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

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SUMMARY

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

INTRODUCTION

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

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

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

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

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

We performed genetic association studies in approximately 0.5 million people from UK Biobank focusing on 61 nonsynonymous variants identified in *MC4R*. Twelve of the 61 were nonsense/frameshift variants; the remainder (n=49) were missense variants whose functional properties were characterized in cells quantifying canonical $G\alpha_s$-mediated cAMP production and the recruitment of β-arrestin to MC4R. In meta-regression analyses using the functional consequence of *MC4R* variants as the predictor, we found that 88% of the variance in the association of different *MC4R* variants with BMI was explained by their effect on β-arrestin recruitment. A subset of individuals (6%, n=28,161) were carriers for gain-of-function alleles that exhibited signaling bias, preferentially increasing β-arrestin recruitment rather than cAMP production. These individuals had significantly lower BMI (P=2x10^{-42}) and up to 50% lower risk of obesity, type 2 diabetes and coronary artery disease. Cumulatively, the characterization of BMI-lowering variants in *MC4R* demonstrates the pivotal role of β-arrestin-mediated MC4R signaling in human energy homeostasis. These findings have relevance for the development of β-arrestin-biased MC4R agonists for weight loss and for the treatment of obesity-associated metabolic disease.

RESULTS

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

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

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

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

We next performed genetic association studies with a series of primary phenotypes recorded in UK Biobank: BMI and obesity, hemodynamic phenotypes known to be affected by MC4R signaling (resting heart rate, systolic and diastolic blood pressure) and risk of type 2 diabetes and coronary artery disease. We found that LoF variants in *MC4R* were associated with higher BMI, and higher odds of obesity, severe obesity, type 2 diabetes and coronary artery disease (**Figure 1E**). These results align with reports of LoF *MC4R* variants identified in cohorts of obese and severely obese individuals (Farooqi et al., 2003; Hinney et al., 2006; Stutzmann et al., 2008; Turcot et al., 2018). In contrast, we found that gain-of-function *MC4R* variants were strongly associated with lower BMI (P=2x10^{-47}) and lower odds of obesity (P=3x10^{-38}), severe obesity (P=1x10^{-09}), type 2 diabetes (P=4x10^{-06}) and coronary artery disease (P=0.02) (**Figure 1E**). GoF, but not LoF variants, were associated with lower diastolic blood pressure and lower resting heart rate (**Table S3**).

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

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

β-arrestin-mediated MC4R signaling plays a pivotal role in human weight regulation

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

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186 **Gain-of-function *MC4R* variants that preferentially signal through β-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 β-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 β-arrestin
194 recruitment and two (L304F, Y332C) showed no evidence of biased signaling (**Figure 3A**). GoF
195 MC4R mutants that led to increased β-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 β-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^2 lower than non-carriers
203 (P=2x10^{-42}; **Figure 3E**), while carriers of two alleles had a BMI that was 0.88 kg/m^2 lower
204 (P=7x10^{-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 β-arrestin biased GoF alleles had an approximately 50% lower risk
206 of obesity (OR, 0.51; P=8x10^{-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

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

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

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

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

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

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

DISCUSSION

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

The discovery of protective human genetic variants

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

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

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

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

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

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

Insights into biased signaling from human variants in GPCRs

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

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

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

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AUTHOR CONTRIBUTIONS

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

DECLARATION OF INTERESTS

The authors declare no competing interests.

MAIN FIGURE TITLES AND LEGENDS

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

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

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

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

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

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

Figure 5. Mechanisms by which V103I MC4R causes a gain-of-function. Schematic of mechanisms by which V103I MC4R (pink dot) results in GoF; G proteins (α,β,γ); MSH, melanocyte stimulating hormone; AC, adenylate cyclase; CRE, cyclic AMP response element; TF, transcription factor.

SUPPLEMENTAL FIGURE TITLES AND LEGENDS

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

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

474 squares) MC4R; mean ± 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-αMSH
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 µm. **(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$^+$ cells from unstimulated (-) and NDP-αMSH 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).
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**.

