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3

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14

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26

27 **Abstract**

28 Ghrelin acts as an endocrine link connecting physiological processes regulating food  
29 intake, body composition, growth, and energy balance. Ghrelin is the only peptide known to  
30 undergo octanoylation. The enzyme mediating this process, ghrelin O-acyltransferase (GOAT),  
31 is expressed in the gastrointestinal tract (GI; primary source of circulating ghrelin) as well as  
32 other tissues. The present study demonstrates that stomach GOAT mRNA levels correlate with  
33 circulating acylated-ghrelin levels in fasted and diet-induced obese mice. In addition, GOAT  
34 was found to be expressed in both the pituitary and hypothalamus (two target tissues of  
35 ghrelin's actions), and regulated in response to metabolic status. Using primary pituitary cell  
36 cultures as a model system to study the regulation of GOAT expression, we found that acylated-  
37 ghrelin, but not desacyl-ghrelin, increased GOAT expression. In addition, growth-hormone-  
38 releasing hormone (GHRH) and leptin increased, while somatostatin (SST) decreased GOAT  
39 expression. The physiologic relevance of these later results is supported by the observation that  
40 pituitary GOAT expression in mice lacking GHRH, SST and leptin showed opposite changes to  
41 those observed after *in vitro* treatment with the corresponding peptides. Therefore, it seems  
42 plausible that these hormones directly contribute to the regulation of pituitary GOAT.  
43 Interestingly, in all the models studied, pituitary GOAT expression paralleled changes in the  
44 expression of a dominant spliced-variant of ghrelin (In2-ghrelin) and therefore this transcript  
45 may be a primary substrate for pituitary GOAT. Collectively, these observations support the  
46 notion that the GI tract is not the only source of acylated-ghrelin, but in fact locally-produced  
47 des-acylated-ghrelin could be converted to acylated-ghrelin within target tissues by locally  
48 active GOAT, to mediate its tissue-specific effects.

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55 **1. Introduction**

56 The primary source of circulating ghrelin is the stomach, where pro-ghrelin can be  
57 modified by the addition of an O-linked octanoyl side group added to its serine-3 residue, via  
58 the actions of ghrelin O-acyltransferase (GOAT) to produce acylated-proghrelin (Kojima et al.,  
59 1999; Ariyasu et al., 2001; Garg, 2007; Gonzalez et al., 2008; Gutierrez et al., 2008; Yang et al.,  
60 2008; Gomez et al., 2009; Sakata et al., 2009). Then, prohormone convertase 1/3 (PC1/3) can  
61 further process pro-ghrelin (acylated or desacylated) to generate the mature 28-amino acid  
62 peptide ghrelin (Garg, 2007). Only a small proportion of circulating ghrelin is acylated, while  
63 the remaining is in the unmodified or des-acylated form, where both forms have biological  
64 effects (van der Lely et al., 2004; Kojima and Kangawa, 2005). Acylated-ghrelin is the primary  
65 endogenous ligand for the growth hormone secretagogue receptor (GHSR), and it is now  
66 recognized that the acyl-ghrelin/GHSR system exerts actions on multiple tissues, many of them  
67 related to regulation of metabolic functions (for review, (van der Lely et al., 2004)). In  
68 particular, acylated-ghrelin works at the level of the hypothalamus as a potent orexigenic signal  
69 (Kojima and Kangawa, 2005), and at the level of the pituitary to modulate hormone release  
70 including augmentation of growth hormone (GH), adrenocorticotropin (ACTH) and prolactin  
71 release (Kojima and Kangawa, 2005; Gahete et al., 2009). In addition, acylated ghrelin acts  
72 systemically to regulate glucose homeostasis and adiposity, where the deletion of the genes  
73 encoding ghrelin or its receptor prevents diet-induced obesity, improves insulin sensitivity and  
74 enhances glucose-stimulated insulin secretion (Yada et al., 2008).

75

76 Although the GI track is considered the major source of acylated ghrelin, other tissues  
77 express ghrelin, PC1/3 and GOAT (Gonzalez et al., 2008; Gutierrez et al., 2008; Yang et al.,  
78 2008) (for review, (van der Lely et al., 2004)). Therefore, it is possible that locally produced  
79 non-acylated proghrelin might be converted to acylated-proghrelin within target tissues by  
80 locally-active GOAT, to mediate tissue specific effects. Local production of acyl-ghrelin is  
81 supported by a recent report demonstrating that acylated ghrelin can be produced by a thyroid

82 and a pituitary cell line that express ghrelin, proconvertase 1/3 and GOAT if n-octanoic acid is  
83 available as a substrate (Takahashi et al., 2009). Given the fact that hypothalamus and pituitary  
84 are targets for acylated-ghrelin, coupled with the fact that these tissues also express ghrelin (for  
85 review, (van der Lely et al., 2004)), GOAT (Gonzalez et al., 2008; Gutierrez et al., 2008; Yang  
86 et al., 2008) and PC1/3 (Dong and Day, 2002; Nillni, 2007), it is possible that local production  
87 of acylated-ghrelin, as well as that produced by the GI tract, may play an important role in  
88 regulating this neuroendocrine axis in response to metabolic signals. Therefore, the goals of the  
89 current study were 1) to determine whether GOAT expression in the stomach correlates with  
90 circulating acylated-ghrelin levels in mice under conditions of metabolic stress (i.e., fasting, and  
91 obese mouse models), 2) to confirm if GOAT is expressed within the hypothalamus and  
92 pituitary and if expression levels are mediated by metabolic stress and 3) to determine whether  
93 primary regulatory factors for the pituitary-metabolic interface can directly regulate GOAT  
94 expression, by using primary mouse pituitary cell cultures as a model system.

