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Human Semaphorin 3 variants link melanocortin circuit development and energy balance

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29 **SUMMARY**

30 Hypothalamic melanocortin neurons play a pivotal role in weight regulation. Here we
31 examined the contribution of Semaphorin 3 (SEMA3) signaling to the development of these
32 circuits. In genetic studies, we found 40 rare variants in *SEMA3A-G* and their receptors
33 (*PLXNA1-4; NRP1/2*) in 573 severely obese individuals; variants disrupted secretion and/or
34 signaling through multiple molecular mechanisms. Rare variants in this set of genes were
35 significantly enriched in 982 severely obese cases compared to 4449 controls. In a zebrafish
36 mutagenesis screen, deletion of 7 genes in this pathway led to increased somatic growth
37 and/or adiposity demonstrating that disruption of Semaphorin 3 signaling perturbs energy
38 homeostasis. In mice, deletion of the Neuropilin-2 receptor in Pro-opiomelanocortin
39 neurons disrupted their projections from the arcuate to the paraventricular nucleus,
40 reduced energy expenditure and caused weight gain. Cumulatively, these studies
41 demonstrate that SEMA3-mediated signaling drives the development of hypothalamic
42 melanocortin circuits involved in energy homeostasis.

43

44

45 INTRODUCTION

46 Neural circuits in the hypothalamus play a critical role in the regulation of energy
47 homeostasis (Elmquist et al., 1998). The hypothalamic melanocortin circuit is formed by
48 leptin-responsive neurons in the arcuate nucleus of the hypothalamus (ARH) expressing
49 either Pro-opiomelanocortin (POMC) or Neuropeptide Y (NPY)/Agouti-Related Protein
50 (AgRP) which project to, and synapse with melanocortin-4 receptor (MC4R) expressing
51 neurons in the paraventricular nucleus of the hypothalamus (PVH). In the nutritionally
52 replete state, ARH POMC neurons release melanocortin peptides including α -melanocyte-
53 stimulating hormone (α -MSH) which act as agonists at MC4R to reduce food intake and
54 increase energy expenditure (Cowley et al., 2001). Genetic disruption of POMC and MC4R
55 leads to severe obesity in rodents (Huszar et al., 1997; Yaswen et al., 1999) and humans
56 (Krude et al., 1998; Vaisse et al., 1998; Yeo et al., 1998), emphasising the critical role of this
57 melanocortin circuit in energy homeostasis.

58

59 Here, we studied the development of hypothalamic melanocortin circuits, focussing on the
60 contribution of the Class 3 Semaphorins (Pasterkamp, 2012) (SEMA3A-G) which direct the
61 development of gonadotropin-releasing hormone (GnRH) neurons into the hypothalamus
62 (Cariboni et al., 2015; Hanchate et al., 2012). Disruption of Sema3 signaling impairs the
63 development of GnRH projections in mice and rare variants that disrupt SEMA3 signaling are
64 associated with hypogonadotropic hypogonadism in humans (Cariboni et al., 2015; Young et
65 al., 2012). We performed genetic studies in people with severe obesity to test whether
66 there was an enrichment of rare potentially functional variants in genes encoding the
67 SEMA3s and their receptors compared to controls. We found 40 rare variants in these genes;
68 34 altered the function of these proteins through multiple molecular mechanisms. To test

69 whether disruption of *Sema3* signaling can perturb energy homeostasis, we performed a
70 CRISPR/Cas9 mutant screen of these genes in zebrafish. We showed that disruption of 7
71 genes caused increased somatic growth and/or adiposity. Using hypothalamic explants from
72 mice, we demonstrated that *Sema3* signaling via *Nrp2* receptors drives the development of
73 *Pomc* projections from the ARH to the PVH. Deletion of *Nrp2* in *Pomc* neurons, reduced the
74 density of *Pomc* projections and caused weight gain in young mice. Together, these findings
75 demonstrate the role of *Sema3* signaling in the development of melanocortin circuits that
76 modulate energy homeostasis, findings that have relevance to the understanding of
77 disorders of human hypothalamic development.

78

79 **RESULTS**

80 **Identification of rare variants in Semaphorin 3 ligands and their receptors in severely** 81 **obese individuals**

82 We hypothesized that if the genes encoding *Sema3*s and their receptors (*SEMA3A-G*, *NRP1-*
83 *2* and *PLXNA1-4*) contribute to the development of neurons that regulate body weight in
84 humans, we might identify functional variants in these genes in people with severe early
85 onset obesity. We examined exome sequencing data from 573 individuals with severe early
86 onset obesity (BMI SDS>3; onset under 10 years of age) recruited to the Genetics Of Obesity
87 Study (GOOS) studied as part of the UK10K consortium (Hendricks et al., 2017; Walter et al.,
88 2015). We found 40 rare variants in these 13 genes (**Table 1**). To test whether there was an
89 enrichment for very rare (minor allele frequency < 0.025%) predicted functional variants in
90 13 genes involved in Semaphorin 3 signaling in severely obese cases compared to controls,
91 we compared exome sequencing data from the final UK10K data release of 982 severely
92 obese individuals (including the 573 individuals in whom the first 40 variants were

93 identified) with that of 4449 healthy controls recruited to the INTERVAL study (Moore et al.,
94 2014). After adjusting for multiple testing, we found that very rare predicted functional
95 variants in this cluster of genes were enriched in severely obese cases compared to controls
96 (OR=1.40, p-adjusted=0.02; **Tables S1 and S2**). Although suggestive, these associations were
97 not statistically significant at the single gene level after adjusting for multiple testing. Given
98 the rarity of variants, larger sample sizes will be needed to test whether the burden of rare
99 variants in specific genes or combinations of genes is greater than expected in severely
100 obese cases versus controls.

101

102 **Rare variants in *SEMA3s* affect their secretion and function in cells**

103 We performed experiments in cells, to dissect the functional consequences of very rare
104 human variants (**Figures 1 and S1; Table S3**). SEMA3A-G are secreted as disulphide bridge-
105 linked dimers and processed by furin (**Figure 1A**). SEMA3s (except SEMA3E) bind to
106 Neuropilin co-receptors (NRP1 and NRP2) in hetero-complexes with PlexinA1-4 (PLXNA1-4)
107 receptors to activate plexin signal-transduction (SEMA3E can signal without NRPs through
108 the class D plexin, PLXND1) (Janssen et al., 2012; Tran et al., 2007). The dimeric SEMA3s
109 form the signalling complex with two PLXNAs and NRPs, the NRPs cross-bracing the
110 interfacing SEMA3s and PLXNAs (Janssen et al., 2012).

111

112 We mapped the 19 variants in *SEMA3s* onto the crystal structure of SEMA3A and homology
113 models of SEMA3B-3G to suggest structural explanations for our findings (**Figure 1A**). To
114 assess whether SEMA3s mutants affect protein secretion, we quantified the amount of
115 secreted SEMA3 detected in the medium of HEK293 cells transiently transfected with Flag-

116 tagged WT or mutant SEMA3 by ELISA. Six mutants decreased protein secretion compared
117 to WT SEMA3 (**Figure 1B**). Most led to increased intracellular retention of mutant SEMA3
118 suggesting that the defect was in secretion rather than synthesis (**Figure S1A**). In contrast, 6
119 mutants led to increased protein secretion (**Figure 1B and S1B**). The *SEMA3G* R728C variant
120 may hinder SEMA3 dimerization by disrupting the formation of an inter-subunit disulfide
121 bridge by the proximal, conserved cysteine residue C726 (**Figures 1A and S1C**).

122

123 To test whether SEMA3 mutants affect receptor-mediated signaling and thus disassembly of
124 the actin cytoskeleton and cellular collapse, U97MG cells were treated with medium from
125 cells transfected with WT or mutant SEMA3s, and the number of collapsed cells counted.
126 Compared to WT SEMA3s, 9 of the 19 SEMA3 mutants affected cell collapse (**Figure 1C**;
127 **Table S3**). Five SEMA3D mutants induced less collapse than WT (**Figure 1C**).

