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# Human gain-of-function *MC4R* variants show signaling bias and protect against obesity

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25 **SUMMARY**

26 The Melanocortin 4 Receptor (MC4R) is a G-protein coupled receptor whose disruption  
27 causes obesity. We functionally characterized 61 *MC4R* variants identified in 0.5 million  
28 people from UK Biobank and examined their associations with Body Mass Index (BMI) and  
29 obesity-related cardiometabolic diseases. We found that the maximal efficacy of  $\beta$ -arrestin  
30 recruitment to MC4R, rather than canonical  $G\alpha_s$ -mediated cyclic adenosine-monophosphate  
31 production, explained 88% of the variance in the association of *MC4R* variants with BMI.  
32 Whilst most *MC4R* variants caused loss-of-function, a subset caused gain-of-function; these  
33 variants were associated with significantly lower BMI, and lower odds of obesity, type 2  
34 diabetes and coronary artery disease. Protective associations were driven by *MC4R* variants  
35 exhibiting signaling bias towards  $\beta$ -arrestin recruitment and increased Mitogen-Activated  
36 Protein Kinase pathway activation. Harnessing  $\beta$ -arrestin-biased MC4R signaling may  
37 represent an effective strategy for weight loss and the treatment of obesity-related  
38 cardiometabolic diseases.

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41

42 **INTRODUCTION**

43  
44 Obesity is associated with type 2 diabetes and coronary artery disease, which together  
45 account for significant morbidity, mortality and substantial health care costs globally  
46 (Heymsfield and Wadden, 2017). Whilst advances in our understanding of the molecular  
47 mechanisms involved in weight regulation have informed the development of new weight  
48 loss therapies, some drugs lack target specificity whilst others affect multiple signaling  
49 pathways downstream of their intended target leading to adverse effects which limit their  
50 long-term use (Bray et al., 2016). Therefore, there is a substantial unmet need for safe and  
51 effective weight loss therapies.

52  
53 G protein-coupled receptors (GPCRs) are targeted by approximately 30% of US Food and Drug  
54 Administration (FDA)-approved medicines highlighting their tractability for drug discovery  
55 (Hauser et al., 2017; Santos et al., 2017). Classically, upon ligand binding GPCRs interact with  
56 heterotrimeric Guanine nucleotide-binding (G) proteins to direct signaling and gene  
57 transcription, a response which is attenuated within minutes when phosphorylated GPCRs  
58 bind  $\beta$ -arrestins which sterically prevent their coupling to G proteins (Rajagopal and Shenoy,  
59 2018). This molecular interaction also promotes the internalization of ligand-bound receptors  
60 to early endosomes, from where GPCRs either recycle rapidly to the cell membrane or  
61 translocate to lysosomes for degradation (Shenoy and Lefkowitz, 2011; Shinyama et al.,  
62 2003).  $\beta$ -arrestins may also directly/indirectly mediate signaling via Mitogen-Activated  
63 Protein Kinase (MAPK)-mediated phosphorylation of extracellular signal-regulated kinase 1/2  
64 (ERK1/2).

65

66 Whilst balanced GPCR agonists signal with comparable efficacy through multiple pathways,  
67 the development of biased agonists which preferentially activate signaling through either G  
68 protein-dependent or G protein-independent  $\beta$ -arrestin-mediated pathways, is emerging as  
69 a powerful way of emphasizing favorable signals whilst de-emphasizing signals that may lead  
70 to adverse effects (Povsic et al., 2017; Rajagopal et al., 2011; Smith et al., 2018). Such targeted  
71 drug discovery relies on the precise delineation of the relative contributions of G proteins  
72 versus  $\beta$ -arrestins to the physiological consequences of GPCR activation.

73

74 Here we focused on the Melanocortin 4 Receptor (MC4R), a brain-expressed  $G\alpha_s$ -coupled  
75 GPCR involved in weight regulation (Fan et al., 1997; Kishi et al., 2003; Mountjoy et al., 1994;  
76 Ollmann et al., 1997). Feeding-induced release of the melanocortin peptides,  $\alpha$ - and  $\beta$ -  
77 melanocyte-stimulating hormone (MSH), leads to activation of MC4R-expressing neurons  
78 resulting in reduced food intake (Cowley et al., 2001; Fan et al., 1997). Targeted deletion of  
79 *Mc4r* in rodents causes weight gain in a gene dosage-dependent manner (Huszar et al., 1997).  
80 In humans, rare heterozygous *MC4R* variants that reduce  $G\alpha_s$ -mediated cyclic adenosine-  
81 monophosphate (cAMP) accumulation in cells have been identified in obese children and  
82 adults in many populations (Vaisse et al., 1998)(Yeo et al., 1998) ([www.mc4r.org.uk](http://www.mc4r.org.uk)). MC4R  
83 deficiency in rodents and humans (Fan et al., 2000; Farooqi et al., 2003) is characterized by  
84 low blood pressure (for the degree of obesity) due to impaired sympathetic nervous system  
85 activation (Greenfield et al., 2009) (Sayk et al., 2010; Simonds et al., 2014; Tallam et al., 2005).  
86 As predicted by these genetic findings, first generation MC4R agonists caused weight loss but  
87 increased blood pressure (BP) (Greenfield et al., 2009), which halted their development. A  
88 second generation MC4R agonist reduced weight in rare patients with obesity due to genetic  
89 disruption of the melanocortin pathway (Clement et al., 2018; Collet et al., 2017; Kuhnen et

90 al., 2016) without affecting BP (Chen et al., 2015; Kievit et al., 2013); however, off-target  
91 effects on the melanocortin-1 receptor (skin pigmentation) may limit its wider use. We  
92 hypothesized that a more refined understanding of MC4R signaling and its impact on clinical  
93 phenotypes in the general population may inform the design of drugs targeting this pathway  
94 to treat common obesity and its complications.

95

96 We performed genetic association studies in approximately 0.5 million people from UK  
97 Biobank focusing on 61 nonsynonymous variants identified in *MC4R*. Twelve of the 61 were  
98 nonsense/frameshift variants; the remainder (n=49) were missense variants whose functional  
99 properties were characterized in cells quantifying canonical  $G\alpha_s$ -mediated cAMP production  
100 and the recruitment of  $\beta$ -arrestin to MC4R. In meta-regression analyses using the functional  
101 consequence of *MC4R* variants as the predictor, we found that 88% of the variance in the  
102 association of different *MC4R* variants with BMI was explained by their effect on  $\beta$ -arrestin  
103 recruitment. A subset of individuals (6%, n=28,161) were carriers for gain-of-function alleles  
104 that exhibited signaling bias, preferentially increasing  $\beta$ -arrestin recruitment rather than  
105 cAMP production. These individuals had significantly lower BMI ( $P=2\times 10^{-42}$ ) and up to 50%  
106 lower risk of obesity, type 2 diabetes and coronary artery disease. Cumulatively, the  
107 characterization of BMI-lowering variants in *MC4R* demonstrates the pivotal role of  $\beta$ -  
108 arrestin-mediated MC4R signaling in human energy homeostasis. These findings have  
109 relevance for the development of  $\beta$ -arrestin-biased MC4R agonists for weight loss and for the  
110 treatment of obesity-associated metabolic disease.

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113

114 **RESULTS**

115 **Genetic variants in *MC4R* found in the general population cause loss- or gain-of-function in**  
116 **cells**

117 We studied 61 independent nonsynonymous variants in *MC4R* (pairwise  $R^2 < 0.01$ ; variant  
118 allele frequency 2%-0.0001%) that were directly genotyped (n=59) or well-imputed (info  
119 score > 0.8; n=2) in 452,300 European ancestry participants from UK Biobank (**Methods; Table**  
120 **S1**), which is a UK population-based cohort of people aged 40-69 years (Bycroft et al., 2018;  
121 Sudlow et al., 2015). Of the 61 *MC4R* variants, 12 were nonsense/frameshift variants and 49  
122 were missense variants (**Table S1**).

123

124 To characterize the functional consequences of all missense variants in *MC4R* (**Figure 1; Table**  
125 **S2**), HEK293 cells were transiently transfected with constructs encoding Wild-Type (WT) or  
126 mutant *MC4Rs*. We measured canonical  $G\alpha_s$ -mediated signaling by quantifying the maximal  
127 efficacy of ligand (NDP- $\alpha$ MSH; melanocyte stimulating hormone)-induced cAMP production  
128 in a time-resolved assay (**Figure 1A**). Additionally, we quantified the interaction between  
129 WT/mutant *MC4R* and  $\beta$ -arrestin-2 using a time-resolved enzyme complementation assay  
130 (**Figure 1B**). We found that 58 of 61 (95%) nonsynonymous *MC4R* variants had functional  
131 consequences; 47 (77%) resulted in a loss-of-function (LoF), 9 variants (15%) resulted in a  
132 significant gain-of-function (GoF), 2 variants (3%) had opposing effects on the two signaling  
133 pathways and 3 (5%) were wild-type like in both assays (**Figure 1A-D; Table S2**). In contrast to  
134 most previous studies of human *MC4R* variants, which have measured the direct/indirect  
135 accumulation of cAMP, we find that the majority of *MC4R* variants present in UK Biobank  
136 affect both cAMP production and the recruitment of  $\beta$ -arrestin-2 to *MC4R*.