95

## 96 **2. Material and Methods**

### 97 *2.1. Animals and cell culture*

98 All experimental procedures were approved by the Animal Care and Use Committees of  
99 the University of Cordoba, University of Illinois at Chicago and the Jesse Brown VA Medical  
100 Center. Mice were housed under standard conditions of light (12-h light, 12-h dark cycle; lights  
101 on at 0700 h) and temperature (22–24 C), with free access to tap water and food (standard  
102 rodent chow; LabDiet, St. Louis, MO; USA; Catalog no. 5008; fat, 17 kcal%, carbohydrate, 56  
103 kcal%; protein, 27 kcal%). Leptin deficient ob/ob mice and their corresponding controls were  
104 purchased from Jackson laboratories, while growth hormone releasing hormone (GHRH),  
105 somatostatin (SST) and neuropeptide Y (NPY) knockout mice were bred in house. Original  
106 breeders were obtained from Dr. Ute Hochgeschwender (SST-KO), Dr. Richard D. Palmiter  
107 (NPY-KO) and Dr. Roberto Salvatori (GHRH-KO) as previously reported (Alba and Salvatori,  
108 2004; Park et al., 2005; Luque et al., 2006; Luque and Kineman, 2006; Luque and Kineman,  
109 2007; Luque et al., 2007b; Luque et al., 2007c). Mice were handled daily at least 1 week prior to

110 euthanasia to acclimate them to personnel and handling procedures and were sacrificed by  
111 decapitation, without anesthesia, under fed conditions unless otherwise specified. Trunk blood  
112 was immediately mixed with MiniProtease inhibitor (Roche, Nutley, NJ) and placed on ice,  
113 centrifuged and plasma was stored at -80°C until analysis of total- and acylated- ghrelin by  
114 ELISA (Linco, St. Charles, MO) following the manufacturer's instructions, including the  
115 addition of hydrochloric acid at a final concentration of 0.05N in order to prevent rapid des-  
116 acylation of ghrelin after the plasma collection. Tissues (stomachs, pituitaries and hypothalami)  
117 were immediately frozen in liquid nitrogen and stored at -80°C until further analysis of mRNA  
118 levels by quantitative real-time RT-PCR (qRT-PCR; see below).

119

## 120 2.2. Influence of metabolic stress on circulating total and acylated ghrelin and stomach, 121 pituitary and hypothalamic GOAT mRNA levels

122 For these studies, serum and tissues previously processed and analyzed for other  
123 endpoints were used, where each study is listed below followed by a brief summary of the  
124 experimental design. **Fasting** – Ten week-old male C57Bl/6J mice were either fed *ad libitum* or  
125 fasted for 12h (food removed at 1900 h), 24h or 48h (food removed at 0700 h) (Luque et al.,  
126 2007c). An additional group of 8-9 week-old male C57Bl/6 x FVBN mice (n=5/group) were  
127 also fed *ad libitum* or fasted for 24h (Kineman et al., 2007). For these and subsequent studies all  
128 tissues and blood were collected between 0700h-0900h. **Diet-induced obesity (DIO)** - Male  
129 C57Bl/6J mice (n = 6–7) were fed a low-fat (LFD; 10% kcal from fat) or a high-fat (HFD; 60%  
130 kcal from fat) diet starting at 4 week of age and killed at 20 wk (Luque and Kineman, 2006).  
131 **Leptin-deficient mice (ob/ob)** – Blood and tissues were analyzed from 10 week old *ob/ob* mice  
132 and their littermates-controls (*ob/?*), as well as from *ob/ob* mice treated with vehicle (control 1),  
133 leptin (7d via osmotic mini-pumps released at a rate of 0.5µl/h, delivering a total of 15.6µg of  
134 leptin each day) or from a group of *ob/ob* mice pair-fed along with the leptin-treated animals  
135 that was used as an additional control (control 2) to match the food intake of leptin-treated mice  
136 (Luque and Kineman, 2006; Luque et al., 2007a) in order to differentiate between direct effects

137 of leptin and those mediated indirectly by leptin-induced reduction in food intake and weight  
138 loss.

139

140 *2.3. Direct regulation of GOAT mRNA levels by acylated-ghrelin, desacyl-ghrelin, GHRH, SST,*  
141 *leptin, NPY, insulin and IGF-I in mouse primary pituitary cell cultures*

142 To examine whether primary regulatory hormones for the pituitary-metabolic interface can  
143 directly control pituitary GOAT expression, pituitaries of 8-12 wk old male C57Bl6 mice  
144 obtained from “Centro de Instrumentación Científica” (University of Granada, Spain) or from  
145 Jackson Laboratories (Bar Harbor, ME, USA)) were enzymatically dispersed (n=3-5 pituitaries  
146 pooled/experiment, 6-7 separate experiments) into single cells and cultured (250,000 cells/well,  
147 24-well plates) in serum containing  $\alpha$ -MEM, as previously described (Luque et al., 2006; Luque  
148 and Kineman, 2006). After 24-48h of culture, media was removed and wells were washed in  
149 serum free media and subsequently treated with mouse acylated-ghrelin, desacyl-ghrelin,  
150 GHRH, NPY, insulin, IGF-I (10nM each), SST-14 (100nM) and mouse leptin (10ng/ml), for  
151 24h (Sigma, Saint Louis, MO or Phoenix, Burlingame, CA; USA), and then total cellular RNA  
152 was extracted for determination of GOAT mRNA levels by qrtRT-PCR (see below). In order to  
153 determine if the direct actions of GHRH, SST, NPY or leptin could be important in maintaining  
154 *in vivo* expression of pituitary GOAT, total RNA obtained from pituitaries of 9-11 week old  
155 GHRH-knockout (KO) (B6129PF1/J), SST-KO mice (C57Bl/6J), NPY-KO mice (129/sv),  
156 leptin-KO (ob/ob; C57Bl/6J) and their respective littermate controls used in other studies (Alba  
157 and Salvatori, 2004; Park et al., 2005; Luque et al., 2006; Luque and Kineman, 2006; Luque and  
158 Kineman, 2007; Luque et al., 2007b; Luque et al., 2007c), were also evaluated from GOAT  
159 mRNA levels by qrtRTPCR.