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

Studies in cellular models

HEK293 (XX female) and suspension HeLa (XX female) cells were cultured in high glucose Dulbecco's modified eagle medium (DMEM, Gibco, 41965) and supplemented with 10% fetal bovine serum (Gibco, 10270, South America origin), 1% GlutaMAX™ (100X) (Gibco, 35050), and 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, P0781). Cells were incubated at 37°C in humidified air containing 5% CO_2 and transfections were performed using Lipofectamine 2000™ (Gibco, 11668) in serum-free Opti-MEM I medium (Gibco, 31985) according to the manufacturer's protocols.

METHOD DETAILS

STUDIES IN HUMANS

Genotype data

We studied 61 nonsynonymous genetic variants in *MC4R* (NCBI Reference NM_005912) that were directly genotyped or well-imputed in UK Biobank (**Table S1**). All participants of UK Biobank with suitable DNA samples underwent genome-wide SNP-array genotyping using the Affymetrix UK BILEVE and UK Biobank Axiom arrays, with imputation to the Haplotype Reference Consortium r1.1 panel (McCarthy et al., 2016) supplemented with the 1000 Genomes phase 3 (Altshuler et al., 2015) and UK10K (Walter et al., 2015) panels, as previously described. (Bycroft et al., 2018). A total of 59 variants were directly genotyped, while 2 genetic variants were imputed and had an imputation quality score greater than 0.8, indicating high-quality imputation. The 61 variants included in this study had pairwise R^2<0.01, consistent with no or negligible linkage disequilibrium.

Genotype quality checks

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

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

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

Genetic association analysis

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

In analyses of the association of GoF or LoF variants, association estimates for each variant of either functional category were pooled using fixed-effect inverse-variance weighted meta-analysis (Burgess et al., 2013). In these analyses, GoF variants were variants with significantly enhanced cAMP production or β-arrestin recruitment compared to wild-type MC4R in experiments. LoF variants were variants with significantly reduced cAMP production or β-arrestin recruitment compared to wild-type MC4R or variants resulting in premature receptor truncation (frameshift or nonsense variants). Variants that were WT-like or had opposite effects on the two pathways (GoF for cAMP production but LoF for β-arrestin recruitment) were excluded from these analyses. Genetic association analyses were performed using STATA v14.2 (StataCorp, College Station, Texas 77845 USA), R v3.2.2 (The R Foundation for 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^2 and severe obesity as BMI greater than or equal to 40 kg/m^2. In obesity
601 association analyses, the control group was the group of people with BMI less than 25 kg/m^2.

602

603 **Meta-regression**

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

Sensitivity and external validation analyses

Associations with BMI of functional variants in *MC4R*: To assess whether genotyping cluster-plot quality was influencing associations with BMI, we conducted sensitivity analyses after (a) exclusion of ultra-rare genetic variants (variant allele frequency < 0.001%; ie. variants that were shown to have generally lower quality cluster-plot scores by Wright et al. (Wright et al., 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* β-arrestin recruitment and *in vitro* cAMP signaling
641 were included in the model as possible predictors, to assess whether *in vitro* β-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 β-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

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

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

Additional genetic association analyses for the V103I *MC4R* variant

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

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

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

STUDIES IN CELLULAR MODELS

Functional characterization of human variants

Cloning and site-directed mutagenesis

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

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_2 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^{-11} to 10^{-6} 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**

