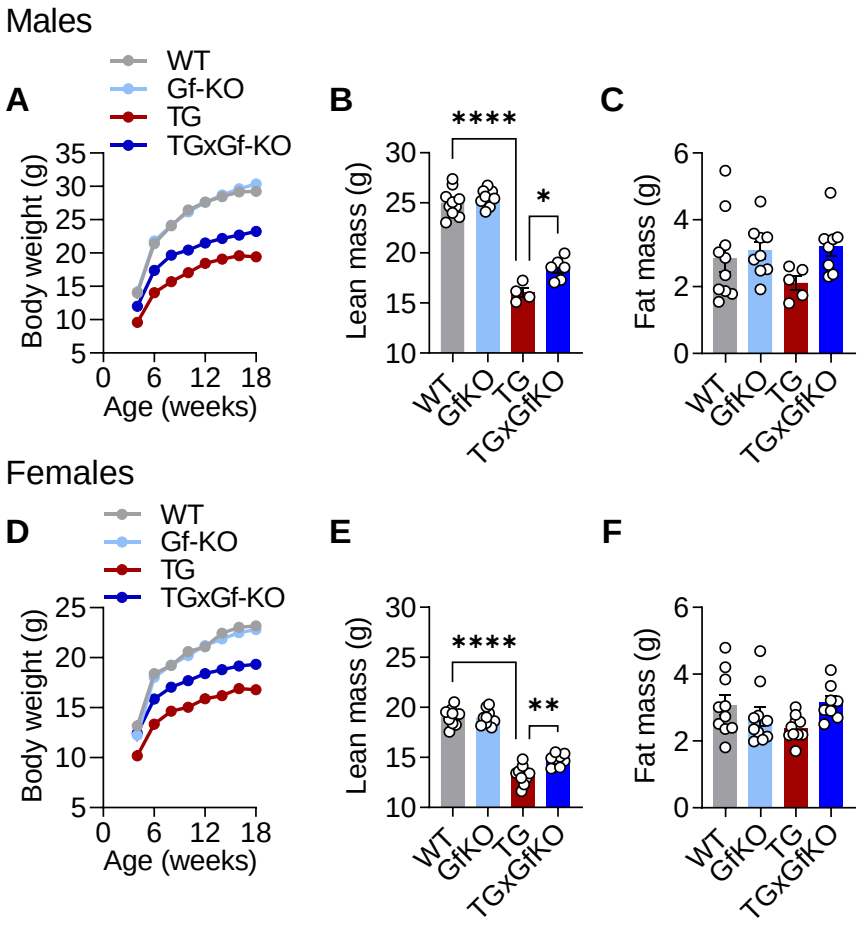


Figure 19. The GDF15-GFRAL axis controls body composition under muscle mitochondrial stress. (**A, D**) Body weight development and (**B, C, E, F**) body composition (lean and fat mass) at 18 wks of age (n=10). Data correspond to 4-18 wks old male (right panel) and female (left panel) mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P*< 0.0001. WT, wildtype; GfKO, *Gfral*-knockout; TG, HSA-*Ucp1* -transgenic; TGxGfKO, HSA-*Ucp1* -transgenic x *Gfral*-KO.

4.3.1 GFRAL signaling controls diurnal food intake under muscle mitochondrial stress

Next, the involvement of GFRAL in the control of GDF15-dependent daytime anorexia in TG mice was evaluated. Interestingly, GFRAL appeared to be as much responsible as GDF15 in the control of daytime-restricted food intake in TG mice (**Fig. 20**). Of note, a nighttime GFRAL dependent increase in food intake of TG male mice was observed (**Fig. 20A, B**), opening the question of whether the GDF15-GFRAL axis is involved in the regulation of circadian food intake behavior by promoting a shift in ingestive behavior from day- to nighttime.

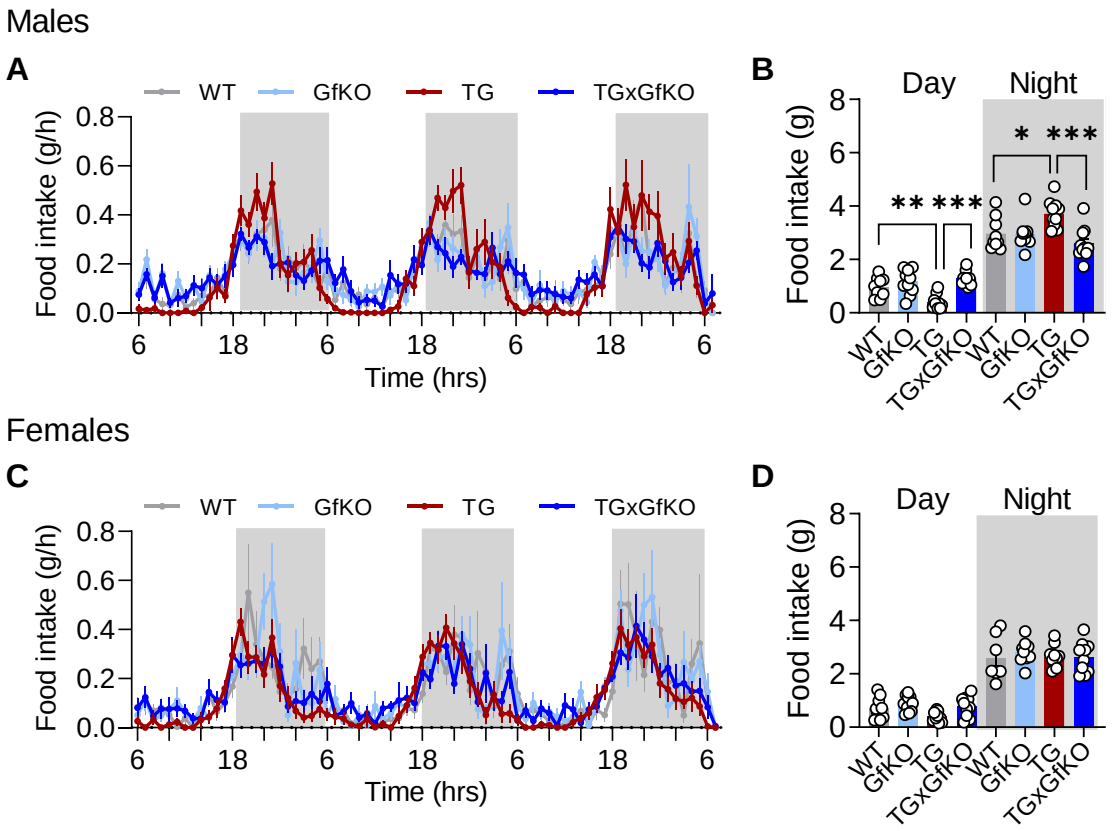


Figure 20. GFRAL mediates GDF15-induced daytime-restricted anorexia under muscle mitochondrial stress. (**A, C**) Longitudinal assessment of food intake during three consecutive days and (**B, D**) quantification of 24 hrs food intake [day vs. night] (n=10). Data correspond to 18-19 wks old male (upper panel) and female (lower panel) mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P*< 0.0001. WT, wildtype; GfKO, *Gfral*-knockout; TG, HSA-*Ucp1* -transgenic; TGxGfKO, HSA-*Ucp1* -transgenic x *Gfral*-KO.

Interestingly, the GDF15-dependent increased metabolic flexibility in TG mice evidenced by an increased RQ amplitude proved to be mediated by GFRAL in both male and female mice (**Fig. 21**). Thus, TGxGfKO mice fully phenocopy TGxGdKO mice on daytime-restricted anorexia and metabolic flexibility, demonstrating that GDF15 released from muscle as myokine signals to the brain via the brainstem-specific GFRAL-receptor to control systemic energy metabolism.

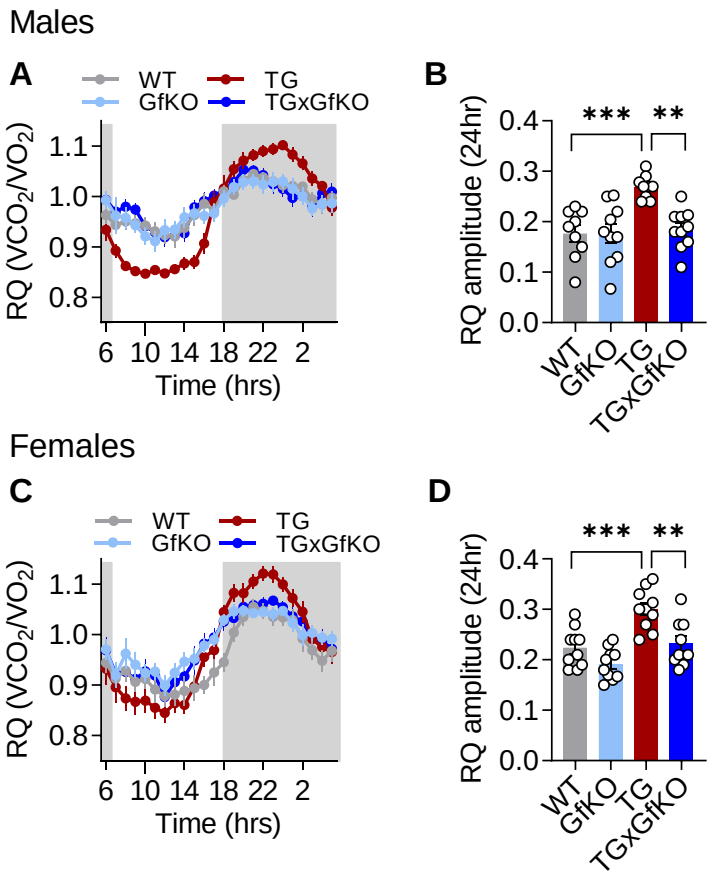


Figure 21. GFRAL signaling induces metabolic flexibility under muscle mitochondrial stress. (**A, C**) Respiratory quotient (RQ) over 24 hrs and (**B, D**) RQ amplitude (n=10). Data correspond to 18-19 wks old male (upper panel) and female (lower panel) mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P*< 0.0001. WT, wildtype; GfKO, *Gfral*-knockout; TG, HSA-*Ucp1* -transgenic; TGxGfKO, HSA-*Ucp1* -transgenic x *Gfral*-KO.

In order to further characterize the daytime anorectic phenotype in TG mice and its dependence on GFRAL signaling, the regulation of the known central appetite drivers in the hypothalamus, POMC and AgRP, was examined. While *Pomc* gene expression was reduced, *Agrp* was increased in TG mice, indicating a state of negative energy balance that was fully reversed upon GFRAL ablation (**Fig. 22A, B, E, F**). Furthermore, plasma levels of total ghrelin, a well-described orexigenic hormone, were increased in TG mice and this effect was again reversed in TGxGfKO mice (**Fig. 22C, G**), while a reduction in the levels of the anorectic hormone leptin in TG mice was also normalized to WT levels in TGxGfKO mice (**Fig. 22D, H**).

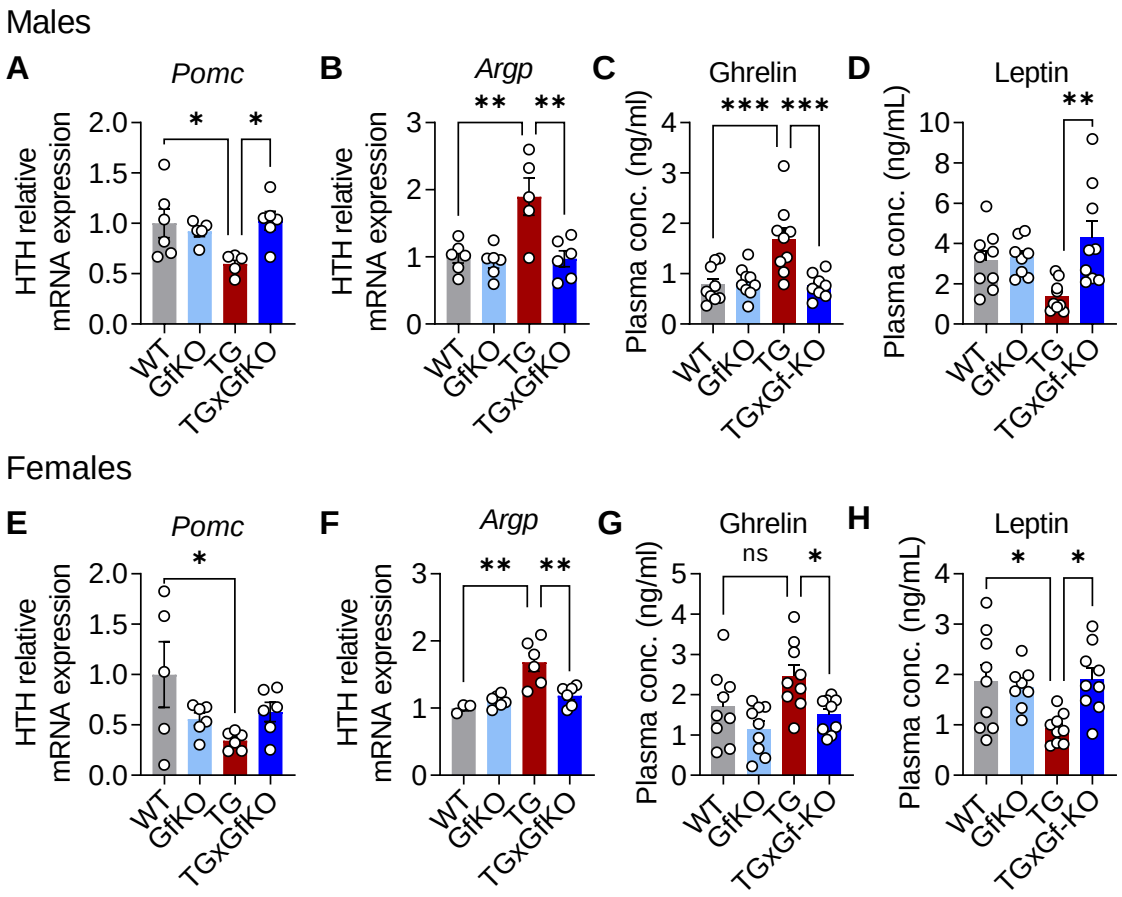


Figure 22. GFRAL signaling induces a state of negative energy balance under muscle mitochondrial stress. (**A, E**) Hypothalamus (HTH) *Pomc* and (**B, F**) *Agrp* gene expression (n=6). (**C, G**) Plasma level of total ghrelin at daytime (n=9). (**D, H**) Plasma level of leptin at daytime (n=9). Data correspond to 20 wks old male (upper panel) and female (lower panel) mice and are expressed as means ± SEM; P-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; GfKO, *Gfral*-knockout; TG, HSA-*Ucp1* -transgenic; TGxGfKO, HSA-*Ucp1* -transgenic x *Gfral*-KO.