128

129 Based on homology modeling, 12 out of 19 variants were predicted to affect secretion
130 and/or cellular collapse due to destabilisation of the Sema domain important for SEMA3-
131 PLXNA-NRP recognition (**Figure 1D**). Paradoxically, four mutants decreased collapse despite
132 increased secretion. SEMA3C R739Q and SEMA3D R265H both locate close to the SEMA3-
133 NRP interface and may thus weaken SEMA3C-NRP1/2 binding. SEMA3D R773G may
134 destabilize the SEMA3-PLXNA-NRP complex by affecting the charge distribution on the basic
135 tail. SEMA3E R167G, located at the SEMA3-PLXNA interface, may directly affect PLXN
136 binding (**Figure 1D**). Two SEMA3B mutants showed decreased secretion, yet increased
137 collapse even after adjustment for the amount of protein secreted (**Figures 1B-C and S1D-E**).
138 In summary 15 of the 19 variants have functional consequences on the protein by affecting
139 secretion and/or collapse in these assays (**Table S3**).

140

141 **Rare variants in *NRP1-2* and *PLXNA1-4* disrupt cell surface localization and function**

142 We examined the molecular mechanisms by which the 21 variants in *PLXNA1-4* and *NRP1-2*
143 might affect their function (**Figures 2 and S2**). HEK293 cells were transfected with N-
144 terminally GLU-GLU-tagged WT and mutant constructs. Surface localization of NRPs and
145 PLXNs on non-permeabilized cells was quantified by ELISA using an anti-GLU-GLU antibody.
146 One NRP2 mutant (A506V) and 17 of the 18 PLXNA mutants significantly decreased cell
147 surface expression compared to WT receptors (**Figures 2A and S2A**). WT PLXNA1, A2 and A4
148 were predominantly localized on the plasma membrane whereas mutant PLXNs with
149 reduced cell surface expression were predominantly found within the endoplasmic
150 reticulum (ER) (**Figure S2B**). Interestingly, both WT and mutant PLXNA3 were localized in the
151 ER (**Figure S2B**). Almost all mutants with decreased cell surface localization reduced cell
152 collapse in cells transfected with NRP or PLXN (**Figure 2B**). In a ligand binding assay, none of
153 the mutants affected the equilibrium dissociation constant of the interaction (**Figure S2C**);
154 only NRP2 A506V decreased total binding (**Figure 2C**), in agreement with its modest
155 decrease in cell surface expression. Co-expression of NRP mutants with each WT PLXN gave
156 similar results as with expression of NRP alone (**Figures 2C and S2C**).

157

158 Structural modeling of the 21 mutants in PLXNAs and NRPs (**Figures 2D-F**) suggested
159 possible explanations for protein misfolding. Six of the 11 mutants lie in the Sema domain of
160 the PlxnA ectodomains; however, they are not at the Sema3 and Nrp-binding surfaces, so
161 are likely to affect structural stability rather than ligand or co-receptor interactions (**Figure**
162 **2D**). Mutants on the PSI and IPT domains may disrupt the stability of these domains (**Figure**

163 **2D)**. PLXNA3 D127N is located on a surface that mediates PlxnA-PlxnA interactions
164 important for receptor auto-inhibition pre-ligand binding (Kong et al., 2016) (**Figures S2D-E**).
165 Six mutants lie in the cytoplasmic Plxn domains and may additionally affect coupling to
166 downstream signaling molecules; PLXNA1 L1278F on the juxta-membrane helices may affect
167 pre-signaling inhibition, PLXNA3 D1710N on the Rap-GAP pocket helices may affect Rap
168 binding, PLXNA1 G1650S and PLXNA4 T1642I on the Rho-GTPase binding domain (RBD) may
169 affect interactions with the Rho-GTPases and PLXNA3 T1679I may disrupt the hydrophobic
170 core of the PlxnA GAP domain (**Figure 2E**). Only 1 out of the 3 mutants found on NRP1 and
171 NRP2 lead to decreased cell collapse and reduced cell surface expression likely by affecting
172 molecular stability (**Figure 2F**).

173

174 **A mutagenesis screen in zebrafish demonstrates that disruption of *semaphorins*, *plexins***
175 **and *neuropilins* alters somatic growth and adiposity**

176 We used zebrafish to test whether altered Sema3 signaling can disturb energy homeostasis,
177 as the hypothalamic neural circuits involved in energy homeostasis are highly conserved
178 (Leibold and Hammerschmidt, 2015; Minchin and Rawls, 2017). Multiple CRISPR guide RNAs
179 (gRNAs) targeting distinct regions of each zebrafish *semaphorin 3*, *neuropilin* and *plexin a*
180 ortholog (**Figures 3A and S3A**) were injected into one-cell stage zebrafish embryos.
181 Disruption of the melanocortin system can influence both somatic growth and adiposity in
182 zebrafish (Sebag et al., 2013; Zhang et al., 2012). Deletions in 7 genes significantly increased
183 somatic growth, body weight and/or % body fat (**Figures 3B and S3B**). Deletion of a
184 duplicate gene for NRP2 (*nrp2b*) increased adiposity and somatic growth (**Figure 3C**). In
185 contrast, deletions of 2 genes decreased % body fat. Cumulatively, these data demonstrate

186 that Semaphorin 3 signaling can affect energy homeostasis, potentially through several
187 different mechanisms.

188

189 **SEMA3s and their receptors are expressed in the hypothalamus during early development**

190 Using RT-qPCR we found that *Nrp1-2* and *PlxnA1-4* mRNAs were detected in the mouse
191 hypothalamus as early as E10 and were highly expressed postnatally, particularly in the ARH
192 (**Figures 4A and S4A**). *NRP1-2* and *PLXNA1-4* mRNAs were also expressed in the human fetal
193 hypothalamus and the hypothalamus of human young adults (**Figure 4A**). *Sema3a*, *Sema3c*,
194 *Sema3e* and *Sema3f* were expressed in the mouse PVH at P10 (**Figure 4A**); *SEMA3C* was the
195 most abundant in the mouse PVH, DMH, ARH, and preoptic area (**Figure S4A**) and in the
196 human fetal and young adult hypothalamus (**Figure 4A**). This temporal pattern of gene
197 expression overlaps with a critical time window for the development of the melanocortin
198 circuits that regulate energy homeostasis. *Pomc* neurons in the ARH are generated on
199 embryonic day (E)11-12, acquire their terminal peptidergic phenotype during mid-late
200 gestation (Padilla et al., 2010) and send axonal projections to their target sites during the
201 first few weeks of postnatal life (Bouret et al., 2004).

202

203 **Sema3s drive the growth of Pomc projections *in vitro***

204 To test whether Sema3s are involved in the development of arcuate *Pomc* projections, we
205 performed co-cultures between ARH explants derived from *Pomc-Cre*; TdTomato mice (to
206 genetically label *Pomc* axons) and HEK293 cell aggregates transfected with human *SEMA3*-
207 encoding vectors (**Figure 4B**). We quantified the density of axons extending toward
208 (proximal) or away (distal) from cell aggregates (**Figure 4B**). Compared with the radial

209 growth seen with HEK293 cells transfected with an empty vector, growth of arcuate Pomc
210 axons was enhanced by HEK293 cell aggregates overexpressing SEMA3A, SEMA3B, SEMA3C,
211 SEMA3D and SEMA3G (**Figure 4C**). Notably, only SEMA3C and SEMA3D affected the growth
212 of arcuate NPY axons (**Figure S4B**). To validate the specificity of our *in vitro* assay, we co-
213 cultured an explant derived from the dorsal root ganglion (DRG) with HEK293 cells
214 overexpressing SEMA3A and confirmed, as previously described (Taniguchi et al., 1997) that
215 SEMA3A inhibited the growth of DRG axons (**Figure S4C**). Together, these observations
216 demonstrate that SEMA3s can signal to ARH axons, including to Pomc axons, and display
217 enhanced growth.

218

219 We repeated co-cultures between mouse ARH explants and HEK293 cell aggregates
220 transfected with a subset of human *SEMA3* variants and evaluated axon growth. Three out
221 of 5 variants tested negatively impacted on the overall growth of arcuate axons; one
222 increased the density of POMC projections (Figure 4D). Interestingly, *SEMA3G* E478D and
223 *SEMA3B* P296L mutants specifically reduced POMC axon growth.