160

161 *2.4. RNA isolation and reverse transcription (RT)*

162 Tissues and cell cultures were processed for recovery of total RNA using the Absolutely  
163 RNA RT-PCR Miniprep Kit with Deoxyribonuclease treatment (Stratagene, La Jolla, CA) as  
164 previously described (Luque et al., 2006; Luque and Kineman, 2006; Luque and Kineman,



165 2007; Luque et al., 2007c). The amount of RNA recovered was determined using the Ribogreen  
166 RNA quantification kit (Molecular Probes, Eugene, OR). Total RNA (1 µg for whole tissue  
167 extract and 0.25µg for primary cell cultures) was reversed transcribed with enzyme and buffers  
168 supplied in the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD). cDNA was  
169 treated with ribonuclease H (1U; MRI Fermentas) and duplicate aliquots (1µl) were amplified  
170 by qrtRT-PCR, in which samples were run against synthetic standards to estimate mRNA copy  
171 number, as described below.

172

### 173 2.5. Quantification of GOAT and ghrelin mRNA levels by qrtRT-PCR

174 Details regarding the development, validation, and application of a qrtRT-PCR to  
175 measure expression levels of mouse transcripts, including ghrelin mRNA levels, have been  
176 reported previously (Luque et al., 2006; Luque and Kineman, 2006; Kineman et al., 2007;  
177 Luque et al., 2007a; Luque and Kineman, 2007; Luque et al., 2007c). Specific primer  
178 sequences, GenBank accession numbers and product sizes for mouse GOAT, ghrelin and  
179 cyclophilin used in this study were as follows: GOAT (Mboat4; forward 5'-  
180 ATTTGTGAAGGGAAGGTGGAG-3' and reverse 5'-CAGGAGAGCAGGGAAAAAGAG-3',  
181 NM\_001126314, 120bp); Ghrelin (forward 5'-TCCAAGAAGCCACCAGCTAA-3' and reverse  
182 5'-AACATCGAAGGGAGCATTGA-3', NM\_021488, 126bp) and cyclophilin (forward 5'-  
183 TGGTCTTTGGGAAGGTGAAAG-3' and reverse 5'- TGTCCACAGTCGGAAATGGT-3',  
184 NM\_008907, 109bp). Primers were selected using Primer 3 software (Rozen and Skaletsky,  
185 2000) with selection parameters set to identify primer sets that: 1) span an intron (when  
186 possible), 2) differ by no more than 1°C in annealing temperature, 3) are at least 20bp in length,  
187 4) have a GC content between 45-55%, but 5) exclude primers that may form primer-dimers.  
188 Sequences of selected primers were used in BLAST (NCBI) searches to check for potential  
189 homology to sequences other than the designated target. Initial screening of primer efficiency  
190 using real-time detection was performed by amplifying 2-fold dilutions of RT products, where  
191 optimal efficiency was demonstrated by a difference of one cycle threshold between dilutions

192 and a clear melting peak followed by a graded temperature-dependent dissociation to verify that  
193 only one product was amplified. The thermocycling profile consisted of one cycle of 95 C for  
194 10 min, 40 cycles of 95 C for 30 sec, 61 C for 1 min, and 72 C for 30 sec. PCR products were  
195 then column-purified (QIAGEN, Valencia, CA) and sequenced to confirm target specificity.  
196 After confirmation of primer efficiency and specificity, the concentration of purified products  
197 was determined using Molecular Probe's Picogreen DNA quantification kit, and PCR products  
198 were serial diluted to obtain standards containing 1,  $10^1$ ,  $10^2$ ,  $10^3$ ,  $10^4$ ,  $10^5$ , and  $10^6$  copies of  
199 synthetic template. Standards were then amplified by real-time PCR, and standard curves were  
200 generated using Stratagene Mx3000p software. The slope of a standard curve for each template  
201 examined was approximately 1, indicating that the efficiency of amplification of our primers  
202 was 100%, meaning that all templates in each cycle were copied. To determine the starting copy  
203 number of cDNA, RT samples were PCR amplified and the signal was compared with that of a  
204 specific standard curve of each transcript run on the same plate. In addition, total RNA samples  
205 that were not reversed transcribed and a no DNA control were run on each plate to control for  
206 genomic DNA contamination and to monitor potential exogenous contamination, respectively.  
207 Also, to control for variations in the amount of RNA used in the RT reaction and the efficiency  
208 of the RT reaction, mRNA copy number of the transcript of interest was adjusted by the mRNA  
209 copy number of cyclophilin A (used as housekeeping gene), where cyclophilin A mRNA levels  
210 did not significantly vary between experimental groups, within tissue type or treatment group  
211 (data not shown).