4.3.2 Mitochondrial stress induced anxiety-like behavior and hypothalamic *Crh* are induced by GFRAL signaling

The next focus was to understand the involvement of the GDF15-GFRAL axis in the control of anxiety-like behavior and CRH induction under muscle mitochondrial stress. In male TG mice, the reduced number of entries into the center during OFT and the increased freezing time during EPM were completely abolished with the loss of GFRAL (**Fig. 23A, B**) while in female mice, the anxiety-like phenotype reported previously (**Fig. 16**) could not be reproduced (**Fig. 23D, E**). Increased post-EPM plasma corticosterone levels of TG mice were in tendency reduced in TGxGfKO male mice (**Fig. 23C**) while female mice showed no differences among genotypes (**Fig. 23F**), confirming the previous results. In summary,

these data indicate that GFRAL signaling is responsible for the increased anxiety-like phenotype in, at least, male TG mice.

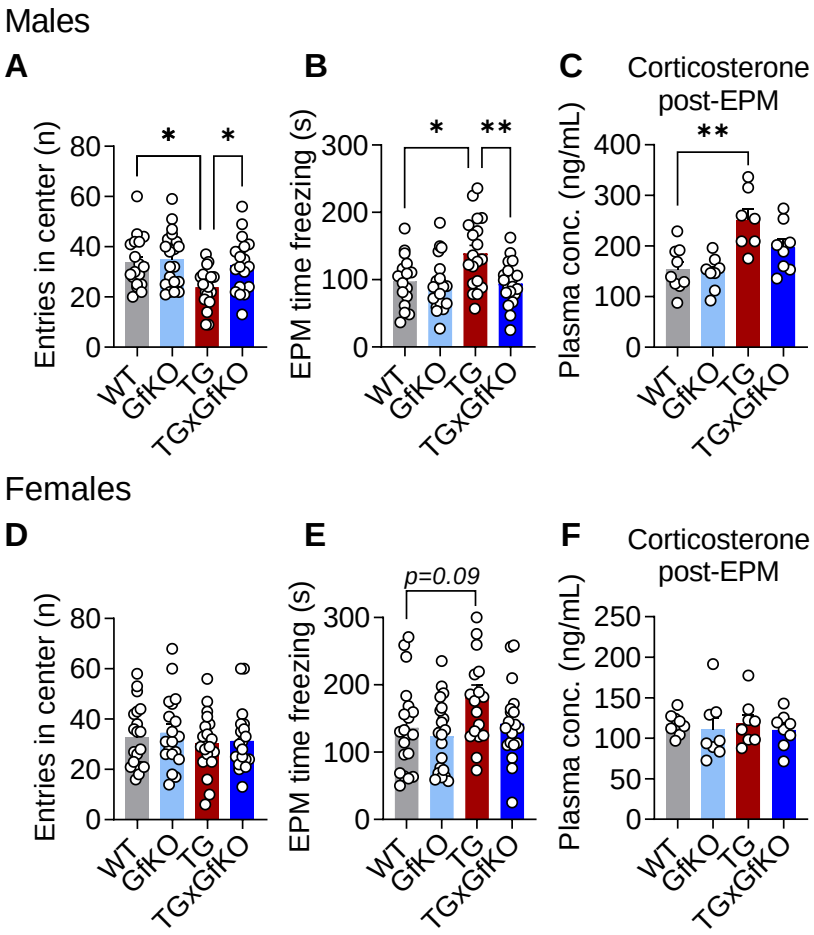


Figure 23. Loss of GFRAL abolishes mitochondrial stress-induced anxiety-like behavior. (**A, D**) Entries in the center during an open field test (n=20). (**B, E**) Time spent freezing during an elevated plus maze (EPM) test (n=17-20). (**C, F**) Plasma corticosterone levels after the EPM (n=8-9). Data correspond to 10-13 wks old male (upper panel) and female (lower panel) mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; GfKO, *Gfral*-knockout; TG, HSA-*Ucp1* -transgenic; TGxGfKO, HSA-*Ucp1* -transgenic x *Gfral*-KO.

Moving forward, the next aim was to understand the dependence of the GDF15-GFRAL axis in the previously reported induction of hypothalamic CRH in TG mice. In both male and female mice, hypothalamic *Crh* induction in TG mice was completely blunted in TGxGfKO mice (**Fig. 24A, D**). The absence of changes in *Pomc* gene expression in the pituitary gland (**Fig. 24B, E**) and circulating corticosterone (**Fig. 24C, F**) further confirmed the independence of CRH induction from the systemic activation of the HPA axis.

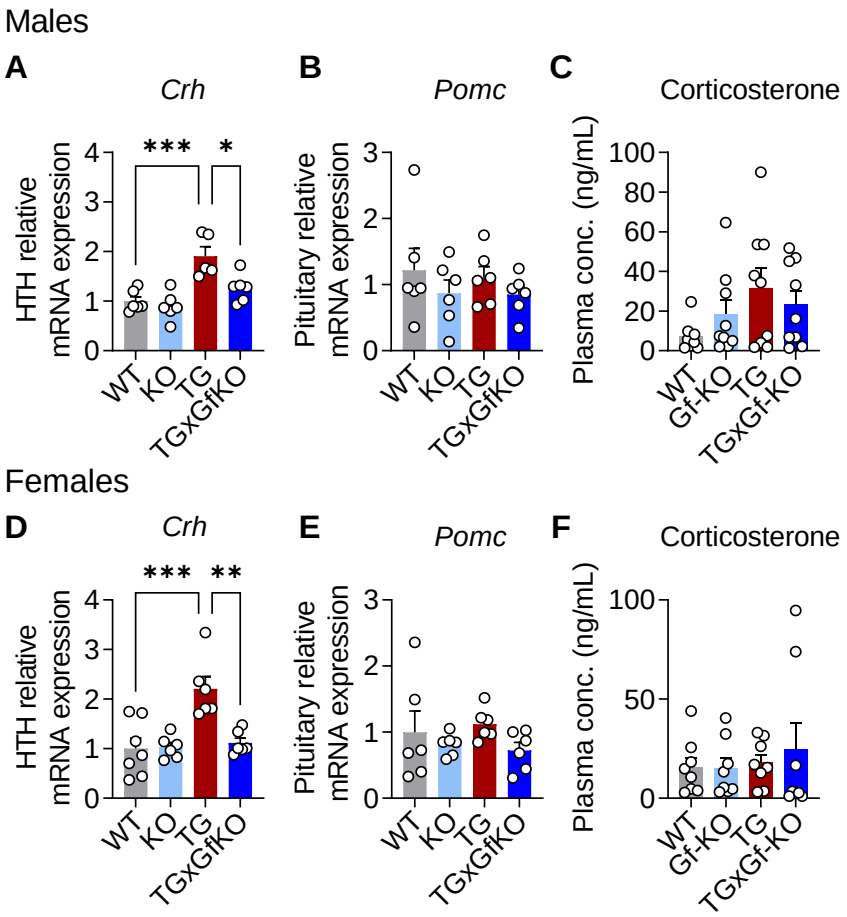


Figure 24. Hypothalamic *Crh* induction under muscle mitochondrial stress in GFRAL dependent. (**A, D**) Hypothalamus (HTH) *Crh* mRNA expression (n=5-7). (**B, E**) Pituitary *Pomc* mRNA expression (n=6). (**C, F**) Plasma corticosterone levels under routine conditions (n=8-9). Data correspond to 20 wks old male (upper panel) and female (lower panel) mice and are expressed as means ± SEM; *P*-value calculated by one-way ANOVA with Tukey's post hoc test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. WT, wildtype; GfKO, *Gfral*-knockout; TG, HSA-*Ucp1* -transgenic; TGxGfKO, HSA-*Ucp1* -transgenic x *Gfral*-KO.

4.3.3 Hypothalamic CRH controls anorectic phenotype in TG mice through CRHR1

CRH is known to be a potent food intake inhibitor. Furthermore, CRH receptor 1 (CRHR1) knockout mice show a light-phase restricted induction of food intake (Muller, Keck et al. 2000) and a reduced anxiety (Timpl, Spanagel et al. 1998), namely the opposite phenotype to what is observed in TG mice. It was therefore hypothesized that the GDF15-GFRAL axis might exert its anorectic action through the induction of CRH-CRHR1 signaling. To test this hypothesis, WT and TG mice were treated with antalarmin, a specific antagonist of CRHR1, and food intake was monitored. Intraperitoneal injection of antalarmin abolished the daytime-restricted anorexia in TG male mice in contrast to vehicle injection (**Fig. 25A**), while it did not have an effect on female mice (**Fig. 25B**). These results indicate that, at least in

male TG mice, CRH signaling through CRHR1 is a downstream mediator of the GDF15-GFRAL axis to control daytime-restricted anorexia.

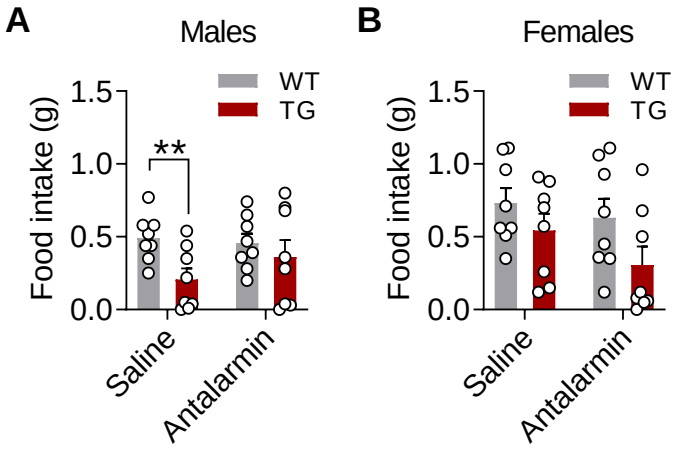


Figure 25. GFRAL-dependent hypothalamic CRH induction controls diurnal food intake under muscle mitochondrial stress. Daytime food intake after intraperitoneal injection of antalarmin, a selective CRHR1 antagonist, in male (**A**) and female (**B**) WT vs. TG mice (n=8). Data correspond to 40 wks old mice and are expressed as means ± SEM; *P*-value calculated by unpaired t-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wildtype; TG, HSA-*Ucp1* -transgenic.

5. Discussion

In the last years, and due to its involvement in a wide range of diseases and stress conditions, there has been an increasing interest in understanding the role and mode of action of GDF15. The discovery of the specific hindbrain receptor GFRAL in 2017 (Emmerson, Wang et al. 2017, Hsu, Crawley et al. 2017, Mullican, Lin-Schmidt et al. 2017, Yang, Chang et al. 2017) has allowed the establishment of GDF15-GFRAL signaling as an anorectic pathway especially through pharmacological studies using rGDF15. Nevertheless, the role of endogenous GDF15 induced by organ dysfunction or stress has been less studied and as such, remains elusive.

This PhD thesis aimed to uncover the role of GDF15 and its receptor GFRAL under one of the conditions that have been most reported to endogenously induce GDF15: mitochondrial stress. Using a mouse model of skeletal muscle specific mitochondrial stress (the TG mouse model) combined with knockout strategies of GDF15 and GFRAL, these studies uncovered (1) the involvement of GDF15 in the control of metabolic remodeling under muscle mitochondrial stress and (2) the control of diurnal food intake by GDF15 leading to daytime-restricted anorexia, potentially due to (3) a diurnal rhythm of expression and secretion of GDF15. Further aiming to understand the molecular mechanisms involved in GDF15-GFRAL modulation of food intake, this study reveals (4) hypothalamic CRH-CRHR1 signaling as a mediator of GDF15-GFRAL-dependent daytime-restricted anorexia. Finally, this work uncovers (5) a so far unknown physiological outcome of the GDF15-GFRAL pathway: the induction of anxiety-like behavior.