224

225 **Neuropilin receptors mediate growth of arcuate Pomc projections towards the PVH and** 226 **away from the VMH**

227 We reconstructed arcuate connections *in vitro* by co-culturing 2 explants derived from
228 different hypothalamic nuclei. When an ARH explant derived from *Pomc*-Cre; TdTomato
229 mice was co-cultured with a PVH explant, the density of fibers directed toward the PVH was
230 substantially greater than that from the opposite side of the ARH, suggesting that the PVH
231 releases diffusible chemotropic factors that promote growth of arcuate Pomc axons (**Figure**
232 **4E**). Substitution of the PVH explants with control HEK293 cells or an explant derived from

233 the cortical cortex (normally not innervated by ARH axons) did not result in any detectable
234 effect on neural projections (**Figures 4E and S4D**), demonstrating a high degree of
235 specificity. Nrp1 or Nrp2 neutralizing antibodies blocked the induction of growth exerted by
236 the PVH on arcuate Pomc axons (**Figure 4E**). The DMH promoted growth of arcuate Pomc
237 axons, but this effect was not blocked with Nrp1 or Nrp2 neutralizing antibodies (**Figure 4E**).
238 In contrast, the VMH inhibited growth of arcuate Pomc axons and this effect was blocked by
239 the addition of Nrp1, but not Nrp2, antibodies (**Figure 4E and S4E**). Thus, Nrp-mediated
240 signaling plays a specific role in establishing Pomc axonal projections to the PVH.

241

242 **Genetic deletion of Neuropilin 2 reduces the density of Pomc projections to the PVH and** 243 **causes weight gain in mice**

244 In the ARH, *Nrp2* mRNA expression was two times higher than that of *Nrp1* (**Figure 4A**). To
245 investigate the role of Nrp2 in Pomc neurons *in vivo*, we crossed mice carrying a *Nrp2*^{loxP}
246 allele (Walz et al., 2002) with mice expressing Cre recombinase in a *Pomc*-specific manner
247 (*Pomc*-Cre) (Balthasar et al., 2004) to generate mice that lack *Nrp2* in Pomc-derived
248 neurons. We repeated the *in vitro* co-culture assay with an ARH explant derived from
249 *Nrp2*^{loxP/loxP} mice that received intra-ARH injections of an AAV-Cre vector and a PVH explant
250 derived from WT mice. Genetic loss of Nrp2 in ARH neurons also blocked growth of axons
251 towards the PVH (**Figure 4F**). As expected, the levels of *Nrp2* mRNA were decreased in the
252 arcuate nucleus of *Pomc*-Cre; *Nrp2*^{loxP/loxP} mice whereas the levels of *Nrp1* were comparable
253 between control and mutant mice (**Figure S5A**); there was a 3-fold reduction in *Nrp2* mRNA
254 in sorted POMC neurons derived from mutant mice (**Figure 5A**). In contrast, there was no
255 significant change in the levels of *Nrp1* and *Nrp2* mRNAs in the pituitary of mutant mice

256 (Figure S5B-C), or in other hypothalamic nuclei and extra-hypothalamic brain regions (Figure
257 S5D).

258

259 *Pomc*-Cre; *Nrp2*^{loxP/loxP} mice were born normally and had body weights indistinguishable
260 from control littermates until 11 weeks of age (Figure 5B) when mutant mice displayed
261 significantly higher body weights (Figure 5B). Oxygen consumption (VO₂), locomotor activity
262 and energy expenditure were reduced in mutant mice compared with control *Nrp2*^{loxP/loxP}
263 mice (Figure 5C-E). Although there was no change in body composition (Figure 5F), there
264 was an increase in adipocyte size in mutant mice (Figure 5G). Food intake and respiratory
265 exchange ratio were not significantly different compared to controls (Figures 5H and I).
266 *Pomc*-Cre; *Nrp2*^{loxP/loxP} mice displayed elevated levels of glucose 15 minutes after a glucose
267 challenge, compared to control mice (Figure 5J). Leptin levels, but not insulin, T3, T4 and
268 corticosterone levels, were significantly elevated in mutant mice (Figures S5E-I).

269

270 Although the overall distribution of αMSH-IR fibers was similar between mutant and control
271 mice, the density of αMSH-IR fibers innervating the neuroendocrine and pre-autonomic
272 parts of the PVH of *Pomc*-Cre; *Nrp2*^{loxP/loxP} mice was 2-3 fold lower than that observed in the
273 *Nrp2*^{loxP/loxP} mice (Figure 5K). The density of αMSH projections to the DMH (Figure 5K) as
274 well as to other major terminal fields (Figure S5J) was comparable between mutant and
275 control mice. Corticotrophin-releasing hormone (*Crh*) and thyrotrophin-releasing hormone
276 (*Trh*) expression in the PVH was increased, but *oxytocin* mRNA levels were unchanged
277 (Figure 5L). The number of *Pomc* mRNA-expressing cells in the ARH of *Pomc*-Cre; *Nrp2*^{loxP/loxP}
278 mice was comparable to that of control mice (Figure 5M and S5K-M). Together, these data

279 indicate that Nrp2 signaling directs the formation of Pomc projections to the PVH with
280 marked target specificity.

281

282 **DISCUSSION**

283 In this study, we identified rare heterozygous variants in *SEMA3s*, their receptors and co-
284 receptors in individuals with severe early-onset obesity. In zebrafish, we showed that
285 deletion of several genes in this pathway increased weight-related phenotypes establishing
286 a role for these molecules in energy homeostasis. There are several potential mechanisms
287 by which these genes might modulate body weight and/or fat mass. In mice, we showed
288 that *Sema3s* acting via *Nrp2* direct the development of *Pomc* projections from the arcuate
289 to the paraventricular nucleus of the hypothalamus. A role for *Nrp1* mediated signaling in
290 pre-adipocyte differentiation has been demonstrated (Ceccarelli et al., 2018). In zebrafish,
291 *plxnd1* has been shown to regulate adipose tissue growth by affecting the formation of the
292 extracellular matrix (Minchin et al., 2015) indicating that Semaphorin 3 signaling may affect
293 non-neural mechanisms that contribute to adiposity.

294

295 **Rare variants in the *SEMA3s*, their receptors and co-receptors**

296 We found multiple rare variants in the genes encoding ligands, receptors and co-receptors
297 involved in *Sema3* signaling by studying severely obese individuals. Variants were not fully
298 penetrant and did not segregate with severe obesity in families demonstrating non-
299 Mendelian inheritance. There are parallels with hypogonadotropic hypogonadism, where
300 incomplete penetrance and variable expressivity within and across families has been
301 observed (Cassatella et al., 2018). As the number of genes implicated in hypogonadotropic
302 hypogonadism has increased, it has become clear that oligogenic inheritance (i.e. more than

303 one gene mutated in the same individual) can in part explain these observations. Indeed,
304 heterozygous variants in *SEMA3A*, *SEMA3E* and *PLXNA1* can contribute to hypogonadotropic
305 hypogonadism in an oligogenic manner with variable penetrance (Cariboni et al., 2015;
306 Hanchate et al., 2012). In this study, whilst variant carriers did not carry additional variants
307 in known obesity genes, it is plausible that other, as yet unidentified, genes may contribute
308 to expression of the obesity phenotype in the individuals studied here.

309

310 There are many challenges when studying rare variants that might contribute to complex
311 traits that do not follow Mendelian patterns of inheritance (Agarwala et al., 2013; Zuk et al.,
312 2014). Statistical burden association tests are used to test whether there is a difference in
313 the load of rare variants predicted to have a functional impact in cases versus controls.
314 Using this approach, we found nominal enrichment for very rare variants in the cluster of
315 genes encoding SEMA3 ligands, receptors and co-receptors when severely obese cases were
316 compared to healthy controls indicating that rare variants within this gene-set may be
317 associated with obesity. However, given the number of rare variants, their presence in cases
318 and in controls, and the complexity of Sema3 signaling, larger genetic studies are needed to
319 more fully test whether the burden of functionally significant variants (as tested in cells) is
320 increased in severely obese individuals compared to controls and whether this association is
321 driven by specific genes/combinations of genes.

322

323 Some of the neurodevelopmental phenotypes observed in variant carriers overlap with
324 those seen in animals (**Table 1**). One male carrying *PLXNA3* D1710N had hypogonadotropic
325 hypogonadism. As *PLXNA3* lies on the X chromosome, this individual would have minimal

326 residual signaling through *PLXNA3*. The true prevalence of hypogonadotropic
327 hypogonadism amongst variant carriers may be underestimated here as several probands
328 were pre-pubertal children.

329

330 *SEMA3* signaling is known to regulate the development of the enteric nervous system in
331 rodents and rare heterozygous loss of function variants in *SEMA3C* and *SEMA3D* have been
332 associated with Hirschsprung's disease in humans (Jiang et al., 2015), a disorder
333 characterised by failure of development of parasympathetic ganglion cells in the large
334 intestine. Five people had severe medication-resistant constipation in childhood with
335 multiple hospital admissions; two individuals had been investigated for Hirschsprung's
336 disease and one required a colostomy for severe dysmotility (*SEMA3D* D640Y). Additional
337 studies in neuronal cells, experimental animal models of human variants and in variant
338 carriers will be needed to directly test the functional impact of these variants on the
339 development of the enteric nervous system.