137

138 **Gain-of-function *MC4R* variants are associated with protection against obesity and its**  
139 **metabolic complications**

140 We next performed genetic association studies with a series of primary phenotypes recorded  
141 in UK Biobank: BMI and obesity, hemodynamic phenotypes known to be affected by *MC4R*  
142 signaling (resting heart rate, systolic and diastolic blood pressure) and risk of type 2 diabetes  
143 and coronary artery disease. We found that LoF variants in *MC4R* were associated with higher  
144 BMI, and higher odds of obesity, severe obesity, type 2 diabetes and coronary artery disease  
145 (**Figure 1E**). These results align with reports of LoF *MC4R* variants identified in cohorts of  
146 obese and severely obese individuals (Farooqi et al., 2003; Hinney et al., 2006; Stutzmann et  
147 al., 2008; Turcot et al., 2018). In contrast, we found that gain-of-function *MC4R* variants were  
148 strongly associated with lower BMI ( $P=2\times 10^{-47}$ ) and lower odds of obesity ( $P=3\times 10^{-38}$ ), severe  
149 obesity ( $P=1\times 10^{-09}$ ), type 2 diabetes ( $P=4\times 10^{-06}$ ) and coronary artery disease ( $P=0.02$ ) (**Figure**  
150 **1E**). GoF, but not LoF variants, were associated with lower diastolic blood pressure and lower  
151 resting heart rate (**Table S3**).

152  
153 Associations with BMI were robust in sensitivity analyses that excluded ultra-rare genetic  
154 variants (variant allele frequency <0.001%) and factored in manually-curated cluster-plot  
155 quality scores (**Methods, Table S4**). The association of LoF variants with BMI was particularly  
156 strong for variants resulting in protein truncation or complete LoF of either pathway *in vitro*  
157 (**Table S4**). For 6 overlapping nonsynonymous variants, associations with BMI were consistent  
158 with external validation data from the GIANT consortium (Locke et al., 2015; Turcot et al.,  
159 2018) (**Table S4**). The association of LoF alleles in *MC4R* with type 2 diabetes was validated  
160 using exome sequencing data from the T2D Knowledge Portal; odds ratio [OR] for carriers of

161 rare LoF variants vs non-carriers, 1.59; 95% confidence interval [CI], 1.22-2.08;  $P=0.0007$ ;  $P$   
162 heterogeneity compared to the estimate in UK Biobank from this study=0.21.

163

## 164 **$\beta$ -arrestin-mediated MC4R signaling plays a pivotal role in human weight regulation**

165 We next used random-effects meta-regression to investigate whether  $\beta$ -arrestin recruitment  
166 or cAMP production explained the variance in the association of different *MC4R* variants with  
167 BMI (**Methods; Figure 2**). We found that  $\beta$ -arrestin recruitment was a statistically-significant  
168 predictor of the association of different *MC4R* variants with BMI ( $P=3\times 10^{-05}$ ) and explained  
169 88% of the variance in these associations (**Figure 2**). Several different sensitivity analyses  
170 supported the robustness of this association, including multivariable models with cAMP  
171 production as an additional predictor, leave-one-out analyses excluding one of the variants in  
172 each iteration, models restricted to rare variants (variant allele frequency  $<0.5\%$ , i.e.  
173 excluding the two variants with the largest weight in the main analysis), models including  
174 nonsense/frameshift variants and models excluding ultra-rare genetic variants and factoring  
175 in manually-curated cluster-plot quality scores (**Methods, Table S5**). In contrast, cAMP  
176 production did not predict the associations of different *MC4R* variants with BMI, either on its  
177 own ( $P=0.19$ ) or when the degree of  $\beta$ -arrestin recruitment was also included in the model  
178 ( $P=0.52$ ; **Table S5**). Increased  $\beta$ -arrestin recruitment also predicted lower estimates of  
179 association with BMI among the 20 variants that were WT-like for cAMP production ( $P=0.02$ ;  
180 **Table S5**). Increased  $\beta$ -arrestin recruitment remained a predictor of BMI-associations when  
181 using the functional category (LoF, WT-like or GoF) rather than the actual experimental value  
182 as predictor ( $P=0.04$ ; **Table S5**); there was evidence that both LoF and GoF variants  
183 contributed to this association (**Table S5**). Taken together, these results suggest that  $\beta$ -  
184 arrestin-mediated MC4R signaling plays a critical role in the regulation of human body weight.



185

186 **Gain-of-function *MC4R* variants that preferentially signal through  $\beta$ -arrestin mediate the**  
187 **protective association with BMI, obesity and its complications**

188 We hypothesized that naturally occurring genetic variants that preferentially affect signaling  
189 through one pathway versus the other (exhibit bias) may provide insights into the  
190 physiological consequences of targeting a specific pathway therapeutically. Among 11  
191 variants resulting in a GoF (including two that were GoF for cAMP but LoF for  $\beta$ -arrestin), five  
192 variants (T11S, T101N, F201L, G231S, R236C) exhibited significant bias towards cAMP  
193 production, four (V103I, I251L, I289L, I317V) exhibited significant bias towards  $\beta$ -arrestin  
194 recruitment and two (L304F, Y332C) showed no evidence of biased signaling (**Figure 3A**). GoF  
195 *MC4R* mutants that led to increased  $\beta$ -arrestin recruitment (but not those that predominantly  
196 increased cAMP production) resulted in enhanced signaling via the Mitogen-Activated Protein  
197 Kinase pathway measured by quantifying Extracellular Signal-Regulated Kinase (ERK1/2)  
198 phosphorylation assayed using Western blotting (**Figure 3B-D**).

199

200 In UK Biobank, approximately 1 in every 16 participants (6.1%; n=27,750) carried one copy of  
201 a  $\beta$ -arrestin-biased GoF allele, while 1 in every 1,102 (0.1%; n=411) carried two alleles.  
202 Carriers of one GoF allele had a BMI that was on average 0.39 kg/m<sup>2</sup> lower than non-carriers  
203 ( $P=2 \times 10^{-42}$ ; **Figure 3E**), while carriers of two alleles had a BMI that was 0.88 kg/m<sup>2</sup> lower  
204 ( $P=7 \times 10^{-05}$ ; **Figure 3E**). The latter is equivalent to ~2.5 kg lower body weight for a person 1.7  
205 metres tall. Carriers of two  $\beta$ -arrestin biased GoF alleles had an approximately 50% lower risk  
206 of obesity (OR, 0.51;  $P=8 \times 10^{-06}$ ; Fig. 3e), type 2 diabetes (OR, 0.52;  $P=0.03$ ; **Figure 3E**) and  
207 coronary artery disease (OR, 0.50;  $P=0.02$ ; **Figure 3E**), compared to non-carriers; carriers of  
208 one allele had intermediate risk (**Figure 3E**). Conversely, carriers of gain-of-function variants

209 exhibiting bias towards cAMP production had similar BMIs, and risks of obesity and cardio-  
210 metabolic disease as non-carriers; these variants were associated with a significant increase  
211 in systolic and a marginal (but non-significant) increase in diastolic blood pressure compared  
212 to non-carriers (**Tables S3 and S6**).

213

214 Associations of  $\beta$ -arrestin-biased GoF alleles in *MC4R* with cardio-metabolic outcomes were  
215 directionally consistent with those observed with a 97-variant polygenic score for lower BMI  
216 derived from a previous genome-wide study (Locke et al., 2015). For coronary artery disease,  
217 per kg/m<sup>2</sup> genetically-lower BMI, OR [95% CI] 0.94 [0.93-0.95] for the 97-variant polygenic  
218 score vs 0.82 [0.72-0.95] for the  $\beta$ -arrestin-biased *MC4R* GoF variants,  $P_{\text{heterogeneity}} = 0.07$ .  
219 Interestingly, *MC4R* GoF variants were more strongly associated with a reduced risk of type 2  
220 diabetes (OR [95% CI] per kg/m<sup>2</sup> genetically-lower BMI, 0.86 [0.85-0.87] for the 97-variant  
221 polygenic score vs 0.72 [0.62-0.83] for the  $\beta$ -arrestin-biased *MC4R* GoF variants,  $P_{\text{heterogeneity}} =$   
222 0.01). Experimental studies in rodents and humans have shown that impaired MC4R signaling  
223 increases insulin secretion (Fan et al., 2000; Greenfield et al., 2009), which may affect the  
224 onset and prevalence of type 2 diabetes in variant carriers through mechanisms that require  
225 further exploration.