5.1 Regulation of GDF15 expression under muscle mitochondrial stress

The regulation of GDF15 at the transcriptional and translational level under different stressors is still not completely understood. The results presented in this thesis show that GDF15 oscillates in a circadian manner in TG mice with the highest peak in plasma concentrations at 10:00 hrs (~0,9 ng/mL) and the lowest at 22:00 hrs (~0,4-0,6 ng/mL) (**Fig. 14**), raising the question of whether the circadian clock is involved in the regulation of GDF15 under mitochondrial stress. In fact, in the literature there is some evidence that supports this hypothesis. In rat uterus endometrial stromal cells (UESCs), *Gdf15* expression was shown to oscillate in a circadian manner (Tasaki, Zhao et al. 2013) and to be repressed by the transcription factor REV-ERBα (Zhao, Isayama et al. 2016), a component of the negative feedback loop of the core clock machinery. Furthermore, in healthy humans, serum GDF15 levels oscillate in a diurnal pattern with a peak around 22:00 hrs (Tsai, Macia et al. 2015, Klein, Nicolaisen et al. 2021), which corresponds to the peak in TG mice at

10:00 hrs considering that mice and humans have opposite activity phases. Nevertheless, the main mechanism proposed for the induction of GDF15 under cellular stress is the activation of the ISR (Chung, Ryu et al. 2017, Patel, Alvarez-Guaita et al. 2019), which was indeed confirmed to be increased in TG mice but not affected in a diurnal manner between 10:00 and 22:00 hrs (**Fig. 13B-D**). While oscillations in plasma GDF15 levels in WT mice were not detected, it is a possibility that there is a basal circadian regulation of *Gdf15* gene expression that is enhanced by the activation of the ISR, leading to a greater amplitude in the oscillations in TG mice. A link between the ISR and the circadian clock has recently been elucidated. In the SCN circadian clock of the brain, also called the central clock, the eIF2α kinase general control nonderepressible 2 (GCN2) rhythmically phosphorylates eIF2α, which in turn promotes the rhythmic mRNA translation of *Atf4*, known to have multiple binding motifs in known clock genes such as *Period* 2 and 3 (*Per2, Per3)*, *Cryptochrome Circadian Regulator* 1 and 2 (*Cry1*, *Cry2)* and *Clock* (Pathak, Liu et al. 2019). This study showed a circadian phosphorylation of eIF2α in WT mice kept in constant darkness with peak at circadian time (CT)6, which would correspond to 10:00 hrs in the experiments presented in this thesis (**Fig. 13C, D**). Since the present study was conducted under normal light-dark cycles, it is a possibility that the peak of eIF2α phosphorylation reported by Pathak, Liu and colleagues might not coincide with 10:00 hrs, since exposure to constant darkness may shift circadian cycles. It is therefore still a possibility that circadian oscillations in the integrated stress response drive the circadian expression of *Gdf15* and this will need to be investigated further.

Interestingly, this study further shows that loss of the hindbrain receptor GFRAL led to slightly higher *Gdf15* mRNA levels in female and GDF15 plasma levels in male mice (**Fig. 18C, E**). While, based on the gender differences, this does not seem to be a robust effect, it is worth mentioning that it could be part of a compensatory mechanism to further perpetuate the stress response elicited by the GDF15-GFRAL axis.

5.2 Effects of the GDF15-GFRAL axis on body composition and peripheral tissues under muscle mitochondrial stress

This study shows evidence for an effect of the activation of the GDF15-GFRAL axis by mitochondrial stress on body composition but, paradoxically, GDF15 and GFRAL loss affected body composition differentially. On one hand, loss of GDF15 led to an accumulation of body fat mass in TG mice while body lean mass was unaffected (**Fig. 8**). On the other hand, GFRAL loss led to an increase in body lean mass in TG mice while body fat mass remained mostly unchanged (**Fig. 19**).

While both GDF15 and GFRAL proved to be responsible for a daytime-restricted anorexia in TG mice (**Fig. 12C, F**, **Fig. 20**), only GFRAL loss proved to induce a nighttime increase of food intake (**Fig. 20**). Of note, a nighttime increase of food intake in TG mice was not observed in mouse study 1 (Results Chapter I), a fact that could have hindered a potential nighttime orexigenic effect of GDF15. One possibility for the differences on nighttime food intake in TG mice between the two studies are differences in experimental conditions, since mice are very sensitive to their environment. In the two studies, mice were kept in different rooms with different hygiene status, which might have affected their gut microbiota, and had different cage enrichment, facts that could have affected their stress levels and potentially their nighttime food intake pattern. These differences in food intake regulation and thus, energy balance, among the two studies could potentially explain the different outcomes of GDF15 and GFRAL ablation regarding body composition. Nevertheless, the possibility that regulation of nighttime food intake in TG mice is independent of GDF15 cannot be ruled out. In this case, GFRAL could act as a receptor for another as yet unidentified factor which would induce food intake at nighttime.

Since GDF15 has been previously shown to have GFRAL-independent effects, possible auto-/paracrine roles for GDF15 under mitochondrial stress should not be excluded. Therefore, the effects of GDF15 on skeletal muscle, the source of GDF15 in TG mice, were studied. Phenotypic characterization of skeletal muscle in TGxGdKO compared to TG mice proved GDF15 not to have a direct effect on muscle mass (**Fig. 6C**), myofiber size (**Fig. 6D, E**) or the induction of the ISR (**Fig. 7**), indicating that it likely does not play an auto-/paracrine role under these specific conditions. It is of course a possibility that this is due to the specific phenotype of the TG mouse model, in which the ectopic expression of UCP1 in mitochondria induces a whole muscle metabolic remodeling including an increased protein turnover and amino acid metabolism with induced serine/glycine and transsulfuration pathways, which are related to an induction of NADPH-generating pathways as an adaptation to oxidative stress (Ost, Keipert et al. 2015).

One of the traits of metabolic remodeling in TG mice is browning of sWAT (Keipert, Ost et al. 2014). This study demonstrates that browning of sWAT under muscle mitochondrial stress is dependent on GDF15 (**Fig 9B, C**). This goes in line with reported effects of GDF15 on WAT under mitochondrial stress in other mouse models (Chung, Ryu et al. 2017, Choi, Jung et al. 2020). Whether the effects of GDF15 on WAT are direct or whether they are mediated via GFRAL will require further investigations. Interestingly, GDF15 has previously been shown to have direct effects on adipocytes. In differentiated 3T3-L1 adipocytes, rGDF15 treatment was shown to induce lipolysis *in vitro* (Chung, Ryu et al. 2017), although these experiments need to be interpreted with care since rGDF15 might have been

contaminated with TGFβ (Olsen, Skjaervik et al. 2017). Furthermore, GDF15-rich conditioned media (CM) of primary myotubes subjected to electrical pulse stimulation (EPS) promoted lipolysis in human adipocytes in culture (Laurens, Parmar et al. 2020). However, the mechanisms mediating these effects are still unknown. As Breit and colleagues suggest in their recent review, it is possible that a soluble form of GFRAL lacking transmembrane and cytoplasmatic domains is expressed in peripheral tissues and mediates these effects, but also an alternative receptor for GDF15 that has not been discovered yet cannot be ruled out (Breit, Tsai et al. 2017). Nevertheless, a possible involvement of the sympathetic nervous system in the induction of browning via GDF15-GFRAL should not be excluded. In a mouse model of cancer cachexia, the GDF15-GFRAL axis was proven to induce lipolysis in adipose tissue through sympathetic innervation (Suriben, Chen et al. 2020). It must be pointed out that TGxGdKO mice exhibited a fat mass expansion (**Fig. 8C, 9A**). Thus, it is a possibility that the loss of browning in these mice is rather a secondary effect of this fat mass expansion than a direct GDF15 effect. This hypothesis might be supported by the fact that fibroblast growth factor 21 (FGF21), another important cytokine in the regulation of energy metabolism which is also induced by skeletal muscle in TG mice (Keipert, Ost et al. 2014), proved to be fully responsible for the browning phenotype in TG mice despite high circulating GDF15 levels (Ost, Coleman et al. 2016).

5.3 Impact of the GDF15-GFRAL axis on diurnal regulation of food intake

The present study demonstrates that the GDF15-GFRAL axis alters food intake in a diurnal manner under muscle mitochondrial stress. On one hand, GDF15 proved to be responsible for a daytime-restricted anorexia in TG mice (**Fig. 12C, F**). On the other hand, GFRAL was shown to mediate daytime GDF15 anorectic effects, but also a nighttime increase in food intake in male mice (**Fig. 20**). Altogether, these data show that the regulation of food intake by the GDF15-GFRAL axis under muscle mitochondrial stress conditions is highly complex and further research is needed in order to fully understand its mechanisms.

Presumably, the fact that GDF15 production and secretion into the blood stream oscillates in a circadian manner in TG mice (**Fig. 14**), could be linked to its diurnal control of food intake. Interestingly, highest plasma levels in TG mice were detected during the light phase around 10:00 hrs (~0,9 ng/mL), when GDF15-dependent anorexia is present. In contrast, lowest GDF15 levels were detected at 22:00 hrs (~0,4-0,6 ng/mL), when GDF15 had no effect on food intake. It is therefore tempting to hypothesize that GDF15 anorectic effects are elicited upon reaching a certain concentration threshold of ~0.7 ng/mL, above which GDF15 is able to restrict food intake, and below which it is not.

This work uncovers CRH as a mediator of the anorectic response of the GDF15-GFRAL axis by signaling through its receptor CRHR1 in male TG mice (**Fig. 25**). Interestingly, hypothalamic CRH mRNA has been shown to present circadian oscillations which are independent of the inhibitory effects of glucocorticoids on its expression (Kwak, Morano et al. 1993). Of note, CRH mRNA expression was shown to increase from morning to afternoon (when GDF15 exerts its anorectic effects) in rats but to decrease near the onset of dark, when plasma glucocorticoid concentration reaches its peak (Kwak, Morano et al. 1993). Furthermore, CRHR1 signaling seems to be, as well, regulated in a diurnal manner. *Crhr1*-knockout mice display a daytime-restricted increase in food intake (Muller, Keck et al. 2000), which likely indicates that CRH signaling through CRHR1 is daytime-restricted. C*rhr1*-knockout mice present, therefore, the opposite phenotype than TG mice with regards to daytime food intake. It seems, thus, that the circadian clock is highly involved in the control of food intake via CRH-CRHR1 pathways. Whether these are responsible for the diurnal control of food intake by the GDF15-GFRAL pathway will need to be addressed in future studies.

It is important to note that, while there is a strong food intake suppression during daytime in TG mice, classical hypothalamic players such as POMC and AgRP and peripheral signals such as ghrelin and leptin show a typical pattern of a negative energy balance (**Fig. 22**) that, in normal circumstances, would drive food consumption. Instead, TG mice display a marked daytime-restricted anorexia that is dependent on the GDF15-GFRAL-CRH axis. It might therefore be hypothesized that the GDF15-GFRAL-CRH axis works independently of the classical hypothalamic pathways to suppress food intake, and that hypothalamic POMC/AgRP signaling as well as peripheral ghrelin and leptin signaling are aimed to compensate for this negative energy balance. The idea that GFRAL-GDF15 signaling overrides homeostatic pathways was already proposed in 2017 by Hsu and colleagues, since rGDF15 treatment reversed hyperphagia in MC4R-knockout rats, a model of obesity (Hsu, Crawley et al. 2017). This goes in line with the idea that, under normal conditions, the homeostatic system maintains energy balance but, under stress conditions, this homeostatic system might be overridden by the activation of “emergency response circuits” (Morton, Meek et al. 2014). Interestingly, the pattern of the molecular players of homeostatic control of food intake described in TG mice is very close to that described in anorexia nervosa (AN) patients, who show high AgRP (Moriya, Takimoto et al. 2006) and ghrelin (Germain, Galusca et al. 2010) levels, reduced leptin levels (Tolle, Kadem et al. 2003) and, importantly, high GDF15 levels (Dostalova, Kavalkova et al. 2010) in the circulation. Furthermore, the central player uncovered by this study as downstream signaling of the GDF15-GFRAL axis, namely CRH, has been suggested to play a role in the pathophysiology of AN due to its known anorectic role and the fact that patients present

elevated CRH levels in the cerebrospinal fluid (Krahn and Gosnell 1989). Taking all this into account, it is intriguing to speculate that the GDF15-GFRAL pathway might play a role in the phenotype observed in AN patients.

While orexigenic effects of GDF15 have been reported in goldfish (Blanco, Bertucci et al. 2020), an induction of food intake through the GDF15-GFRAL axis had, until now, not been reported in mammals. The work presented here reports nighttime specific orexigenic effects of GFRAL signaling under muscle mitochondrial stress (**Fig. 20A, B**). Nevertheless, this effect seems to be sex-specific, since female TG mice did not show a nighttime increase of food intake (**Fig. 20C, D**), which could be due to the fact that in female mice estrogen is a potent inhibitor of food intake through the induction of leptin (Fungfuang, Terada et al. 2013), which could be antagonizing these GFRAL-dependent orexigenic pathways. As previously discussed, there is the possibility that another factor with an orexigenic role works through GFRAL to promote food intake in male mice, but this and other possible regulatory factors of GFRAL signaling need to be further explored in future research. These data, nevertheless, highlight the importance of considering the diurnal rhythm of animals for this kind of studies. The activation of the GDF15-GFRAL pathway is widely reported to elicit an anorectic response, but this notion is based on studies that have been conducted at daytime, when humans are active but mice are inactive. Therefore, studies are not performed at the phase at which they consume most of their food. It will thus be interesting to further study GDF15-GFRAL effects during the dark phase, when rodents are active, in order to further elucidate the complexity of this signaling pathway.

