

1 GHSR-1a is not Required for Ghrelin's Anti-inflammatory and Fat-sparing Effects in Cancer

2 Cachexia

3 RUNNING TITLE: Ghrelin Prevents Fat Atrophy in Cachexia

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34

35 ACRONYM:

36 GHSR-1a: Growth Hormone Secretagogue Receptor 1a

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41 Brief summary: Ghrelin ameliorates WAT inflammation, fat atrophy and anorexia in LLC-induced

42 cachexia. GHSR-1a is required for ghrelin's orexigenic effect but not for its anti-inflammatory or

43 fat-sparing effects.

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45

46 ABSTRACT

47 Adipose tissue (AT) atrophy is a hallmark of cancer cachexia contributing to increased
48 morbidity/mortality. Ghrelin has been proposed as a treatment for cancer cachexia partly by
49 preventing AT atrophy. However, the mechanisms mediating ghrelin's effects are incompletely
50 understood, including the extent to which its only known receptor, GHSR-1a, is required for these
51 effects. This study characterizes the pathways involved in AT atrophy in the Lewis Lung Carcinoma
52 (LLC)-induced cachexia model and those mediating the effects of ghrelin in *Ghsr*^{+/+} and *Ghsr*^{-/-} mice.
53 We show that LLC causes AT atrophy by inducing anorexia, and increasing AT inflammation,
54 thermogenesis and energy expenditure. These changes were greater in *Ghsr*^{-/-}. Ghrelin
55 administration prevented LLC-induced anorexia only in *Ghsr*^{+/+}, but prevented WAT inflammation
56 and atrophy in both genotypes, although its effects were greater in *Ghsr*^{+/+}. LLC-induced increases
57 in BAT inflammation, WAT and BAT thermogenesis, and energy expenditure were not affected by
58 ghrelin. In conclusion, ghrelin ameliorates WAT inflammation, fat atrophy and anorexia in
59 LLC-induced cachexia. GHSR-1a is required for ghrelin's orexigenic effect but not for its
60 anti-inflammatory or fat-sparing effects.

61

62 INTRODUCTION

63 Every year, over 1,500,000 individuals in the US are diagnosed with cancer. Cachexia (involuntary
64 loss of muscle and adipose tissue) is present in up to 80% of cancer patients, is strongly associated
65 with higher morbidity and mortality, and is reported as the direct cause of death in 20-40% of these
66 patients (Dewys, Begg et al., 1980, Fearon, Strasser et al., 2011). Adipose tissue, once considered
67 only a high-energy fuel reserve, has emerged recently as an active metabolic organ modulating
68 inflammation, energy expenditure and food intake in non-cancer settings (You & Nicklas, 2006).
69 Accelerated loss of adipose tissue plays an important role in cancer cachexia contributing
70 significantly to the increased morbidity and mortality seen in this setting (Fouladiun, Korner et al.,
71 2005).

72

73 Increased inflammation is common in the setting of cancer (Garcia, Garcia-Touza et al., 2005) and
74 is associated with adipose tissue wasting in human studies (Lerner, Hayes et al., 2015). White
75 adipose tissue (WAT) is a significant source of inflammatory cytokines accounting for more than 30%
76 of circulating interleukin (IL)-6 (Michaud, Boulet et al., 2014) and this and other inflammatory
77 cytokines have been linked to WAT atrophy in the setting of cancer (Petruzzelli, Schweiger et al.,
78 2014, Tsoli & Robertson, 2013, Tsoli, Swarbrick et al., 2016). Also, a phenotypic switch from WAT to
79 brown adipose tissue (BAT) known as “browning” is thought to contribute to the overall increase in
80 energy expenditure and WAT atrophy seen in cancer cachexia (Petruzzelli et al., 2014).
81 Nevertheless, the mechanisms regulating adipose tissue atrophy and dysfunction in this setting are
82 incompletely understood.

83

84 Ghrelin, originally identified as the endogenous ligand for the growth hormone secretagogue
85 receptor (GHSR)-1a, has emerged as a pleiotropic hormone that regulates body weight, body

86 composition and energy expenditure (Muller & Tschop, 2013). In non-cancer models, it has been
87 shown to increase food intake by activating neuropeptide Y and agouti-related peptide-secreting
88 neurons in the hypothalamus and to have direct effects on adipocytes (Kos, Harte et al., 2009,
89 Muller & Tschop, 2013, Perez-Tilve, Heppner et al., 2011). Ghrelin has also been proposed as a
90 promising target for cancer cachexia and it has been shown to prevent fat atrophy in tumor-bearing
91 animals and in patients with cancer cachexia (Chen, Splenser et al., 2015, Garcia, Boccia et al.,
92 2015, Garcia, Scherer et al., 2013b). However, the mechanisms mediating these effects are
93 incompletely understood. Interestingly, emerging data suggest that some of these effects are
94 independent of the only ghrelin receptor identified to date, GHSR-1a (Kojima, Hosoda et al., 1999,
95 Smith, Van der Ploeg et al., 1997).

96

97 The objectives of this study were to characterize the pathways involved in adipose tissue atrophy in
98 the Lewis Lung Carcinoma (LLC)-induced cachexia model and to determine the pathways mediating
99 the effects of ghrelin on adipose tissue in this setting, including the relative contribution of GHSR-1a.

100

101

102 RESULTS

103 We utilized C57/BL6 congenic mice with (*Ghsr*^{+/+}) or without GHSR-1a (*Ghsr*^{-/-}). Five to
104 seven-month-old male *Ghsr*^{+/+} and *Ghsr*^{-/-} mice were inoculated with 1x10⁶ heat-killed (HK, control)
105 or live LLC cells in the right flank. When the tumor was palpable (approximately 1 wk after
106 implantation), tumor-bearing mice were injected with vehicle (saline solution, tumor-vehicle, TV) or
107 ghrelin (0.8 mg/kg, tumor-ghrelin, TG) subcutaneously (s.q.) twice/day, while HK mice were injected
108 with vehicle until the end of the experiments (2 weeks after the tumor became palpable). Body
109 weight and fat mass were measured by nuclear magnetic resonance (NMR) before tumor
110 implantation and 2 weeks after tumors were noted. Brown adipose tissue (BAT) and inguinal and
111 epididymal white adipose tissue (iWAT, eWAT) were collected and weighed upon sacrificing animals
112 2 weeks after tumors were noted. We confirmed that *Ghsr*^{-/-} mice did not express *Ghsr* globally by
113 genotyping. Also, there was no expression of *Ghsr* in neither iWAT or BAT on either genotype
114 (Supplemental Fig.1).

115

116 **Ghrelin prevents tumor-induced weight loss and adipose tissue atrophy only partially via**
117 **GHSR-1a.**

118 LLC tumor implantation induced significant decreases in carcass weight in both genotypes;
119 although, the decrease was more profound in *Ghsr*^{-/-} than in *Ghsr*^{+/+} mice (Fig. 1A, genotype effect:
120 $p < 0.001$). The same pattern was seen in whole body fat mass measured by NMR (Fig. 1B,
121 genotype effect: $p = 0.002$) as well as in iWAT and eWAT pad weights measured upon dissection
122 (Fig. 1C, genotype effect on iWAT: $p = 0.043$). These changes were fully prevented by ghrelin
123 administration in *Ghsr*^{+/+} tumor-bearing animals and partially prevented in *Ghsr*^{-/-} animals. *Ghsr*^{-/-}
124 mice exhibited significantly less food intake *versus* *Ghsr*^{+/+} mice during daytime (genotype effect: $p =$
125 0.018) and tumor-bearing mice showed less food intake than controls, although this difference only

126 reached significant for the TG group at nighttime (Figure 1D). LLC-induced decreases in food intake
127 were prevented by ghrelin during daytime (6am – 6pm) only in *Ghsr*^{+/+}.

128

129 **Ghrelin attenuates tumor-induced inflammation in iWAT but not in iBAT or in circulation.**

130 In *Ghsr*^{+/+} animals, protein level for the pro-inflammatory cytokines IL-1 β and TNF in iWAT were
131 increased in tumor-bearing mice and ghrelin prevented these increases (Fig 2A, C). IL-6 and the
132 macrophage marker monocyte chemoattractant protein-1 (MCP-1), a key chemokine responsible for
133 migration and infiltration of monocytes/macrophages (Deshmane, Kremlev et al., 2009), followed a
134 similar pattern although the differences did not reach statistical significance (Fig. 2B, D).