226

### 227 **The most frequent gain-of-function *MC4R* variant (V103I) leads to increased cell surface** 228 **expression of mutant receptors**

229 To explore the potential mechanisms by which increased  $\beta$ -arrestin recruitment leads to a  
230 GoF rather than to a LoF as might be predicted, we studied V103I *MC4R*, the commonest  
231 nonsynonymous variant (variant allele frequency, 2%) found in UK Biobank which exhibits  
232 significant bias towards  $\beta$ -arrestin-mediated signaling (**Figure 3A**;  $P=0.004$ ). Previously, we

233 and others have reported associations of V103I *MC4R* with lower BMI and obesity risk (Geller  
234 et al., 2004; Gu et al., 1999; Heid et al., 2005; Stutzmann et al., 2007; Young et al., 2007),  
235 which were confirmed in this analysis (**Table S7**). In meta-analyses of genetic association  
236 studies including over 600,000 people, we now find that V103I *MC4R* is associated with lower  
237 risk of type 2 diabetes ( $P=7\times 10^{-07}$ ) and of coronary artery disease ( $P=0.003$ ; **Table S7**). V103I  
238 *MC4R* was also associated with lower diastolic blood pressure and resting heart rate, but not  
239 with any adverse disease outcomes in an exploratory phenome-wide association analysis of  
240 353 frequent clinical diagnoses in UK Biobank (**Figure S1**).

241

242 In contrast to previous studies (Gu et al., 1999; Hinney et al., 2006), we found that V103I  
243 *MC4R* increased ligand-induced cAMP production in a time-resolved assay (**Figure 4A, Figure**  
244 **S2A and Table S8**).  $\beta$ -arrestin recruitment by V103I *MC4R* was significantly increased in  
245 response to both synthetic and endogenous ligands; an effect that was sustained over 60  
246 minutes (**Figure 4B, Figure S2B and Table S8**). The magnitude and duration of ligand-induced  
247 ERK1/2 phosphorylation was also increased ( $P=0.009$ ) (**Figure 4C-D, Figure S2C-D**). Using  
248 confocal microscopy, we demonstrated that whilst WT *MC4Rs* translocated from the  
249 membrane into the cytoplasm upon agonist stimulation, V103I *MC4R* remained at the cell  
250 surface (**Figure 4E, Figure S2E**). These findings were replicated in a Fluorescence-Activated  
251 Cell Sorting (FACS) assay where cell surface expression of WT *MC4R* decreased by 23% upon  
252 ligand stimulation ( $P=0.003$ ; **Figure 4F**), whilst there was no change in expression of V103I  
253 *MC4R* (**Figure 4F, Figure S2F**). Further studies will be needed to investigate whether these  
254 findings may be explained by impaired internalization or accelerated recycling of V103I  
255 *MC4Rs* leading to an accumulation of V103I *MC4R* at the cell surface and a gain-of function  
256 (**Figure 5**).

257

## 258 **DISCUSSION**

259 By combining genetic studies in over 0.5 million people with detailed functional  
260 characterization of identified *MC4R* variants in cells, we demonstrated that  $\beta$ -arrestin-biased  
261 gain-of-function *MC4R* variants are associated with lower risk of obesity and its cardio-  
262 metabolic complications in the general population. We found that that almost all naturally  
263 occurring nonsynonymous variants in *MC4R* affect signaling and that the degree of  $\beta$ -arrestin  
264 recruitment to MC4R accounts for a large proportion of the variation in genetic association of  
265 these *MC4R* variants with BMI in the general population indicating that MC4R signaling  
266 through  $\beta$ -arrestin is critical for its role in the regulation of body weight. Approximately 6% of  
267 European ancestry individuals in the general UK population carry  $\beta$ -arrestin-biased GoF  
268 variants which are associated with up to 50% lower risk of obesity and its metabolic  
269 complications, but are not associated with increased BP and HR. These findings provide strong  
270 human genetic evidence to inform the development of  $\beta$ -arrestin-biased MC4R agonists for  
271 weight loss and the treatment of obesity-associated metabolic disease.

272

### 273 **The discovery of protective human genetic variants**

274 It is well-established that human genetic studies can inform the understanding of disease  
275 mechanisms and the development of new therapeutics. This concept is illustrated by the rapid  
276 development of new lipid-lowering drugs guided by studies of loss-of-function and gain-of-  
277 function coding variants in *PCSK9* (Cohen et al., 2006), *LPA* (Clarke et al., 2009), *APOC3* (Crosby  
278 et al., 2014; Jorgensen et al., 2014) and *ANGPTL3* (Dewey et al., 2017; Musunuru et al., 2010)  
279 and by the higher probability of successful drug development for targets supported by human  
280 genetic evidence (Nelson et al., 2015; Plenge et al., 2013).

281

282 In addition to studies of genetic variants that cause/are associated with disease/risk of  
283 disease, an alternative approach gaining traction in several fields, is the study of “resilient”  
284 individuals (e.g. smokers who remain healthy) or extremely elderly and healthy individuals  
285 (centenarians) (Friend and Schadt, 2014; Govindaraju et al., 2015; Harper et al., 2015). Several  
286 protective alleles have been identified to date (Liu et al., 1996; Myocardial Infarction Genetics  
287 Consortium et al., 2014). Some of these are rare and ancestry-specific, for example, a LoF  
288 allele within the amyloid- $\beta$  precursor protein (*APP*)-coding region in Icelanders reduces  
289 amyloid- $\beta$  aggregation and may offer protection against Alzheimer’s disease (Goate, 2006).  
290 Scandinavian carriers of variants in *SLC30A8* (Solute carrier family 30, member 8) are  
291 significantly less likely to develop type 2 diabetes even if obese (Flannick et al., 2014);  
292 associations that have been replicated in people from other ancestries. However, the  
293 discovery of low frequency variants associated with protection from common complex  
294 diseases is contingent upon sample size, with large numbers of affected individuals and  
295 controls being required to generate sufficient power to detect these associations. Here, by  
296 studying data on BMI and metabolic diseases in 0.5 million participants in UK Biobank and by  
297 focusing on a gene known to be involved in the regulation of weight and harboring a large  
298 number of low frequency variants, we find BMI-lowering genetic variants that are prevalent  
299 in a significant proportion (6%) of European ancestry individuals.

300

301 Our study has demonstrated the value of testing the functional consequences of variants  
302 identified in large scale genetic association studies, in particular as gain-of-function variants  
303 cannot be reliably identified or predicted using *in silico* algorithms. Here, by measuring the  
304 functional consequences of all missense *MC4R* variants in cells, we demonstrate strong

305 associations for gain-of-function variants with lower risk of obesity and show that loss-of-  
306 function variants are associated with obesity and diabetes risk in the general population. Loss-  
307 of-function *MC4R* variants were first identified in people with hyperphagia and severe early-  
308 onset obesity 20 years ago (Vaisse et al., 1998)(Yeo et al., 1998) and subsequently, over 300  
309 rare variants that reduce cAMP accumulation have been identified, mostly in obese people  
310 (Collet et al., 2017; Stutzmann et al., 2008) ([www.mc4r.org.uk](http://www.mc4r.org.uk)). A recent study in the general  
311 population identified an association of the known Y35X/D37V haplotype with higher BMI  
312 (Turcot et al., 2018). However, this and other studies have not detected significant  
313 associations for other *MC4R* LoF variants with obesity in the general population (Hinney et  
314 al., 2006). We suggest that these discordant findings may be partly explained by the rarity of  
315 these variants but also by the fact that some rare variants, including several predicted  
316 damaging by *in silico* algorithms, have minimal impact on cAMP signaling but do, as shown in  
317 this study, impact on  $\beta$ -arrestin recruitment which has previously not been studied. Our study  
318 highlights the value of combining detailed and comprehensive functional characterization of  
319 variants with large-scale genetic analyses.