5.4 Physiological responses to endogenous *versus* recombinant GDF15

An important aspect that arises from this work is the different physiological outcome of chronic, endogenous GDF15 signaling compared to rGDF15 treatment.

The fact that treatment with supraphysiological doses of rGDF15 reduces food intake and in consequence body weight has been proven by a number of studies up to date. Nevertheless, only few studies have addressed the effects of the GDF15-GFRAL pathway on food intake of endogenously produced GDF15 at physiological concentrations. Recently, Klein et al. could show that endogenously produced GDF15 after strenuous exercise (~0,25 ng/mL) does not affect cumulative food intake in mice (Klein, Nicolaisen et al. 2021). Furthermore, in a mouse model of NASH, high GDF15 circulating levels (~0,4 ng/mL) did not affect 24 hrs food intake (Kim, Kim et al. 2018). Importantly, none of these studies analyzed the light and dark phase restricted food intake individually. The present study is the first to show a diurnal regulation of food intake in TG mice with endogenous induction of GDF15 (with peak at ~0,9 ng/mL at daytime) (**Fig. 14**) that drives a daytime-

restricted anorexia (**Fig. 12C, F**). Whether endogenously produced GDF15 drives a daytime-restricted anorexia that is undetected when measuring total 24 hrs food intake in other models of endogenous GDF15 induction will need to be further investigated. However, the fact that the studies mentioned above did not report food intake effects of GDF15 induction could also have to do with the already discussed possible threshold effect, since GDF15 concentrations were, in the models cited here, below those observed in TG mice.

The main physiological outcomes described for the treatment with rGDF15 are nausea and vomiting (emesis) (Borner, Shaulson et al. 2020, Borner, Wald et al. 2020) as well as conditioned taste aversion (Patel, Alvarez-Guaita et al. 2019, Borner, Wald et al. 2020, Worth, Shoop et al. 2020), which goes in line with an induced malaise state. Nausea and emesis have been reported after either central (3-60 pmol) and subcutaneous (20-200 µg/kg) rGDF15 administration in rats (Borner, Shaulson et al. 2020, Borner, Wald et al. 2020). Again, while no comparison can be done with centrally administered rGDF15 effects, peripheral administration of rGDF15 was substantially higher than what is found in physiological and pathophysiological conditions. The data presented in this thesis demonstrate that muscle mitochondrial stress-derived GDF15 at pathophysiological concentrations does not elicit nausea in TG mice (**Fig. 15**). Based on these results, it could be speculated that supraphysiological doses of rGDF15 might be responsible for eliciting this physiological response as a secondary effect. Recently, single-nucleus RNA sequencing revealed that GFRAL-expressing neurons co-express glucagon-like peptide 1 receptor (Zhang, Kaye et al. 2020), which has previously been linked to nausea since this is the most common side effect GLP1R agonists used as anti-diabetic drugs (Filippatos, Panagiotopoulou et al. 2014). Thus, a possible explanation for rGDF15-induced nausea could be an overactivation of GFRAL/GLP1R expressing neurons. Furthermore, the AP has been regarded since its discovery as a center for the control of nausea and emesis (Miller and Leslie 1994). Along the previous lines, a neuronal overactivation of this area could promote nausea as a secondary effect of supraphysiological rGDF15 doses. This could reflect the situation in pregnant women, which may have up to 100 times higher serum GDF15 levels than what is found in normal physiological conditions, being those elevated levels highly associated with nausea and vomiting (Moore, Brown et al. 2000, Petry, Ong et al. 2018).

Lastly, a very recent study by Cimino et al. has shown an activation of the HPA axis by both exogenous and endogenous GDF15 (Cimino, Kim et al. 2021). In this study, the authors show a GFRAL dependent hypothalamic CRH mRNA induction together with increased corticosterone levels 1 and 4 hrs after treatment with rGDF15 which led to ~ 35 and 20 ng/mL circulating rGDF15 levels 1 and 4 hrs after treatment, respectively. Furthermore,

cisplatin- and tunicamycin-induced GDF15 (~1,3-1,5 ng/mL) increased corticosterone levels 6 hrs after treatment. Therefore, the authors conclude that GDF15-GFRAL signaling induces the activation of the HPA-axis. The data presented in this thesis challenges this notion, since muscle mitochondrial stress-induced endogenous, chronic GDF15-GFRAL signaling does not lead to an induction of corticosterone in TG mice, although it does induce hypothalamic CRH mRNA levels (**Fig. 24**). These different outcomes might be due to, again, supraphysiological doses elicited by rGDF15 injection or, in the case of cisplatin and tunicamycin-induced GDF15, a toxin-induced hypersensitivity of the HPA axis. It is therefore of importance to assess the activation of the HPA axis and corticosterone levels in other models of endogenous activation of the GDF15-GFRAL axis in order to draw meaningful conclusions.

Finally, the fact that elevated GDF15 in TG mice does not elicit the same responses as rGDF15 or acutely induced endogenous GDF15 could reflect an adaptation of TG mice to chronically elevated GDF15 levels, which should be considered when aiming to understand GDF15's mode of action under different conditions or pathologies.

5.5 Relevance of the GDF15-GFRAL axis in the control of anxiety-like behavior

Probably the most novel aspect regarding the role of the GDF15-GFRAL axis that arises from this work is its involvement in the induction of anxiety-like behavior. This work shows that the activation of the GFRAL receptor in TG mice leads to an increased anxiety-like phenotype under muscle mitochondrial stress (**Fig. 23**). Since it has previously been reported that *Gdf15*-knockout mice display a decreased anxiety-like phenotype (Low, Ambikairajah et al. 2017), it could be assumed that the effects of GFRAL reported in this thesis are as well GDF15-mediated. Nevertheless, a GDF15-independent GFRAL signaling to regulate anxiety cannot be completely ruled out by these results.

Importantly, these results show that a disturbance in a peripheral organ (i.e. skeletal muscle mitochondrial stress), can lead to elaborate behavioral changes by acting on the brain through the activation of GFRAL, uncovering a muscle-brain crosstalk to control mood-related behavior. Although only few studies have addressed the prevalence of psychological disorders in MD patients, a correlation between these two has been established. MD patients often display psychiatric conditions such as major depression or social anxiety symptoms independent of disease progression (Mancuso, Orsucci et al. 2013), and as such, the need for psychiatric screening in MD patients has been recently addressed (Parikh, Goldstein et al. 2017). Considering that MD patients have been reported to present elevated plasma GDF15 levels (Yatsuga, Fujita et al. 2015), the involvement of GDF15-GFRAL signaling in the psychological outcomes of these patients needs to be further

investigated. This could potentially be extended to other pathologies such as AN, which is also tightly linked to psychological disorders such as depression, anxiety and obsessionality (Pollice, Kaye et al. 1997). Since, as discussed before, TG mice present an anorexia-nervosa like phenotype, it is tempting to hypothesize that the GDF15-GFRAL axis might mediate psychological disorders linked to this pathology in humans.

Although a link between the activation of the GDF15-GFRAL axis and the induction of anxiety-like behavior can be concluded from this work, a molecular mechanism for this link remains elusive. These results indicate that the GDF15-GFRAL axis leads to the induction of hypothalamic CRH mRNA (**Fig. 24A, D**). As previously discussed, CRH has a potent role in the control of food intake, but it also does influence anxiety-like behavior. There is increasing evidence that CRH-expressing neurons in the PVH (CRH^{PVH} neurons) are central players in linking stress and anxiety behavior by allowing shifts between active and passive innate behavior (Daviu, Fuzesi et al. 2020). Nevertheless, CRH immunoreactive fibers are also found in the cortex, amygdala, hippocampus and hindbrain in non-human primates, regions involved in the regulation of emotional behavior (for review see Risbrough and Stein 2006). It is believed that CRHR1 mostly mediates the anxiogenic effects of CRH, since administration of antalarmin (as previously described, a CRH1R antagonist) reduces anxiety-like responses (Habib, Weld et al. 2000) and *Crhr1*-knockout mice display a reduced anxiety-like behavior (Timpl, Spanagel et al. 1998). Therefore, it is a possibility that CRH induction by the GDF15-GFRAL pathway in the PVH but as well in other brain regions not only mediates daytime anorexia (**Fig. 25**) but also the anxiogenic effects observed in TG mice through CRHR1. Although the role of corticotropin releasing factor receptor 2 (CRHR2) in the modulation of anxiety-like behavior is less clear since both anxiolytic and anxiogenic effects have been described for this receptor (Kishimoto, Radulovic et al. 2000, Hammack, Schmid et al. 2003), its possible involvement in the modulation of emotional behavior by the GDF15-GFRAL pathway cannot be ruled out.

While CRH could be the central mediator of the anxiety-like response elicited by the GDF15-GFRAL axis, a role of the HPA-axis during the stress response should not be excluded, since male TG mice displayed higher post-EPM corticosterone levels that, although not significantly, tended to be dependent on GFRAL (**Fig. 23C**). While at basal, undisturbed levels, TG mice do not present an activation of the HPA-axis (**Fig. 24C, F**), it is a possibility that increased basal CRH levels lead to an increased responsiveness of the HPA-axis in TG mice subjected to stress, therefore leading to the observed anxiety-like phenotype. It would be therefore important to assess anxiety-linked behaviors in undisturbed TG mice in their home cage to narrow down the mechanisms eliciting the anxiety-like stress response.

It has to be noted that the experiments presented in this thesis indicating an involvement of the GDF15-GFRAL axis were performed at daytime, when GDF15 plasma levels peak in TG mice and elicit their anorectic effects (**Fig.12C, F**, **Fig. 14**, **Fig. 20**). Nevertheless, while the anorectic effects of the GDF15-GFRAL pathway are gone at nighttime, it will be important to further investigate nighttime specific anxiogenic phenotype in these mice, since night is their active phase and probably the most clinically relevant in this context.

5.6 GDF15: two sides of the same coin

One of the main questions that still remains unresolved about GDF15 deals with whether it is a rescue signal that the organism activates in states of stress, or whether it is rather a detrimental side effect of the stress response.

The GDF15-GFRAL axis is involved in the pathogenesis of different metabolic diseases such as obesity, type 2 diabetes (T2D) or anorexia cachexia, and therefore it has been proposed as a potential therapeutic target. Treatment with rGDF15 improves glucose and insulin tolerance and reduces adiposity in HFD-fed and normal mice (Macia, Tsai et al. 2012, Tsai, Zhang et al. 2018). Furthermore, metformin, the most commonly used anti-diabetic drug, has been proven to exert its effects through the induction of the GDF15-GFRAL axis, mainly by the reduction of body weight (Day, Ford et al. 2019, Coll, Chen et al. 2020). Paradoxically, obesity and T2D patients present elevated serum GDF15 levels (Adela and Banerjee 2015, Xiong, Walker et al. 2017). In HFD-fed mice, inhibition of the GFRAL-GDF15 pathway led to a rapid body weight gain, indicating, as pointed out by Breit and colleagues (Breit, Brown et al. 2020), that physiological levels in obesity might be involved in counteracting the development of the pathology. On the other extreme, antibody-mediated inhibition of the GDF15-GFRAL pathway has proven successful to reverse cancer cachexia in mice (Suriben, Chen et al. 2020), showing that activation of this pathway might as well be detrimental for the organism in some pathological conditions.

The results from this thesis show two sides of the same coin for the function of the GDF15-GFRAL pathway under muscle mitochondrial stress. On one hand, activation of this pathway leads to a systemic metabolic remodeling with beneficial metabolic outcomes such as sWAT browning (**Fig. 9**), improved glucose metabolism (**Fig. 10**) and increased metabolic flexibility (**Fig. 11**). These beneficial effects of GDF15 on energy metabolism have also been reported by others working with different models of mitochondrial dysfunction directed at different organs (Chung, Ryu et al. 2017, Choi, Jung et al. 2020, Kang, Choi et al. 2021). On the other hand, this work uncovers that chronic activation of this signaling is responsible for a marked daytime-restricted AN-like phenotype (**Fig. 22**) and, importantly, an increased anxiety-like phenotype (**Fig. 23**).

As GDF15's anorectic role is well established in other mouse models, one might hypothesize that the systemic metabolic remodeling in TG mice elicited by GDF15 is a consequence of its effects on food intake. Interestingly, the GDF15 induced daytime-restricted anorexia of TG mice resembles a time restricted feeding, which has been reported in mice and humans to have beneficial health outcomes (Hatori, Vollmers et al. 2012, Sutton, Beyl et al. 2018). Nevertheless, the consequences or side effects of this improved metabolic effects via time-restricted food intake reduction must be considered. This thesis uncovers, for the first time, the induction of anxiety-like behavior by the GDF15-GFRAL pathway, which argues against an activation of the GDF15-GFRAL pathway as a potential therapeutic target. The question of the biological relevance of this finding arises. Is anxiety-like behavior elicited by the GDF15-GFRAL pathway an important outcome of the stress response, or is it a secondary side effect? This is a question that will need to be addressed in future studies, since it might influence the therapeutic potential of targeting the GDF15-GFRAL pathway for the treatment of metabolic diseases.