135 Interestingly, in *Ghsr*^{-/-} mice LLC-induced IL-6 level increases in iWAT appear to be dampened;
136 whereas, MCP-1 levels were not affected by LLC or by ghrelin. Immunohistochemistry staining
137 shows complete co-localization of IL-6 and TNF with F4/80, a marker of macrophages in mice,
138 demonstrating that the source of these cytokines in iWAT are macrophages (Fig 2 E-F). High
139 resolution images of immunohistochemistry staining in iWAT are demonstrated in Supplemental Fig.
140 2.

141

142 In BAT, all the inflammatory markers were generally lower than in WAT. IL-1 β was increased in both
143 genotypes (Fig. 3A) and MCP-1 only in *Ghsr*^{-/-} (Fig. 3D). Ghrelin did not significantly affect these
144 changes. IL-6 and TNF levels were not significantly different between groups (Fig. 3B-C).

145 Nevertheless, immunohistochemistry analysis shows similar results as in iWAT suggesting that IL-6
146 and TNF in BAT were also derived exclusively from macrophages (Fig. 3 E-F). High resolution
147 images of immunohistochemistry staining in BAT are demonstrated in Supplemental Fig. 3. Plasma
148 cytokine and MCP-1 levels followed a different pattern than those seen in adipose tissue being
149 increased by LLC and not modified by ghrelin (Supplemental Fig. 4).

150

151 **Ghrelin does not prevent the increases in UCP-1 induced by LLC in iWAT or BAT**

152 Thermogenesis in BAT is activated by uncoupling protein-1 (UCP-1) by de-coupling oxidative
153 phosphorylation from ATP synthesis and dissipating heat in the inner mitochondrial membrane
154 (Puigserver, Wu et al., 1998). A similar process has been reported in WAT which has been
155 described as “fat browning” with transformation of “white” to “beige” adipocytes (Rosen &
156 Spiegelman, 2014, Wu, Bostrom et al., 2012). To test the effect of LLC and the role of ghrelin and
157 GHSR-1a on this pathway, we quantified UCP-1 levels in iWAT and BAT using
158 immunohistochemistry (IHC) by normalizing the positively-stained area to the total cross-sectional
159 area of the adipose tissue. Tumor implantation induced increases in UCP-1 expression in iWAT and
160 BAT in both genotypes and these increases were more pronounced in *Ghsr*^{-/-} than in *Ghsr*^{+/+} (Fig 4
161 A-D, genotype effect in BAT: $p = 0.005$). In iWAT, the LLC-induced UCP-1 increase only reached
162 significance in the tumor-bearing *Ghsr*^{-/-} mice and no significant effect of ghrelin was observed. In
163 BAT, the positively stained UCP-1 area increased with tumor implantation from 22% to 59% in
164 *Ghsr*^{+/+} and from 35% to 70% in *Ghsr*^{-/-} mice. However, no effect of ghrelin on reducing UCP-1 in
165 BAT was observed.

166

167 **Tumor-induced increases in energy expenditure (EE) are not prevented by ghrelin**

168 Tumor implantation increased EE and this difference was of greater magnitude in *Ghsr*^{-/-} animals
169 when the heat production was adjusted for lean body mass (LBM, Fig 5 A-C; endpoint EE
170 normalized to baseline level, genotype effect: $p = 0.013$; average EE at endpoint, genotype effect: p
171 = 0.010). We also analyzed the raw EE data (kcal/h) by analysis of covariance (ANCOVA) with LBM
172 as a covariate as recommended by Tschop et al. (Tschop, Speakman et al., 2011). A significant
173 strain difference ($p = 0.001$) was also detected using this method where *Ghsr*^{-/-} mice showed higher

174 EE levels in response to LLC tumor implantation when compared to *Ghsr^{+/+}*. Animals
175 co-administered ghrelin were not statistically different from vehicle-treated, tumor-bearing animals.
176 Tumor implantation also decreased spontaneous locomotor activity in both genotypes and ghrelin
177 administration did not prevent these changes (Fig 5 D-F). The respiratory quotient (RQ), was
178 significantly decreased by tumor implantation and was not affected by genotype or ghrelin
179 administration (Fig 5 G-I).
180

181 DISCUSSION

182 Adipose tissue atrophy is a central component of the cancer anorexia and cachexia syndrome
183 (CACS) leading to increased morbidity and mortality (Das, Eder et al., 2011). Recently, emerging
184 roles for inflammation, WAT browning and increased BAT thermogenesis have been demonstrated
185 in this setting (Daas, Rizeq et al., 2018, Dalal, 2019, Han, Meng et al., 2018, Kir, White et al., 2014,
186 Kliewer, Ke et al., 2015, Petruzzelli et al., 2014, Rohm, Schafer et al., 2016, Rohm, Zeigerer et al.,
187 2019, Wang, Zhu et al., 2019); however, the pathways involved and their potential as therapeutic
188 targets are not well-known. Ghrelin and agonists of its only known receptor, GHSR-1a, show
189 potential to ameliorate CACS at least in part by preventing fat atrophy, but the specific mechanisms
190 mediating these effects have not been fully characterized. Given that there are no FDA-approved
191 treatments for cancer cachexia and that several clinical trials targeting this pathway have failed to
192 meet their primary endpoints (Garcia et al., 2015, Temel, Abernethy et al., 2016), there is a pressing
193 need to improve our understanding of the mechanisms of action of ghrelin in this setting. In this
194 study we show that ghrelin prevents LLC tumor-induced weight loss, fat atrophy and WAT
195 inflammation without affecting tumor-induced BAT inflammation, WAT browning, and increased BAT
196 uncoupling and whole-body energy expenditure. We confirmed that its orexigenic effects are
197 GHSR-1a-dependent, and also show that other novel GHSR-1a-independent mechanisms are
198 involved given the partial improvements in fat atrophy and WAT inflammation seen in ghrelin-treated,
199 *Ghsr*^{-/-} animals. Also, this is the first report of macrophages as the source of IL-6 and TNF in both
200 WAT and BAT in the setting of CACS.

201

202 Weight loss and survival rates are correlated with IL-6 levels in cancer patients (Garcia et al., 2005,
203 Moses, Maingay et al., 2009, Scott, McMillan et al., 1996). These observations and several
204 mechanistic studies support the premise that inflammation plays a central role in CACS. Increases

205 in IL-1 β and TNF contribute to anorexia (Baracos, Martin et al., 2018, Braun, Zhu et al., 2011, Khatib,
206 Gaidhane et al., 2018), and TNF and IL-6 promote lipolysis and inhibit lipogenesis in WAT leading to
207 weight loss (Fearon, Glass et al., 2012, Han et al., 2018, Jeanson, Carriere et al., 2015, Jung & Choi,
208 2014, Ruan, Hacothen et al., 2002). In non-cancer settings, one third of the circulating IL-6 is
209 produced by WAT (Mohamed-Ali, Goodrick et al., 1997) and most of this WAT-derived IL-6 comes
210 from the stroma-vascular fraction composed of endothelial cells, monocytes/macrophages,
211 myocytes, and fibroblasts (Fain, Madan et al., 2004), although it can also be derived from
212 adipocytes (Fain, 2006). Macrophages in WAT are known to be the source of proinflammatory
213 cytokines in conditions leading to AT hypertrophy including obesity (Di Gregorio, Yao-Borengasser
214 et al., 2005, Divoux, Tordjman et al., 2010, Lumeng, Deyoung et al., 2007) but this has not been
215 previously shown in CACS. Here we show that LLC tumor implantation induces an increase in
216 inflammatory cytokines in circulation as well as in BAT and WAT. Moreover, these AT cytokines
217 appear to be derived exclusively from macrophages residing in these tissues. Adipose tissue
218 atrophy in cancer patients with CACS has been associated with an increase in subcutaneous AT
219 macrophages (Batista, Henriques et al., 2016, de Matos-Neto, Lima et al., 2015, Henriques, Sertie
220 et al., 2017) and tissue inflammation (Batista, Oliven et al., 2013, de Matos-Neto et al., 2015,
221 Henriques et al., 2017). Although, macrophage infiltration has also been described in WAT from
222 tumor-bearing rodents (Henriques et al., 2017, Machado, Costa Rosa et al., 2004, Petruzzelli et al.,
223 2014), to our knowledge this is the first report of macrophages as the source of pro-inflammatory
224 cytokines in adipose tissue in CACS. These findings may explain why AT remains an important
225 source of pro-inflammatory cytokines even when the adipocyte mass is significantly reduced in this
226 setting. Also, this may be clinically relevant to cancer patients since knowing the source of
227 inflammation may allow us to target these pathways more effectively (Henriques, Lopes et al.,
228 2018).