320

321 While population-based studies may tend to underestimate the phenotypic consequences of  
322 genetic variants, as participants tend to be healthier than individuals in the general population  
323 from which they are sourced, studies of severe clinical cases may overestimate them (Wright  
324 et al., 2019). This may partly explain the smaller impact on BMI for *MC4R* LoF variants in  
325 population-based cohorts as opposed to cohorts of severely obese people (Farooqi et al.,  
326 2003). Furthermore, for a relatively modest difference in BMI (0.4 to 0.9 kg/m<sup>2</sup>), we observed  
327 that  $\beta$ -arrestin-biased GoF alleles in *MC4R* were associated with a large difference in risk of  
328 cardio-metabolic disease outcomes (up to 50% lower risk), more than expected from

329 observational epidemiology studies (Emerging Risk Factors et al., 2011). This is likely to reflect  
330 the life-long nature of exposure to lower levels of the risk factor (BMI) due to genotype, as  
331 opposed to short-term exposure in observational studies or clinical trials. Typical examples of  
332 this phenomenon are genetic variants associated with small differences in low-density-  
333 lipoprotein cholesterol that are associated with a large reduction in cardiovascular risk (Cohen  
334 et al., 2006; Ference et al., 2015).

335

### 336 **Insights into biased signaling from human variants in GPCRs**

337 With advances in GPCR biology and in our understanding of structure activity relationships  
338 (Whalen et al., 2011), the potential to develop biased agonists that differentially activate  
339 signaling pathways is beginning to be realized. Experiments demonstrating that morphine has  
340 greater analgesic properties and causes less respiratory depression and constipation in  $\beta$ -  
341 arrestin-2 knockout mice have paved the way for trials of a small-molecule mu-opioid  
342 receptor agonist which stimulates nearly undetectable levels of  $\beta$ -arrestin recruitment  
343 compared to morphine (Wadman, 2017). However, cell-type specific effects on the  
344 differential propagation of signaling responses can affect the interpretation of  
345 pharmacological studies resulting in a need to establish which signaling pathway leads to the  
346 desired therapeutic effect *in vivo* (Gundry et al., 2017). By demonstrating that gain-of-  
347 function  $\beta$ -arrestin-biased *MC4R* alleles in the population are associated with up to a 50%  
348 lower risk of obesity and type 2 diabetes, our studies demonstrate that naturally occurring  
349 genetic variants in a GPCR can be used to characterize the physiological consequences of  
350 biased signaling in humans. This approach is likely to have broader relevance. By analyzing  
351 data from over 68,000 individuals, Hauser et al. have recently shown that there is substantial  
352 variation in genes encoding 108 GPCRs that are targeted by known drugs (Hauser et al., 2018).

353 Combining genetic predictions with experiments in cells, they showed that specific variants  
354 in the mu-opioid and Cholecystinin-A receptors could affect therapeutic responses *invitro*  
355 which they hypothesized might predict clinical response *in vivo*. Our data suggests that the  
356 phenotypic consequences of genetic variants that exhibit natural signaling bias for a given  
357 pathway may serve as a “blueprint” for the likely consequences of preferentially modulating  
358 that pathway pharmacologically with a biased agonist. This approach may be generalizable to  
359 other GPCRs and thus to the development of a broad spectrum of drug targets.

360

361 In summary, our work has shown that dissecting the molecular mechanisms underpinning  
362 genetic associations with disease, and with protection from disease, can advance our  
363 understanding of how to most effectively target specific GPCRs for the treatment of common  
364 complex diseases such as obesity and its cardio-metabolic complications.

365

366

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368

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383 the decision to submit the manuscript for publication.

384

### 385 **AUTHOR CONTRIBUTIONS**

386 LL, JM, EMO, CL, NJW and ISF conceived and designed the studies and wrote the paper; LL,  
387 CL, SJS, JL, NB, NK, VK, DH, IDS, EW, FRD, JRBP, CL and NJW designed, performed and  
388 interpreted the genetic association analyses; JM, EMO, BB, VA and ISF designed, performed  
389 and interpreted the molecular studies. All authors contributed to the paper and approved the  
390 final version.

391

### 392 **DECLARATION OF INTERESTS**

393 The authors declare no competing interests.

394

### 395 **MAIN FIGURE TITLES AND LEGENDS**

396 **Figure 1. Gain-of-function *MC4R* variants are associated with protection from obesity and**  
397 **its complications. (A)** Maximal efficacy of NDP- $\alpha$ MSH-induced cAMP production and **(B)**  $\beta$ -  
398 arrestin recruitment for mutant *MC4Rs*. Data represented as mean (95% CI) of 4-12  
399 independent experiments; each mutant expressed as % WT. Variants classified as Gain-of-  
400 Function (GoF) (orange), Loss-of-Function (LoF) (blue) or WT-like (grey) based on statistically  
401 significant differences between WT and mutant (unpaired single-sample *t*-test). **(C)** *MC4R*

402 protein highlighting amino acids affected by variants. \*†Residues affected by >1 variant;  
403 \*R165W (LoF) and R165Q (LoF); †G231S (GoF) and G231V (WT-like). **(D)** Counts for GoF (n=9)  
404 and LoF (n=47) variants included in genetic association analyses. Five variants with opposite  
405 effects in the two assays or WT-like results were excluded. **(E)** Genetic associations of GoF  
406 and LoF *MC4R* variants with BMI, obesity and its complications. OR, odds ratio; CI, confidence  
407 interval; BMI, body mass index, n, number of participants. See also Tables S1-S4.

408

409 **Figure 2.  $\beta$ -arrestin recruitment by different *MC4R* variants explains a significant proportion**  
410 **of the variance in their association with BMI.** Model (top) and results (bottom) from a meta-  
411 regression analysis in which  $\beta$ -arrestin recruitment for each *MC4R* mutant was the predictor  
412 and association estimates for BMI were the outcome. Circles represent each variant, with  
413 circle size proportional to the weight of each variant in the model. The enlarged box shows  
414 the area where variants with the largest weight clustered. CI, confidence interval; BMI, body  
415 mass index. Sensitivity analyses excluding the two variants with the largest weight and leave-  
416 one-out analyses are in Table S5.

417

418 **Figure 3. Gain-of-Function *MC4R* variants that exhibit bias towards  $\beta$ -arrestin-mediated**  
419 **signaling protect against obesity and its complications. (A)** Signaling bias for 11 GoF *MC4R*  
420 mutants calculated as ratio (95% CI) of geometric means for maximal activity of  $\beta$ -arrestin to  
421 cAMP; data from 4-12 experiments. The null hypothesis of no bias (ratio=1) was tested using  
422 unpaired two-sample *t*-test. Variants were classified as biased towards  $\beta$ -arrestin (green),  
423 cAMP (purple) or unbiased (grey). **(B)** Representative Western blots and **(C)** quantification of  
424 ERK1/2 phosphorylation (expressed as % WT) before (-) and after (+) NDP- $\alpha$ MSH stimulation  
425 of GoF *MC4R* mutants; Epidermal Growth Factor [EGF] used as a positive control; vinculin as

426 a loading control. Data represented as mean  $\pm$  SEM from 3-8 independent experiments;  
427 statistical significance of differences between WT and mutant (unpaired single-sample *t*-test).  
428 **(D)** Meta-regression analysis showing that greater bias for  $\beta$ -arrestin recruitment predicts  
429 greater ERK1/2 phosphorylation for GoF variants (depicted as circles with size proportional to  
430 precision in ERK1/2 phosphorylation estimates). **(E)** Associations with BMI (Body Mass Index),  
431 obesity, severe obesity, type 2 diabetes, coronary artery disease, resting heart rate (RHR) in  
432 beats/minute (bpm), systolic and diastolic blood pressure (SBP and DBP) in millimetres of  
433 mercury (mmHg) by carrier status for  $\beta$ -arrestin biased GoF *MC4R* alleles. OR, odds ratio; CI,  
434 confidence interval; IQR, interquartile range, n, number of participants. See also Tables S6  
435 and S7.

436

437 **Figure 4. Effects of V103I MC4R on signaling and receptor internalization.** Dose response  
438 curves for  $\alpha$ MSH,  $\beta$ MSH and NDP- $\alpha$ MSH-induced **(A)** cAMP production and **(B)**  $\beta$ -arrestin  
439 recruitment for V103I compared to WT *MC4R* and mock transfected cells; expressed as % WT.  
440 Data represented as mean  $\pm$  SEM from 4 independent experiments. Representative Western  
441 blots and quantification of ERK1/2 phosphorylation by WT and V103I *MC4R* before (-) and  
442 after (+) stimulation by **(C)**  $\alpha$ MSH and  $\beta$ MSH and **(D)** NDP- $\alpha$ MSH in a time-course experiment;  
443 Epidermal Growth Factor [EGF] used as a positive control; vinculin as a loading control. **(E)**  
444 Confocal microscopy and **(F)** receptor internalization quantified by FACS for cells expressing  
445 WT and V103I *MC4R* before (-) and after (+) stimulation by NDP- $\alpha$ MSH. Scale bars, 50  $\mu$ m; 10  
446  $\mu$ m (inset). Data represented as mean  $\pm$  SEM from 3-8 independent experiments; statistical  
447 significance of differences between WT and mutant analyzed with unpaired single-sample *t*-  
448 test **(A, B, C, D, F)**; \**p* < 0.05; \*\**p* < 0.01. AUC; area under the curve. See also Figures S1 and  
449 2 and Table S8.