212

## 213 2.6. Data presentation and statistical analysis

214 Samples from all groups within an experiment were processed at the same time. The  
215 effects of fasting and leptin replacement were assessed by one-way ANOVA followed by a  
216 Newman-Keuls test for multiple comparisons, while the effects of obesity, genotype and the *in*  
217 *vitro* effects of acylated-ghrelin, desacyl-ghrelin, GHRH, SST, NPY, leptin, insulin and IGF-I  
218 were assessed by Student's t-test.  $P < 0.05$  was considered significant. All data are expressed as  
219 means  $\pm$  SEM. The *in vivo* effects of fasting/obesity/genotype were obtained from a minimum

220 of 5 animals per group. Results from *in vitro* studies were obtained from 3-7 separate,  
221 independent experiments (3-5 wells/treatment) carried out in different days and with different  
222 cells preparation. Endpoints displaying heterogeneity of variance were log transformed prior to  
223 analysis. All statistical analyses were performed using the GB-STAT software package  
224 (Dynamic Microsystems, Inc. Silver Spring, MD, USA).

225

### 226 **3. Results and Discussion**

#### 227 *3.1. Quantification of GOAT mRNA levels in mouse stomachs, pituitaries and hypothalami*

228 Using qRT-PCR, we found that GOAT mRNA levels were 5-fold greater in the  
229 stomach as compared to the pituitary and hypothalamus (Table 1). These results are consistent  
230 with other reports showing relative expression levels of GOAT in both human (Gutierrez et al.,  
231 2008) and rodent (Gonzalez et al., 2008; Yang et al., 2008; Sakata et al., 2009) tissues.  
232 Interestingly, the expression level of GOAT transcripts in the mouse tissues analyzed in the  
233 current study (table 1) paralleled the mRNA levels of ghrelin in the same tissues (Stomach >>  
234 pituitary = hypothalamus), as previously reported (Kineman et al., 2007).

235 Since it has been previously reported that the enzyme prohormone convertase 1/3 which  
236 is required for the conversion of preprohormones, including preproghrelin to its 28 amino acid  
237 form, is also expressed in the pituitary and hypothalamus (Dong and Day, 2002; Nillni, 2007),  
238 as well as the GI tract (Macro et al., 1996), it is possible that the source of acylated-ghrelin  
239 within the pituitary and hypothalamus may include locally produced ghrelin, in addition to that  
240 found in the circulation. Therefore, the current study compared the impact of metabolic stress  
241 (fasting and obesity) on circulating ghrelin (acylated and total) levels, as well as GOAT and  
242 ghrelin mRNA levels in the stomach, pituitary and hypothalamus. Specifically, Figure 1  
243 illustrates the impact of fasting, diet-induced obesity (DIO) and obesity caused by leptin  
244 deficiency (*ob/ob* mice) on circulating acylated- and total-ghrelin levels and stomach GOAT  
245 mRNA levels (Panels A, B and C, respectively), as well as pituitary and hypothalamic GOAT  
246 mRNA levels (Panels D, E and F, respectively). It should be noted that, in some cases we have  
247 previously published the regulation of stomach, pituitary and hypothalamic ghrelin mRNA

248 levels in these model systems (Kineman et al., 2007; Luque et al., 2007a; Luque et al., 2007c).  
249 These results are summarized in Figure 1 as the relative changes in ghrelin mRNA levels (=, ↑,  
250 ↓), as compared to controls, as shown below each graph of tissue-specific GOAT expression.

251

### 252 3.2. Effects of nutrient deprivation on GOAT/ghrelin axis.

253 As shown in Figure 1, panel A, fasting did not alter circulating levels of total-ghrelin or  
254 stomach ghrelin expression, but did increase circulating acylated-ghrelin (12 and 24h) and  
255 stomach GOAT mRNA levels (24h). The impact of 24h fasting on stomach GOAT mRNA  
256 levels was confirmed in tissues taken from an independent set of male mice from another study  
257 (Kineman et al., 2007), using two different primer sets for amplification of the GOAT  
258 transcripts (supplemental Fig. 1). These results differ from those of a recent report by Kirchner  
259 et al., (Kirchner et al., 2009) showing that fasting (12h, 24h and 36h) significantly increased  
260 total-ghrelin levels and tended to increase acylated-ghrelin levels, but unexpectedly suppressed  
261 stomach GOAT mRNA in C57Bl/6 male mice. It is possible that such differences may be  
262 related to the time of food withdrawal, time of sample collection, type of standard rodent chow  
263 provided and/or the method of euthanasia which were not clearly defined in that study. In fact,  
264 Kirchner et al., (Kirchner et al., 2009) observed a diurnal pattern of circulating ghrelin and  
265 stomach ghrelin/GOAT mRNA levels, as well as an association between stomach GOAT  
266 expression and the type of dietary lipids supplied. It is also possible that the differences  
267 observed are related to the analytical techniques applied, where circulating ghrelin levels were  
268 assessed by commercial assays in the current study, but in the case of Kirchner et al., (Kirchner  
269 et al., 2009), were assessed by MALDI-TOF mass spectrometry of immunoprecipitated ghrelin.  
270 Finally, with respect to GOAT mRNA levels, although both the current study and that of  
271 Kirchner et al., (Kirchner et al., 2009) used real-time RT-PCR, the primer sets used and the  
272 characteristics and properties of the primers were not the same (length of primers, GC content,  
273 etc.). Nonetheless, our current observations are consistent with other reports showing a clear rise  
274 in acylated-ghrelin levels in fasted humans and other species (for review: (Casanueva and  
275 Dieguez, 2002; Gottero et al., 2004; Kojima and Kangawa, 2005; Williams and Cummings,