5.7 Limitations of the study

This study aimed to understand the physiological relevance of the activation of the GDF15-GFRAL pathway under muscle-specific mitochondrial stress. For this, GDF15-overexpressing TG mice in combination with GDF15 and GFRAL knockout mouse models were employed.

TG mice have, since birth, an increased level of skeletal muscle mitochondrial UCP1 expression, leading to lifelong induced mitochondrial stress levels. This mutation leads, therefore, to chronic expression of GDF15. Further along these lines, TG mice do not only present an induction of the GDF15-GFRAL axis but other physiological and molecular adaptations to mitochondrial uncoupling that could affect the regulation of this pathway. Therefore, it would be important to assess in future research whether the results from this study can be translated to other mouse models of mitochondrial stress or even in other conditions in which GDF15 is induced endogenously which represent a more pathological approach such as cancer cachexia or obesity.

The inconsistency of the female data regarding the effects of the GDF15-GFRAL pathway on anxiety-like responses, constitutes another limitation of this study. While in mouse study 3 (Results Chapter II) an increased anxiety-like phenotype could be observed in female TG mice (**Fig. 16D, E**), this could not be reproduced in mouse study 4 (**Fig. 23D, E**), therefore not allowing conclusions on the effects of this pathway in TG female mice. Of note, estrus cycle of females mice, which could have a potential effect on behavioral outcomes (Chari, Griswold et al. 2020), was not checked or matched prior to experiments. Thus, more

controlled future experiments on female mice will have to be carried out to draw final conclusions on their susceptibility to anxiety-like behavior induced by the GDF15-GFRAL pathway. Along these lines, while CRHR1 could be confirmed as a mediator of the anorectic response elicited by the GDF15-GFRAL pathway in male mice, this could not be reproduced in female mice (**Fig. 25**). Nevertheless, female mice showed a strong GDF15-GFRAL-dependent *Crh* mRNA induction (**Fig. 24D**), indicating that CRH is probably also responsible for their daytime-restricted anorexia, but this will need to be further investigated in future research.

6. Conclusion and future perspectives

Currently, considerable effort is being made in order to understand the role, mode of action, and importantly, the biological significance of the GDF15-GFRAL pathway. Nonetheless, the wide range of pathologies and conditions that induce GDF15 makes it a hard task to narrow down general GDF15-GFRAL downstream effects.

This study focused on understanding the role of the endogenous activation of the GDF15-GFRAL signaling pathway under mitochondrial stress conditions making use of TG mice with muscle-targeted mitochondrial stress. In a first approach aiming to elucidate the metabolic role of GDF15 under these conditions, this study identifies GDF15 as a mediator of mitochondrial stress-induced metabolic adaptations in TG mice. Furthermore, this work identifies a diurnal control of food intake (i.e. daytime-restricted anorexia) in TG mice for which the GDF15-GFRAL pathway proves to be responsible, possibly mediated by diurnal fluctuations of GDF15 synthesis and circulating levels. Furthermore, the work presented in this PhD thesis uncovers hypothalamic CRH as a downstream effector of the GDF15-GFRAL pathway to mediate daytime-restricted anorexia in TG mice, identifying for the first time a mechanism for GDF15-GFRAL-induced anorexia. Finally, this study uncovers a so-far unknown role of the GDF15-GFRAL axis, the induction of anxiety-like behavior, opening new avenues of research regarding the consequences of the modulation of this pathway.

The results of this study shed new light into the biology and mode of action of GDF15 and its receptor GFRAL. First, they uncover a GDF15-dependent diurnal pattern of a shift in ingestive behavior, bringing up the importance of acknowledging circadian rhythms when aiming to understand the role of endogenously produced GDF15. Second, these results highlight the different behavioral and metabolic outcomes of the endogenous, chronic activation of this pathway when compared with pharmacological studies using rGDF15, and as such, the need for more studies addressing GDF15-GFRAL mode of action under different endogenous stressors. Third, this thesis identifies anxiety as a consequence of the activation of the GDF15-GFRAL axis. This should stimulate translational research aiming to understand whether this signaling pathway is involved in the often-reported association between metabolic disorders such as mitochondrial disease and anorexia nervosa with mood-related disorders.

7. References

Abdalla, M. M. (2017). "Central and peripheral control of food intake." Endocr Regul **51**(1): 52-70.

Adela, R. and S. K. Banerjee (2015). "GDF-15 as a Target and Biomarker for Diabetes and Cardiovascular Diseases: A Translational Prospective." J Diabetes Res **2015**: 490842.

Baek, S. J., J. M. Horowitz and T. E. Eling (2001). "Molecular cloning and characterization of human nonsteroidal anti-inflammatory drug-activated gene promoter. Basal transcription is mediated by Sp1 and Sp3." J Biol Chem **276**(36): 33384-33392.

Baek, S. J., J. S. Kim, J. B. Nixon, R. P. DiAugustine and T. E. Eling (2004). "Expression of NAG-1, a transforming growth factor-beta superfamily member, by troglitazone requires the early growth response gene EGR-1." J Biol Chem **279**(8): 6883-6892.

Baek, S. J., K. S. Kim, J. B. Nixon, L. C. Wilson and T. E. Eling (2001). "Cyclooxygenase inhibitors regulate the expression of a TGF-beta superfamily member that has proapoptotic and antitumorigenic activities." Mol Pharmacol **59**(4): 901-908.

Bauskin, A. R., D. A. Brown, S. Junankar, K. K. Rasiah, S. Eggleton, M. Hunter, T. Liu, D. Smith, T. Kuffner, G. J. Pankhurst, H. Johnen, P. J. Russell, W. Barret, P. D. Stricker, J. J. Grygiel, J. G. Kench, S. M. Henshall, R. L. Sutherland and S. N. Breit (2005). "The propeptide mediates formation of stromal stores of PROMIC-1: role in determining prostate cancer outcome." Cancer Res **65**(6): 2330-2336.

Becker, C., A. Kukat, K. Szczepanowska, S. Hermans, K. Senft, C. P. Brandscheid, P. Maiti and A. Trifunovic (2018). "CLPP deficiency protects against metabolic syndrome but hinders adaptive thermogenesis." EMBO Rep **19**(5).

Benoit, S. C., T. E. Thiele, S. C. Heinrichs, P. A. Rushing, K. A. Blake and R. J. Steeley (2000). "Comparison of central administration of corticotropin-releasing hormone and urocortin on food intake, conditioned taste aversion, and c-Fos expression." Peptides **21**(3): 345-351.

Blanco, A. M., J. I. Bertucci, C. Velasco and S. Unniappan (2020). "Growth differentiation factor 15 (GDF-15) is a novel orexigen in fish." Mol Cell Endocrinol **505**: 110720.

Bootcov, M. R., A. R. Bauskin, S. M. Valenzuela, A. G. Moore, M. Bansal, X. Y. He, H. P. Zhang, M. Donnellan, S. Mahler, K. Pryor, B. J. Walsh, R. C. Nicholson, W. D. Fairlie, S. B. Por, J. M. Robbins and S. N. Breit (1997). "MIC-1, a novel macrophage inhibitory cytokine,

is a divergent member of the TGF-beta superfamily." Proc Natl Acad Sci U S A **94**(21): 11514-11519.

Borner, T., E. D. Shaulson, M. Y. Ghidewon, A. B. Barnett, C. C. Horn, R. P. Doyle, H. J. Grill, M. R. Hayes and B. C. De Jonghe (2020). "GDF15 Induces Anorexia through Nausea and Emesis." Cell Metab **31**(2): 351-362 e355.

Borner, T., H. S. Wald, M. Y. Ghidewon, B. Zhang, Z. Wu, B. C. De Jonghe, D. Breen and H. J. Grill (2020). "GDF15 Induces an Aversive Visceral Malaise State that Drives Anorexia and Weight Loss." Cell Rep **31**(3): 107543.

Breit, S. N., D. A. Brown and V. W. Tsai (2020). "The GDF15-GFRAL Pathway in Health and Metabolic Disease: Friend or Foe?" Annu Rev Physiol.

Breit, S. N., V. W. Tsai and D. A. Brown (2017). "Targeting Obesity and Cachexia: Identification of the GFRAL Receptor-MIC-1/GDF15 Pathway." Trends Mol Med **23**(12): 1065-1067.

Chari, T., S. Griswold, N. A. Andrews and M. Fagiolini (2020). "The Stage of the Estrus Cycle Is Critical for Interpretation of Female Mouse Social Interaction Behavior." Front Behav Neurosci **14**: 113.

Choi, M. J., S.-B. Jung, S. E. Lee, S. G. Kang, J. H. Lee, M. J. Ryu, H. K. Chung, J. Y. Chang, Y. K. Kim, H. J. Hong, H. Kim, H. J. Kim, C.-H. Lee, A. Mardinoglu, H.-S. Yi and M. Shong (2020). "An adipocyte-specific defect in oxidative phosphorylation increases systemic energy expenditure and protects against diet-induced obesity in mouse models." Diabetologia **63**(4): 837-852.

Chung, H. K., D. Ryu, K. S. Kim, J. Y. Chang, Y. K. Kim, H. S. Yi, S. G. Kang, M. J. Choi, S. E. Lee, S. B. Jung, M. J. Ryu, S. J. Kim, G. R. Kweon, H. Kim, J. H. Hwang, C. H. Lee, S. J. Lee, C. E. Wall, M. Downes, R. M. Evans, J. Auwerx and M. Shong (2017). "Growth differentiation factor 15 is a myomitokine governing systemic energy homeostasis." J Cell Biol **216**(1): 149-165.

Cimino, I., A. P. Coll and G. S. H. Yeo (2017). "GDF15 and energy balance: homing in on a mechanism." Nat Med **23**(10): 1119-1120.

Cimino, I., H. Kim, Y. C. L. Tung, K. Pedersen, D. Rimmington, J. A. Tadross, S. N. Kohnke, A. Neves-Costa, A. Barros, S. Joaquim, D. Bennett, A. Melvin, S. M. Lockhart, A. J. Rostron, J. Scott, H. Liu, K. Burling, P. Barker, M. R. Clatworthy, E. C. Lee, A. J. Simpson, G. S. H. Yeo, L. F. Moita, K. K. Bence, S. B. Jorgensen, A. P. Coll, D. M. Breen and S. O'Rahilly

(2021). "Activation of the hypothalamic-pituitary-adrenal axis by exogenous and endogenous GDF15." Proc Natl Acad Sci U S A **118**(27).

Coll, A. P., M. Chen, P. Taskar, D. Rimmington, S. Patel, J. A. Tadross, I. Cimino, M. Yang, P. Welsh, S. Virtue, D. A. Goldspink, E. L. Miedzybrodzka, A. R. Konopka, R. R. Esponda, J. T. Huang, Y. C. L. Tung, S. Rodriguez-Cuenca, R. A. Tomaz, H. P. Harding, A. Melvin, G. S. H. Yeo, D. Preiss, A. Vidal-Puig, L. Vallier, K. S. Nair, N. J. Wareham, D. Ron, F. M. Gribble, F. Reimann, N. Sattar, D. B. Savage, B. B. Allan and S. O'Rahilly (2020). "GDF15 mediates the effects of metformin on body weight and energy balance." Nature **578**(7795): 444-448.

Coll, A. P., M. Chen, P. Taskar, D. Rimmington, S. Patel, J. A. Tadross, I. Cimino, M. Yang, P. Welsh, S. Virtue, D. A. Goldspink, E. L. Miedzybrodzka, A. R. Konopka, R. R. Esponda, J. T. J. Huang, Y. C. L. Tung, S. Rodriguez-Cuenca, R. A. Tomaz, H. P. Harding, A. Melvin, G. S. H. Yeo, D. Preiss, A. Vidal-Puig, L. Vallier, K. S. Nair, N. J. Wareham, D. Ron, F. M. Gribble, F. Reimann, N. Sattar, D. B. Savage, B. B. Allan and S. O'Rahilly (2019). "GDF15 mediates the effects of metformin on body weight and energy balance." Nature.

Couplan, E., C. Gelly, M. Goubern, C. Fleury, B. Quesson, M. Silberberg, E. Thiaudiere, P. Mateo, M. Lonchampt, N. Levens, C. De Montrion, S. Ortmann, S. Klaus, M. D. Gonzalez-Barroso, A. M. Cassard-Doulcier, D. Ricquier, A. X. Bigard, P. Diolez and F. Bouillaud (2002). "High level of uncoupling protein 1 expression in muscle of transgenic mice selectively affects muscles at rest and decreases their IIb fiber content." J Biol Chem **277**(45): 43079-43088.

Daviu, N., T. Fuzesi, D. G. Rosenegger, N. P. Rasiah, T. L. Sterley, G. Peringod and J. S. Bains (2020). "Paraventricular nucleus CRH neurons encode stress controllability and regulate defensive behavior selection." Nat Neurosci **23**(3): 398-410.