340

341 **Insights into the molecular mechanisms disrupted by rare human variants**

342 Many of the *SEMA3* variants reduced secretion and/or receptor-mediated signaling. In
343 blinded studies, four mutants that had functional consequences on secretion and/or
344 signaling in cells affected the density of neuronal projections, while the one WT-like variant
345 (*SEMA3D* D640Y) studied, did not affect neuronal projections (**Table S3**). Paradoxically,
346 some mutants exhibited increased secretion in cells. Experiments in chick DRGs have shown
347 that the concentration of *SEMA3*s can determine which signaling pathway is activated and

348 thereby influence the extent of growth cone collapse within a <100 ng/ml to >625 ng/ml
349 range (Manns et al., 2012). As such, mutants that both increase and decrease secretion
350 might disrupt axon guidance *in vivo*. Understanding how ligand concentration (as altered by
351 human variants) impacts on axon growth may provide insights into the development of
352 hypothalamic and extra-hypothalamic circuits in the human brain.

353

354 Mutant PLXN receptors were predominantly found within the endoplasmic reticulum rather
355 than at the cell surface; structural modelling of the 21 mutants in *PLXNAs* and *NRPs*
356 suggested several explanations for the misfolding of mutant receptors. The downstream
357 consequences of aberrant receptor expression and signaling are challenging to predict as
358 different NRP-PLXN complexes mediate signaling by different combinations of Semaphorins
359 in different brain regions. For example, experiments in neurons suggest that *Sema3A* signals
360 via *Nrp1-PlxnA4* complexes, whereas *Nrp2-PlxnA3* complexes mediate responses to
361 *Sema3F*. Studies in *PlxnA3* null mice show that *PlxnA3* can mediate the effects of *Sema3A*
362 and *Sema3F* (Cheng et al., 2001). However, neurons from *PlxnA3* null mice only partially lose
363 responses to *Sema3A* implying a degree of compensation through other receptor
364 complexes, whereas hippocampal neurons from these mice lose essentially all
365 responsiveness to *Sema3F* implying there is no redundant receptor for this specific
366 response.

367

368 In preliminary studies, we modelled the effects of two NRP1 and -2 variants on
369 melanocortin neural circuits in zebrafish. Compared to WT NRP2, A506V NRP2 resulted in a
370 significant further reduction in the density of α -MSH labeled fibers in the preoptic area

371 (teleost homolog of the PVH) (**Figure S3C**). Compared to WT NRP1, the R237Q NRP1 mutant
372 resulted in a further reduction in the density of AgRP immunoreactive fibres innervating
373 the anterior tuberal nucleus (teleost homolog of the VMH) (**Figure S3C**). Given the
374 challenges associated with dissecting the impact of these molecules on neuronal circuits and
375 weight related phenotypes, deletion/reactivation studies of components of the signaling
376 pathway in specific neuronal populations will be needed to determine the extent to which
377 other components of the signaling complex can compensate for the partial lack of
378 expression of mutant receptors *in vivo*.

379

380 **The role of SEMA3 signaling in the development of hypothalamic neural circuits**

381 Our data suggest that Sema3 signaling in developing Pomc neurons contributes to the
382 regulation of energy homeostasis and glucose homeostasis. The relatively modest effect of
383 *Nrp2* deletion in Pomc neurons on body weight is not surprising, as only Pomc axonal
384 projections to the PVH were disrupted in this mouse model. Our findings align with
385 experiments specifically disrupting leptin signalling in ARH Pomc neurons (Berglund et al.,
386 2012). Further in depth exploration of the degree of compensation/plasticity associated
387 with targeted experimental manipulations will be needed. Further characterisation of the
388 impact of human variants in mature neurons where they can affect the maturation and
389 density of dendritic spines, synaptogenesis and synaptic plasticity (Orr et al., 2017) may
390 inform understanding of the mechanisms that underlie human disorders characterised by
391 hypothalamic dysfunction.

392

393 **AUTHOR CONTRIBUTIONS**

394 AAvdK, SGB and ISF conceived and designed the studies and wrote the paper; SGB and ISF
395 supervised the project; AAvdK, JMK and EH contributed to the clinical studies and with RB,
396 SOR and ISF contributed to the recruitment of the GOOS cohort; EMO, LKJS, MCB and SP
397 performed the molecular studies and YK and EYJ the structural studies of human variants.
398 AAvdK, AEH, EGB, VM, IB and ISF contributed to the human genetic studies as part of work
399 supported by the UK10K consortium and INTERVAL. SC, SP, RBS and SGB performed and/or
400 supervised the experiments in mice; PT, SER, JENM performed and/or supervised the
401 experiments in zebrafish.

402

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434

435 **DECLARATION OF INTERESTS**

436 The authors declare no competing interests.

437

438

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605

607 **MAIN FIGURE TITLES AND LEGENDS**

608 **FIGURE 1. Rare human variants in *SEMA3A-G* disrupt protein secretion and signaling**

609 **(A)** Structural modeling of *SEMA3* variants. Upper panel: *SEMA3* variants on a schematic
610 representation (mouse *Sema3A* numbering). SS, signal sequence; Sema, semaphorin
611 domain; PSI, Plexin-Semaphorin-Integrin domain; conserved furin cleavage sites indicated by
612 scissors; conserved cysteines that form *SEMA3A-G* dimers (orange line). Lower Panel:
613 *SEMA3A-G* mutants mapped onto human *SEMA3A* structure (increase, blue; decrease, red;
614 no effect, grey; on U87MG cell collapse). Sema and PSI domains on mouse *Sema3A* crystal
615 structure (PDB: 4GZ8); Ig domain, model combining human *SEMA4D* (PDB: 1OLZ) and mouse
616 *Sema3A* (PDB: 4GZ8) structural data; c-terminal basic domain, schematic. **(B)** ELISA analysis
617 of C-FLAG-tagged WT/mutant *SEMA3A-G* secreted in the medium (A.U; arbitrary units). **(C)**
618 Effect of WT/mutant *SEMA3A-G* on cell collapse normalized to amount of semaphorin
619 secreted. **(D)** Structural analysis of *SEMA3* mutants affecting cell collapse (increased, blue;
620 decreased, red). Mutants are mapped on the crystal structure of the mouse *Sema3A-Nrp1-*
621 *PlxnA2* complex (PDB: 4GZA). Data represented as mean \pm SEM from at least 3 independent
622 experiments; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for all experiments. Scale bars, 100 μ m. See
623 also Figure S1 and Table S3.

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628 **FIGURE 2. Rare human variants in *Neuropilins 1-2 and Plexins A1-4* disrupt cellular**
629 **localisation and signaling**

630 **(A)** Cell surface localization for WT/mutant NRP1-2 and PLXNA1-4 by ELISA. **(B)** Effect of
631 WT/mutant NRP1-2 and PLXNA1-4 on semaphorin-induced cell collapse. **(C)** Total binding
632 (Bmax) in cells expressing WT/mutant NRP1-2 or co-expressing NRP1-2 mutants and WT
633 PLXNA1-4. **(D)** Structural modeling of PLXNA mutants. Upper panel: PLXNA1-4 variants
634 shown on schematic (mouse PlxnA1 numbering). SS, signal sequence; PSI, Plexin-
635 Semaphorin-Integrin; IPT, Ig domain. Lower panel: PLXNA mutants mapped onto the crystal
636 structure of mouse PlxnA1 ectodomain (PDB: 5L56). **(E)** PLXNA mutants mapped onto the
637 crystal structure of mouse PlxnA3 intracellular domain (PDB: 3IG3). **(F)** Upper panel: NRP
638 variants shown on the schematic (mouse Nrp1 numbering). Lower panel: NRP1-2 variants
639 mapped onto the crystal structure of mouse Nrp1 (PDB: 4GZ9). The membrane-proximal
640 MAM (meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu) domain of Nrp
641 is represented schematically. RBD, Rho GTPase-binding domain; GAP, GTPase activating
642 protein; TM, transmembrane; JM, Juxtamembrane. The neuropilin ectodomain comprises
643 two CUB domains (a1 and a2), two coagulation factor V/VIII homology domains (b1 and b2)
644 and a MAM domain, L, linker. In D-F, variants causing decreased surface expression (red), no
645 effect on surface expression (grey), decreased surface expression as well as decreased cell
646 collapse (blue) are shown. Data represented as mean \pm SEM from at least 3 independent
647 experiments; *P<0.05, **P<0.01 and ***P<0.001. See also Figure S2 and Table S3.