229

230 Previously, we have shown that activation of GHSR-1a by ghrelin or GHSR-1a agonists (GHS)
231 increases food intake and body weight (13, 39, 40). Our group and others also have shown that
232 ghrelin reduces fat oxidation and lipolysis and increases lipogenesis and adiposity in a rodent model
233 of cisplatin-induced cachexia by a combination of food intake-dependent and independent
234 mechanisms (Chen et al., 2015, Garcia et al., 2013b, Porporato, Filigheddu et al., 2013). Ghrelin is
235 thought to have anti-inflammatory effects in other settings (Deboer, Zhu et al., 2008, Dixit, Schaffer
236 et al., 2004, Tsubouchi, Yanagi et al., 2014) but this is not yet clear in CACS. Some reports suggest
237 an anti-inflammatory effect of native ghrelin administration, but this was not confirmed in other
238 studies using GHSR-1a agonists (Chen et al., 2015, Garcia, Friend et al., 2013a). In the current
239 study, we report that ghrelin modulates inflammation in a tissue-specific manner. Ghrelin did not
240 prevent tumor-induced increases in circulating inflammatory cytokines or in BAT IL-1 β or MCP-1
241 protein levels. However, it mitigated LLC-induced inflammation in WAT. This effect was seen in both
242 genotypes although it was clearer in wild type animals partly because *Ghsr*^{-/-} mice appear to be
243 resistant to tumor-induced inflammation. GHSR-1a is not expressed in adipocytes (Sun, Garcia et
244 al., 2007) but is present in macrophages (Ma, Lin et al., 2013) and our findings are consistent with a
245 previous report showing that old, non-tumor-bearing *Ghsr*^{-/-} mice have reduced macrophage
246 infiltration, a shift on macrophage differentiation towards a more anti-inflammatory phenotype, and
247 decreased inflammation in adipose tissue (Lin, Lee et al., 2016). However, a GHSR-1a-independent
248 effect of ghrelin on macrophages is also possible as it has been proposed in other settings (Avallone,
249 Demers et al., 2006, Bulgarelli, Tamiazzo et al., 2009, Lucchi, Costa et al., 2017). Taken together,
250 our data is consistent with a WAT-specific, anti-inflammatory effect of ghrelin that is partly GHSR-1a
251 dependent. This is clinically relevant as GHSR-1a agonists are in clinical development for CACS
252 and their effect on these GHSR-1a independent pathways is not known (Garcia et al., 2015). Also,

253 the differences we report between serum, WAT and BAT levels underscore the limitations of relying
254 exclusively on circulating cytokine levels when trying to determine the potential role of inflammation
255 in other tissues.

256

257 Energy expenditure is an important mechanism in the regulation of body weight and is increased in
258 CACS (Garcia et al., 2013a, Kir, Komaba et al., 2016, Rohm et al., 2019). Factors contributing to EE
259 include physical activity and resting EE (REE) (Silver, Dietrich et al., 2007, Vazeille, Jouinot et al.,
260 2017) and adipose tissue can lead to an increase in REE by uncoupling oxidative phosphorylation in
261 mitochondria thereby releasing heat through activation of a proton leak (Nicholls, 1976,
262 Okamatsu-Ogura, Kitao et al., 2007). In WAT, browning has been noted in multiple cancer cachexia
263 models with adipocytes showing an upregulation of the main regulator of thermogenesis, UCP1
264 (Dong, Lin et al., 2018, Vaitkus & Celi, 2017). In BAT, increased thermogenesis has been reported in
265 cachectic animals (Kir et al., 2014) independently of decreased food intake or their ability to
266 maintain their body temperature (Tsolis, Moore et al., 2012). Proinflammatory cytokines have been
267 suggested as key drivers of WAT browning (Han et al., 2018, Petruzzelli et al., 2014) and of BAT
268 thermogenesis through activation of sympathetic nervous system or targeting BAT directly (Arruda,
269 Milanski et al., 2010, Dascombe, Rothwell et al., 1989, Li, Klein et al., 2002, Tsoli et al., 2012). Here
270 we show that LLC-tumor implantation led to an increase in total EE in spite of a significant decrease
271 in physical activity, suggesting an increase in REE. This was associated with an increase in UCP-1
272 expression in WAT (browning) and in BAT. Moreover, these effects were more marked in *Ghsr*^{-/-} mice
273 suggesting a protective role of GHSR-1a in this setting. These results agree with previous reports in
274 aged, non-tumor-bearing *Ghsr*^{-/-} showing higher levels of thermogenesis and energy expenditure
275 when compared to aged-matched, wild-type mice (Lin, Saha et al., 2011). The effect of ghrelin or
276 GHSR1a agonists on energy expenditure is unclear with some studies showing a decrease in EE

277 (Borner, Loi et al., 2016, Villars, Pietra et al., 2017) while others showed no effect (Adachi, Takiguchi
278 et al., 2010, Tschop, Smiley et al., 2000, Vestergaard, Djurhuus et al., 2008). In this study, we did
279 not see a significant effect of ghrelin on preventing LLC-induced fat browning, BAT thermogenesis,
280 increased REE or decreased physical activity in the setting of CACS despite the fact that ghrelin
281 prevented fat and weight loss and anorexia. We hypothesize that differences in the models, route of
282 administration and treatment regimen and agents used (LLC mice vs. C26 mice or hepatoma model
283 in rats, administration via s.q. vs. oral gavage vs. osmotic mini pump, ghrelin vs. GHSR1a agonists)
284 could account for these discrepancies. More studies will be needed to test this hypothesis.

285

286 Macrophage infiltration contributes to the high levels of inflammatory cytokines (TNF, IL-6, and IL-1 β)
287 in BAT in conditions associated with AT hypertrophy such as high fat diet (Roberts-Toler, O'Neill et
288 al., 2015, van den Berg, van Dam et al., 2017) or obesity (Alcala, Calderon-Dominguez et al., 2017,
289 Calderon-Dominguez, Mir et al., 2016). In CACS the aforementioned tumor-induced inflammation is
290 thought to play an important role in BAT thermogenesis (Petruzzelli et al., 2014, Tsoli et al., 2012);
291 however, the source of inflammation in BAT is not known. Similar to WAT, we found that BAT IL-6
292 and TNF come exclusively from macrophages in the setting of cachexia. However, their expression
293 in BAT were lower than in WAT and no significant changes were found in response to tumor
294 implantation or ghrelin. We found a significant tumor-effect on increasing IL-1 β levels in BAT
295 although ghrelin did not prevent this increase, suggesting tissue-specific differences in inflammation
296 between BAT and WAT in response to tumor and ghrelin. Taken together, these results are
297 important because they show that tumor-induced WAT browning and BAT thermogenesis are
298 associated with significant increases in REE and appear to be independent of inflammation given
299 that downregulating inflammation does not prevent uncoupling in WAT and that BAT IL6 and TNF
300 levels were not upregulated upon tumor implantation. In addition, our data suggests that WAT is a

301 significant source of inflammatory cytokines, which express the highest levels of IL-1 β , IL-6, and
302 TNF when compared to BAT and circulating levels.

303

304 There were limitations to our approach. This study was not set up to establish the safety of ghrelin
305 administration in the setting of cancer. Nevertheless, none of the studies published to date using
306 ghrelin or GHSR-1a agonists in mice or humans have shown an increase in tumor progression
307 (Sever, White et al., 2016). Also, the experiments were not designed to characterize other
308 mechanisms contributing to the protective role of GHSR-1a in this setting. Lastly, our data suggest
309 that there is an alternative receptor for ghrelin although identification of this receptor remains elusive
310 and is the focus of other studies.

311

312 In summary, ghrelin prevents LLC tumor-induced body weight and fat loss by a combination of
313 GHSR-1a-dependent mechanisms including preventing anorexia, and other mechanisms that are
314 partly GHSR-1a-independent. The increase in inflammation in AT induced by tumor implantation is
315 prevented by ghrelin only in WAT; however, tumor-induced WAT browning, and increased BAT
316 inflammation, uncoupling and whole body energy expenditure are not prevented by ghrelin even
317 when the presence of GHSR-1a appears to contribute to maintaining energy balance in this setting.
318 Tumor-induced WAT browning and BAT thermogenesis are associated with significant increases in
319 REE and these seem to be independent of inflammation given that downregulating it does not
320 prevent these changes. These results are clinically relevant because they show that ghrelin
321 ameliorates WAT inflammation, fat atrophy and anorexia in CACS in spite of not having a discernible
322 effect on energy expenditure, WAT browning or BAT inflammation and thermogenesis. Our data fills
323 an important gap in the knowledge regarding the mechanisms of action of ghrelin in the setting of
324 cancer cachexia and should inform the design of future preclinical and clinical studies targeting this

325 pathway.