Day, E. A., R. J. Ford, B. K. Smith, P. Mohammadi-Shemirani, M. R. Morrow, R. M. Gutgesell, R. Lu, A. R. Raphenya, M. Kabiri, A. G. McArthur, N. McInnes, S. Hess, G. Pare, H. C. Gerstein and G. R. Steinberg (2019). "Metformin-induced increases in GDF15 are important for suppressing appetite and promoting weight loss." Nature Metabolism **1**(12): 1202-+.

Day, E. A., R. J. Ford, B. K. Smith, P. Mohammadi-Shemirani, M. R. Morrow, R. M. Gutgesell, R. Lu, A. R. Raphenya, M. Kabiri, A. G. McArthur, N. McInnes, S. Hess, G. Paré, H. C. Gerstein and G. R. Steinberg (2019). "Metformin-induced increases in GDF15 are important for suppressing appetite and promoting weight loss." Nature Metabolism **1**(12): 1202-1208.

Dickmeis, T. (2009). "Glucocorticoids and the circadian clock." Journal of Endocrinology **200**(1): 3-22.

Dominguez-Gonzalez, C., C. Badosa, M. Madruga-Garrido, I. Marti, C. Paradas, C. Ortez, J. Diaz-Manera, A. Berardo, J. Alonso-Perez, S. Trifunov, D. Cuadras, S. G. Kalko, C. Blazquez-Bermejo, Y. Camara, R. Marti, F. Mavillard, M. A. Martin, J. Montoya, E. Ruiz-Pesini, J. Villarroya, R. Montero, F. Villarroya, R. Artuch, M. Hirano, A. Nascimento and C. Jimenez-Mallebrera (2020). "Growth Differentiation Factor 15 is a potential biomarker of therapeutic response for TK2 deficient myopathy." Sci Rep **10**(1): 10111.

Dostalova, I., P. Kavalkova, H. Papezova, D. Domluvilova, V. Zikan and M. Haluzik (2010). "Association of macrophage inhibitory cytokine-1 with nutritional status, body composition and bone mineral density in patients with anorexia nervosa: the influence of partial realimentation." Nutrition & Metabolism **7**.

Edwards, G. L. and R. C. Ritter (1981). "Ablation of the area postrema causes exaggerated consumption of preferred foods in the rat." Brain Res **216**(2): 265-276.

Emmerson, P. J., F. Wang, Y. Du, Q. Liu, R. T. Pickard, M. D. Gonciarz, T. Coskun, M. J. Hamang, D. K. Sindelar, K. K. Ballman, L. A. Foltz, A. Muppidi, J. Alsina-Fernandez, G. C. Barnard, J. X. Tang, X. Liu, X. Mao, R. Siegel, J. H. Sloan, P. J. Mitchell, B. B. Zhang, R. E. Gimeno, B. Shan and X. Wu (2017). "The metabolic effects of GDF15 are mediated by the orphan receptor GFRAL." Nat Med **23**(10): 1215-1219.

Fejzo, M. S., D. Arzy, R. Tian, K. MacGibbon and P. M. Mullin (2018). "Evidence GDF15 Plays a Role in Familial and Recurrent Hyperemesis Gravidarum." Geburtshilfe Und Frauenheilkunde **78**(9): 866-870.

Fejzo, M. S., O. V. Sazonova, J. F. Sathirapongsasuti, I. B. Hallgrimsdottir, V. Vacic, K. W. MacGibbon, F. P. Schoenberg, N. Mancuso, D. J. Slamon, P. M. Mullin and T. andMe Research (2018). "Placenta and appetite genes GDF15 and IGFBP7 are associated with hyperemesis gravidarum." Nat Commun **9**(1): 1178.

Filippatos, T. D., T. V. Panagiotopoulou and M. S. Elisaf (2014). "Adverse Effects of GLP-1 Receptor Agonists." Rev Diabet Stud **11**(3-4): 202-230.

Frontera, W. R. and J. Ochala (2015). "Skeletal muscle: a brief review of structure and function." Calcif Tissue Int **96**(3): 183-195.

Fujita, Y., M. Ito, T. Kojima, S. Yatsuga, Y. Koga and M. Tanaka (2015). "GDF15 is a novel biomarker to evaluate efficacy of pyruvate therapy for mitochondrial diseases." Mitochondrion **20**: 34-42.

Fungfuang, W., M. Terada, N. Komatsu, C. Moon and T. R. Saito (2013). "Effects of estrogen on food intake, serum leptin levels and leptin mRNA expression in adipose tissue of female rats." Lab Anim Res **29**(3): 168-173.

Gao, Q. and T. L. Horvath (2008). "Neuronal control of energy homeostasis." FEBS Lett **582**(1): 132-141.

Germain, N., B. Galusca, D. Grouselle, D. Frere, S. Billard, J. Epelbaum and B. Estour (2010). "Ghrelin and obestatin circadian levels differentiate bingeing-purging from restrictive anorexia nervosa." J Clin Endocrinol Metab **95**(6): 3057-3062.

Gil, C. I., M. Ost, J. Kasch, S. Schumann, S. Heider and S. Klaus (2019). "Role of GDF15 in active lifestyle induced metabolic adaptations and acute exercise response in mice." Sci Rep **9**(1): 20120.

Gjerstad, J. K., S. L. Lightman and F. Spiga (2018). "Role of glucocorticoid negative feedback in the regulation of HPA axis pulsatility." Stress **21**(5): 403-416.

Godoy, L. D., M. T. Rossignoli, P. Delfino-Pereira, N. Garcia-Cairasco and E. H. de Lima Umeoka (2018). "A Comprehensive Overview on Stress Neurobiology: Basic Concepts and Clinical Implications." Front Behav Neurosci **12**: 127.

Habib, K. E., K. P. Weld, K. C. Rice, J. Pushkas, M. Champoux, S. Listwak, E. L. Webster, A. J. Atkinson, J. Schulkin, C. Contoreggi, G. P. Chrousos, S. M. McCann, S. J. Suomi, J. D. Higley and P. W. Gold (2000). "Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates." Proc Natl Acad Sci U S A **97**(11): 6079-6084.

Hammack, S. E., M. J. Schmid, M. L. LoPresti, A. Der-Avakian, M. A. Pellymounter, A. C. Foster, L. R. Watkins and S. F. Maier (2003). "Corticotropin releasing hormone type 2 receptors in the dorsal raphe nucleus mediate the behavioral consequences of uncontrollable stress." J Neurosci **23**(3): 1019-1025.

Hankin, M. E., H. M. Theile and A. W. Steinbeck (1977). "An evaluation of laboratory tests for the detection and differential diagnosis of Cushing's syndrome." Clin Endocrinol (Oxf) **6**(3): 185-196.

Hatori, M., C. Vollmers, A. Zarrinpar, L. DiTacchio, E. A. Bushong, S. Gill, M. Leblanc, A. Chaix, M. Joens, J. A. Fitzpatrick, M. H. Ellisman and S. Panda (2012). "Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet." Cell Metab **15**(6): 848-860.

Heinrichs, S. C., F. Menzaghi, E. M. Pich, R. L. Hauger and G. F. Koob (1993). "Corticotropin-releasing factor in the paraventricular nucleus modulates feeding induced by neuropeptide Y." Brain Res **611**(1): 18-24.

Herz, C. T. and F. W. Kiefer (2019). "Adipose tissue browning in mice and humans." J Endocrinol **241**(3): R97-R109.

Hsiao, E. C., L. G. Koniaris, T. Zimmers-Koniaris, S. M. Sebald, T. V. Huynh and S. J. Lee (2000). "Characterization of growth-differentiation factor 15, a transforming growth factor beta superfamily member induced following liver injury." Mol Cell Biol **20**(10): 3742-3751.

Hsu, J. Y., S. Crawley, M. Chen, D. A. Ayupova, D. A. Lindhout, J. Higbee, A. Kutach, W. Joo, Z. Gao, D. Fu, C. To, K. Mondal, B. Li, A. Kekatpure, M. Wang, T. Laird, G. Horner, J. Chan, M. McEntee, M. Lopez, D. Lakshminarasimhan, A. White, S. P. Wang, J. Yao, J. Yie, H. Matern, M. Solloway, R. Haldankar, T. Parsons, J. Tang, W. D. Shen, Y. Alice Chen, H. Tian and B. B. Allan (2017). "Non-homeostatic body weight regulation through a brainstem-restricted receptor for GDF15." Nature **550**(7675): 255-259.

Impey, S., S. R. McCorkle, H. Cha-Molstad, J. M. Dwyer, G. S. Yochum, J. M. Boss, S. McWeeney, J. J. Dunn, G. Mandel and R. H. Goodman (2004). "Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions." Cell **119**(7): 1041-1054.

Johnen, H., S. Lin, T. Kuffner, D. A. Brown, V. W. Tsai, A. R. Bauskin, L. Wu, G. Pankhurst, L. Jiang, S. Junankar, M. Hunter, W. D. Fairlie, N. J. Lee, R. F. Enriquez, P. A. Baldock, E. Corey, F. S. Apple, M. M. Murakami, E. J. Lin, C. Wang, M. J. During, A. Sainsbury, H. Herzog and S. N. Breit (2007). "Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1." Nat Med **13**(11): 1333-1340.

Jung, S. B., M. J. Choi, D. Ryu, H. S. Yi, S. E. Lee, J. Y. Chang, H. K. Chung, Y. K. Kim, S. G. Kang, J. H. Lee, K. S. Kim, H. J. Kim, C. S. Kim, C. H. Lee, R. W. Williams, H. Kim, H. K. Lee, J. Auwerx and M. Shong (2018). "Reduced oxidative capacity in macrophages results in systemic insulin resistance." Nat Commun **9**(1): 1551.

Kang, S. G., M. J. Choi, S. B. Jung, H. K. Chung, J. Y. Chang, J. T. Kim, Y. E. Kang, J. H. Lee, H. J. Hong, S. M. Jun, H. J. Ro, J. M. Suh, H. Kim, J. Auwerx, H. S. Yi and M. Shong (2021). "Differential roles of GDF15 and FGF21 in systemic metabolic adaptation to the mitochondrial integrated stress response." iScience **24**(3): 102181.

Keipert, S., S. Klaus, G. Heldmaier and M. Jastroch (2010). "UCP1 ectopically expressed in murine muscle displays native function and mitigates mitochondrial superoxide production." Biochim Biophys Acta **1797**(2): 324-330.

Keipert, S., D. Lutter, B. O. Schroeder, D. Brandt, M. Ståhlman, T. Schwarzmayr, E. Graf, H. Fuchs, M. H. de Angelis, M. H. Tschöp, J. Rozman and M. Jastroch (2020). "Endogenous FGF21-signaling controls paradoxical obesity resistance of UCP1-deficient mice." Nature communications **11**(1): 624-624.

Keipert, S. and M. Ost (2021). "Stress-induced FGF21 and GDF15 in obesity and obesity resistance." Trends in Endocrinology & Metabolism.

Keipert, S., M. Ost, K. Johann, F. Imber, M. Jastroch, E. M. van Schothorst, J. Keijer and S. Klaus (2014). "Skeletal muscle mitochondrial uncoupling drives endocrine cross-talk through the induction of FGF21 as a myokine." Am J Physiol Endocrinol Metab **306**(5): E469-482.

Keipert, S., A. Voigt and S. Klaus (2011). "Dietary effects on body composition, glucose metabolism, and longevity are modulated by skeletal muscle mitochondrial uncoupling in mice." Aging Cell **10**(1): 122-136.

Kelly, J. A., M. S. Lucia and J. R. Lambert (2009). "p53 controls prostate-derived factor/macrophage inhibitory cytokine/NSAID-activated gene expression in response to cell density, DNA damage and hypoxia through diverse mechanisms." Cancer Lett **277**(1): 38-47.

Kempf, T., M. Eden, J. Strelau, M. Naguib, C. Willenbockel, J. Tongers, J. Heineke, D. Kotlarz, J. Xu, J. D. Molkentin, H. W. Niessen, H. Drexler and K. C. Wollert (2006). "The transforming growth factor-beta superfamily member growth-differentiation factor-15 protects the heart from ischemia/reperfusion injury." Circ Res **98**(3): 351-360.

Kempf, T., A. Guba-Quint, J. Torgerson, M. C. Magnone, C. Haefliger, M. Bobadilla and K. C. Wollert (2012). "Growth differentiation factor 15 predicts future insulin resistance and impaired glucose control in obese nondiabetic individuals: results from the XENDOS trial." Eur J Endocrinol **167**(5): 671-678.

Kempf, T., A. Zarbock, C. Widera, S. Butz, A. Stadtmann, J. Rossaint, M. Bolomini-Vittori, M. Korf-Klingebiel, L. C. Napp, B. Hansen, A. Kanwischer, U. Bavendiek, G. Beutel, M. Hapke, M. G. Sauer, C. Laudanna, N. Hogg, D. Vestweber and K. C. Wollert (2011). "GDF-15 is an inhibitor of leukocyte integrin activation required for survival after myocardial infarction in mice." Nat Med **17**(5): 581-588.

Kim, K. H., Y. T. Jeong, H. Oh, S. H. Kim, J. M. Cho, Y. N. Kim, S. S. Kim, D. H. Kim, K. Y. Hur, H. K. Kim, T. Ko, J. Han, H. L. Kim, J. Kim, S. H. Back, M. Komatsu, H. Chen, D. C. Chan, M. Konishi, N. Itoh, C. S. Choi and M. S. Lee (2013). "Autophagy deficiency leads to

protection from obesity and insulin resistance by inducing Fgf21 as a mitokine." Nat Med **19**(1): 83-92.