450

451 **Figure 5. Mechanisms by which V103I MC4R causes a gain-of-function.** Schematic of  
452 mechanisms by which V103I MC4R (pink dot) results in GoF; G proteins ( $\alpha,\beta,\gamma$ ); MSH,  
453 melanocyte stimulating hormone; AC, adenylate cyclase; CRE, cyclic AMP response element;  
454 TF, transcription factor.

455

456

#### 457 **SUPPLEMENTAL FIGURE TITLES AND LEGENDS**

458

459 **Figure S1. Phenome-wide scan of the associations of V103I MC4R with 353 common**  
460 **diagnoses.** Analyses were performed in European ancestry participants of UK Biobank. Only  
461 diagnosis codes with >500 cases were included. Diagnosis codes were grouped in 19 broad  
462 categories. Each triangle represents the association for a given diagnosis code. Full triangles  
463 that are upward-pointing represent associations in a risk increasing direction, while open  
464 triangles that are downward-pointing represent associations in a protective direction. The  
465 horizontal broken line represents the statistical significance threshold of  $P < 0.00014$ ,  
466 corresponding to a Bonferroni correction for 353 diagnoses. Related to Figure 4.

467

468 **Figure S2. Effects of V103I MC4R on cAMP production,  $\beta$ -arrestin recruitment, MAPK**  
469 **pathway activation and cell surface expression.** Representative real-time measurement of  
470 **(A) cAMP (B)  $\beta$ -arrestin recruitment** upon NDP- $\alpha$ MSH stimulation. **(C)** Time-course  
471 quantification and **(D)** respective area under the curve (AUC) of ERK1/2 phosphorylation  
472 assessed by Homogenous Time-Resolved Resonance Energy Transfer (HTRF)-based sandwich  
473 immunoassay (Cisbio, 64AERPET); data expressed as % WT. WT (open squares), V103I (solid

474 squares) MC4R; mean  $\pm$  standard error; (n=4); statistical significance of differences between  
475 WT and mutant analyzed in an unpaired single-sample t-test; \*\*p < 0.01. **(E)** Confocal  
476 microscopy on COS-7 cells expressing WT and V103I MC4R unstimulated and upon NDP- $\alpha$ MSH  
477 stimulation. MC4R in red (Anti-FLAG (M2) antibody), plasma membrane in green (wheat germ  
478 agglutinin) and nuclei in blue (DAPI). Scale bars, 50  $\mu$ m. **(F)** Effects of V103I MC4R on receptor  
479 internalization quantified by FACS. Representative data on receptor internalization using HeLa  
480 cells transiently transfected with FLAG-MC4R WT or V103I. Mock-transfected cells were used  
481 as controls for non-specific binding of the antibody (FLAG-PE; negative control). Mean  
482 fluorescence intensity (MFI) represents the amount of MC4R present at the cell surface;  
483 receptor internalization was quantified using the mean fluorescence intensity (MFI) values of  
484 FLAG-PE<sup>+</sup> cells from unstimulated (-) and NDP- $\alpha$ MSH stimulated WT and V103I MC4R. Related  
485 to Figure 4.

486

## 487 **STAR METHODS**

### 488 **CONTACT FOR REAGENT AND RESOURCE SHARING**

489 Further information and requests for resources and reagents should be directed to and will  
490 be fulfilled by the Lead Contact, Sadaf Farooqi ([isf20@cam.ac.uk](mailto:isf20@cam.ac.uk)).

491

### 492 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

#### 493 **Studies in humans**

494 UK Biobank is a prospective population-based cohort study of people aged 40-69 years who  
495 were recruited in 2006-2010 from 22 centres located in urban and rural areas across the  
496 United Kingdom (Sudlow et al., 2015). Participants' characteristics are reported in **Table S1**.

497 UK Biobank has received ethical approval from the North West Multicentre Research Ethics  
498 Committee and participants gave written informed consent.

499

500 **Studies in cellular models**

501 HEK293 (XX female) and suspension HeLa (XX female) cells were cultured in high glucose  
502 Dulbecco's modified eagle medium (DMEM, Gibco, 41965) and supplemented with 10% fetal  
503 bovine serum (Gibco, 10270, South America origin), 1% GlutaMAX™ (100X) (Gibco, 35050),  
504 and 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, P0781). Cells were  
505 incubated at 37°C in humidified air containing 5% CO<sub>2</sub> and transfections were performed  
506 using Lipofectamine 2000™ (Gibco, 11668) in serum-free Opti-MEM I medium (Gibco, 31985)  
507 according to the manufacturer's protocols.

508

509

## 510 **METHOD DETAILS**

### 511 **STUDIES IN HUMANS**

#### 512 **Genotype data**

513 We studied 61 nonsynonymous genetic variants in *MC4R* (NCBI Reference NM\_005912) that  
514 were directly genotyped or well-imputed in UK Biobank (**Table S1**). All participants of UK  
515 Biobank with suitable DNA samples underwent genome-wide SNP-array genotyping using the  
516 Affymetrix UK BILEVE and UK Biobank Axiom arrays, with imputation to the Haplotype  
517 Reference Consortium r1.1 panel (McCarthy et al., 2016) supplemented with the 1000  
518 Genomes phase 3 (Altshuler et al., 2015) and UK10K (Walter et al., 2015) panels, as previously  
519 described. (Bycroft et al., 2018). A total of 59 variants were directly genotyped, while 2 genetic  
520 variants were imputed and had an imputation quality score greater than 0.8, indicating high-  
521 quality imputation. The 61 variants included in this study had pairwise  $R^2 < 0.01$ , consistent  
522 with no or negligible linkage disequilibrium.

523

#### 524 **Genotype quality checks**

525 Genotype quality control in UK Biobank followed guidelines that have been published in detail  
526 elsewhere (Bycroft et al., 2018). In brief, DNA samples were assigned to genotype batch using  
527 an automated sample selection algorithm to ensure random assignment relative to baseline  
528 characteristics. Genotyping underwent a number of quality control procedures including (a)  
529 routine quality checks carried out during the process of sample retrieval, DNA extraction, and  
530 genotype calling; (b) checks and filters for genotype batch effects, plate effects, departures  
531 from Hardy-Weinberg equilibrium, sex effects, array effects, and discordance across control  
532 replicates; (c) individual and genetic variant call rate filters. Wright et al. (Wright et al., 2019)

533 have proposed that the expert manual review of genotype cluster plots may help distinguish  
534 lower vs higher quality genotyped variants in UK Biobank, particularly for rare alleles. We  
535 adopted a similar scoring approach. In the aforementioned study, cluster plots for each  
536 genotyping-batch were merged into one single cluster plot. In this study, instead, we  
537 reviewed cluster-plots by genotyping-batch, which reflects the data units parsed by the  
538 genotyping algorithm and is less likely to be influenced by batch effects or variation in  
539 fluorescence signal. Cluster plots were generated using evoker-lite  
540 (<https://github.com/dlrice/evoker-lite>) using the default configuration for UK Biobank data.  
541 This plots the clusters in the groupings and on the axes that are used by the clustering  
542 algorithm. Each variant is plotted for each genotyping batch separately, using x axis (contrast  
543 between signals A and B) =  $\log_2(A/B)$ , and Y axis (signal strength) =  $\log_2(A*B)/2$ . Two  
544 independent expert laboratory team members reviewed the cluster plots of each batch for  
545 each of the rare variants in *MC4R* included in the study. Blind to each other and to the  
546 association results, they scored the cluster-plot quality of each variant as low (score=0, most  
547 cluster plots display low-quality, defined for instance by carriers being called at the edge of a  
548 the cluster of non-carriers without contrast separation or with very low signal strength),  
549 intermediate (score=1, the majority of cluster plots display high quality, defined by separation  
550 of clusters and signal strength of carriers close to average) or high (score=2, all or almost all  
551 cluster plots display high-quality). Individual scoring was highly consistent with ~80% of  
552 variants receiving the same exact score and only 1 variant receiving a high-score by one scorer  
553 and a low-score by the second scorer (resolved with scoring by a third independent scorer).  
554 The results of the individual scoring were summed into an overall cluster-plot quality score  
555 and variants defined as low-quality cluster plot score if the combined score was 0 or 1,  
556 intermediate-quality if the combined score was 2, high-quality if the combined score was 3 or



557 4. One variant had a low-quality cluster-plot score (V166I), while two had intermediate-  
558 quality scores (G55D and F202L) and all other variants had high quality cluster-plot scores.