326

327 METHODS

328 **Animals**

329 Five to seven-month-old male C57BL/6J growth hormone (GH) secretagogue receptor wild type
330 (*Ghsr*^{+/+}) and knockout (*Ghsr*^{-/-}) congenic mice were used for all experiments. Briefly the *Ghsr*^{+/+} and
331 *Ghsr*^{-/-} mice were originally from Dr. Roy G. Smith Ph.D's laboratory (Sun, Butte et al., 2008) and the
332 *Ghsr*^{-/-} mice were backcrossed with C57BL/6J for at least 10 generations to minimize selective
333 genetic traits. The mice used in the study were off springs of these congenic mice and were bred in
334 the Animal Research Facilities in Veterans Affairs Puget Sound Health Care System. Mice were
335 individually housed, acclimated to their cages and human handling for 1 week before the
336 experiments and maintained on a 12/12 light/dark cycle (lights on at 6AM). All experiments were
337 conducted with the approval of the Institutional Animal Care and Use Committee at VA Puget Sound
338 Health Care System and were in compliance with the NIH Guidelines for Use and Care of
339 Laboratory Animals. Sample sizes of each experiment are shown in the figure legends.

340

341 **Tumor implantation and ghrelin administration**

342 The procedures of tumor implantation (TI) and ghrelin intervention were described previously (Chen
343 et al., 2015). In brief, mice were injected subcutaneously (s.q.) with Lewis lung carcinoma (LLC)
344 cells (1×10^6 cells, CRL1642, American Type Culture Collection, Manassas, VA) into the right flank
345 or with equal volume and number of heat-killed LLC cells (HK). Approximately 7 days after tumor
346 implantation (TI), when the tumor was palpable (~1cm in diameter), the tumor-bearing mice were
347 treated with either acylated ghrelin (AS-24160, Anaspect, Fremont, CA) at a dose of 0.8 mg/kg or
348 vehicle (0.9% sodium chloride, 8881570121, COVIDIEN, Dublin, Ireland), s.q., twice daily, while

349 mice in HK group received vehicle (saline, same volume), s.q., twice daily for two weeks.
350 Mice were euthanized by CO₂ on Day 21 after TI, approximately 2 weeks after TN. Blood samples
351 were collected and then processed into plasma. Fat pads including iWAT, eWAT, and BAT, as well as
352 tumors were collected during dissection. The timeline of the study is demonstrated in Supplemental
353 Fig. 5.

354

355 **Body weight, food intake, and body composition**

356 Body weight and food intake were assessed daily starting before TI (baseline) until endpoint.
357 Parameters of body composition, including LBM and fat mass (FM) were measured by nuclear
358 magnetic resonance (NMR, Bruker optics, The Woodlands, TX) and identified at the baseline before
359 tumor implantation, when tumor was noted, and 2 weeks after tumor noted before terminating the
360 experiment (endpoint).

361

362 **Comprehensive laboratory animal monitoring system (CLAMS™)**

363 The Comprehensive Laboratory Animal Monitoring System (CLAMS™, Columbus Instruments,
364 Columbus, OH) was used to identify metabolic parameters of the animals as we previously
365 described (Guillory, Chen et al., 2017). *Ghsr*^{+/+} and *Ghsr*^{-/-} mice were individually housed in CLAMS
366 cages for 96 hours before TI as well as at the endpoint (see the Supplemental Fig. 5, timeline for the
367 study). The first 12 hours of CLAMS was considered as the acclimation phase and the data for the
368 next 72 hours were analyzed. Oxygen consumption (VO₂) (mL/h), carbon dioxide production (VCO₂)
369 (mL/h), and locomotor activity (infrared beam-break counts) were recorded automatically by the
370 CLAMS system every 20 min. The respiratory exchange ratio (RQ) and energy expenditure (EE, or
371 heat generation) were calculated from VO₂ and VCO₂ gas exchange data as follows: RQ =
372 VCO₂/VO₂ and EE = (3.815 + 1.232 × RQ) × VO₂, respectively. Energy expenditure was then

373 normalized to LBM for statistical analysis using two-way analysis of variance (ANOVA). Alternatively,
374 we also analyzed EE value by ANCOVA with LBM as a covariate. Locomotor activity was measured
375 on x- and z-axes by the counts of beam-breaks during the recording period. The data shown in the
376 results was summarized as the mean of every 6 hours in a 72-hour-period.

377

378 **Electrochemiluminescence immunoassay**

379 Inflammatory cytokines IL-1 β , IL-6, and TNF- α and macrophage marker MCP-1 in iWAT, BAT, and
380 serum were detected by U-PLEX Biomarker Group1 (ms) Assays which are developed by Meso
381 Scale Diagnostics (K15069L-1, MSD, Rockville, MD). A protocol provided by manufacturer was
382 used for this assay. In brief, each plate was prepared by overnight coating with the multiplex coating
383 solution at 4 °C, which contained linker-coupled biotinylated antibodies. Standards and serum
384 samples were diluted with Diluent 41 into 2-fold and loaded onto the coated plate on the next day.
385 For iWAT and BAT samples, 150ug of the protein lysate was diluted with Diluent 41 and loaded onto
386 each well. The plate was incubated at room temperature (RT) with shaking for 2h followed by 3
387 times of wash in phosphate buffered saline with .05% Tween 20 (PBS/T). Sulfo-tag labeled
388 detection antibody was then added to plates and incubated for 2.5h. After another 3 washes in
389 PBS/T, Read Buffer T(2x) was added and the plate was read on MSD Sector Imager (MSD).

390

391 **Immunohistochemistry**

392 The iWAT and BAT were mounted with OCT (VWR 25608-930, VWR, Radnor, PA) and flash frozen
393 in liquid nitrogen-chilled isopentane immediately after tissue collection. The OCT-mounted iWAT
394 and BAT blocks were sliced at 14 μ m using a Cryostat (Leica CM3050S, Nussloch, Germany) at
395 -40°C. Before the process of staining, slides were dehydrated at RT for 30 minutes followed by
396 incubating in methanol for 15 minutes at -20 °C. To identify the colocalization of F4/80 and IL-6 or

397 TNF α in iWAT and BAT, slides were blocked with 10% donkey serum for 1 hour at RT and followed
398 by incubating in primary antibodies (F4/80 Monoclonal Antibody 1:100, MF48000, Thermo Fisher
399 Scientific; Anti-IL-6 antibody 1:100, ab6672, Abcam; TNF alpha monoclonal antibody, FITC,
400 eBioscience™ 1:200, 11-7349-82, Thermo Fisher Scientific) at 4°C for overnight. After 3 washes in
401 PBS, the slides were incubated by the corresponding secondary antibodies (Alexa Fluor 594
402 donkey anti-rat IgG, A21209, or Alexa Fluor 488 donkey anti-rat IgG, A21208, for F4/80; Texas Red
403 goat anti-rabbit IgG, T-2767, for IL-6) for 2 hours at RT and followed by incubating in 1:1000 DAPI
404 (62248, Thermo Fisher Scientific) in PBS for 1min. The slides were then mounted by Prolong Gold
405 AntiFade reagent (P36930, Thermo Fisher Scientific) with coverslips. To identify UCP1 in iWAT and
406 BAT, slides were incubated with 3% hydrogen peroxide (323381, Sigma-Aldrich, St. Louis, MO) for
407 30 min and then in 2.5% normal horse serum for 1hr. Then the slides were incubated with UCP1
408 Polyclonal Antibody (PA1-24894, Thermo Fisher Scientific) diluted 1:200 in 2.5% normal horse
409 serum at 4°C for overnight. On the following day, signals were visualized using SignalStain® Boost
410 IHC Detection Reagent (8114, Cell Signaling) and the SignalStain® DAB Substrate kit (8059, Cell
411 Signaling). The stained slides were dehydrated by 70%, 90%, 100% ethanol, and 100% xylene
412 sequentially and mounted with coverslips by using Permount (SP15-100, Thermo Fisher Scientific).
413 All stained slides were imaged by Nikon NiE microscope at 20x (iWAT) or 40x (BAT). The positive
414 cells (immunofluorescence) or positive area (DAB stain) in the section were quantified and
415 normalized to the total area of the section (mm²) using ImageJ analysis software (National Institutes
416 of Health, <http://rsb.info.nih.gov/ij/>).