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650 **FIGURE 3. Disruption of *semaphorin 3s*, *neuropilins* and *plexins* alters energy homeostasis**
651 **in Zebrafish**

652 **(A)** Unrooted phylogenetic trees of *Sema3*, *PlxnA* and *Nrp* genes. Genes from zebrafish
653 (dotted lines) and mouse and human (solid lines) were used to construct the trees. Where
654 zebrafish genes have been duplicated, a letter is used to identify paralogs. Scale bars,
655 number of substitutions per amino acid site. **(B)** Heatmap showing change in length, weight
656 and % body fat in deletion mutants relative to Cas9-only control fish; decrease (blue),
657 increase (orange) in the phenotype of mutants relative to control fish (for the natural log
658 fold change); *genes not screened; *agrp*, positive control. **(C)** Length (mm), weight (mg) and
659 % body fat in *nrp2a* and *nrp2b* mutant fish relative to Cas9-only injected control fish. Data
660 represented as mean \pm SEM. * $P < 0.05$; *** $P < 0.001$ in one-sample t-tests. Representative
661 images of Nile Red stained zebrafish showing increased adiposity and size of *nrp2b* mutant
662 fish (right). Scale bar, 1mm. See also Figure S3.

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670 **FIGURE 4. Class 3 Semaphorins and their receptors are expressed in the developing**
671 **hypothalamus and direct innervation of the paraventricular nucleus of the hypothalamus**
672 **by arcuate Pomc axons**

673 **(A)** Expression of Neuropilin (*NRP1-2*), PlexinA (*PLXNA1-A4*), Semaphorin (*SEMA3A-G*) mRNA
674 in the hypothalamus (HYPO) of mouse fetuses at embryonic day E10/12/14, in hypothalamic
675 nuclei of P10 mice (ARH-arcuate; PVH-paraventricular nucleus of the hypothalamus), in the
676 hypothalamus from human fetuses at 14 weeks of gestational age (GA) and from human
677 young adults; values relative to GAPDH expression shown. **(B)** In a co-culture system to
678 evaluate neural growth, the average density of neurites in the proximal and distal parts of
679 the ARH explant (with respect to the target tissue, e.g. PVH) is compared to quantify the
680 density of axons extending toward (proximal) or away (distal) from cell aggregates. **(C)** ARH
681 explants from *Pomc-Cre*; TdTomato mice were co-cultured with an aggregate of HEK293
682 cells overexpressing Sema3A-G and immunostained with TUJ1 (neuron-specific class III beta-
683 tubulin). **(D)** Quantitative analysis of TUJ1⁺ (upper panel) and Pomc⁺ (lower panel) axons
684 derived from arcuate explants co-cultured with an aggregate of HEK293 cells overexpressing
685 *SEMA3A-G* mutants (*P<0.05 versus mock; #P<0.05 versus WT). ARH, arcuate nucleus. **(E)**
686 ARH explants derived from *Pomc-Cre*, TdTomato mice were co-cultured with explants
687 containing the PVH, DMH, or VMH and Nrp1 or Nrp2 blocking antibodies (α). Data
688 represented as mean \pm SEM. *P<0.05 versus mock; Δ , P<0.05 versus PVH IgG; \$, P<0.05
689 versus VMH IgG. **(F)** ARH explants derived from *Nrp2*^{loxP/loxP} mice that received intra-ARH
690 injections of an AAV-Cre vector with explants containing the PVH. Genetic loss of Nrp2 in the
691 ARH causes a significant reduction in ARH axon growth. **P<0.01 vs control. Scale bars,
692 250 μ m (C); 100 μ m (D). See also Figure S4.

693

694 **FIGURE 5. Loss of neuropilin 2 signaling in Pomc neurons causes reduced energy**
695 **expenditure and weight gain in mice and disrupts arcuate Pomc projections to the PVH**
696 **(A)** Expression of *Nrp1* and *Nrp2* mRNA in sorted POMC⁺ neurons of adult *Nrp2*^{loxP/loxP} and
697 *Pomc-Cre; Nrp2*^{loxP/loxP} mice; values relative to GAPDH expression shown. **(B)** Body weight,
698 **(C)** oxygen consumption, **(D)** locomotor activity, **(E)** energy expenditure, **(F)** body
699 composition, **(G)** adipocyte area, **(H)** average food intake, **(I)** respiratory exchange rate (RER)
700 and **(J)** glucose tolerance test and area under the curve (AUC) of adult *Nrp2*^{loxP/loxP} (control)
701 and *Pomc-Cre; Nrp2*^{loxP/loxP} (mutant) mice. **(K)** Microphotographs and quantification of the
702 density of α -melanocyte-stimulating hormone (α MSH)-immunoreactive (IR) fibers
703 innervating the neuroendocrine paraventricular nucleus of the hypothalamus (PVHpml and
704 PVHmpd), pre-autonomic PVH (postPVH) and DMH of adult *Nrp2*^{loxP/loxP} and *Pomc-Cre;*
705 *Nrp2*^{loxP/loxP} mice. **(L)** Relative expression of corticotropin-releasing factor (*Crh*), thyrotropin-
706 releasing hormone (*Trh*), and oxytocin (*Oxt*) mRNA in the PVN of the hypothalamus of adult
707 *Nrp2*^{loxP/loxP} and *Pomc-Cre; Nrp2*^{loxP/loxP} mice. **(M)** Microphotographs and quantification of
708 Pomc expressing neurons and relative levels of Pomc mRNA in the ARH of adult *Nrp2*^{loxP/loxP}
709 and *Pomc-Cre; Nrp2*^{loxP/loxP} mice. Data represented as mean \pm SEM; *P<0.05 and **P<0.01
710 versus *Nrp2*^{loxP/loxP}. ARH, arcuate nucleus of the hypothalamus; DMH, dorsomedial nucleus
711 of the hypothalamus; PVH, paraventricular nucleus of the hypothalamus; PVHmpd, dorsal
712 component of the medial parvicellular PVH; PVHpml, lateral magnocellular PVH; post PVH,
713 posterior part of the PVH; V3, third ventricle. Scale bars, 100 μ m. See also Figure S5.

714 **MAIN TABLE TITLE AND LEGEND**

715 **TABLE 1: Phenotypes of *SEMA3A-G*, *PLXNA1-4* and *NRP1-2* variant carriers**

716 All variants were in found in heterozygous form unless indicated (*denotes homozygous);
717 gastrointestinal motility disorders presented as severe therapy-resistant constipation.
718 Abbreviations: yrs (years); BMI (body mass index); BMI SDS (BMI standard deviation score
719 for children. **Two participants harboured two variants. See also Table S1 and S2.

Gene	Variant	Age (yrs)	BMI (kg/m ²)	BMI SDS	Endocrine	Neuro-developmental	Gut	Other
NRP1; PLXNA3 **	R237Q*; T1679I	2.8	29.3	5.41				
NRP2	V573L	34.1	50.0	-				
NRP2	A506V	3.9	23.8	4.15		autism, speech delay		
NRP2	A506V	11.3	24.1	2		severe migraine		
PLXNA1	L1278F	6.3	28.7	4.53		behavioural problems		
PLXNA1	G1650S	11.9	38.7	3.7				
PLXNA1	R378H	21.0	34.8	2.89		hypotonia autism, speech delay		
PLXNA2	T515M	14.0	35.9	3.32				
PLXNA2	N788I	6.3	26.0	3.65				
PLXNA2	W25X	11.0	34.2	3.48				
PLXNA2	R1668Q	33.2	63.4	-		Asperger's syndrome, speech delay		
PLXNA2	A436V	6.2	26.0	3.7				
PLXNA3	V879M	53.7	49.0	-	hypothyroidism		severe constipation	
PLXNA3	R1116C	3.5	23.3	3.71				
PLXNA3	D1710N*	13.9	29.4	2.72	hypog. hypogonadism			
PLXNA3; PLXNA3 **	D127N; R351H	6.9	26.8	3.59				
PLXNA4	V245G	15.3	58.6	4.63	hypothyroidism	nocturnal enuresis	severe constipation	
PLXNA4	G643D	5.5	27.2	4.19				recurrent infections
PLXNA4	T1642I	11.1	32.8	3.32				
PLXNA4	R70Q	9.5	26.6	3.03		behavioural problems		
SEMA3A	K600M	2.3	29.5	5.5	hypothyroidism			
SEMA3A	R350T	7.7	30.2	3.8	hypothyroidism			
SEMA3B	F355L	10.0	28.4	3.16				
SEMA3B	P296L	13.2	33.4	3.14		epilepsy, narcolepsy, conductive hearing loss, impaired pain sensation, behavioural problems, speech delay		recurrent infections
SEMA3C	R739Q	15.5	43.6	3.82	hypothyroidism	Asperger's syndrome	severe constipation	
SEMA3D	R773G	11.9	41.3	3.82		nocturnal enuresis		
SEMA3D	R265H	13.0	35.8	3.4				
SEMA3D	T397A	8.9	36.1	4.02				
SEMA3D	Y199S	14.4	37.4	3.44				
SEMA3D	N444S	34.1	41.1	-				
SEMA3D	D380H	16.0	56.4	4.56	hypothyroidism			
SEMA3D	D640Y	7.5	29.2	4.01		autism, learning difficulties, speech delay	severe constipation	