Coupling between MC4R and β-arrestin 2 was monitored using a NanoBiT™ protein: protein interaction assay (Promega®, M2014). WT/mutant MC4Rs were cloned into pBiT1.1-C [TK/LgBiT] vector and β-arrestin 2 into pBiT2.1-N [TK/SmBiT] vector. Assays were performed in HEK293 cells seeded in poly-D-lysine-coated, white 96-well plates (40,000 cells/well) transiently transfected with 50 ng/well of each of the two constructs as specified previously. For the negative control, SmBiT-β-arrestin 2 construct was substituted with NanoBiT™ negative control vector (HaloTag-SmBiT). Positive control consisted of SmBiT-PRKACA and LgBiT-PRKAR2A vectors. Following transfection, cells were maintained overnight in cell culture medium as specified previously. The next day, 30 minutes prior to the assay, culture medium was substituted for 100 µl/well serum-free Opti-MEM I medium (Gibco, 31985). Nano-luciferase activity was measured at 37°C and 5% CO_2 using Spark 10M microplate reader (Tecan). After initial measurement of the background signal, 25 µl/well Nano-Glo® Live Cell Assay System (Promega®, N2013) was added and cells were equilibrated while basal luciferase activity was measured for 10 minutes (1 minute intervals). Subsequently, cells were stimulated with 10 µl of 13.5x stock solution of the MC4R agonist NDP-αMSH (final concentration 1 µM), and chemiluminescent signal was quantified for 45 minutes (30 seconds intervals). The area under the curve (AUC) was calculated for each MC4R mutant using the average value for the negative control as the baseline and the total peak of each curve. For data normalization, the AUC from the negative control was set as 0% and the AUC from WT MC4R was set as 100%. Results are from 4-11 independent experiments. For V103I MC4R, dose-response curves using NDP-αMSH, αMSH and βMSH were plotted from total peak area under the curve values calculated for each agonist concentration, ranging from 10^{-11} to 10^{-6} M. Then, sigmoidal dose-response curves with variable slope (three-parameter logistic

regression) were plotted. Normalized data were merged and presented as sum curves. Results are from 3-5 independent experiments.

Western-blotting

For these experiments, 1.5 x10^5 HEK293 cells were seeded in a poly-D-lysine coated 24-well plate and transfected the next day, when cells had reached 80% confluency, with 250 ng of MC4R constructs. Cells were serum starved overnight and stimulated for indicated periods of time with either NDP-αMSH (1 μM), αMSH (1 μM) or βMSH (1 μM). Additional stimulation with recombinant human epidermal growth factor (EGF, 10 ng/mL, Invitrogen, PHG0311, 5 minutes) was also performed as a positive control. Then, cells were washed once with PBS and lysed in radio-immunoprecipitation assay buffer (RIPA) (Sigma, R0278) supplemented with protease and phosphatase inhibitors (Roche cOmplete™, Mini Protease Inhibitor Cocktail, 11836153001; Roche PhosSTOP™, PHOSS-RO). After being harvested from the wells and centrifuged at 14,000 rpm for 15 minutes, the samples were prepared for electrophoresis (resuspended in 1x Bolt™ LDS sample buffer (Thermo, B0007) and 1x Bolt™ reducing agent (Thermo, B0009) and heated for 10 minutes at 85°C). Equal amounts of protein were used and protein electrophoresis was performed using Bolt™ 4-12% Bis-Tris Plus gels (Thermo, NW04125BOX) and transfered onto nitrocellulose membrane using an iBLOT™ (Thermo, IB301001). After blocking with 5% bovine serum albumin (BSA) solution in Tris-buffered saline (TBS) supplemented with 0.1% Tween® 20 (TBS-T) for 1 hour at room temperature, membranes were probed overnight at 4°C using a Rabbit anti-p44/42 mitogen-activated protein kinase (MAPK) (extracellular signal–regulated kinases, Erk1/2) (137F5) at 1:1000 dilution (Cell Signaling Technology, 4695), a Rabbit anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) at 1:1000 dilution (Cell Signaling Technology, 9101) or a Rabbit-Anti-Vinculin

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

ELISA for cell surface expression

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

Laboratories, BUF062) was used to detect HRP activity. The reaction was terminated with 0.2 M H_2SO_4. Color reaction product was transferred to another 96-well plate prior to measurement of absorbance at 450 nm using Infinite M1000 PRO microplate reader (Tecan®). Six technical replicates were performed for each mutant in a given assay. Data were normalized to mean absorbance for pcDNA3.1(+) mock-transfected cells. Results are from four independent experiments. Statistical significance of differences between WT and mutant were estimated by unpaired *t*-test.

Confocal Microscopy

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

Fluorescence-activated cell sorting (FACS)

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

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

QUANTIFICATION AND STATISTICAL ANALYSIS

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

SUPPLEMENTAL TABLES TITLES AND LEGENDS

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

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

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

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

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

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

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

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

867 **REFERENCES**

868 Bray, G.A., Fruhbeck, G., Ryan, D.H., and Wilding, J.P. (2016). Management of obesity. Lancet
869 *387*, 1947-1956.

870 Breit, A., Buch, T.R., Boekhoff, I., Solinski, H.J., Damm, E., and Gudermann, T. (2011).
871 Alternative G protein coupling and biased agonism: new insights into melanocortin-4 receptor
872 signalling. Molecular and cellular endocrinology *331*, 232-240.