417

418 **Statistics**

419 Two-way ANOVA was performed to identify differences between genotypes (*Ghsr*^{+/+} vs. *Ghsr*^{-/-})
420 across treatments (HK, TV, and TG) followed by Fisher's LSD post hoc test. For inflammatory

421 cytokines, Kruskal-Wallis test was performed to identify the differences between groups. For energy
422 expenditure, ANCOVA was also used for analysis in addition to ANOVA with LBM as a covariate to
423 identify differences between genotypes across treatments followed by Fisher's LSD post hoc test.
424 Values are presented in mean \pm SEM. All statistical testing was performed using IBM SPSS version
425 18 software. Significant difference was set at *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

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440 AUTHOR CONTRIBUTIONS

441 HL, JL, BG, and JMG designed the study. HL, JL, PZ, JAC, JKY, YH and BA conducted experiments
442 and acquired data. HL, JL, BG, JAC, PZ, and IL handled the mice in the study. HL, JL, BG, JAC, PZ,
443 IL, BA, MS, and AT collected tissue. HL, JL, BA, MS, and AT analyzed data. HL, JL, and JMG wrote
444 the manuscript. All authors reviewed and approved the final version of the manuscript.

445 REFERENCES

- 446 Adachi S, Takiguchi S, Okada K, Yamamoto K, Yamasaki M, Miyata H, Nakajima K, Fujiwara Y,
447 Hosoda H, Kangawa K, Mori M, Doki Y (2010) Effects of ghrelin administration after total
448 gastrectomy: a prospective, randomized, placebo-controlled phase II study. *Gastroenterology* 138:
449 1312-20
- 450 Alcala M, Calderon-Dominguez M, Bustos E, Ramos P, Casals N, Serra D, Viana M, Herrero L
451 (2017) Increased inflammation, oxidative stress and mitochondrial respiration in brown adipose
452 tissue from obese mice. *Sci Rep* 7: 16082
- 453 Arruda AP, Milanski M, Romanatto T, Solon C, Coope A, Alberici LC, Festuccia WT, Hirabara SM,
454 Ropelle E, Curi R, Carnevali JB, Vercesi AE, Velloso LA (2010) Hypothalamic actions of tumor
455 necrosis factor alpha provide the thermogenic core for the wastage syndrome in cachexia.
456 *Endocrinology* 151: 683-94
- 457 Avallone R, Demers A, Rodrigue-Way A, Bujold K, Harb D, Anghel S, Wahli W, Marleau S, Ong H,
458 Tremblay A (2006) A growth hormone-releasing peptide that binds scavenger receptor CD36 and
459 ghrelin receptor up-regulates sterol transporters and cholesterol efflux in macrophages through a
460 peroxisome proliferator-activated receptor gamma-dependent pathway. *Mol Endocrinol* 20: 3165-78
- 461 Baracos VE, Martin L, Korc M, Guttridge DC, Fearon KCH (2018) Cancer-associated cachexia. *Nat*
462 *Rev Dis Primers* 4: 17105
- 463 Batista ML, Jr., Henriques FS, Neves RX, Olivani MR, Matos-Neto EM, Alcantara PS, Maximiano LF,
464 Otoch JP, Alves MJ, Seelaender M (2016) Cachexia-associated adipose tissue morphological
465 rearrangement in gastrointestinal cancer patients. *J Cachexia Sarcopenia Muscle* 7: 37-47
- 466 Batista ML, Jr., Olivani M, Alcantara PS, Sandoval R, Peres SB, Neves RX, Silverio R, Maximiano LF,
467 Otoch JP, Seelaender M (2013) Adipose tissue-derived factors as potential biomarkers in cachectic
468 cancer patients. *Cytokine* 61: 532-9

- 469 Borner T, Loi L, Pietra C, Giuliano C, Lutz TA, Riediger T (2016) The ghrelin receptor agonist HM01
470 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia
471 syndrome in tumor-bearing rats. *Am J Physiol Regul Integr Comp Physiol* 311: R89-96
- 472 Braun TP, Zhu X, Szumowski M, Scott GD, Grossberg AJ, Levasseur PR, Graham K, Khan S,
473 Damaraju S, Colmers WF, Baracos VE, Marks DL (2011) Central nervous system inflammation
474 induces muscle atrophy via activation of the hypothalamic-pituitary-adrenal axis. *J Exp Med* 208:
475 2449-63
- 476 Bulgarelli I, Tamiasso L, Bresciani E, Rapetti D, Caporali S, Lattuada D, Locatelli V, Torsello A (2009)
477 Desacyl-ghrelin and synthetic GH-secretagogues modulate the production of inflammatory
478 cytokines in mouse microglia cells stimulated by beta-amyloid fibrils. *J Neurosci Res* 87: 2718-27
- 479 Calderon-Dominguez M, Mir JF, Fucho R, Weber M, Serra D, Herrero L (2016) Fatty acid
480 metabolism and the basis of brown adipose tissue function. *Adipocyte* 5: 98-118
- 481 Chen JA, Splenser A, Guillory B, Luo J, Mendiratta M, Belinova B, Halder T, Zhang G, Li YP, Garcia
482 JM (2015) Ghrelin prevents tumour- and cisplatin-induced muscle wasting: characterization of
483 multiple mechanisms involved. *J Cachexia Sarcopenia Muscle* 6: 132-43
- 484 Daas SI, Rizeq BR, Nasrallah GK (2018) Adipose tissue dysfunction in cancer cachexia. *J Cell*
485 *Physiol* 234: 13-22
- 486 Dalal S (2019) Lipid metabolism in cancer cachexia. *Ann Palliat Med* 8: 13-23
- 487 Das SK, Eder S, Schauer S, Diwokoy C, Temmel H, Guertl B, Gorkiewicz G, Tamilarasan KP, Kumari
488 P, Trauner M, Zimmermann R, Vesely P, Haemmerle G, Zechner R, Hoefler G (2011) Adipose
489 triglyceride lipase contributes to cancer-associated cachexia. *Science* 333: 233-8
- 490 Dascombe MJ, Rothwell NJ, Sagay BO, Stock MJ (1989) Pyrogenic and thermogenic effects of
491 interleukin 1 beta in the rat. *Am J Physiol* 256: E7-11
- 492 de Matos-Neto EM, Lima JD, de Pereira WO, Figueredo RG, Riccardi DM, Radloff K, das Neves RX,

493 Camargo RG, Maximiano LF, Tokeshi F, Otoch JP, Goldszmid R, Camara NO, Trinchieri G, de
494 Alcantara PS, Seelaender M (2015) Systemic Inflammation in Cachexia - Is Tumor Cytokine
495 Expression Profile the Culprit? *Front Immunol* 6: 629

496 Deboer MD, Zhu X, Levasseur PR, Inui A, Hu Z, Han G, Mitch WE, Taylor JE, Halem HA, Dong JZ,
497 Datta R, Culler MD, Marks DL (2008) Ghrelin treatment of chronic kidney disease: improvements in
498 lean body mass and cytokine profile. *Endocrinology* 149: 827-35

499 Deshmane SL, Kremlev S, Amini S, Sawaya BE (2009) Monocyte chemoattractant protein-1
500 (MCP-1): an overview. *J Interferon Cytokine Res* 29: 313-26

501 Dewys WD, Begg C, Lavin PT, Band PR, Bennett JM, Bertino JR, Cohen MH, Douglass HO, Jr.,
502 Engstrom PF, Ezdinli EZ, Horton J, Johnson GJ, Moertel CG, Oken MM, Perlia C, Rosenbaum C,
503 Silverstein MN, Skeel RT, Sponzo RW, Tormey DC (1980) Prognostic effect of weight loss prior to
504 chemotherapy in cancer patients. Eastern Cooperative Oncology Group. *Am J Med* 69: 491-7

505 Di Gregorio GB, Yao-Borengasser A, Rasouli N, Varma V, Lu T, Miles LM, Ranganathan G,
506 Peterson CA, McGehee RE, Kern PA (2005) Expression of CD68 and macrophage chemoattractant
507 protein-1 genes in human adipose and muscle tissues: association with cytokine expression, insulin
508 resistance, and reduction by pioglitazone. *Diabetes* 54: 2305-13

509 Divoux A, Tordjman J, Lacasa D, Veyrie N, Hugol D, Aissat A, Basdevant A, Guerre-Millo M, Poitou
510 C, Zucker JD, Bedossa P, Clement K (2010) Fibrosis in human adipose tissue: composition,
511 distribution, and link with lipid metabolism and fat mass loss. *Diabetes* 59: 2817-25

512 Dixit VD, Schaffer EM, Pyle RS, Collins GD, Sakthivel SK, Palaniappan R, Lillard JW, Jr., Taub DD
513 (2004) Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by
514 human monocytes and T cells. *J Clin Invest* 114: 57-66

515 Dong M, Lin J, Lim W, Jin W, Lee HJ (2018) Role of brown adipose tissue in metabolic syndrome,
516 aging, and cancer cachexia. *Front Med* 12: 130-138