559

#### 560 **Genetic association analysis**

561 Association of genotypes with outcome phenotypes were estimated using linear or logistic  
562 regression models, as appropriate for outcome type and analytical design. To minimize  
563 genetic confounding, association analyses were restricted to European ancestry individuals,  
564 identified by combining k-means clustering of genetic principal components with self-  
565 reported ancestry. To control for relatedness, analyses were either clustered using family  
566 structure data (third degree relatives) and adjusted for 40 genetic principal components or  
567 performed using linear mixed-effects models adjusting for a genomic kinship matrix. All  
568 analyses were adjusted for age, sex and genotyping array.

569

570 In analyses of the association of GoF or LoF variants, association estimates for each variant of  
571 either functional category were pooled using fixed-effect inverse-variance weighted meta-  
572 analysis (Burgess et al., 2013). In these analyses, GoF variants were variants with significantly  
573 enhanced cAMP production or  $\beta$ -arrestin recruitment compared to wild-type MC4R in  
574 experiments. LoF variants were variants with significantly reduced cAMP production or  $\beta$ -  
575 arrestin recruitment compared to wild-type MC4R or variants resulting in premature receptor  
576 truncation (frameshift or nonsense variants). Variants that were WT-like or had opposite  
577 effects on the two pathways (GoF for cAMP production but LoF for  $\beta$ -arrestin recruitment)  
578 were excluded from these analyses. Genetic association analyses were performed using  
579 STATA v14.2 (StataCorp, College Station, Texas 77845 USA), R v3.2.2 (The R Foundation for  
580 Statistical Computing), BOLT-LMM v2.3.2 (Loh et al., 2018; Loh et al., 2015).

581

## 582 **Phenotype definitions**

583 Primary outcomes of interest were BMI, obesity, type 2 diabetes and coronary artery disease.

584 We also investigated associations with hemodynamic phenotypes known to be affected by

585 MC4R signaling (Greenfield et al., 2009), i.e. resting heart rate, systolic and diastolic blood

586 pressure. BMI was calculated as weight in kilograms divided by height in meters squared.

587 Height was measured using a Seca 240cm tape, while weight was measured using a Tanita

588 BC418MA body composition analyzer. Systolic, diastolic blood pressure and resting heart rate

589 were measured at baseline using an Omron blood pressure monitor and following a

590 standardized procedure (<http://biobank.ctsu.ox.ac.uk/crystal/docs/Bloodpressure.pdf>). Type

591 2 diabetes was defined on the basis of self-reported physician diagnosis at nurse interview or

592 digital questionnaire, age at diagnosis older than 36 years (to exclude likely type 1 diabetes

593 cases), use of oral anti-diabetic medications or electronic records of hospital admissions or

594 death reporting type 2 diabetes as diagnosis or cause of death (International Statistical

595 Classification of Diseases and Related Health Problems Tenth Revision [ICD-10] code E11).

596 Coronary artery disease was defined as either (a) myocardial infarction or coronary disease

597 documented in the participant's medical history at the time of enrolment by a trained nurse

598 or (b) hospitalization or death involving acute myocardial infarction or its complications (i.e.

599 ICD-10 codes I21, I22 or I23). Obesity was defined on the basis of BMI greater than or equal

600 to 30 kg/m<sup>2</sup> and severe obesity as BMI greater than or equal to 40 kg/m<sup>2</sup>. In obesity

601 association analyses, the control group was the group of people with BMI less than 25 kg/m<sup>2</sup>.

602

## 603 **Meta-regression**

604 The potential for *in vitro* measures of  $\beta$ -arrestin recruitment or cAMP production to explain  
605 the variance (i.e. between-genetic-variants variance) in the association of different *MC4R*  
606 genetic variants with BMI was investigated using random-effects meta-regression. In these  
607 models, the predictors were the relative  $E_{\max}$  for  $\beta$ -arrestin recruitment and/or cAMP  
608 signaling of a given *MC4R* variant allele compared to wild-type (on a natural log-scale)  
609 measured *in vitro* as described below. Values of the outcome were the associations of each  
610 genetic variant with BMI (in kg/m<sup>2</sup> per copy of variant allele), estimated in 450,708 European  
611 ancestry participants in UK Biobank using linear mixed models adjusted for age, sex and a  
612 genomic kinship matrix. For significant predictors, the percentage of total variance in the  
613 outcome explained by a given predictor (e.g. *in vitro*  $\beta$ -arrestin recruitment) was calculated.  
614 Similar meta-regression analyses were conducted (1) in the overall set of 49 missense variants  
615 using the functional category of  $\beta$ -arrestin recruitment (ie. LoF, WT-like or GoF) to assess  
616 whether the different functional categories of genetic variants predicted their association  
617 with BMI; (2) in a subset of 20 missense variants that were found to be wild-type-like for cAMP  
618 signaling to assess whether *in vitro*  $\beta$ -arrestin recruitment predicted their association with  
619 BMI; and (3) in a subset of 11 gain-of-function variants to assess whether bias towards  $\beta$ -  
620 arrestin recruitment *in vitro* predicted their level of signaling via the ERK/MAPK pathway.

621

## 622 **Sensitivity and external validation analyses**

623 Associations with BMI of functional variants in *MC4R*: To assess whether genotyping cluster-  
624 plot quality was influencing associations with BMI, we conducted sensitivity analyses after (a)  
625 exclusion of ultra-rare genetic variants (variant allele frequency < 0.001%; ie. variants that  
626 were shown to have generally lower quality cluster-plot scores by Wright et al. (Wright et al.,  
627 2019); (b) exclusion of variants from point (a) plus any variant with low overall cluster-plot

628 quality score; (c) exclusion of variants from points (a-b) plus any variant with intermediate  
629 cluster-plot quality score; (d) exclusion of variants from points (a-c) plus any variant where  
630 the combined cluster-plot quality score was below 4 (ie. the maximum possible score).

631

632 Meta-regression analyses: The main analysis (**Figure 2**) included all 49 missense variants in  
633 *MC4R* found in European ancestry participants of UK Biobank. Over 50 sensitivity analyses  
634 were conducted to assess the robustness of the results of the main analysis (**Table S5**). First,  
635 we conducted 49 leave-one-out analyses where each missense variant was excluded at a  
636 given iteration to assess if a single variant was driving the association observed in the main  
637 analysis. Then, we conducted an analysis of rare variants only (i.e. excluding the low-  
638 frequency V103I and I251L variants, which had the largest weight in the main analysis) to  
639 assess whether V103I and I251L were driving the association. Then, we conducted a  
640 multivariable analysis in which both *in vitro*  $\beta$ -arrestin recruitment and *in vitro* cAMP signaling  
641 were included in the model as possible predictors, to assess whether *in vitro*  $\beta$ -arrestin  
642 recruitment was an independent predictor from cAMP signaling. Then, we conducted an  
643 analysis including all 61 nonsynonymous genetic variants in *MC4R* found in European ancestry  
644 participants of UK Biobank, to assess whether the association was influenced by the focus of  
645 our main analysis on missense variants directly expressed *in vitro*. For this analysis, the level  
646 of  $\beta$ -arrestin recruitment of nonsense/frameshift variants of *MC4R* was assumed to be 1% of  
647 wild-type. Finally, to assess whether genotyping cluster-plot quality was influencing the  
648 association, we conducted analyses after (a) exclusion of ultra-rare genetic variants (variant  
649 allele frequency < 0.001%); (b) exclusion of variants from point (a) plus any variant with low  
650 overall cluster-plot quality score; (c) exclusion of variants from points (a-b) plus any variant  
651 with intermediate cluster-plot quality score; (d) exclusion of variants from points (a-c) plus

652 any variant where the combined cluster-plot quality score was below 4 (ie. the maximum  
653 possible score).

654

655 External validation: We attempted to validate genetic associations using available external  
656 datasets. For 6 overlapping nonsynonymous variants in *MC4R*, we meta-analyses our BMI-  
657 association results with those from up to 550,000 participants in the GIANT consortium (Locke  
658 et al., 2015; Turcot et al., 2018). We also used publically-accessible data from exome  
659 sequencing of 9121 type 2 diabetes cases and 9335 controls from the T2D Knowledge portal  
660 (URL: <http://www.type2diabetesgenetics.org/> accessed 15th February 2019) to test the  
661 association between LoF variants in *MC4R* and type 2 diabetes.