873 Bycroft, C., Freeman, C., Petkova, D., Band, G., Elliott, L.T., Sharp, K., Motyer, A., Vukcevic, D.,
874 Delaneau, O., O'Connell, J.*, et al.* (2018). The UK Biobank resource with deep phenotyping and
875 genomic data. Nature *562*, 203-209.

876 Chen, K.Y., Muniyappa, R., Abel, B.S., Mullins, K.P., Staker, P., Brychta, R.J., Zhao, X., Ring, M.,
877 Psota, T.L., Cone, R.D.*, et al.* (2015). RM-493, a melanocortin-4 receptor (MC4R) agonist,
878 increases resting energy expenditure in obese individuals. The Journal of clinical
879 endocrinology and metabolism *100*, 1639-1645.

880 Clarke, R., Peden, J.F., Hopewell, J.C., Kyriakou, T., Goel, A., Heath, S.C., Parish, S., Barlera, S.,
881 Franzosi, M.G., Rust, S.*, et al.* (2009). Genetic Variants Associated with Lp(a) Lipoprotein Level
882 and Coronary Disease. New Engl J Med *361*, 2518-2528.

883 Clement, K., Biebermann, H., Farooqi, I.S., Van der Ploeg, L., Wolters, B., Poitou, C., Puder, L.,
884 Fiedorek, F., Gottesdiener, K., Kleinau, G.*, et al.* (2018). MC4R agonism promotes durable
885 weight loss in patients with leptin receptor deficiency. Nature medicine.

886 Cohen, J.C., Boerwinkle, E., Mosley, T.H., and Hobbs, H.H. (2006). Sequence variations in
887 PCSK9, low LDL, and protection against coronary heart disease. New Engl J Med *354*, 1264-
888 1272.

889 Collet, T.H., Dubern, B., Mokrosinski, J., Connors, H., Keogh, J.M., Mendes de Oliveira, E.,
890 Henning, E., Poitou-Bernert, C., Oppert, J.M., Tounian, P.*, et al.* (2017). Evaluation of a

melanocortin-4 receptor (MC4R) agonist (Setmelanotide) in MC4R deficiency. Molecular metabolism *6*, 1321-1329.

Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdan, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low, M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature *411*, 480-484.

Crosby, J., Peloso, G.M., Auer, P.L., Crosslin, D.R., Stitziel, N.O., Lange, L.A., Lu, Y.C., Tang, Z.Z., Zhang, H., Hindy, G*., et al.* (2014). Loss-of-Function Mutations in APOC3, Triglycerides, and Coronary Disease. New Engl J Med *371*, 22-31.

da Silva, A.A., do Carmo, J.M., Kanyicska, B., Dubinion, J., Brandon, E., and Hall, J.E. (2008). Endogenous melanocortin system activity contributes to the elevated arterial pressure in spontaneously hypertensive rats. Hypertension *51*, 884-890.

Dewey, F.E., Gusarova, V., Dunbar, R.L., O'Dushlaine, C., Schurmann, C., Gottesman, O., McCarthy, S., Van Hout, C.V., Bruse, S., Dansky, H.M*., et al.* (2017). Genetic and Pharmacologic Inactivation of ANGPTL3 and Cardiovascular Disease. New Engl J Med *377*, 211-221.

Emerging Risk Factors, C., Wormser, D., Kaptoge, S., Di Angelantonio, E., Wood, A.M., Pennells, L., Thompson, A., Sarwar, N., Kizer, J.R., Lawlor, D.A*., et al.* (2011). Separate and combined associations of body-mass index and abdominal adiposity with cardiovascular disease: collaborative analysis of 58 prospective studies. Lancet *377*, 1085-1095

Fan, T., Dinulescu, D.M., Butler, A.A., Zhou, J., Marks, D.L., and Cone, R.D. (2000). The central melanocortin system can directly regulate serum insulin levels. Endocrinology *141*, 3072-3079.

Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J., and Cone, R.D. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature *385*, 165-168.

914 Farooqi, I.S., Yeo, G.S., Keogh, J.M., Aminian, S., Jebb, S.A., Butler, G., Cheetham, T., and
915 O'Rahilly, S. (2000). Dominant and recessive inheritance of morbid obesity associated with
916 melanocortin 4 receptor deficiency. J Clin Invest *106*, 271-279.
917 Farooqi, I.S., Keogh, J.M., Yeo, G.S., Lank, E.J., Cheetham, T., and O'Rahilly, S. (2003). Clinical
918 spectrum of obesity and mutations in the melanocortin 4 receptor gene. The New England
919 Journal of Medicine *348*, 1085-1095.
920 Ference, B.A., Majeed, F., Penumetcha, R., Flack, J.M., and Brook, R.D. (2015). Effect of
921 naturally random allocation to lower low-density lipoprotein cholesterol on the risk of
922 coronary heart disease mediated by polymorphisms in NPC1L1, HMGCR, or both: a 2 x 2
923 factorial Mendelian randomization study. Journal of the American College of Cardiology *65*,
924 1552-1561.
925 Flannick, J., Thorleifsson, G., Beer, N.L., Jacobs, S.B., Grarup, N., Burtt, N.P., Mahajan, A.,
926 Fuchsberger, C., Atzmon, G., Benediktsson, R.*, et al.* (2014). Loss-of-function mutations in
927 SLC30A8 protect against type 2 diabetes. Nature genetics *46*, 357-363.
928 Friend, S.H., and Schadt, E.E. (2014). Translational genomics. Clues from the resilient. Science
929 *344*, 970-972.
930 Geller, F., Reichwald, K., Dempfle, A., Illig, T., Vollmert, C., Herpertz, S., Siffert, W., Platzer, M.,
931 Hess, C., Gudermann, T.*, et al.* (2004). Melanocortin-4 receptor gene variant I103 is negatively
932 associated with obesity. American Journal of Human Genetics *74*, 572-581.
933 Ghamari-Langroudi, M., Digby, G.J., Sebag, J.A., Millhauser, G.L., Palomino, R., Matthews, R.,
934 Gillyard, T., Panaro, B.L., Tough, I.R., Cox, H.M.*, et al.* (2015). G-protein-independent coupling
935 of MC4R to Kir7.1 in hypothalamic neurons. Nature *520*, 94-98.
936 Goate, A. (2006). Segregation of a missense mutation in the amyloid beta-protein precursor
937 gene with familial Alzheimer's disease. Journal of Alzheimer's disease : JAD *9*, 341-347.

938 Govindaraju, D., Atzmon, G., and Barzilai, N. (2015). Genetics, lifestyle and longevity: Lessons
939 from centenarians. Applied & translational genomics *4*, 23-32.
940 Greenfield, J.R., Miller, J.W., Keogh, J.M., Henning, E., Satterwhite, J.H., Cameron, G.S., Astruc,
941 B., Mayer, J.P., Brage, S., See, T.C.*, et al.* (2009). Modulation of Blood Pressure by Central
942 Melanocortinergic Pathways. New Engl J Med *360*, 44-52.
943 Gu, W., Tu, Z., Kleyn, P.W., Kissebah, A., Duprat, L., Lee, J., Chin, W., Maruti, S., Deng, N.,
944 Fisher, S.L.*, et al.* (1999). Identification and functional analysis of novel human melanocortin-
945 4 receptor variants. Diabetes *48*, 635-639.
946 Gundry, J., Glenn, R., Alagesan, P., and Rajagopal, S. (2017). A Practical Guide to Approaching
947 Biased Agonism at G Protein Coupled Receptors. Frontiers in neuroscience *11*, 17.
948 Harper, A.R., Nayee, S., and Topol, E.J. (2015). Protective alleles and modifier variants in
949 human health and disease. Nature reviews Genetics *16*, 689-701.
950 Hauser, A.S., Attwood, M.M., Rask-Andersen, M., Schioth, H.B., and Gloriam, D.E. (2017).
951 Trends in GPCR drug discovery: new agents, targets and indications. Nature reviews Drug
952 discovery *16*, 829-842.
953 Hauser, A.S., Chavali, S., Masuho, I., Jahn, L.J., Martemyanov, K.A., Gloriam, D.E., and Babu,
954 M.M. (2018). Pharmacogenomics of GPCR Drug Targets. Cell *172*, 41-54 e19.
955 Heid, I.M., Vollmert, C., Hinney, A., Doring, A., Geller, F., Lowel, H., Wichmann, H.E., Illig, T.,
956 Hebebrand, J., and Kronenberg, F. (2005). Association of the 103I MC4R allele with decreased
957 body mass in 7937 participants of two population based surveys. Journal of medical genetics
958 *42*, e21.
959 Heymsfield, S.B., and Wadden, T.A. (2017). Mechanisms, Pathophysiology, and Management
960 of Obesity. The New England journal of medicine *376*, 254-266.