517 Fain JN (2006) Release of interleukins and other inflammatory cytokines by human adipose tissue is
518 enhanced in obesity and primarily due to the nonfat cells. *Vitam Horm* 74: 443-477

519 Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW (2004) Comparison of the release of
520 adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and
521 subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* 145: 2273-82

522 Fearon K, Strasser F, Anker SD, Bosaeus I, Bruera E, Fainsinger RL, Jatoi A, Loprinzi C,
523 MacDonald N, Mantovani G, Davis M, Muscaritoli M, Ottery F, Radbruch L, Ravasco P, Walsh D,
524 Wilcock A, Kaasa S, Baracos VE (2011) Definition and classification of cancer cachexia: an
525 international consensus. *Lancet Oncol* 12: 489-95

526 Fearon KC, Glass DJ, Guttridge DC (2012) Cancer cachexia: mediators, signaling, and metabolic
527 pathways. *Cell Metab* 16: 153-66

528 Fouladiun M, Korner U, Bosaeus I, Daneryd P, Hyltander A, Lundholm KG (2005) Body composition
529 and time course changes in regional distribution of fat and lean tissue in unselected cancer patients
530 on palliative care--correlations with food intake, metabolism, exercise capacity, and hormones.
531 *Cancer* 103: 2189-98

532 Garcia JM, Boccia RV, Graham CD, Yan Y, Duus EM, Allen S, Friend J (2015) Anamorelin for
533 patients with cancer cachexia: an integrated analysis of two phase 2, randomised,
534 placebo-controlled, double-blind trials. *Lancet Oncol* 16: 108-16

535 Garcia JM, Friend J, Allen S (2013a) Therapeutic potential of anamorelin, a novel, oral ghrelin
536 mimetic, in patients with cancer-related cachexia: a multicenter, randomized, double-blind,
537 crossover, pilot study. *Support Care Cancer* 21: 129-37

538 Garcia JM, Garcia-Touza M, Hijazi RA, Taffet G, Epner D, Mann D, Smith RG, Cunningham GR,
539 Marcelli M (2005) Active ghrelin levels and active to total ghrelin ratio in cancer-induced cachexia. *J*
540 *Clin Endocrinol Metab* 90: 2920-6

541 Garcia JM, Scherer T, Chen JA, Guillory B, Nassif A, Papusha V, Smiechowska J, Asnicar M,
542 Buettner C, Smith RG (2013b) Inhibition of cisplatin-induced lipid catabolism and weight loss by
543 ghrelin in male mice. *Endocrinology* 154: 3118-29

544 Guillory B, Chen JA, Patel S, Luo JH, Splenser A, Mody A, Ding M, Baghaie S, Anderson B,
545 Lankova B, Halder T, Hernandez Y, Garcia JM (2017) Deletion of ghrelin prevents aging-associated
546 obesity and muscle dysfunction without affecting longevity. *Aging Cell* 16: 859-869

547 Han J, Meng Q, Shen L, Wu G (2018) Interleukin-6 induces fat loss in cancer cachexia by promoting
548 white adipose tissue lipolysis and browning. *Lipids Health Dis* 17: 14

549 Henriques F, Lopes MA, Franco FO, Knobl P, Santos KB, Bueno LL, Correa VA, Bedard AH,
550 Guilherme A, Birbrair A, Peres SB, Farmer SR, Batista ML, Jr. (2018) Toll-Like Receptor-4
551 Disruption Suppresses Adipose Tissue Remodeling and Increases Survival in Cancer Cachexia
552 Syndrome. *Sci Rep* 8: 18024

553 Henriques FS, Sertie RAL, Franco FO, Knobl P, Neves RX, Andreotti S, Lima FB, Guilherme A,
554 Seelaender M, Batista ML, Jr. (2017) Early suppression of adipocyte lipid turnover induces
555 immunometabolic modulation in cancer cachexia syndrome. *FASEB J* 31: 1976-1986

556 Jeanson Y, Carriere A, Casteilla L (2015) A New Role for Browning as a Redox and Stress Adaptive
557 Mechanism? *Front Endocrinol (Lausanne)* 6: 158

558 Jung UJ, Choi MS (2014) Obesity and its metabolic complications: the role of adipokines and the
559 relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty
560 liver disease. *Int J Mol Sci* 15: 6184-223

561 Khatib MN, Gaidhane A, Gaidhane S, Quazi ZS (2018) Ghrelin as a Promising Therapeutic Option
562 for Cancer Cachexia. *Cell Physiol Biochem* 48: 2172-2188

563 Kir S, Komaba H, Garcia AP, Economopoulos KP, Liu W, Lanske B, Hodin RA, Spiegelman BM
564 (2016) PTH/PTHrP Receptor Mediates Cachexia in Models of Kidney Failure and Cancer. *Cell*

- 565 *Metab* 23: 315-23
- 566 Kir S, White JP, Kleiner S, Kazak L, Cohen P, Baracos VE, Spiegelman BM (2014) Tumour-derived
567 PTH-related protein triggers adipose tissue browning and cancer cachexia. *Nature* 513: 100-4
- 568 Kliewer KL, Ke JY, Tian M, Cole RM, Andridge RR, Belury MA (2015) Adipose tissue lipolysis and
569 energy metabolism in early cancer cachexia in mice. *Cancer Biol Ther* 16: 886-97
- 570 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999) Ghrelin is a
571 growth-hormone-releasing acylated peptide from stomach. *Nature* 402: 656-60
- 572 Kos K, Harte AL, O'Hare PJ, Kumar S, McTernan PG (2009) Ghrelin and the differential regulation of
573 des-acyl (DSG) and oct-anoyl ghrelin (OTG) in human adipose tissue (AT). *Clin Endocrinol (Oxf)* 70:
574 383-9
- 575 Lerner L, Hayes TG, Tao N, Krieger B, Feng B, Wu Z, Nicoletti R, Chiu MI, Gyuris J, Garcia JM
576 (2015) Plasma growth differentiation factor 15 is associated with weight loss and mortality in cancer
577 patients. *J Cachexia Sarcopenia Muscle* 6: 317-24
- 578 Li G, Klein RL, Matheny M, King MA, Meyer EM, Scarpace PJ (2002) Induction of uncoupling
579 protein 1 by central interleukin-6 gene delivery is dependent on sympathetic innervation of brown
580 adipose tissue and underlies one mechanism of body weight reduction in rats. *Neuroscience* 115:
581 879-889
- 582 Lin L, Lee JH, Buras ED, Yu K, Wang R, Smith CW, Wu H, Sheikh-Hamad D, Sun Y (2016) Ghrelin
583 receptor regulates adipose tissue inflammation in aging. *Aging (Albany NY)* 8: 178-91
- 584 Lin L, Saha PK, Ma X, Henshaw IO, Shao L, Chang BH, Buras ED, Tong Q, Chan L, McGuinness
585 OP, Sun Y (2011) Ablation of ghrelin receptor reduces adiposity and improves insulin sensitivity
586 during aging by regulating fat metabolism in white and brown adipose tissues. *Aging Cell* 10:
587 996-1010
- 588 Lucchi C, Costa AM, Giordano C, Curia G, Piat M, Leo G, Vinet J, Brunel L, Fehrentz JA, Martinez J,

- 589 Torsello A, Biagini G (2017) Involvement of PPARgamma in the Anticonvulsant Activity of EP-80317,
590 a Ghrelin Receptor Antagonist. *Front Pharmacol* 8: 676
- 591 Lumeng CN, Deyoung SM, Bodzin JL, Saltiel AR (2007) Increased inflammatory properties of
592 adipose tissue macrophages recruited during diet-induced obesity. *Diabetes* 56: 16-23
- 593 Ma X, Lin L, Yue J, Pradhan G, Qin G, Minze LJ, Wu H, Sheikh-Hamad D, Smith CW, Sun Y (2013)
594 Ghrelin receptor regulates HFCS-induced adipose inflammation and insulin resistance. *Nutr*
595 *Diabetes* 3: e99
- 596 Machado AP, Costa Rosa LF, Seelaender MC (2004) Adipose tissue in Walker 256 tumour-induced
597 cachexia: possible association between decreased leptin concentration and mononuclear cell
598 infiltration. *Cell Tissue Res* 318: 503-14
- 599 Michaud A, Boulet MM, Veilleux A, Noel S, Paris G, Tchernof A (2014) Abdominal subcutaneous and
600 omental adipocyte morphology and its relation to gene expression, lipolysis and adipocytokine
601 levels in women. *Metabolism* 63: 372-81
- 602 Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppel SW (1997)
603 Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J*
604 *Clin Endocrinol Metab* 82: 4196-200
- 605 Moses AG, Maingay J, Sangster K, Fearon KC, Ross JA (2009) Pro-inflammatory cytokine release
606 by peripheral blood mononuclear cells from patients with advanced pancreatic cancer: relationship
607 to acute phase response and survival. *Oncol Rep* 21: 1091-5
- 608 Muller TD, Tschop MH (2013) Ghrelin - a key pleiotropic hormone-regulating systemic energy
609 metabolism. *Endocr Dev* 25: 91-100
- 610 Nicholls DG (1976) The bioenergetics of brown adipose tissue mitochondria. *FEBS Letters* 61:
611 103-110
- 612 Okamatsu-Ogura Y, Kitao N, Kimura K, Saito M (2007) Brown fat UCP1 is not involved in the febrile