SEMA3E	R167G	14.1	40.5	3.72				
SEMA3E	K711N	7.1	23.1	2.75				
SEMA3F	E88K	2.8	34.8	6.4			severe constipation	
SEMA3G	R561W	3.7	21.3	2.98				recurrent infections
SEMA3G	R728C	13.9	36.1	3.36				
SEMA3G	E478D	15.5	43.8	3.9				
SEMA3G	A86S	14.9	34.6	3.11				

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736 **SUPPLEMENTAL FIGURE TITLES AND LEGENDS**

737 Supplemental information including 5 Figures and 3 Tables can be found with this article
738 online.

739

740 **Figure S1. Functional characterization of rare human variants in SEMA3A-G. Related to**
741 **Figure 1.**

742 **(A)** Total expression of C-FLAG-tagged SEMA3A-G by ELISA analysis (A.U, arbitrary units). **(B)**
743 Western blotting of total cellular and secreted SEMA3A-G. **(C)** Dimerization analysis using
744 reducing and non-reducing western blotting of total cellular and secreted SEMA3G. **(D)**
745 Collapse efficiency was assessed by counting the proportion of collapsed cells 30 minutes
746 following addition of the indicated WT Semaphorin to the culture medium. **(E)** Effect of
747 SEMA3A-G on cell collapse unadjusted for the amount of semaphorin secreted. Data are
748 presented as mean \pm SEM from at least 3 independent experiments; *P<0.05, **P<0.01 and
749 ***P<0.001.

750

751 **Figure S2. Functional characterization of rare human variants in Neuropilins 1-2 and**
752 **Plexins A1-4. Related to Figure 2.**

753 Total expression of WT and mutant NRP1-2 and PLXNA1-4 by ELISA on permeabilised HEK-
754 293 cells (A.U, arbitrary units). **(B)** Confocal microscopy of Cos-7 cells showing the co-
755 localization of transiently expressed WT and mutant PLXNA1-4 (green) with plasma
756 membrane (magenta) and endoplasmic reticulum (red) markers. Scale bars represent 10
757 μ m. **(C)** Saturation receptor-ligand binding assay. Cells expressing WT/mutant NRP1-2 or co-
758 expressing mutant NRP1-2 and WT PLXNA1-4 were incubated with increasing amounts of

759 recombinant human SEMA3C and the equilibrium dissociation constant (kd) of the
760 interaction and the total binding (Bmax) were calculated. **(D)** Structural model of SEMA3s
761 signalling via the PLXNA receptors and co-receptor NRP1 or 2. **(E)** Variant *PXLNA2* D127N
762 locates on the PLXNA-PLXNA interface important for pre-signalling auto-inhibition. Data are
763 presented as mean \pm SEM from at least 3 independent experiments; *P<0.05, **P<0.01 and
764 ***P<0.001.

765

766 **Figure S3. Generation and characterisation of semaphorin-neuropilin-plexin deletion**
767 **mutants in Zebrafish. Related to Figure 3.**

768 **(A)** Schematic illustrating the mutagenesis strategy to target *sema3*, *plxna* and *nrp* genes in
769 zebrafish. Two-five sgRNAs were generated to mutagenize each zebrafish gene. Only sgRNAs
770 verified to induce mutagenesis were injected into one-cell stage zebrafish embryos.
771 Zebrafish were raised to ~30 days post fertilization and fish length (mm), weight (mg) and %
772 body fat were quantified. **(B)** Results on fish length (mm), weight (mg) and % body fat for all
773 deletion mutants (summarised in Figure 3B). **(C)** Microphotographs and quantification of the
774 density of α -melanocyte-stimulating hormone (α MSH) (red) and agouti-related peptide
775 (AgRP) (green) immunoreactive (IR) fibers innervating the preoptic area (POA), anterior
776 tuberal nucleus of hypothalamus (ATN), and lateral hypothalamic nucleus (LH) of 35-day-old
777 wild-type zebrafish overexpressing NRP1 and NRP2; *P<0.05 in one-sample t-tests.

778

779 **Figure S4. Expression of Class 3 Semaphorins and their receptors in the developing**
780 **hypothalamus and specificity of co-culture assays. Related to Figure 4.**

781 **(A)** Expression of Semaphorin (*Sema3A-G*), Neuropilin (*Nrp1-2*), PlexinA (*PlxnA1-A4*) mRNA
782 in microdissected hypothalamic nuclei of P10 mice; compared to expression of GAPDH. **(B)**
783 Quantitative analysis of NPY⁺ axons derived from arcuate explants co-cultured with an
784 aggregate of HEK-293 cells overexpressing Sema3A-G. **(C)** Representative image showing an
785 isolated explant derived from the dorsal root ganglion (DRG) and co-cultured with an
786 aggregate of HEK-293 cells overexpressing Sema3A. **(D)** Representative image showing an
787 ARH explant co-cultured with a cortical explant. **(E)** Representative image showing ARH
788 explants derived from *Pomc-Cre*, TdTomato mice co-cultured with explants containing the
789 VMH in the presence of *Nrp1* blocking antibodies (α). Data are represented as mean \pm SEM.
790 *P<0.05 and **P<0.01 versus mock. ARH, arcuate nucleus of the hypothalamus; DMH,
791 dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; POA, preoptic
792 area; SCN, suprachiasmatic nucleus; VMH, ventromedial nucleus of the hypothalamus.

793

794 **Figure S5. Metabolic and neuroanatomical characterization of *Pomc-Cre; Nrp2*^{loxP/loxP} mice.**

795 **Related to Figure 5. (A)** Expression of *Nrp1* and *Nrp2* mRNA in the arcuate nucleus of the
796 hypothalamus of adult *Nrp2*^{loxP/loxP} and *Pomc-Cre; Nrp2*^{loxP/loxP} mice; values relative to
797 GAPDH expression are shown. **(B)** Relative levels of *Nrp1* in the pituitary of adult *Nrp2*^{loxP/loxP}
798 (control) and *Pomc-Cre; Nrp2*^{loxP/loxP} (mutant) mice. *Nrp2* mRNA expression in **(C)** the
799 pituitary, and **(D)** ventromedial nucleus of the hypothalamus (VMH), dorsomedial nucleus of
800 the hypothalamus (DMH), lateral hypothalamic area (LHA), paraventricular nucleus of the
801 thalamus (PVT), hippocampus (HIP), cortex (Cx), and medial amygdala (MEA) of *Nrp2*^{loxP/loxP}
802 (control) and *Pomc-Cre; Nrp2*^{loxP/loxP} (mutant) mice. **(E)** Serum leptin, **(F)** insulin, **(G)**
803 triiodothyronine (T3), **(H)** thyroxine (T4), and **(I)** corticosterone levels of adult *Nrp2*^{loxP/loxP}
804 and *Pomc-Cre; Nrp2*^{loxP/loxP} mice. **(J)** Representative confocal images showing α -melanocyte-

805 stimulating hormone (α MSH)-immunoreactive (IR) fibers in the brain of adult *Nrp2*^{loxP/loxP}
806 and *Pomc-Cre; Nrp2*^{loxP/loxP} mice. Relative levels of **(K)** Agouti-related peptide (*Agrp*), **(L)**
807 neuropeptide Y (*Npy*), and **(M)** leptin receptor (*Leprb*) mRNA in the arcuate nucleus of the
808 hypothalamus of adult *Nrp2*^{loxP/loxP} and *Pomc-Cre; Nrp2*^{loxP/loxP} mice. Data are presented as
809 mean \pm SEM. *P<0.05, **P<0.01 versus *Nrp2*^{loxP/loxP} mice. Aq, aqueduct; ARH, arcuate
810 nucleus of the hypothalamus; CEA, central nucleus of the amygdala; vIPAG, ventrolateral
811 periaqueductal grey matter; PBN, parabrachial nucleus; PVT, paraventricular nucleus of the
812 thalamus; SCN, suprachiasmatic nucleus; VMH, ventromedial nucleus of the hypothalamus;
813 V3, third ventricle. Scale bars, 100 μ m.