961 Hinney, A., Bettecken, T., Tarnow, P., Brumm, H., Reichwald, K., Lichtner, P., Scherag, A.,
962 Nguyen, T.T., Schlumberger, P., Rief, W.*, et al.* (2006). Prevalence, spectrum, and functional
963 characterization of melanocortin-4 receptor gene mutations in a representative population-
964 based sample and obese adults from Germany. The Journal of clinical endocrinology and
965 metabolism *91*, 1761-1769.
966 Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu,
967 W., Kesterson, R.A., Boston, B.A., Cone, R.D.*, et al.* (1997). Targeted disruption of the
968 melanocortin-4 receptor results in obesity in mice. Cell *88*, 131-141.
969 Jorgensen, A.B., Frikke-Schmidt, R., Nordestgaard, B.G., and Tybjaerg-Hansen, A. (2014). Loss-
970 of-Function Mutations in APOC3 and Risk of Ischemic Vascular Disease. New Engl J Med *371*,
971 32-41.
972 Kievit, P., Halem, H., Marks, D.L., Dong, J.Z., Glavas, M.M., Sinnayah, P., Pranger, L., Cowley,
973 M.A., Grove, K.L., and Culler, M.D. (2013). Chronic Treatment With a Melanocortin-4 Receptor
974 Agonist Causes Weight Loss, Reduces Insulin Resistance, and Improves Cardiovascular
975 Function in Diet-Induced Obese Rhesus Macaques. Diabetes *62*, 490-497.
976 Kishi, T., Aschkenasi, C.J., Lee, C.E., Mountjoy, K.G., Saper, C.B., and Elmquist, J.K. (2003).
977 Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. The
978 Journal of comparative neurology *457*, 213-235.
979 Kuhnen, P., Clement, K., Wiegand, S., Blankenstein, O., Gottesdiener, K., Martini, L.L., Mai, K.,
980 Blume-Peytavi, U., Gruters, A., and Krude, H. (2016). Proopiomelanocortin Deficiency Treated
981 with a Melanocortin-4 Receptor Agonist. New Engl J Med *375*, 240-246.
982 Li, Y.Q., Shrestha, Y., Pandey, M., Chen, M., Kablan, A., Gavrilova, O., Offermanns, S., and
983 Weinstein, L.S. (2016). G(q/11)alpha and G(s)alpha mediate distinct physiological responses
984 to central melanocortins. J Clin Invest *126*, 40-49.