Kim, K. H., S. H. Kim, D. H. Han, Y. S. Jo, Y. H. Lee and M. S. Lee (2018). "Growth differentiation factor 15 ameliorates nonalcoholic steatohepatitis and related metabolic disorders in mice." Sci Rep **8**(1): 6789.

Kim, K. H. and M. S. Lee (2021). "GDF15 as a central mediator for integrated stress response and a promising therapeutic molecule for metabolic disorders and NASH." Biochim Biophys Acta Gen Subj **1865**(3): 129834.

Kishimoto, T., J. Radulovic, M. Radulovic, C. R. Lin, C. Schrick, F. Hooshmand, O. Hermanson, M. G. Rosenfeld and J. Spiess (2000). "Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2." Nat Genet **24**(4): 415-419.

Klaus, S., B. Rudolph, C. Dohrmann and R. Wehr (2005). "Expression of uncoupling protein 1 in skeletal muscle decreases muscle energy efficiency and affects thermoregulation and substrate oxidation." Physiol Genomics **21**(2): 193-200.

Klein, A. B., T. S. Nicolaisen, N. Ortenblad, K. D. Gejl, R. Jensen, A. M. Fritzen, E. L. Larsen, K. Karstoft, H. E. Poulsen, T. Morville, R. E. Sahl, J. W. Helge, J. Lund, S. Falk, M. Lyngbaek, H. Ellingsgaard, B. K. Pedersen, W. Lu, B. Finan, S. B. Jorgensen, R. J. Seeley, M. Kleinert, B. Kiens, E. A. Richter and C. Clemmensen (2021). "Pharmacological but not physiological GDF15 suppresses feeding and the motivation to exercise." Nat Commun **12**(1): 1041.

Krahn, D. D. and B. A. Gosnell (1989). "Corticotropin-releasing hormone: possible role in eating disorders." Psychiatr Med **7**(4): 235-245.

Kwak, S. P., M. I. Morano, E. A. Young, S. J. Watson and H. Akil (1993). "Diurnal CRH mRNA rhythm in the hypothalamus: decreased expression in the evening is not dependent on endogenous glucocorticoids." Neuroendocrinology **57**(1): 96-105.

Laurens, C., A. Parmar, E. Murphy, D. Carper, B. Lair, P. Maes, J. Vion, N. Boulet, C. Fontaine, M. Marques, D. Larrouy, I. Harant, C. Thalamas, E. Montastier, S. Caspar-Bauguil, V. Bourlier, G. Tavernier, J. L. Grolleau, A. Bouloumie, D. Langin, N. Viguerie, F. Bertile, S. Blanc, I. de Glisezinski, D. O'Gorman and C. Moro (2020). "Growth and differentiation factor 15 is secreted by skeletal muscle during exercise and promotes lipolysis in humans." JCI Insight **5**(6).

Lee, S. H., J. S. Kim, K. Yamaguchi, T. E. Eling and S. J. Baek (2005). "Indole-3-carbinol and 3,3'-diindolylmethane induce expression of NAG-1 in a p53-independent manner." Biochem Biophys Res Commun **328**(1): 63-69.

Lehtonen, J. M., M. Auranen, N. Darin, K. Sofou, L. Bindoff, O. Hikmat, J. Uusimaa, P. Vieira, M. Tulinius, T. Lönnqvist, I. F. de Coo, A. Suomalainen and P. Isohanni (2021). "Diagnostic value of serum biomarkers FGF21 and GDF15 compared to muscle sample in mitochondrial disease." Journal of Inherited Metabolic Disease **44**(2): 469-480.

Li, J. J., J. Liu, K. Lupino, X. Liu, L. Zhang and L. Pei (2018). "Growth Differentiation Factor 15 Maturation Requires Proteolytic Cleavage by PCSK3, -5, and -6." Mol Cell Biol **38**(21).

Li, P. X., J. Wong, A. Ayed, D. Ngo, A. M. Brade, C. Arrowsmith, R. C. Austin and H. J. Klamut (2000). "Placental transforming growth factor-beta is a downstream mediator of the growth arrest and apoptotic response of tumor cells to DNA damage and p53 overexpression." J Biol Chem **275**(26): 20127-20135.

Lovas, K. and E. S. Husebye (2007). "Continuous subcutaneous hydrocortisone infusion in Addison's disease." Eur J Endocrinol **157**(1): 109-112.

Low, J. K., A. Ambikairajah, K. Shang, D. A. Brown, V. W. Tsai, S. N. Breit and T. Karl (2017). "First Behavioural Characterisation of a Knockout Mouse Model for the Transforming Growth Factor (TGF)-beta Superfamily Cytokine, MIC-1/GDF15." PLoS One **12**(1): e0168416.

Luft, R., D. Ikkos, G. Palmieri, L. Ernster and B. Afzelius (1962). "A case of severe hypermetabolism of nonthyroid origin with a defect in the maintenance of mitochondrial respiratory control: a correlated clinical, biochemical, and morphological study." J Clin Invest **41**: 1776-1804.

Macia, L., V. W.-W. Tsai, A. D. Nguyen, H. Johnen, T. Kuffner, Y.-C. Shi, S. Lin, H. Herzog, D. A. Brown, S. N. Breit and A. Sainsbury (2012). "Macrophage Inhibitory Cytokine 1 (MIC-1/GDF15) Decreases Food Intake, Body Weight and Improves Glucose Tolerance in Mice on Normal & Obesogenic Diets." PLOS ONE **7**(4): e34868.

Macia, L., V. W. Tsai, A. D. Nguyen, H. Johnen, T. Kuffner, Y. C. Shi, S. Lin, H. Herzog, D. A. Brown, S. N. Breit and A. Sainsbury (2012). "Macrophage inhibitory cytokine 1 (MIC-1/GDF15) decreases food intake, body weight and improves glucose tolerance in mice on normal & obesogenic diets." PLoS One **7**(4): e34868.

Mancuso, M., D. Orsucci, E. C. Ienco, E. Pini, A. Choub and G. Siciliano (2013). "Psychiatric involvement in adult patients with mitochondrial disease." Neurol Sci **34**(1): 71-74.

Maresca, A., V. Del Dotto, M. Romagnoli, C. La Morgia, L. Di Vito, M. Capristo, M. L. Valentino, V. Carelli and E.-M. S. Group (2020). "Expanding and validating the biomarkers for mitochondrial diseases." J Mol Med (Berl).

Miller, A. D. and R. A. Leslie (1994). "The area postrema and vomiting." Front Neuroendocrinol **15**(4): 301-320.

Miller, C. C., P. V. Holmes and G. L. Edwards (2002). "Area postrema lesions elevate NPY levels and decrease anxiety-related behavior in rats." Physiol Behav **77**(1): 135-140.

Montero, R., D. Yubero, J. Villarroya, D. Henares, C. Jou, M. A. Rodriguez, F. Ramos, A. Nascimento, C. I. Ortez, J. Campistol, B. Perez-Duenas, M. O'Callaghan, M. Pineda, A. Garcia-Cazorla, J. C. Oferil, J. Montoya, E. Ruiz-Pesini, S. Emperador, M. Meznaric, L. Campderros, S. G. Kalko, F. Villarroya, R. Artuch and C. Jimenez-Mallebrera (2016). "GDF-15 Is Elevated in Children with Mitochondrial Diseases and Is Induced by Mitochondrial Dysfunction." PLoS One **11**(2): e0148709.

Moore, A. G., D. A. Brown, W. D. Fairlie, A. R. Bauskin, P. K. Brown, M. L. Munier, P. K. Russell, L. A. Salamonsen, E. M. Wallace and S. N. Breit (2000). "The transforming growth factor-ss superfamily cytokine macrophage inhibitory cytokine-1 is present in high concentrations in the serum of pregnant women." J Clin Endocrinol Metab **85**(12): 4781-4788.

Moriya, J., Y. Takimoto, K. Yoshiuchi, T. Shimosawa and A. Akabayashi (2006). "Plasma agouti-related protein levels in women with anorexia nervosa." Psychoneuroendocrinology **31**(9): 1057-1061.

Morton, G. J., T. H. Meek and M. W. Schwartz (2014). "Neurobiology of food intake in health and disease." Nat Rev Neurosci **15**(6): 367-378.

Muller, M. B., M. E. Keck, S. Zimmermann, F. Holsboer and W. Wurst (2000). "Disruption of feeding behavior in CRH receptor 1-deficient mice is dependent on glucocorticoids." Neuroreport **11**(9): 1963-1966.

Mullican, S. E., X. Lin-Schmidt, C. N. Chin, J. A. Chavez, J. L. Furman, A. A. Armstrong, S. C. Beck, V. J. South, T. Q. Dinh, T. D. Cash-Mason, C. R. Cavanaugh, S. Nelson, C. Huang, M. J. Hunter and S. M. Rangwala (2017). "GFRAL is the receptor for GDF15 and the ligand promotes weight loss in mice and nonhuman primates." Nat Med **23**(10): 1150-1157.

Neschen, S., Y. Katterle, J. Richter, R. Augustin, S. Scherneck, F. Mirhashemi, A. Schurmann, H. G. Joost and S. Klaus (2008). "Uncoupling protein 1 expression in murine

skeletal muscle increases AMPK activation, glucose turnover, and insulin sensitivity in vivo." Physiol Genomics **33**(3): 333-340.

Nunnari, J. and A. Suomalainen (2012). "Mitochondria: in sickness and in health." Cell **148**(6): 1145-1159.

Olsen, O. E., A. Skjaervik, B. F. Stordal, A. Sundan and T. Holien (2017). "TGF-beta contamination of purified recombinant GDF15." PLoS One **12**(11): e0187349.

Ost, M., V. Coleman, A. Voigt, E. M. van Schothorst, S. Keipert, I. van der Stelt, S. Ringel, A. Graja, T. Ambrosi, A. P. Kipp, M. Jastroch, T. J. Schulz, J. Keijer and S. Klaus (2016). "Muscle mitochondrial stress adaptation operates independently of endogenous FGF21 action." Mol Metab **5**(2): 79-90.

Ost, M., S. Keipert, E. M. van Schothorst, V. Donner, I. van der Stelt, A. P. Kipp, K. J. Petzke, M. Jove, R. Pamplona, M. Portero-Otin, J. Keijer and S. Klaus (2015). "Muscle mitohormesis promotes cellular survival via serine/glycine pathway flux." FASEB J **29**(4): 1314-1328.

Ost, M., F. Werner, J. Dokas, S. Klaus and A. Voigt (2014). "Activation of AMPKalpha2 is not crucial for mitochondrial uncoupling-induced metabolic effects but required to maintain skeletal muscle integrity." PLoS One **9**(4): e94689.

Paralkar, V. M., A. L. Vail, W. A. Grasser, T. A. Brown, H. Xu, S. Vukicevic, H. Z. Ke, H. Qi, T. A. Owen and D. D. Thompson (1998). "Cloning and characterization of a novel member of the transforming growth factor-beta/bone morphogenetic protein family." J Biol Chem **273**(22): 13760-13767.

Parikh, S., A. Goldstein, A. Karaa, M. K. Koenig, I. Anselm, C. Brunel-Guitton, J. Christodoulou, B. H. Cohen, D. Dimmock, G. M. Enns, M. J. Falk, A. Feigenbaum, R. E. Frye, J. Ganesh, D. Griesemer, R. Haas, R. Horvath, M. Korson, M. C. Kruer, M. Mancuso, S. McCormack, M. J. Raboisson, T. Reimschisel, R. Salvarinova, R. P. Saneto, F. Scaglia, J. Shoffner, P. W. Stacpoole, C. M. Sue, M. Tarnopolsky, C. Van Karnebeek, L. A. Wolfe, Z. Z. Cunningham, S. Rahman and P. F. Chinnery (2017). "Patient care standards for primary mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society." Genet Med **19**(12).

Patel, S., A. Alvarez-Guaita, A. Melvin, D. Rimmington, A. Dattilo, E. L. Miedzybrodzka, I. Cimino, A. C. Maurin, G. P. Roberts, C. L. Meek, S. Virtue, L. M. Sparks, S. A. Parsons, L. M. Redman, G. A. Bray, A. P. Liou, R. M. Woods, S. A. Parry, P. B. Jeppesen, A. J. Kolnes, H. P. Harding, D. Ron, A. Vidal-Puig, F. Reimann, F. M. Gribble, C. J. Hulston, I. S. Farooqi, P. Fafournoux, S. R. Smith, J. Jensen, D. Breen, Z. Wu, B. B. Zhang, A. P. Coll, D. B.

Savage and S. O'Rahilly (2019). "GDF15 Provides an Endocrine Signal of Nutritional Stress in Mice and Humans." Cell Metab **29**(3): 707-718 e708.