276 2005; Cummings, 2006)), including mice (Perreault et al., 2004; Luque et al., 2006; Luque et  
277 al., 2007c; Zizzari et al., 2007), as well as an increase in stomach GOAT mRNA levels in rats  
278 subjected to 21-days of caloric-restriction (Gonzalez et al., 2008). However, it should be noted  
279 that like Kirchner *et al.*, Liu *et al.* failed to detect a rise in circulating acylated ghrelin in fasted  
280 humans (Liu et al., 2008). It should also be emphasized that in the current study the fasting-  
281 induced rise in acylated-ghrelin was observed at 12 and 24h, but began to fall after 48h, while  
282 the fasting-induced rise in GOAT mRNA was only observed at 24h. These results suggest that  
283 an increase in GOAT expression levels in the stomach may only in part contribute to the rise in  
284 circulating acyl-ghrelin, where enhanced GOAT activity and/or substrate availability may  
285 precede that process. However, after 48h of fasting circulating acylated-ghrelin levels and  
286 GOAT mRNA levels did not differ from controls (Fig. 1, panel A), where 48h of fasting in a  
287 mouse represent a severe catabolic state (Luque et al., 2007c). This observation is consistent  
288 with reports showing that the proportion of circulating acylated-ghrelin falls after long-term  
289 fasting in humans (61.5h-fasting) (Liu et al., 2008) and rats (48h-fasting) (Toshinai et al., 2001;  
290 Gonzalez et al., 2008) and with the recent observation indicating that 48h of fasting did not  
291 significantly alter stomach GOAT mRNA expression in rats (Gonzalez et al., 2008; Takahashi  
292 et al., 2009). Taken together our current results suggest that changes in stomach GOAT  
293 expression may contribute in part to fasting-induced changes in circulating acylated-ghrelin  
294 levels, a hypothesis previously put forth by other laboratories (Gonzalez et al., 2008; Gualillo et  
295 al., 2008). However, given the only other report examining this relationship in mice gave  
296 different results (Kirchner et al., 2009), and the fact that there are many potential levels of  
297 GOAT regulation (transcriptional, translational, enzyme activity level, substrate availability), it  
298 will be important for other laboratories to validate and confirm these findings.

299

### 300 3.3. Effects of obesity on GOAT/ghrelin axis.

301 As shown in Figure 1, panel B, diet-induced obesity tended (50% reduction,  $p=0.13$ ) to  
302 suppress total ghrelin levels in mice without altering acylated-ghrelin levels, consistent with a  
303 significant decline in stomach ghrelin expression, while GOAT mRNA levels remained

304 unchanged. In contrast, obesity as a result of leptin-deficiency (ob/ob mice, Fig. 1 panel C) led  
305 to a significant decline in circulating acylated-ghrelin levels (and a downward trend in total-  
306 ghrelin levels), changes which were accompanied by a significant decrease in stomach ghrelin  
307 but not GOAT mRNA levels, similar to DIO mice. This is the first report to explore the impact  
308 of DIO on stomach GOAT and ghrelin expression, while a previous study showed similar  
309 results for stomach GOAT and ghrelin mRNA levels in ob/ob mice (Kirchner et al., 2009). In  
310 addition, others have reported a significant decline in total- or desacyl-ghrelin in DIO (Ueno et  
311 al., 2007) and obese Ay (Nonogaki et al., 2006) mice and a reduction in total- and acylated-  
312 ghrelin levels in obese humans (Casanueva and Dieguez, 2002; Gottero et al., 2004; Ghigo et  
313 al., 2005; Kojima and Kangawa, 2005), where DIO-induced alterations in acylated-ghrelin  
314 levels may be dependent on the time of day sampled (Perreault et al., 2004). Taken together  
315 these results indicate that the decline in circulating total-ghrelin levels in the obese state could  
316 be in part due to a decline in stomach ghrelin gene expression, however alterations in circulating  
317 acylated-ghrelin may be related to factors independent of GOAT expression, such as the rate of  
318 degradation of acylated-ghrelin, GOAT enzyme activity levels or substrate (ghrelin or fatty  
319 acid) availability.

320

321 *3.4. Impact of metabolic stress (fasting and obesity) on hypothalamic and pituitary GOAT*  
322 *expression.*

323 As shown in Figure 1, panel D, fasting also enhanced expression of GOAT at both the  
324 hypothalamic [at 48h (p=0.0016)] and pituitary [24h (p=0.017) and 12h (p=0.051)] level.  
325 Interestingly, it should be noted that the timing of the GOAT mRNA rise was different from that  
326 observed in the stomach (Fig. 1, panel A). Conversely, DIO (Fig. 1, panel E) and obesity caused  
327 by leptin-deficiency (Fig. 1, panel F) did not alter hypothalamic GOAT expression, but did  
328 result in a significant decline in pituitary GOAT mRNA levels. As in the stomach, hypothalamic  
329 and pituitary GOAT expression did not always parallel local changes in ghrelin expression.  
330 Taken together, these results suggest local production (ghrelin gene expression) and/or  
331 modification (via GOAT gene expression) of ghrelin may contribute to regulation of

332 hypothalamic and pituitary function independent of circulating ghrelin levels produced by the  
333 stomach which would be supported by a recent study indicating that various cell lines, including  
334 the mouse pituitary cell line AtT20, can produce acylated-ghrelin when both ghrelin and GOAT  
335 are present in the cells and when substrate (fatty acid) is available in the culture medium  
336 (Takahashi et al., 2009)

337

338 *3.5. Direct regulation of GOAT mRNA levels by ghrelin, desacyl-ghrelin, GHRH, SST, leptin,*  
339 *NPY, insulin and IGF-I in mouse primary pituitary cell cultures.*