985 Liu, R., Paxton, W.A., Choe, S., Ceradini, D., Martin, S.R., Horuk, R., MacDonald, M.E.,
986 Stuhlmann, H., Koup, R.A., and Landau, N.R. (1996). Homozygous defect in HIV-1 coreceptor
987 accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell *86*, 367-
988 377.
989 Locke, A.E., Kahali, B., Berndt, S.I., Justice, A.E., Pers, T.H., Day, F.R., Powell, C., Vedantam, S.,
990 Buchkovich, M.L., Yang, J.*, et al.* (2015). Genetic studies of body mass index yield new insights
991 for obesity biology. Nature *518*, 197-206.
992 Loh, P.R., Tucker, G., Bulik-Sullivan, B.K., Vilhjalmsson, B.J., Finucane, H.K., Salem, R.M.,
993 Chasman, D.I., Ridker, P.M., Neale, B.M., Berger, B.*, et al.* (2015). Efficient Bayesian mixed-
994 model analysis increases association power in large cohorts. Nature genetics *47*, 284-290.
995 Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B., and Cone, R.D. (1994). Localization of
996 the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the
997 brain. Mol Endocrinol *8*, 1298-1308.
998 Musunuru, K., Pirruccello, J.P., Do, R., Peloso, G.M., Guiducci, C., Sougnez, C., Garimella, K.V.,
999 Fisher, S., Abreu, J., Barry, A.J.*, et al.* (2010). Exome Sequencing, ANGPTL3 Mutations, and
1000 Familial Combined Hypolipidemia. New Engl J Med *363*, 2220-2227.
1001 Myocardial Infarction Genetics Consortium, I., Stitziel, N.O., Won, H.H., Morrison, A.C.,
1002 Peloso, G.M., Do, R., Lange, L.A., Fontanillas, P., Gupta, N., Duga, S.*, et al.* (2014). Inactivating
1003 mutations in NPC1L1 and protection from coronary heart disease. The New England journal
1004 of medicine *371*, 2072-2082.
1005 Nelson, M.R., Tipney, H., Painter, J.L., Shen, J., Nicoletti, P., Shen, Y., Floratos, A., Sham, P.C.,
1006 Li, M.J., Wang, J.*, et al.* (2015). The support of human genetic evidence for approved drug
1007 indications. Nature genetics *47*, 856-860.

1008 Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I., and Barsh, G.S. (1997).
1009 Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein.
1010 Science *278*, 135-138.
1011 Plenge, R.M., Scolnick, E.M., and Altshuler, D. (2013). Validating therapeutic targets through
1012 human genetics. Nature reviews Drug discovery *12*, 581-594.
1013 Povsic, T.J., Scott, R., Mahaffey, K.W., Blaustein, R., Edelberg, J.M., Lefkowitz, M.P., Solomon,
1014 S.D., Fox, J.C., Healy, K.E., Khakoo, A.Y.*, et al.* (2017). Navigating the Future of Cardiovascular
1015 Drug Development-Leveraging Novel Approaches to Drive Innovation and Drug Discovery:
1016 Summary of Findings from the Novel Cardiovascular Therapeutics Conference. Cardiovascular
1017 drugs and therapy / sponsored by the International Society of Cardiovascular
1018 Pharmacotherapy *31*, 445-458.
1019 Rajagopal, S., Ahn, S., Rominger, D.H., Gowen-MacDonald, W., Lam, C.M., Dewire, S.M.,
1020 Violin, J.D., and Lefkowitz, R.J. (2011). Quantifying ligand bias at seven-transmembrane
1021 receptors. Molecular pharmacology *80*, 367-377.
1022 Rajagopal, S., and Shenoy, S.K. (2018). GPCR desensitization: Acute and prolonged phases.
1023 Cellular signalling *41*, 9-16.
1024 Santos, R., Ursu, O., Gaulton, A., Bento, A.P., Donadi, R.S., Bologa, C.G., Karlsson, A., Al-
1025 Lazikani, B., Hersey, A., Oprea, T.I.*, et al.* (2017). A comprehensive map of molecular drug
1026 targets. Nature reviews Drug discovery *16*, 19-34.
1027 Sayk, F., Heutling, D., Dodt, C., Iwen, K.A., Wellhoner, J.P., Scherag, S., Hinney, A., Hebebrand,
1028 J., and Lehnert, H. (2010). Sympathetic function in human carriers of melanocortin-4 receptor
1029 gene mutations. The Journal of clinical endocrinology and metabolism *95*, 1998-2002.