613 and thermogenic responses to IL-1beta in mice. *Am J Physiol Endocrinol Metab* 292: E1135-9

614 Perez-Tilve D, Heppner K, Kirchner H, Lockie SH, Woods SC, Smiley DL, Tschop M, Pfluger P

615 (2011) Ghrelin-induced adiposity is independent of orexigenic effects. *FASEB J* 25: 2814-22

616 Petruzzelli M, Schweiger M, Schreiber R, Campos-Olivas R, Tsoi M, Allen J, Swarbrick M,

617 Rose-John S, Rincon M, Robertson G, Zechner R, Wagner EF (2014) A switch from white to brown

618 fat increases energy expenditure in cancer-associated cachexia. *Cell Metab* 20: 433-47

619 Porporato PE, Filigheddu N, Reano S, Ferrara M, Angelino E, Gnocchi VF, Prodam F, Ronchi G,

620 Fagoonee S, Fornaro M, Chianale F, Baldanzi G, Surico N, Sinigaglia F, Perroteau I, Smith RG, Sun

621 Y, Geuna S, Graziani A (2013) Acylated and unacylated ghrelin impair skeletal muscle atrophy in

622 mice. *J Clin Invest* 123: 611-22

623 Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM (1998) A Cold-Inducible

624 Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis. *Cell* 92: 829-839

625 Roberts-Toler C, O'Neill BT, Cypess AM (2015) Diet-induced obesity causes insulin resistance in

626 mouse brown adipose tissue. *Obesity (Silver Spring)* 23: 1765-70

627 Rohm M, Schafer M, Laurent V, Ustunel BE, Niopek K, Algire C, Hautzinger O, Sijmonsma TP, Zota

628 A, Medrikova D, Pellegata NS, Ryden M, Kulyte A, Dahlman I, Arner P, Petrovic N, Cannon B, Amri

629 EZ, Kemp BE, Steinberg GR et al. (2016) An AMP-activated protein kinase-stabilizing peptide

630 ameliorates adipose tissue wasting in cancer cachexia in mice. *Nat Med* 22: 1120-1130

631 Rohm M, Zeigerer A, Machado J, Herzig S (2019) Energy metabolism in cachexia. *EMBO Rep* 20

632 Rosen ED, Spiegelman BM (2014) What we talk about when we talk about fat. *Cell* 156: 20-44

633 Ruan H, Hacohen N, Golub TR, Van Parijs L, Lodish HF (2002) Tumor necrosis factor-alpha

634 suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1

635 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. *Diabetes* 51: 1319-36

636 Scott HR, McMillan DC, Crilly A, McArdle CS, Milroy R (1996) The relationship between weight loss

637 and interleukin 6 in non-small-cell lung cancer. *Brit J Cancer* 73: 1560-1562

638 Sever S, White DL, Garcia JM (2016) Is there an effect of ghrelin/ghrelin analogs on cancer? A
639 systematic review. *Endocr Relat Cancer* 23: R393-409

640 Silver HJ, Dietrich MS, Murphy BA (2007) Changes in body mass, energy balance, physical function,
641 and inflammatory state in patients with locally advanced head and neck cancer treated with
642 concurrent chemoradiation after low-dose induction chemotherapy. *Head Neck* 29: 893-900

643 Smith RG, Van der Ploeg LH, Howard AD, Feighner SD, Cheng K, Hickey GJ, Wyvratt MJ, Jr.,
644 Fisher MH, Nargund RP, Patchett AA (1997) Peptidomimetic regulation of growth hormone secretion.
645 *Endocr Rev* 18: 621-645

646 Sun Y, Butte NF, Garcia JM, Smith RG (2008) Characterization of adult ghrelin and ghrelin receptor
647 knockout mice under positive and negative energy balance. *Endocrinology* 149: 843-50

648 Sun Y, Garcia JM, Smith RG (2007) Ghrelin and growth hormone secretagogue receptor expression
649 in mice during aging. *Endocrinology* 148: 1323-9

650 Temel JS, Abernethy AP, Currow DC, Friend J, Duus EM, Yan Y, Fearon KC (2016) Anamorelin in
651 patients with non-small-cell lung cancer and cachexia (ROMANA 1 and ROMANA 2): results from
652 two randomised, double-blind, phase 3 trials. *The Lancet Oncology* 17: 519-531

653 Tschop M, Smiley DL, Heiman ML (2000) Ghrelin induces adiposity in rodents. *Nature* 407: 908-13

654 Tschop MH, Speakman JR, Arch JR, Auwerx J, Bruning JC, Chan L, Eckel RH, Farese RV, Jr.,
655 Galgani JE, Hambly C, Herman MA, Horvath TL, Kahn BB, Kozma SC, Maratos-Flier E, Muller TD,
656 Munzberg H, Pfluger PT, Plum L, Reitman ML et al. (2011) A guide to analysis of mouse energy
657 metabolism. *Nat Methods* 9: 57-63

658 Tsoli M, Moore M, Burg D, Painter A, Taylor R, Lockie SH, Turner N, Warren A, Cooney G, Oldfield B,
659 Clarke S, Robertson G (2012) Activation of thermogenesis in brown adipose tissue and
660 dysregulated lipid metabolism associated with cancer cachexia in mice. *Cancer Res* 72: 4372-82

- 661 Tsoli M, Robertson G (2013) Cancer cachexia: malignant inflammation, tumorkines, and metabolic
662 mayhem. *Trends Endocrinol Metab* 24: 174-83
- 663 Tsoli M, Swarbrick MM, Robertson GR (2016) Lipolytic and thermogenic depletion of adipose tissue
664 in cancer cachexia. *Semin Cell Dev Biol* 54: 68-81
- 665 Tsubouchi H, Yanagi S, Miura A, Matsumoto N, Kangawa K, Nakazato M (2014) Ghrelin relieves
666 cancer cachexia associated with the development of lung adenocarcinoma in mice. *Eur J*
667 *Pharmacol* 743: 1-10
- 668 Vaitkus JA, Celi FS (2017) The role of adipose tissue in cancer-associated cachexia. *Exp Biol Med*
669 *(Maywood)* 242: 473-481
- 670 van den Berg SM, van Dam AD, Rensen PC, de Winther MP, Lutgens E (2017) Immune Modulation
671 of Brown(ing) Adipose Tissue in Obesity. *Endocr Rev* 38: 46-68
- 672 Vazeille C, Jouinot A, Durand JP, Neveux N, Boudou-Rouquette P, Huillard O, Alexandre J, Cynober
673 L, Goldwasser F (2017) Relation between hypermetabolism, cachexia, and survival in cancer
674 patients: a prospective study in 390 cancer patients before initiation of anticancer therapy. *Am J Clin*
675 *Nutr* 105: 1139-1147
- 676 Vestergaard ET, Djurhuus CB, Gjedsted J, Nielsen S, Moller N, Holst JJ, Jorgensen JO, Schmitz O
677 (2008) Acute effects of ghrelin administration on glucose and lipid metabolism. *J Clin Endocrinol*
678 *Metab* 93: 438-44
- 679 Villars FO, Pietra C, Giuliano C, Lutz TA, Riediger T (2017) Oral Treatment with the Ghrelin
680 Receptor Agonist HM01 Attenuates Cachexia in Mice Bearing Colon-26 (C26) Tumors. *Int J Mol Sci*
681 18
- 682 Wang YX, Zhu N, Zhang CJ, Wang YK, Wu HT, Li Q, Du K, Liao DF, Qin L (2019) Friend or foe:
683 Multiple roles of adipose tissue in cancer formation and progression. *J Cell Physiol*
- 684 Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P,

685 Schaart G, Huang K, Tu H, van Marken Lichtenbelt WD, Hoeks J, Enerback S, Schrauwen P,
686 Spiegelman BM (2012) Beige adipocytes are a distinct type of thermogenic fat cell in mouse and
687 human. *Cell* 150: 366-76
688 You T, Nicklas BJ (2006) Chronic inflammation: role of adipose tissue and modulation by weight loss.
689 *Curr Diabetes Rev* 2: 29-37
690

691 FIGURE LEGENDS

692 Figure 1. Effects of ghrelin on body weight, fat mass, and food intake in LLC-induced cachexia. HK:
693 heat-killed + vehicle; TV: tumor + vehicle; TG: tumor + ghrelin. Changes in (A) body weight (carcass
694 weight, $n = 8-10$) and (B) fat body mass by NMR expressed as % change from baseline ($n = 8-10$).
695 (C) Fat pad mass normalized to baseline NMR fat mass (mg/g, $n = 4-6$). (D) Average cumulative
696 food intake (FI) normalized to baseline FI (g/g, black areas represent food intake in the nighttime,
697 and the bottom areas in the bars represent food intake in the daytime, $n = 4-6$). * $p < 0.05$ compared
698 to HK within the same genotype. # $p < 0.05$ compared to TV within the same genotype. In panel D,
699 differences in daytime are shown at the lower part of the bars; differences in nighttime are shown at
700 the upper part of the bars. Genotype effects are shown in p -values above the corresponding figures
701 ($p < 0.05$). Data are shown as mean \pm SE.