340         Given the dynamic changes in pituitary GOAT expression in both fasting (up-  
341 regulation; Fig. 1, panel D) and obesity (down-regulation; Fig. 1, panel E and F), we used  
342 primary mouse pituitary cell cultures as a model system to explore which central or systemic  
343 factors might mediate these changes. First, to confirm that mouse primary pituitary cells  
344 maintain differentiated function (ie. GOAT expression) after dispersion and culture, the absolute  
345 mRNA levels (copy numbers/0.05  $\mu$ g total RNA) of GOAT were compared between whole  
346 tissue extracts and extracts prepared from pituitary cultures 24h after incubation in serum-free  
347 media, and the results are shown in Table 1. Transcript levels did not significantly vary between  
348 *in vivo* and *in vitro* samples, indicating that the cell preparation and culture conditions were  
349 appropriate to maintain GOAT expression. Based on reports from our laboratory and others  
350 showing that the levels and/or actions of ghrelin, GHRH and NPY are up-regulated in fasting  
351 (Henry et al., 2001; Park et al., 2005; Luque et al., 2006; Luque et al., 2007c; Kirchner et al.,  
352 2009) and down-regulated in obesity (Tannenbaum et al., 1990; Ahmad et al., 1993; Maccario  
353 et al., 1999; Lin et al., 2000; Perreault et al., 2004; Luque and Kineman, 2006; Nonogaki et al.,  
354 2006; Luque et al., 2007a) while those for SST, leptin, insulin and IGF-I are down-regulated in  
355 fasting (Henry et al., 2001; Frystyk, 2004; Park et al., 2005; Luque et al., 2006; Gonzalez et al.,  
356 2008) and up-regulated in obesity (Zhou et al., 1997; Frystyk, 2004; Luque and Kineman, 2006;  
357 Luque et al., 2007b; Luque et al., 2008), we incubated primary pituitary cell cultures with these  
358 hormones for 24h and measured the impact on GOAT mRNA levels. As shown in Figure 2, top  
359 panel, acylated-ghrelin clearly stimulated GOAT expression compared to vehicle-treated

360 controls (set at 100%). In order to confirm that this effect on GOAT mRNA levels was directly  
361 exerted by acylated-ghrelin and not by desacyl-ghrelin (as result of a des-octanoylation of  
362 acylated-ghrelin in the pituitary cell cultures after 24h of incubation), pituitary cell cultures were  
363 treated with desacyl-ghrelin for 24h. The results clearly indicate that treatment with desacyl-  
364 ghrelin did not alter GOAT mRNA levels. In addition, as shown in figure 2 (top panel), GHRH  
365 and leptin stimulated and SST inhibited pituitary GOAT expression, while NPY, insulin and  
366 IGF-I had no effect, compared to vehicle-treated controls (set at 100%). In order to determine, if  
367 these direct actions might also be important in maintaining pituitary GOAT expression *in vivo*,  
368 we examined pituitary expression of GOAT in available mouse models that lack GHRH, SST,  
369 NPY and leptin. Accordingly, we found an opposite effect of GHRH-KO, SST-KO and leptin-  
370 KO on pituitary GOAT expression, as compared to that observed after *in vitro* treatment with  
371 the corresponding hormone (Fig. 2, bottom panel). It is also interesting to note that when the  
372 impact of leptin replacement was examined in the ob/ob model (Luque et al., 2007a), we  
373 observed that pituitary and stomach GOAT mRNA levels were greater than that observed in  
374 pair-fed controls (Supplemental Figure 2), which is consistent with the report of Gonzalez et al.,  
375 (Gonzalez et al., 2008) showing that administration of exogenous leptin markedly increased  
376 expression levels of GOAT in the stomach of fasted rats. Taken together these results indicate  
377 that acylated-ghrelin may in fact regulate its own production at the level of the pituitary by  
378 increasing the expression of GOAT. In addition, these results clearly demonstrate that GHRH,  
379 SST and leptin are key regulators of pituitary GOAT expression, an observation which may in  
380 part explain changes in pituitary GOAT expression observed in response to metabolic extremes.  
381 Intriguingly, the substrate of GOAT activity in the mouse pituitary may not be limited to native  
382 ghrelin being locally produced within the pituitary, because our laboratory has identified a  
383 spliced mRNA ghrelin variant containing exon2, intron2 and exon3, but lacking exon1, 4 and 5  
384 (In2-ghrelin variant; (Kineman et al., 2007)) which is expressed at higher levels than the native  
385 ghrelin transcript in the pituitary ( $139 \pm 24$  copies for In2-ghrelin variant versus  $18 \pm 3$  copies  
386 for native-ghrelin/ $0.05\mu\text{g}$  RNA). If this variant is translated, it would encode a protein that  
387 would include the first 36 amino acid of the pre-proghrelin, where residues 24-28 represent the



388 first five amino acids of mature ghrelin and thus contain the Ser-3 acylation site. It is interesting  
389 to note that when we compared the impact of metabolic stress (fasting and DIO) or GHRH, SST  
390 and leptin deficiency on pituitary expression of GOAT, native ghrelin and In2-ghrelin, the  
391 levels of GOAT expression paralleled those of In2-ghrelin variant but not native ghrelin, in all  
392 cases (summarized in Table 2, and details presented in Supplemental Figure 3). Based on these  
393 observations it seems reasonable to propose that the In2-ghrelin variant could be a primary  
394 substrate for GOAT in the anterior pituitary gland.