662

#### 663 **Additional genetic association analyses for the V103I *MC4R* variant**

664 The association of the V103I *MC4R* gain-of-function variant with risk of type 2 diabetes and  
665 coronary artery disease was estimated in meta-analyses of large-scale genetic association  
666 studies. The type 2 diabetes association meta-analysis included 68,906 cases and 551,079  
667 controls from the DIAGRAM (Morris et al., 2012), EPIC-InterAct (InterAct et al., 2011) and UK  
668 Biobank (Sudlow et al., 2015) studies. The coronary artery disease association meta-analysis  
669 included 85,697 cases and 550,908 controls from the CARDIoGRAMplusC4D (Nikpay et al.,  
670 2015) and UK Biobank (Sudlow et al., 2015) studies. Association estimates from each study  
671 were combined fixed-effect inverse-variance weighted meta-analysis, as done previously with  
672 similar data sources (Lotta et al., 2016).

673

674 We also conducted a phenome-wide analysis of the association of the V103I *MC4R* variant  
675 with 353 clinical diagnoses using electronic health records from European ancestry

676 participants of UK Biobank. Diagnoses were defined on the basis of the first three digits of  
677 ICD-10 entry codes (for instance, “F20” for a diagnosis of schizophrenia). For a given diagnosis,  
678 we considered individuals as cases if the code corresponding to the diagnosis was entered as  
679 primary or secondary diagnosis in any of their hospital admission records or as a primary or  
680 secondary cause of death in their death certificate. Individuals without the code served as  
681 controls. To minimize the burden of multiple testing and reduce the risk of false positives, we  
682 (a) considered only diagnoses with a total number of cases greater than 500, and (b) used a  
683 Bonferroni corrected  $p < 0.00014$  (corresponding to  $0.05/353$ ) as statistical significance  
684 threshold.

685

## 686 **STUDIES IN CELLULAR MODELS**

### 687 **Functional characterization of human variants**

688

#### 689 **Cloning and site-directed mutagenesis**

690 MC4R cDNA constructs containing an N-terminal FLAG tag in pCDNA3.1(+) vector (Invitrogen)  
691 were used throughout the study. Site-directed mutagenesis was performed using QuikChange  
692 II XL kit (Agilent Technologies, 200516) according to the manufacturer’s protocols. All  
693 constructs were verified with Sanger sequencing. In order to characterize the functional  
694 consequences of MC4R mutants we performed assays in transiently transfected HEK293  
695 (ATCC) and suspension HeLa cells, kindly provided by Dr Kevin Moreau (University of  
696 Cambridge).

697

#### 698 **Time-resolved cAMP measurement assay**

699 Measurement of ligand-induced cAMP generation in HEK293 cells transiently expressing  
700 WT/mutant MC4R was performed using the GloSensor™ cAMP biosensor (Promega)  
701 according to manufacturer's protocols. Briefly, 40,000 cells were seeded in white 96-well  
702 poly-D-lysine-coated plates. After 24 hours, cells were then transfected with both 100 ng/well  
703 of pGloSensor™-20F cAMP plasmid (Promega, E1171) and 30 ng/well of plasmid encoding  
704 either WT/mutant MC4R. The day after transfection, cell media were replaced by 90 µl of  
705 fresh full DMEM with 2% v/v GloSensor™ cAMP Reagent (Promega, E1290) and incubated for  
706 120 min at 37°C. Firefly luciferase activity was measured at 37°C and 5% CO<sub>2</sub> using Spark 10M  
707 microplate reader (Tecan). After initial measurement of the baseline signal for 10 min (1  
708 minute intervals), cells were stimulated with 10 µl of 10x stock solution of the MC4R agonist  
709 NDP-αMSH (final concentration 1 µM) and real-time chemiluminescent signal was quantified  
710 for 45 minutes (30 seconds intervals). In each experiment, a negative control using mock  
711 transfected cells (empty pcDNA3.1(+) plasmid) and a positive control where cells were  
712 stimulated with 10 µM forskolin were assayed. The area under the curve (AUC) for cAMP  
713 production was calculated for each MC4R mutant using the baseline signal and the total peak  
714 of each curve. For data normalization, the AUC from mock transfected cells was set as 0% and  
715 the AUC from WT MC4R was set as 100%. Results represent 4-12 independent experiments.  
716 For V103I MC4R, dose-response curves using NDP-αMSH, αMSH and βMSH were plotted  
717 from total peak area under the curve values calculated for each agonist concentration,  
718 ranging from 10<sup>-11</sup> to 10<sup>-6</sup> M. Then, sigmoidal dose-response curves with variable slope (three-  
719 parameter logistic regression) were plotted. Normalized data were merged and presented as  
720 sum curves. Results are from 4 independent experiments.

721

## 722 **Time-resolved β-arrestin recruitment assay**

723 Coupling between MC4R and  $\beta$ -arrestin 2 was monitored using a NanoBiT™ protein: protein  
724 interaction assay (Promega®, M2014). WT/mutant MC4Rs were cloned into pBiT1.1-C  
725 [TK/LgBiT] vector and  $\beta$ -arrestin 2 into pBiT2.1-N [TK/SmBiT] vector. Assays were performed  
726 in HEK293 cells seeded in poly-D-lysine-coated, white 96-well plates (40,000 cells/well)  
727 transiently transfected with 50 ng/well of each of the two constructs as specified previously.  
728 For the negative control, SmBiT- $\beta$ -arrestin 2 construct was substituted with NanoBiT™  
729 negative control vector (HaloTag-SmBiT). Positive control consisted of SmBiT-PRKACA and  
730 LgBiT-PRKAR2A vectors. Following transfection, cells were maintained overnight in cell  
731 culture medium as specified previously. The next day, 30 minutes prior to the assay, culture  
732 medium was substituted for 100  $\mu$ l/well serum-free Opti-MEM I medium (Gibco, 31985).  
733 Nano-luciferase activity was measured at 37°C and 5% CO<sub>2</sub> using Spark 10M microplate reader  
734 (Tecan). After initial measurement of the background signal, 25  $\mu$ l/well Nano-Glo® Live Cell  
735 Assay System (Promega®, N2013) was added and cells were equilibrated while basal luciferase  
736 activity was measured for 10 minutes (1 minute intervals). Subsequently, cells were  
737 stimulated with 10  $\mu$ l of 13.5x stock solution of the MC4R agonist NDP- $\alpha$ MSH (final  
738 concentration 1  $\mu$ M), and chemiluminescent signal was quantified for 45 minutes (30 seconds  
739 intervals). The area under the curve (AUC) was calculated for each MC4R mutant using the  
740 average value for the negative control as the baseline and the total peak of each curve. For  
741 data normalization, the AUC from the negative control was set as 0% and the AUC from WT  
742 MC4R was set as 100%. Results are from 4-11 independent experiments. For V103I MC4R,  
743 dose-response curves using NDP- $\alpha$ MSH,  $\alpha$ MSH and  $\beta$ MSH were plotted from total peak area  
744 under the curve values calculated for each agonist concentration, ranging from 10<sup>-11</sup> to 10<sup>-6</sup>  
745 M. Then, sigmoidal dose-response curves with variable slope (three-parameter logistic



746 regression) were plotted. Normalized data were merged and presented as sumcurves. Results  
747 are from 3-5 independent experiments.