702

703 Figure 2. Effects of ghrelin on LLC-induced changes in inflammation and macrophages in iWAT. HK:
704 heat-killed + vehicle; TV: tumor + vehicle; TG: tumor + ghrelin. Protein levels of inflammatory
705 markers (A) IL-1 β , (B) IL-6, and (C) TNF; and (D) macrophage marker MCP-1 in iWAT (pg/mg). * $p <$
706 0.05 ; ** $p < 0.01$ compared to HK within the same genotype. # $p < 0.05$ compared to TV within the
707 same genotype. No genotype difference was detected. Data are shown as mean \pm SE. $n =$
708 $6-7$ /group. (E-F) Colocalization of inflammation and macrophages in iWAT. (E) Representative
709 images of colocalization of inflammatory marker IL-6 and macrophage marker F4/80 in iWAT (IL-6 in
710 Texas red; F4/80 in FITC green; nuclei in DAPI blue). (F) Representative images of colocalization of
711 inflammatory marker TNF and macrophage marker F4/80 in iWAT (TNF in FITC green; F4/80 in
712 Texas red; nuclei in DAPI blue). Positively stained inflammatory markers and colocalizations with
713 macrophages are indicated by the white arrows. Scale bars, $100 \mu\text{m}$.

714

715 Figure 3. Effects of ghrelin on LLC-induced changes in inflammation and macrophages in BAT. HK:
716 heat-killed + vehicle; TV: tumor + vehicle; TG: tumor + ghrelin. Protein levels of inflammatory
717 markers (A) IL-1 β , (B) IL-6, and (C) TNF; and (D) macrophage marker MCP-1 in iWAT (pg/mg). * $p <$
718 0.05; ** $p <$ 0.01; *** $p <$ 0.001 compared to HK within the same genotype. # $p <$ 0.05; ### $p <$ 0.001
719 compared to TV within the same genotype. No genotype difference was detected. Data are shown
720 as mean \pm SE. $n = 6-7$ /group. (E-F) Colocalization of inflammation and macrophages in BAT. (E)
721 Representative images of colocalization of inflammatory marker IL-6 and macrophage marker F4/80
722 in BAT (IL-6 in Texas red; F4/80 in FITC green; nuclei in DAPI blue). (F) Representative images of
723 colocalization of inflammatory marker TNF and macrophage marker F4/80 in BAT (TNF in FITC
724 green; F4/80 in Texas red; nuclei in DAPI blue). Positively stained inflammatory markers and
725 colocalizations with macrophages are indicated by the white arrows. Scale bars, 100 μ m.

726

727 Figure 4. Expression of UCP-1 in iWAT and BAT. HK: heat-killed + vehicle; TV: tumor + vehicle; TG:
728 tumor + ghrelin. (A) Representative IHC images of UCP-1 in iWAT. (B) UCP-1 positive area is
729 expressed as % of the total analyzed area in iWAT ($n = 4-6$). (C) Representative IHC images of
730 UCP-1 in BAT. (D) UCP-1 positive area is expressed as % of the total analyzed area in BAT ($n = 4-6$).
731 * $p <$ 0.05; ** $p <$ 0.01; *** $p <$ 0.001 compared to HK within the same genotype. Genotype effects
732 are shown as p -values above the corresponding figures ($p <$.05). Data are shown as mean \pm SE.
733 Scale bars, 200 μ m.

734

735 Figure 5. Indirect calorimetry measurements by CLAMS. HK: heat-killed + vehicle; TV: tumor +
736 vehicle; TG: tumor + ghrelin. (A-C) Energy expenditure adjusted by LBM is expressed (A) compared
737 to the baseline; (B) every 6 hours; and (C) average of every 6 hours. (D-F) Ambulatory activity is
738 expressed (D) compared to baseline; (E) every 6 hours; and (F) daily (black areas represent night

739 activity in each group). (G-I) Respiratory Quotient (RQ) is expressed (G) compared to baseline; (H)
740 every 6 hours; and (I) average of every 6 hours. * $p < 0.05$ compared to HK within the same genotype.
741 Genotype effects are shown in p -values above the corresponding figures ($p < 0.05$). N = 4 for HK
742 groups and N = 6 for the rest of the groups. Data are shown as mean \pm SE.

743

744 Supplemental Fig. 1. Gene expression of *Ghsr* in brain, iWAT, and BAT in *Ghsr*^{+/+} and ^{-/-} mice. Data
745 is expressed as box-and-whisker plot showing the median (middle line), mean (middle cross), upper
746 and lower quartiles (box), maximum and minimum (whiskers). Relative gene expression was
747 determined by normalization to *Gapdh*. N = 4/group. *Ghsr* was only detected in brain in *Ghsr*^{+/+}
748 mice. No *Ghsr* expression was detected in any tissue in *Ghsr*^{-/-} or adipose tissue in *Ghsr*^{+/+} mice.

749

750 Supplemental Fig. 2. High resolution images of immunohistochemistry staining in iWAT. (A)
751 Representative images of colocalization of inflammatory marker IL-6 and macrophage marker F4/80
752 in iWAT (IL-6 in Texas red; F4/80 in FITC green; nuclei in DAPI blue). (B) Representative images of
753 colocalization of inflammatory marker TNF and macrophage marker F4/80 in iWAT (TNF in FITC
754 green; F4/80 in Texas red; nuclei in DAPI blue). Positively stained inflammatory markers and
755 colocalizations with macrophages are indicated by the white arrows. Scale bars, 100 μ m.

756

757 Supplemental Fig. 3. High resolution images of immunohistochemistry staining in BAT. (A)
758 Representative images of colocalization of inflammatory marker IL-6 and macrophage marker F4/80
759 in BAT (IL-6 in Texas red; F4/80 in FITC green; nuclei in DAPI blue). (B) Representative images of
760 colocalization of inflammatory marker TNF and macrophage marker F4/80 in BAT (TNF in FITC
761 green; F4/80 in Texas red; nuclei in DAPI blue). Positively stained inflammatory markers and
762 colocalizations with macrophages are indicated by the white arrows. Scale bars, 100 μ m.

763

764 Supplemental Fig. 4. Effects of ghrelin on LLC-induced protein-level changes in inflammation (IL-1 β ,
765 IL-6, and TNF) and macrophages (MCP-1) in plasma (pg/mg, n = 11-14). *, **: different than HK
766 within the same genotype (*: p < .05; **: p < .01). Genotype effects are shown in p-values above the
767 corresponding figures (p < .05). Data are shown as mean \pm SE.

768

769 Supplemental Fig. 4. Timeline of current study. *Ghsr*^{+/+} and ^{-/-} mice were injected with LLC (T, 1 \times
770 10⁶ cells, s.q.) into the right flank or with equal volume and number of heat-killed LLC cells (HK).
771 Approximately 7 days after tumor implantation, when the tumor was palpable (day 0), the
772 tumor-bearing mice were treated with either acylated ghrelin, 0.8 mg/kg (TG) or vehicle (0.9%
773 sodium chloride, TV), s.q., twice daily, while mice in HK group received vehicle (saline, same
774 volume), s.q., twice daily for two weeks. Body composition were identified by NMR before tumor
775 implantation (7 days before tumor noted, baseline) and weekly till the endpoint. All the mice were
776 individually housed in CLAMS cages for 96 hours before TI (11-7 days before tumor noted, baseline)
777 as well as at the endpoint (day 10-14 after tumor noted).