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818 **STAR METHODS**

819 **CONTACT FOR REAGENT AND RESOURCE SHARING**

820 Further information and requests for resources and reagents should be directed to and will
821 be fulfilled by the Lead Contact, Sadaf Farooqi (isf20@cam.ac.uk).

822

823 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

824 **Studies in humans**

825 The Genetics of Obesity Study (GOOS) is a cohort of 7,000 individuals with severe early-
826 onset obesity; age of obesity onset is less than 10 years (Farooqi et al., 2003; Wheeler et al.,
827 2013). Severe obesity is defined as a body mass index (weight in kilograms divided by the
828 square of the height in meters) standard deviation score greater than 3 (standard deviation
829 scores calculated according to the United Kingdom reference population). The mean age of
830 the subjects with variants was 13.0 ± 1.6 years. 28 were female and 11 were male, mean
831 BMI or BMI SDS did not differ between males and females (35.3 ± 3.4 kg/m² vs 34.9 ± 1.8
832 kg/m²; 3.8 ± 0.2 SDS vs 3.8 ± 0.2 SDS). All studies were approved by the Cambridge Local
833 Research Ethics Committee and each subject (or their parent for those under 16 years)
834 provided written informed consent; minors provided oral consent. Healthy blood donors
835 from the INTERVAL project were used as controls (Moore et al., 2014). All participants gave
836 informed written consent.

837

838 **Studies in cellular models**

839 HEK293 (XX female) and COS7 (XY male) cells were cultured in high glucose Dulbecco's
840 modified eagle medium (DMEM, Gibco, 41965) supplemented with 10% fetal bovine serum

841 (FBS, Gibco, 10270, South America origin), 1% GlutaMAX™ (100X) (Gibco, 35050), and 100
842 units/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, P0781). U87MG (XY male)
843 cells were cultured in MEM with non-essential amino acids (Sigma) supplemented with 10%
844 FBS (Gibco), 1mM Sodium pyruvate, 1% GlutaMAX™ (100X) (Gibco, 35050), and 100
845 units/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, P0781). HUVECs (XX,
846 female) cells were cultured in M199 (with Glutamine) (Sigma-Aldrich, M4530),
847 supplemented with 20% FBS (Gibco), 1% GlutaMAX™ (100X) (Gibco, 35050), 100 units/mL
848 penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, P0781), 1% Vitamins- MEM-EAGLE
849 Vitamin Solution (Gibco, 11120052). HUVEC cells were grown in flasks coated with gelatin
850 (Sigma-Aldrich, G1890). U87MG and HUVEC cells were a kind gift from Gera Neufeld (the
851 Rappaport Institute). Cells were incubated at 37°C in humidified air containing 5% CO₂ and
852 transfections were performed using Lipofectamine 2000™ (Gibco, 11668) in serum-free
853 Opti-MEM I medium (Gibco, 31985) according to the manufacturer's protocols.

854

855 **Studies in zebrafish**

856 All zebrafish experiments were conducted in accordance with the Animals (Scientific
857 Procedures) Act 1986, and following UK Home Office approval (License #: P0662C816). Using
858 CRISPR/Cas9, deletion mutants of zebrafish homologs of Semaphorin3s, PlexinA1-4 and
859 Nrp1-2 were generated and characterised by their length and weight. Zebrafish were reared
860 at equivalent densities and the analyses were conducted prior to any overt sexual
861 differentiation.

862

863

864 **Studies in mice**

865 All animal procedures were conducted in compliance with and approved by the IACUC of
866 the Saban Research Institute of the Children's Hospital of Los Angeles. Animals were housed
867 under specific pathogen-free conditions, maintained in a temperature-controlled room with
868 a 12 h light/dark cycle, and provided *ad libitum* access to water and standard laboratory
869 chow (Special Diet Services). To genetically label Pomc fibers for *in vitro* studies, *Pomc*-Cre
870 mice (Balthasar et al., 2004) were crossed with a ROSA-TdTomato reporter line (Madisen et
871 al., 2010). To generate Pomc-specific Nrp2 knockout (*Pomc*-Cre; *Nrp2*^{loxP/loxP}) mice, *Pomc*-Cre
872 mice were mated to mice carrying a loxP-flanked Nrp2 allele (*Nrp2*^{loxP/loxP}) (Walz et al.,
873 2002). Breeding colonies were maintained by mating *Pomc*-Cre; *Nrp2*^{loxP/+} mice to
874 *Nrp2*^{loxP/loxP} mice. Cre-negative *Nrp2*^{loxP/loxP} were used as controls. All mice were generated in
875 a C57BL/6 background and only male mice were studied.

876

877 **METHOD DETAILS**

878 **STUDIES IN HUMANS**

879 **Sequencing, variant calling, and quality control**

880 Details about sequencing and variant calling for the SCOOP (severely obese) cases
881 (Hendricks et al., 2017), as part of the UK10K exomes, and the INTERVAL controls have been
882 reported previously (Singh et al., 2016). Briefly, single-sample variant calling using GATK
883 Haplotype Caller (v3.2) was performed on the union of Agilent v3 and v5 targets plus a 100
884 base pair flanking region on 9795 UK10K and INTERVAL samples, including SCOOP cases
885 (N=982) and INTERVAL controls (N=4499). The called variants were then merged into 200

886 sample batches and were joint-called using Genotype VCFs and default settings (DePristo et
887 al., 2011; Van der Auwera et al., 2013). To ensure high-quality variant calls across all
888 datasets and sequencing batches, only variants with at least 7x coverage in at least 80% of
889 samples were called. We applied further variants QC keeping only variants with a calibrated
890 VQSR tranche above 99.75% sensitivity, missingness < 20%, Hardy-Weinberg
891 equilibrium χ^2 p-value > 10E-8, mean genotype quality ≥ 30 , and variants in low-complexity
892 regions as described previously (Li, 2014). Further, individual genotypes were set to missing
893 if any of the following was true: GQ<30, alternate allele read depth (DP1)<2, allelic balance
894 (AB) <0.2, or AB>0.8. We used VerifyBamID (v1.0) (Jun et al., 2012) and a threshold of $\geq 3\%$ to
895 identify contaminated samples, principal components calculated from the 1000Genomes
896 Phase I integrated call set (Abecasis et al., 2010) using EIGENSTRAT v4.2 (Price et al., 2006)
897 to identify non-Europeans, and pairwise identity by descent estimates from PLINK
898 v1.07 (Purcell et al., 2007) with a threshold of ≥ 0.125 to identify related
899 individuals. Contaminated, non-European, and related samples were removed resulting
900 in 927 SCOOP cases and 4,057 INTERVAL controls for analysis.

901

902 **Gene-set enrichment**

903 We performed gene-set enrichment similar to previous analyses described in (Purcell et al.,
904 2014; Singh et al., 2016). Briefly, using PLINK/SEQ (Purcell et al., 2007) we calculated
905 individual gene region burden test-statistics for an enrichment in cases compared to
906 controls of very rare (MAF<0.025%) variants meeting one of two predicted functional
907 requirements: functional or loss of function, or only loss of function. We then used the SMP
908 utility to calculate the gene set enrichment while controlling for exome-wide differences

909 between cases and controls. Twenty thousand case control permutations were used to
910 estimate the empirical gene set enrichment p-value on which a Bonferroni adjustment for
911 two tests (i.e. functional and LoF, and LoF only) was applied to arrive at the adjusted p-
912 value.

913

914 **FUNCTIONAL CHARACTERIZATION OF RARE HUMAN VARIANTS**

915 **Cloning and site-directed mutagenesis**

916 The cDNA constructs used throughout the study were made by site-directed mutagenesis
917 using QuikChange II XL kit (Agilent Technologies, 200516) according to the manufacturer's
918 protocols. SEMA3s cDNA constructs contained a C-terminal Myc/DDK tag; NRP1-2 and
919 PLXNA1-4 cDNA constructs contained a N-terminal Glu-Glu tag and C-terminal Myc/DDK tag;
920 all constructs were ligated into pCMV6-Entry vector (Origene) using Sgf I/Mlu I site. All
921 constructs were verified by Sanger sequencing.