Patel, S., A. Alvarez-Guaita, A. Melvin, D. Rimmington, A. Dattilo, E. L. Miedzybrodzka, I. Cimino, A. C. Maurin, G. P. Roberts, C. L. Meek, S. Virtue, L. M. Sparks, S. A. Parsons, L. M. Redman, G. A. Bray, A. P. Liou, R. M. Woods, S. A. Parry, P. B. Jeppesen, A. J. Kolnes, H. P. Harding, D. Ron, A. Vidal-Puig, F. Reimann, F. M. Gribble, C. J. Hulston, I. S. Farooqi, P. Fafournoux, S. R. Smith, J. Jensen, D. Breen, Z. D. Wu, B. B. Zhang, A. P. Coll, D. B. Savage and S. O'Rahilly (2019). "GDF15 Provides an Endocrine Signal of Nutritional Stress in Mice and Humans." Cell Metabolism **29**(3): 707-+.

Pathak, S. S., D. Liu, T. Li, N. de Zavalia, L. Zhu, J. Li, R. Karthikeyan, T. Alain, A. C. Liu, K. F. Storch, R. J. Kaufman, V. X. Jin, S. Amir, N. Sonenberg and R. Cao (2019). "The eIF2alpha Kinase GCN2 Modulates Period and Rhythmicity of the Circadian Clock by Translational Control of Atf4." Neuron **104**(4): 724-735 e726.

Patsalos, A., L. Halasz, M. A. Medina-Serpas, W. K. Berger, B. Daniel, P. Tzerpos, M. Kiss, G. Nagy, C. Fischer, Z. Simandi, T. Varga and L. Nagy (2022). "A growth factor-expressing macrophage subpopulation orchestrates regenerative inflammation via GDF-15." J Exp Med **219**(1).

Pereyra, A. S., A. Rajan, C. R. Ferreira and J. M. Ellis (2020). "Loss of Muscle Carnitine Palmitoyltransferase 2 Prevents Diet-Induced Obesity and Insulin Resistance despite Long-Chain Acylcarnitine Accumulation." Cell reports **33**(6): 108374-108374.

Petry, C. J., K. K. Ong, K. A. Burling, P. Barker, S. F. Goodburn, J. R. B. Perry, C. L. Acerini, I. A. Hughes, R. C. Painter, G. B. Afink, D. B. Dunger and S. O'Rahilly (2018). "Associations of vomiting and antiemetic use in pregnancy with levels of circulating GDF15 early in the second trimester: A nested case-control study." Wellcome Open Res **3**: 123.

Pollice, C., W. H. Kaye, C. G. Greeno and T. E. Weltzin (1997). "Relationship of depression, anxiety, and obsessionality to state of illness in anorexia nervosa." Int J Eat Disord **21**(4): 367-376.

Price, C. J., T. D. Hoyda and A. V. Ferguson (2008). "The area postrema: a brain monitor and integrator of systemic autonomic state." Neuroscientist **14**(2): 182-194.

Rabasa, C. and S. L. Dickson (2016). "Impact of stress on metabolism and energy balance." Current Opinion in Behavioral Sciences **9**: 71-77.

Ratnam, N. M., J. M. Peterson, E. E. Talbert, K. J. Ladner, P. V. Rajasekera, C. R. Schmidt, M. E. Dillhoff, B. J. Swanson, E. Haverick, R. D. Kladney, T. M. Williams, G. W. Leone, D.

J. Wang and D. C. Guttridge (2017). "NF-kappaB regulates GDF-15 to suppress macrophage surveillance during early tumor development." J Clin Invest **127**(10): 3796-3809.

Richardson, R. D., K. Omachi, R. Kermani and S. C. Woods (2002). "Intraventricular insulin potentiates the anorexic effect of corticotropin releasing hormone in rats." Am J Physiol Regul Integr Comp Physiol **283**(6): R1321-1326.

Risbrough, V. B. and M. B. Stein (2006). "Role of corticotropin releasing factor in anxiety disorders: a translational research perspective." Horm Behav **50**(4): 550-561.

Russell, O. M., G. S. Gorman, R. N. Lightowlers and D. M. Turnbull (2020). "Mitochondrial Diseases: Hope for the Future." Cell **181**(1): 168-188.

Sabatini, P. V., H. Frikke-Schmidt, J. Arthurs, D. Gordian, A. Patel, A. C. Rupp, J. M. Adams, J. Wang, S. Beck Jorgensen, D. P. Olson, R. D. Palmiter, M. G. Myers, Jr. and R. J. Seeley (2021). "GFRAL-expressing neurons suppress food intake via aversive pathways." Proc Natl Acad Sci U S A **118**(8).

Suriben, R., M. Chen, J. Higbee, J. Oeffinger, R. Ventura, B. Li, K. Mondal, Z. Gao, D. Ayupova, P. Taskar, D. Li, S. R. Starck, H. H. Chen, M. McEntee, S. D. Katewa, V. Phung, M. Wang, A. Kekatpure, D. Lakshminarasimhan, A. White, A. Olland, R. Haldankar, M. J. Solloway, J. Y. Hsu, Y. Wang, J. Tang, D. A. Lindhout and B. B. Allan (2020). "Antibody-mediated inhibition of GDF15-GFRAL activity reverses cancer cachexia in mice." Nat Med.

Sutton, E. F., R. Beyl, K. S. Early, W. T. Cefalu, E. Ravussin and C. M. Peterson (2018). "Early Time-Restricted Feeding Improves Insulin Sensitivity, Blood Pressure, and Oxidative Stress Even without Weight Loss in Men with Prediabetes." Cell Metab **27**(6): 1212-1221 e1213.

Tasaki, H., L. Zhao, K. Isayama, H. Chen, Y. Nobuhiko, S. Yasufumi, S. Hashimoto and M. A. Hattori (2013). "Profiling of circadian genes expressed in the uterus endometrial stromal cells of pregnant rats as revealed by DNA microarray coupled with RNA interference." Front Endocrinol (Lausanne) **4**: 82.

Tataranni, P. A., D. E. Larson, S. Snitker, J. B. Young, J. P. Flatt and E. Ravussin (1996). "Effects of glucocorticoids on energy metabolism and food intake in humans." Am J Physiol **271**(2 Pt 1): E317-325.

Timpl, P., R. Spanagel, I. Sillaber, A. Kresse, J. M. Reul, G. K. Stalla, V. Blanquet, T. Steckler, F. Holsboer and W. Wurst (1998). "Impaired stress response and reduced anxiety

in mice lacking a functional corticotropin-releasing hormone receptor 1." Nat Genet **19**(2): 162-166.

Tolle, V., M. Kadem, M. T. Bluet-Pajot, D. Frere, C. Foulon, C. Bossu, R. Dardennes, C. Mounier, P. Zizzari, F. Lang, J. Epelbaum and B. Estour (2003). "Balance in ghrelin and leptin plasma levels in anorexia nervosa patients and constitutionally thin women." J Clin Endocrinol Metab **88**(1): 109-116.

Tsai, V. W., L. Macia, C. Feinle-Bisset, R. Manandhar, A. Astrup, A. Raben, J. K. Lorenzen, P. T. Schmidt, F. Wiklund, N. L. Pedersen, L. Campbell, A. Kriketos, A. Xu, Z. Pengcheng, W. Jia, P. M. Curmi, C. N. Angstmann, K. K. Lee-Ng, H. P. Zhang, C. P. Marquis, Y. Husaini, C. Beglinger, S. Lin, H. Herzog, D. A. Brown, A. Sainsbury and S. N. Breit (2015). "Serum Levels of Human MIC-1/GDF15 Vary in a Diurnal Pattern, Do Not Display a Profile Suggestive of a Satiety Factor and Are Related to BMI." PLoS One **10**(7): e0133362.

Tsai, V. W., H. P. Zhang, R. Manandhar, K. K. M. Lee-Ng, H. Lebhar, C. P. Marquis, Y. Husaini, A. Sainsbury, D. A. Brown and S. N. Breit (2018). "Treatment with the TGF-b superfamily cytokine MIC-1/GDF15 reduces the adiposity and corrects the metabolic dysfunction of mice with diet-induced obesity." Int J Obes (Lond) **42**(3): 561-571.

Tyynismaa, H., C. J. Carroll, N. Raimundo, S. Ahola-Erkkila, T. Wenz, H. Ruhanen, K. Guse, A. Hemminki, K. E. Peltola-Mjosund, V. Tulkki, M. Oresic, C. T. Moraes, K. Pietilainen, I. Hovatta and A. Suomalainen (2010). "Mitochondrial myopathy induces a starvation-like response." Hum Mol Genet **19**(20): 3948-3958.

Uehara, Y., H. Shimizu, K. Ohtani, N. Sato and M. Mori (1998). "Hypothalamic corticotropin-releasing hormone is a mediator of the anorexigenic effect of leptin." Diabetes **47**(6): 890-893.

Vila, G., M. Riedl, C. Anderwald, M. Resl, A. Handisurya, M. Clodi, G. Prager, B. Ludvik, M. Krebs and A. Luger (2011). "The Relationship between Insulin Resistance and the Cardiovascular Biomarker Growth Differentiation Factor-15 in Obese Patients." Clinical Chemistry **57**(2): 309-316.

Wallentin, L., B. Zethelius, L. Berglund, K. M. Eggers, L. Lind, B. Lindahl, K. C. Wollert and A. Siegbahn (2013). "GDF-15 for prognostication of cardiovascular and cancer morbidity and mortality in men." PLoS One **8**(12): e78797.

Wang, X., Z. Yang, H. Tian, Y. Li, M. Li, W. Zhao, C. Zhang, T. Wang, J. Liu, A. Zhang, D. Shen, C. Zheng, J. Qi, D. Zhao, J. Shi, L. Jin, J. Rao and W. Zhang (2017). "Circulating MIC-1/GDF15 is a complementary screening biomarker with CEA and correlates with liver metastasis and poor survival in colorectal cancer." Oncotarget **8**(15): 24892-24901.

Worth, A. A., R. Shoop, K. Tye, C. H. Feetham, G. D'Agostino, G. T. Dodd, F. Reimann, F. M. Gribble, E. C. Beebe, J. D. Dunbar, J. T. Alexander-Chacko, D. K. Sindelar, T. Coskun, P. J. Emmerson and S. M. Luckman (2020). "The cytokine GDF15 signals through a population of brainstem cholecystokinin neurons to mediate anorectic signalling." Elife **9**.

Xiong, Y., K. Walker, X. Min, C. Hale, T. Tran, R. Komorowski, J. Yang, J. Davda, N. Nuanmanee, D. Kemp, X. Wang, H. Liu, S. Miller, K. J. Lee, Z. Wang and M. M. Veniant (2017). "Long-acting MIC-1/GDF15 molecules to treat obesity: Evidence from mice to monkeys." Sci Transl Med **9**(412).

Yang, L., C. C. Chang, Z. Sun, D. Madsen, H. Zhu, S. B. Padkjaer, X. Wu, T. Huang, K. Hultman, S. J. Paulsen, J. Wang, A. Bugge, J. B. Frantzen, P. Norgaard, J. F. Jeppesen, Z. Yang, A. Secher, H. Chen, X. Li, L. M. John, B. Shan, Z. He, X. Gao, J. Su, K. T. Hansen, W. Yang and S. B. Jorgensen (2017). "GFRAL is the receptor for GDF15 and is required for the anti-obesity effects of the ligand." Nat Med **23**(10): 1158-1166.

Yatsuga, S., Y. Fujita, A. Ishii, Y. Fukumoto, H. Arahata, T. Kakuma, T. Kojima, M. Ito, M. Tanaka, R. Saiki and Y. Koga (2015). "Growth differentiation factor 15 as a useful biomarker for mitochondrial disorders." Ann Neurol **78**(5): 814-823.

Yau, Y. H. and M. N. Potenza (2013). "Stress and eating behaviors." Minerva Endocrinol **38**(3): 255-267.

Zhang, C., J. A. Kaye, Z. Cai, Y. Wang, S. L. Prescott and S. D. Liberles (2020). "Area Postrema Cell Types that Mediate Nausea-Associated Behaviors." Neuron.

Zhao, L., K. Isayama, H. Chen, N. Yamauchi, Y. Shigeyoshi, S. Hashimoto and M. A. Hattori (2016). "The nuclear receptor REV-ERBalpha represses the transcription of growth/differentiation factor 10 and 15 genes in rat endometrium stromal cells." Physiol Rep **4**(2).

List of Publications

Research articles

Igual Gil, C., M. Ost, J. Kasch, S. Schumann, S. Heider and S. Klaus (2019). "Role of GDF15 in active lifestyle induced metabolic adaptations and acute exercise response in mice." Sci Rep 9(1): 20120

*Ost, M., **C. *Igual Gil**, V. Coleman, S. Keipert, S. Efstathiou, V. Vidic, M. Weyers and S. Klaus (2020). "Muscle-derived GDF15 drives diurnal anorexia and systemic metabolic remodeling during mitochondrial stress." EMBO Rep: e48804.

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Igual Gil, C., B.M., Coull, W. Jonas, R. Lippert, M. Ost and S. Klaus. “Mitochondrial stress-induced GDF15-GFRAL axis promotes diurnal anorexia and anxiety-like behavior in mice”. Under review.

Reviews

Klaus, S., **C. Igual Gil** and M. Ost (2021). "Regulation of diurnal energy balance by mitokines." Cell Mol Life Sci

Curriculum Vitae

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Declaration of academic honesty

I hereby declare that this PhD thesis entitled “Role of the GDF15-GFRAL pathway under skeletal muscle mitochondrial stress” is solely my own work and no sources other than specified were used during the writing process. I confirm that this work has not been previously submitted to any other university for obtaining a degree.

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