395         Although more experiments are required to fully elucidate the physiologic significance  
396 of our findings in the pituitary, our data give credence to the possibility that local production  
397 and/or modification of ghrelin (desacylated to acylated via GOAT gene expression) may  
398 contribute to the fine regulation of pituitary function in response to metabolic stress. For  
399 example, circulating GH levels are known to rise in response to fasting in the mouse, and this is  
400 associated with an increase in pituitary expression of GH, GHRH-R and GHS-R and a decline  
401 in SST receptor expression (rat and mouse; (Luque et al., 2007a; Luque et al., 2008)). The acute  
402 rise in systemic acylated-ghrelin, in combination with an increased sensitivity of the pituitary to  
403 ghrelin and GHRH actions, might initiate local production of acylated-ghrelin by augmentation  
404 of ghrelin and GOAT expression, thereby acting as a positive ultrashort feedback loop to  
405 enhance or facilitate GH release. In contrast, GH output is suppressed in obesity, and effect  
406 which is believed to be due in part to enhanced SST tone [for review see (Luque et al., 2008)]  
407 and a decline in pituitary of GHRH-R, GHS-R and GH (Luque and Kineman, 2006; Luque et  
408 al., 2008). In this context, our data would support the idea that part of the negative effects of  
409 SST on somatotrope function could include direct suppression of GOAT expression and a  
410 decline of locally produced acylated-ghrelin, further contributing to the decline in GH release  
411 observed with weight gain.

412         In summary, we report herein a series of novel analyses on the regulation of the mouse  
413 GOAT/ghrelin system at the circulating-stomach-pituitary-hypothalamic levels by energy status  
414 and relevant metabolic cues in the mouse. By applying a combination of studies involving *in*  
415 *vivo* models (fasting, obesity and knockout mice) and primary pituitary cell cultures, our results

416 further characterize the impact of changes in body energy stores on the expression of GOAT in  
417 different tissues and define the role of key regulators, such as acylated-ghrelin itself, GHRH,  
418 SST and leptin on the control of GOAT expression in the mouse. The fact that GOAT mRNA  
419 levels are oppositely regulated in extreme metabolic states (fasting, DIO) and its regulation is  
420 tissue-dependent suggests that this enzyme may be of biological relevance in coordinating the  
421 neuroendocrine response to metabolic stress. Although much work remains to be done to fully  
422 understand how GOAT fits into the control of energy homeostasis, our results reinforce the  
423 contention that GOAT/acylated-ghrelin system could operate as a novel molecular conduit for  
424 the integration of energy balance, metabolism and pituitary function.

425

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442

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**Table 1:** Absolute cDNA copy number/0.05 $\mu$ g total RNA of GOAT gene transcripts in the stomach, pituitary and hypothalamus of male C57Bl/6 mice, as determined by quantitative real-time RT-PCR.

	<u>GOAT mRNA copy #</u>
<u>Whole tissue:</u>	
- <b>Stomach</b>	357 $\pm$ 46
- <b>Pituitary</b>	67 $\pm$ 8
- <b>Hypothalamus</b>	73 $\pm$ 5
<u>Primary pituitary cell culture:</u>	63 $\pm$ 2

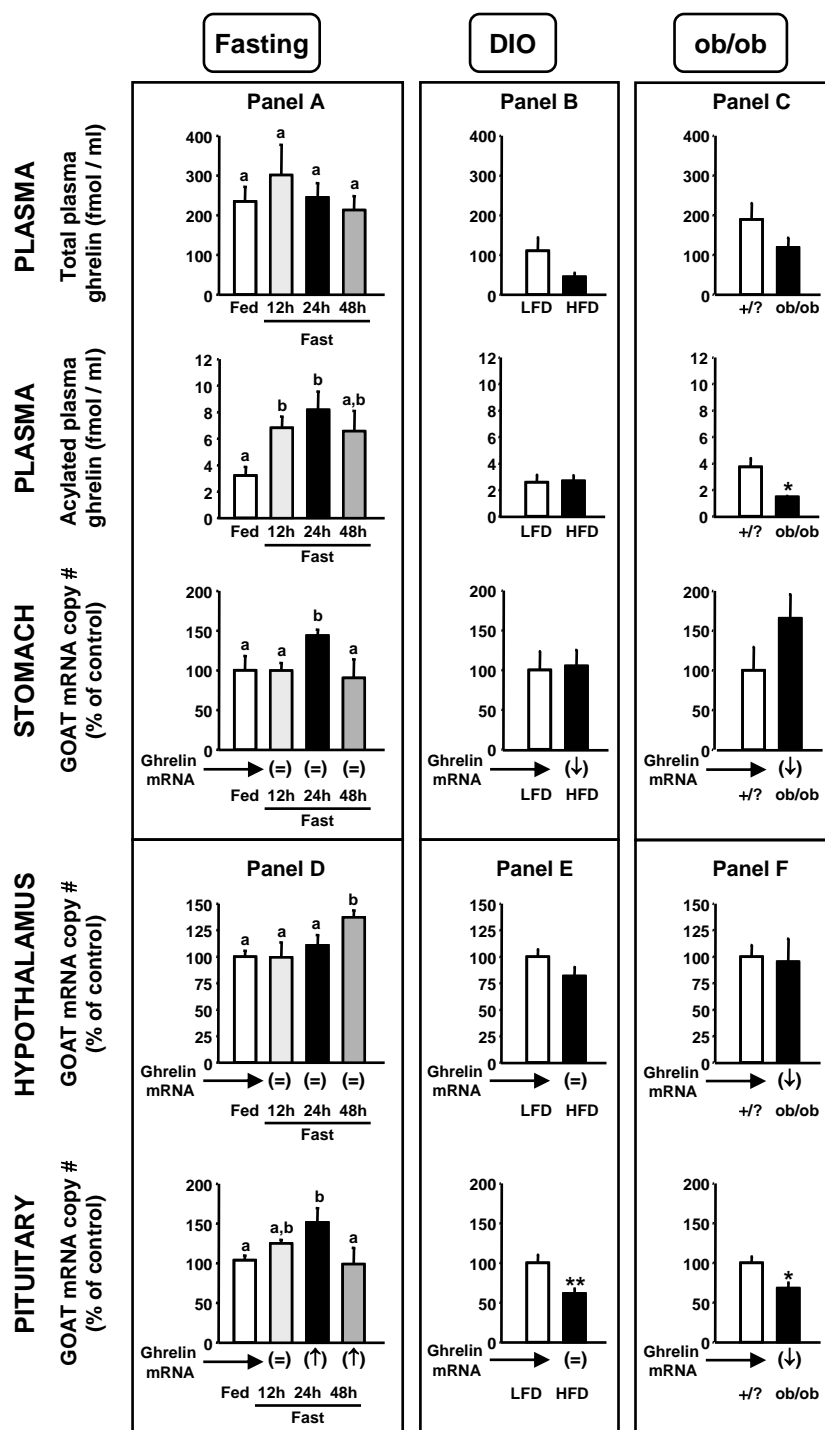
Values represent means  $\pm$  SEM (n=27-43 control tissues or control culture-wells/experimental or treatment group)