1030 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S.,
1031 Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source platform for biological-
1032 image analysis. Nature methods *9*, 676-682.
1033 Shenoy, S.K., and Lefkowitz, R.J. (2011). beta-Arrestin-mediated receptor trafficking and
1034 signal transduction. Trends in pharmacological sciences *32*, 521-533.
1035 Shinyama, H., Masuzaki, H., Fang, H., and Flier, J.S. (2003). Regulation of melanocortin-4
1036 receptor signaling: agonist-mediated desensitization and internalization. Endocrinology *144*,
1037 1301-1314.
1038 Simonds, S.E., Pryor, J.T., Ravussin, E., Greenway, F.L., Dileone, R., Allen, A.M., Bassi, J.,
1039 Elmquist, J.K., Keogh, J.M., Henning, E., *et al.* (2014). Leptin mediates the increase in blood
1040 pressure associated with obesity. Cell *159*, 1404-1416.
1041 Smith, J.S., Lefkowitz, R.J., and Rajagopal, S. (2018). Biased signalling: from simple switches to
1042 allosteric microprocessors. Nature reviews Drug discovery *17*, 243-260.
1043 Stutzmann, F., Tan, K., Vatin, V., Dina, C., Jouret, B., Tichet, J., Balkau, B., Potoczna, N., Horber,
1044 F., O'Rahilly, S., *et al.* (2008). Prevalence of melanocortin-4 receptor deficiency in Europeans
1045 and their age-dependent penetrance in multigenerational pedigrees. Diabetes *57*, 2511-2518.
1046 Stutzmann, F., Vatin, V., Cauchi, S., Morandi, A., Jouret, B., Landt, O., Tounian, P., Levy-
1047 Marchal, C., Buzzetti, R., Pinelli, L., *et al.* (2007). Non-synonymous polymorphisms in
1048 melanocortin-4 receptor protect against obesity: the two facets of a Janus obesity gene.
1049 Human molecular genetics *16*, 1837-1844.
1050 Sudlow, C., Gallacher, J., Allen, N., Beral, V., Burton, P., Danesh, J., Downey, P., Elliott, P.,
1051 Green, J., Landray, M., *et al.* (2015). UK biobank: an open access resource for identifying the
1052 causes of a wide range of complex diseases of middle and old age. PLoS medicine *12*,
1053 e1001779.

1054 Tallam, L.S., Stec, D.E., Willis, M.A., da Silva, A.A., and Hall, J.E. (2005). Melanocortin-4
1055 receptor-deficient mice are not hypertensive or salt-sensitive despite obesity,
1056 hyperinsulinemia, and hyperleptinemia. Hypertension *46*, 326-332.
1057 Turcot, V., Lu, Y., Highland, H.M., Schurmann, C., Justice, A.E., Fine, R.S., Bradfield, J.P., Esko,
1058 T., Giri, A., Graff, M.*, et al.* (2018). Protein-altering variants associated with body mass index
1059 implicate pathways that control energy intake and expenditure in obesity. Nature genetics
1060 *50*, 26-41.
1061 Vaisse, C., Clement, K., Guy-Grand, B., and Froguel, P. (1998). A frameshift mutation in human
1062 MC4R is associated with a dominant form of obesity [letter]. Nature genetics *20*, 113-114.
1063 Wadman, M. (2017). 'Biased' opioids could yield safer pain relief. Science *358*, 847-848.
1064 Whalen, E.J., Rajagopal, S., and Lefkowitz, R.J. (2011). Therapeutic potential of beta-arrestin-
1065 and G protein-biased agonists. Trends in molecular medicine *17*, 126-139.
1066 Wright, C.F., West, B., Tuke, M., Jones, S.E., Patel, K., Laver, T.W., Beaumont, R.N., Tyrrell, J.,
1067 Wood, A.R., Frayling, T.M.*, et al.* (2019). Assessing the Pathogenicity, Penetrance, and
1068 Expressivity of Putative Disease-Causing Variants in a Population Setting. American journal of
1069 human genetics *104*, 275-286.
1070 Yeo, G.S., Farooqi, I.S., Aminian, S., Halsall, D.J., Stanhope, R.G., and O'Rahilly, S. (1998). A
1071 frameshift mutation in MC4R associated with dominantly inherited human obesity. Nature
1072 genetics *20*, 111-112.
1073 Young, E.H., Wareham, N.J., Farooqi, S., Hinney, A., Hebebrand, J., Scherag, A., O'Rahilly, S.,
1074 Barroso, I., and Sandhu, M.S. (2007). The V1031 polymorphism of the MC4R gene and obesity:
1075 population based studies and meta-analysis of 29 563 individuals. International journal of
1076 obesity *31*, 1437-1441.
1077

1078

1079