748

#### 749 **Western-blotting**

750 For these experiments,  $1.5 \times 10^5$  HEK293 cells were seeded in a poly-D-lysine coated 24-well  
751 plate and transfected the next day, when cells had reached 80% confluency, with 250 ng of  
752 MC4R constructs. Cells were serum starved overnight and stimulated for indicated periods of  
753 time with either NDP- $\alpha$ MSH (1  $\mu$ M),  $\alpha$ MSH (1  $\mu$ M) or  $\beta$ MSH (1  $\mu$ M). Additional stimulation  
754 with recombinant human epidermal growth factor (EGF, 10 ng/mL, Invitrogen, PHG0311, 5  
755 minutes) was also performed as a positive control. Then, cells were washed once with PBS  
756 and lysed in radio-immunoprecipitation assay buffer (RIPA) (Sigma, R0278) supplemented  
757 with protease and phosphatase inhibitors (Roche cOmplete™, Mini Protease Inhibitor  
758 Cocktail, 11836153001; Roche PhosSTOP™, PHOSS-RO). After being harvested from the wells  
759 and centrifuged at 14,000 rpm for 15 minutes, the samples were prepared for electrophoresis  
760 (resuspended in 1x Bolt™ LDS sample buffer (Thermo, B0007) and 1x Bolt™ reducing agent  
761 (Thermo, B0009) and heated for 10 minutes at 85°C). Equal amounts of protein were used  
762 and protein electrophoresis was performed using Bolt™ 4-12% Bis-Tris Plus gels (Thermo,  
763 NW04125BOX) and transferred onto nitrocellulose membrane using an iBLOT™ (Thermo,  
764 IB301001). After blocking with 5% bovine serum albumin (BSA) solution in Tris-buffered saline  
765 (TBS) supplemented with 0.1% Tween® 20 (TBS-T) for 1 hour at room temperature,  
766 membranes were probed overnight at 4°C using a Rabbit anti-p44/42 mitogen-activated  
767 protein kinase (MAPK) (extracellular signal-regulated kinases, Erk1/2) (137F5) at 1:1000  
768 dilution (Cell Signaling Technology, 4695), a Rabbit anti-Phospho-p44/42 MAPK (Erk1/2)  
769 (Thr202/Tyr204) at 1:1000 dilution (Cell Signaling Technology, 9101) or a Rabbit-Anti-Vinculin

770 [EPR8185] at 1:5000 dilution (Abcam, ab129002), all prepared in the blocking buffer. Cells  
771 were washed three times with TBS-T for 10 minutes at room temperature with gentle shaking.  
772 They were then incubated with secondary antibody, Goat anti-rabbit IgG-HRP (Dako, P0448)  
773 diluted 1:2500 in 2% BSA in TBS-T for 1 hour at room temperature. Bands were developed  
774 using enhanced chemiluminescence (ECL) substrate (Promega, W1015) and images were  
775 captured with an ImageQuant LAS 4000 (GE Healthcare). The band intensity of Western blots  
776 was quantified using FIJI (Schindelin et al., 2012). For data normalization, unstimulated WT  
777 MC4R readouts were set as baseline (0%), and maximum WT MC4R ERK1/2 phosphorylation  
778 upon agonist stimulation was set as 100%. ERK1/2 phosphorylation data was plotted as  
779 pERK1/2/ tERK1/2. Results are from three to eight independent experiments.

780

#### 781 **ELISA for cell surface expression**

782 Relative cell surface expression of MC4R mutants was assessed in transiently transfected  
783 HEK293 cells. 40,000 cells/well in a clear 96-well plates coated with poly-D-lysine and  
784 transfected the following day (30 ng cDNA/well). 24 hours after transfection, cells were fixed  
785 with 3.7% paraformaldehyde (15 minutes) at room temperature (RT) and washed three times  
786 with phosphate-buffered saline (PBS). Subsequently, non-specific binding sites were blocked  
787 with 3% non-fat dry milk in 50 mM Tris-PBS pH 7.4 (blocking buffer) for 1 hour at RT. Next,  
788 cells were incubated with a mouse monoclonal anti-FLAG (M2) antibody (Sigma-Aldrich,  
789 F1804) (dilution 1:1000 in blocking buffer) overnight at 4°C followed by triple washing with  
790 PBS and incubation with polyclonal goat anti-mouse immunoglobulins conjugated with  
791 horseradish peroxidase (HRP) (Dako, P0447) (1:1250 in 1.5% non-fat dry milk in 50 mM Tris-  
792 PBS pH 7.4) for 2 hours at RT. Finally, plates were washed three times with PBS and the high  
793 performance chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB CORE+, Bio-Rad

794 Laboratories, BUF062) was used to detect HRP activity. The reaction was terminated with 0.2  
795 M H<sub>2</sub>SO<sub>4</sub>. Color reaction product was transferred to another 96-well plate prior to  
796 measurement of absorbance at 450 nm using Infinite M1000 PRO microplate reader (Tecan®).  
797 Six technical replicates were performed for each mutant in a given assay. Data were  
798 normalized to mean absorbance for pcDNA3.1(+) mock-transfected cells. Results are from  
799 four independent experiments. Statistical significance of differences between WT and mutant  
800 were estimated by unpaired *t*-test.

801

### 802 **Confocal Microscopy**

803 150,000 HEK293 cells were seeded onto glass coverslips in 12-well plates and transfected with  
804 250 ng of FLAG-tagged MC4R constructs. 24 h after transfection, cells were serum starved for  
805 2 h and then stimulated for 15 minutes with NDP- $\alpha$ MSH (1  $\mu$ M) at 37°C. Cells were then fixed  
806 with 4% formaldehyde in PBS for 10 minutes at room temperature and washed three times  
807 for 5 minutes with phosphate-buffered saline (PBS). After permeabilization with 0.1% Triton™  
808 X-100 in PBS for 5 minutes, cells were incubated with phycoerythrin (PE)-conjugated anti-  
809 DYKDDDDK tag antibody, clone L5 (Biolegend, 637310) diluted 1:100 in blocking buffer. Slides  
810 were imaged using a Leica SP8 confocal microscope and images processed using FIJI. Results  
811 are from three independent experiments.

812

### 813 **Fluorescence-activated cell sorting (FACS)**

814 80,000 suspension HeLa cells were seeded in 6-well CytoOne® plates (USA Scientific, CC7672-  
815 7506) and transfected with 250 ng of FLAG-tagged MC4R constructs. 24 hours after  
816 transfection, cells were serum starved for 2 hours and then stimulated for 15 minutes with  
817 NDP- $\alpha$ MSH (1  $\mu$ M) at 37°C. Cells were washed once with serum-free medium, high glucose

818 DMEM, no phenol red (DMEM, Gibco, 31053), followed by fixation with 4% formaldehyde in  
819 PBS for 10 minutes at room temperature. After washing three times with PBS, cells were  
820 incubated at 4°C for 30 minutes with PE-conjugated anti-DYKDDDDK tag antibody, clone L5  
821 (Biolegend, 637310) diluted 1:100 in serum-free medium. After an extra wash step, cells were  
822 analyzed on FACS Accuri™ C6 (BD Biosciences). Flow cytometry data analysis and mean  
823 fluorescence intensity (MFI) values were calculated by FlowJo analysis software (Tree Star) on  
824 live-gated cells (minimum of 20,000 cells). Percent internalization was calculated based on  
825 MFI values as follows: % internalization =  $(1 - \text{MFI NDP-}\alpha\text{MSH stimulated} / \text{MFI unstimulated})$   
826 x 100. Results are from four independent experiment.

827

## 828 **QUANTIFICATION AND STATISTICAL ANALYSIS**

829 Results were analyzed using GraphPad Prism 7 (Graph Pad Software), STATA v14.2 (StataCorp,  
830 College Station, Texas 77845 USA), R v3.2.2 (The R Foundation for Statistical Computing). For  
831 cAMP,  $\beta$ -arrestin and ERK1/2 assays, the difference between WT and mutant MC4Rs was  
832 tested using an unpaired single-sample t-test assigning a value of 100% for WT. The signaling  
833 bias for GoF variants of *MC4R* was estimated by calculating the ratio of geometric means for  
834  $E_{\max}$   $\beta$ -arrestin to  $E_{\max}$  cAMP and its 95% confidence interval using unpaired two-sample *t*-  
835 test, with a null hypothesis of no bias (i.e. ratio=1). Studies in cellular models are from at least  
836 3 independent experiments. All p-values reported in this manuscript are from 2-sided  
837 statistical tests. A  $P < 0.05$  was considered statistically significant. In figures, statistical  
838 significance is represented as \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

839

840

841

842 **SUPPLEMENTAL TABLES TITLES AND LEGENDS**

843

844 **Table S1.** Characteristics of European ancestry participants of UK Biobank and of the  
845 nonsynonymous genetic variants in *MC4R* included in this study. Related to Figure 1.

846

847 **Table S2.** Functional characterization of *MC4R* variants identified in UK Biobank. Related to  
848 Figure 1.

849

850 **Table S3.** Association of gain-of-function and loss-of-function variants in *MC4R* with blood  
851 pressure and resting heart rate. Related to Figure 1.

852

853 **Table S4.** Associations with Body Mass Index in sensitivity and validation analyses. Related to  
854 Figure 1.

855

856 **Table S5.** Meta-regression analyses investigating whether *in vitro* cAMP production or  $\beta$ -  
857 arrestin recruitment explain variance in the associations of *MC4R* variants with BMI. Related  
858 to Figure 2.

859

860 **Table S6.** Associations of cAMP-biased gain-of-function variants in *MC4R* with continuous  
861 traits and disease outcomes. Related to Figure 3.

862

863 **Table S7.** Association of the gain-of-function variant V103I *MC4R* with continuous traits and  
864 disease outcomes. Related to Figure 4.

865

866 **Table S8.** Functional characterization of V103I MC4R. Related to Figure 4.

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