**Table 2:** Relative effect of metabolic condition (fasting, obesity) or inactivating mutations in growth hormone releasing hormone (GHRH), somatostatin (SST) or leptin genes on pituitary GOAT, ghrelin and In2-ghrelin variant mRNA levels, as determined by quantitative real-time RT-PCR. This summary represents data presented in the current report, combined with previously published reports (Kineman et al., 2007; Luque et al., 2007a; Luque et al., 2007c).

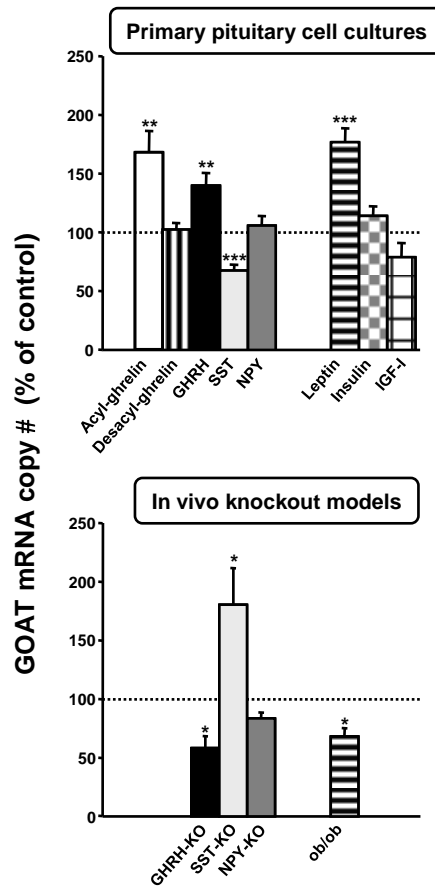
	<u>GOAT mRNA</u>	<u>Native ghrelin</u>	<u>In2-ghrelin</u>
<b>Fasting 24h</b>	↑	↑	↑
<b>Diet induced obese</b>	↓	No change	↓
<b>GHRH-KO</b>	↓	No change	↓
<b>SST-KO</b>	↑	↑	↑*
<b>Leptin-KO (ob/ob)</b>	↓	↓	↓

\* Differences observed only in females.





**Figure 1:** Circulating total- and acylated-ghrelin and stomach, hypothalamus and pituitary GOAT mRNA levels in: mice fed ad libitum or fasted for 12-, 24-, and 48-h (Panel A and D), mice fed a low-fat diet (LFD control group) or high-fat diet (HFD obese group) (Panel B and E) and lean controls (./?) and obese (ob/ob) mice (Panel C and F). Values are shown as mean  $\pm$  SEM ( $n=5-8$  mice/group) and expressed as percent of the respective control group (set at 100%). Group means that do not share a common letter (a,b) significantly differ ( $p<0.05$ ; Panel A and D). Asterisks (\*,  $p<0.05$ , \*\*,  $p<0.01$ ) indicate values that significantly differ from lean control LFD or ./? values. The tissue-specific regulation of ghrelin mRNA levels in these mice models as previously reported (Kineman et al., 2007; Luque et al., 2007a; Luque et al., 2007c) are shown below each graph of tissue-specific GOAT expression and are provided as the relative changes (=,  $\uparrow$ ,  $\downarrow$ ) as compared to controls.



**Figure 2: Top panel:** Effect of 24-h treatment with acylated-ghrelin, growth hormone releasing hormone (GHRH), somatostatin (SST), neuropeptide Y (NPY), leptin, insulin and IGF-I on GOAT mRNA levels in mouse primary pituitary cell cultures. Data represent the mean  $\pm$  SEM (n=3-7 experiment performed in different cell preparations; 3-4 wells/treatment). **Bottom panel:** GOAT mRNA expression levels in pituitaries of growth hormone releasing hormone knockout (GHRH-KO), somatostatin knockout (SST-KO), neuropeptide Y knockout (NPY-KO) and ob/ob (leptin deficient) mice. Values are shown as mean  $\pm$  SEM and expressed as percent of intact control mice (set at 100%; n=5-9 mice/group). Asterisks (\*, p<0.05, \*\*, p<0.01, \*\*\*, p<0.001) indicate values that significantly differ from respective controls (shown by the dotted line set at 100%).