922

923 **SEMA3s secretion, cell surface and total cell ELISA**

924 Secreted SEMA3s were detected in the medium of transfected HEK293 cells grown in 96
925 well plates. Medium from these cells was transferred to MaxiSorp plates and bound SEMA3
926 immunodetected with an anti-Flag antibody. Cell surface antigen was quantified on
927 transfected non-permeabilised live HEK293 cells in 96 well plates using an anti-Glu-Glu
928 antibody. Total cell antigen was detected in transfected HEK293 cells in 96-well plates after
929 fixation and permeabilisation using an anti-Flag antibody.

930

931 **Secreted Semaphorin ELISA**

932 50,000 HEK293 cells were seeded into each well of a poly-D-lysine coated 96-well plate.
933 After 24hr the cell medium was exchanged for Opti-MEM (Gibco) and cells transfected. Cell
934 medium was exchanged once more with 50 µl of Opti-MEM containing a 1:400 dilution of
935 Protease inhibitors (Sigma). Forty-eight hours after transfection, cell medium from two
936 identically transfected 96-well plates was pooled together (100 µl total volume) with the
937 addition of 25mM HEPES pH7.5 and centrifuged at 1500rpm for 5 min. The top 80 µl of
938 medium was transferred to black MaxiSorp plates (NUNC), the plates were sealed and
939 incubated overnight at 4°C with gentle agitation. Cell medium was removed and plates were
940 washed thrice for 10 min with gentle agitation before blocking and immunodetection as
941 described in the ELISA procedure below using the anti-Flag antibody and QuantaBlu
942 Fluorogenic Peroxidase Substrate Kit (Thermo).

943

944 **Cell surface and total protein ELISA**

945 40,000 HEK293 cells were seeded into each well of a poly-D-lysine coated 96-well plate and
946 transfected the following day. Forty-eight hours after transfection cells were washed once
947 with PBS and processed for the detection of either cell surface or total cellular antigens. Cell
948 surface protein was detected by blocking live cells on ice (3% dry milk, 50mM Tris, pH 7.4 in
949 PBS) for 30 mins and then incubation with anti-Glu-Glu antibody (Covance) in blocking
950 buffer on ice for 2 hr. Cells were washed thrice for 10 min on ice with PBS and then fixed
951 (3.7% formaldehyde in PBS) for 10 min on ice and then 10 min at room temperature. Cells
952 were washed thrice for 10 min with PBS, blocked again for 30 min and incubated with
953 secondary antibody as described for the total cellular antigen detection procedure. Total

954 cellular antigens were detected in fixed (3.7% formaldehyde in PBS) and permeabilised
955 (0.1% triton in PBS for 5 mins) cells after blocking for 1hr and incubation with anti-flag
956 antibody (Sigma) for 2 hr in blocking buffer. Cells were washed thrice for 10 min with PBS,
957 incubated with anti-mouse HRP antibody (BioRad) (1.5% dry milk, 50mM Tris, pH 7.4 in PBS)
958 for 1 hr, washed thrice more for 10 min with PBS before incubation with HRP substrate
959 (TMB, BioRad) and measurement of absorbance at 450nm.

960

961 **Cell collapse assays**

962 SEMA3-induced cell collapse was tested in U87MG cells grown on a gelatin coated surface.
963 Cells were exposed to SEMA3-conditioned cell medium and collapsed cells micrographed
964 after 30 min and counted manually. Receptor mutants were tested by transfecting COS7
965 cells with *PLXNA*, *NRP*, and GFP, followed by exposure to purified SEMA3C at 2 µg /ml for 30
966 minutes. Collapsed cells were micrographed under blue laser and collapse quantified using
967 Image J.

968

969 Semaphorin expression vectors were transfected into HEK293 cells at 60-90% confluency in
970 10cm dishes. Growth medium was replaced with serum-free medium after 12 hours. After
971 48 hours the medium, containing secreted semaphorin, was harvested, aliquoted, and snap
972 frozen in liquid nitrogen followed by storage at -80°C. The cell contraction assay with
973 mutant ligands was carried out as reported previously (Sabag et al., 2014). U87MG cells
974 were used for SEMA3A, SEMA3B, SEMA3D, SEMA3F, and SEMA3G induced collapse. HUVEC
975 cells were used with SEMA3C and SEMA3E. The morning of the assay 10^5 cells/well were
976 seeded into a 12-well plate coated with gelatin (Sigma, UK). Cells were allowed to attach to
977 the plate surface for 5 hours. Semaphorin-containing medium was then added to the wells

978 at a 1:2 dilution and cells kept at 37°C for 30 min. Following collapse cells the plates were
979 photographed under the microscope and collapsed cells counted individually (at least 100
980 cells per well). To test cell collapse mediated by mutant receptors, COS7 cells were seeded
981 at 2×10^5 cells/well in a gelatin coated 6-well plate, and the following day co-transfected with
982 PlxnA1-4, Nrp1-2, and GFP. After 24 hours purified, recombinant, human SEMA3C (R&D
983 Systems, UK) was added at a concentration of 2 µg/ml and incubated at 37°C for 30 min.
984 Following collapse, cells were photographed under the microscope and collapsed cells were
985 counted using image recognition software on ImageJ (NIH, Bethesda). Collapse efficiency
986 was assessed by counting the proportion of collapsed cells 30 minutes following addition of
987 the WT semaphorin to the culture medium.

988

989 **Ligand binding assay**

990 Cells expressing *NRP1-2* or co-expressing *NRP1-2* and *PLXNA1-4* were incubated with
991 recombinant human SEMA3C-Fc chimera and binding was quantified using an anti-human
992 IgG (Fc specific) antibody. HEK293 cells (40,000 cells/well) were seeded in a poly-D-lysine
993 coated 96-well plate. After 24 hours, 70-80% confluent cells were transiently transfected
994 with WT or mutant NRP1/2 alone or co-transfected with NRP1/2 (WT and mutant) and WT
995 PLXNA1-4. For NRP and PLXNA constructs, the amount of DNA used was 30 and 60 ng,
996 respectively. Twenty four hours after transfection, cells were blocked with 1% BSA in
997 serum-free media (SFM) for 30 min at 37°C then incubated with recombinant human
998 SEMA3C Fc chimera (0.2 – 10 nM) (rhSEMA3c-Fc, R&D System, catalogue number 5570-S3-
999 050) in SFM supplemented with 1% BSA for 30 min at 37°C. Following 5 washes with
1000 Dulbecco's Phosphate Buffered Saline (DPBS), cells were fixed with 3.7% formaldehyde at
1001 room temperature (RT) for 15 min, washed three times with PBS and subsequently blocked

1002 for 30 min at room temperature (1% BSA in 50 mM Tris-Phosphate buffer pH 7.4). For
1003 binding detection, a biotinylated goat-anti-human IgG (Fc specific) (Sigma-Aldrich,
1004 SAB3701279) diluted 1:10000 in blocking buffer was incubated for 1 hour at RT.
1005 Subsequently, the wells were washed three times with PBS and a peroxidase-conjugated
1006 Streptavidin (Thermo) (dilution 1:12000) was added to the wells and incubated for 30
1007 minutes at RT. After three washes, bound SEMA3C was quantified by the addition of 90 μ l of
1008 TMB (Biorad) per well, and stopped by 60 μ l of 0.2M H₂SO₄. Absorbance at 450 nm was
1009 measured using an Infinite M1000 PRO microplate reader (Tecan). Specific binding was
1010 determined by subtracting non-specific binding (cells transfected with empty vector
1011 [pCMV6]) from total binding. Specific binding was plotted and k_d and B_{max} determined using
1012 Prism 6.07 (GraphPad) software.

1013

1014 **Reducing and non-reducing Immunoblotting**

1015 HEK293 cells were seeded in 10 cm dishes and transfected the following day once the cells
1016 had reached 90% confluency. Six hours after transfection the cell medium was replaced with
1017 serum-free medium containing 1:400 dilution of protease inhibitors. 48 hr after transfection
1018 the cell medium was supplemented with additional protease inhibitors. 72 hr after
1019 transfection the cell medium was harvested from the dishes, centrifuged at 1500 rpm for 5
1020 min and the sample was prepared for electrophoresis (resuspended in 1x BOLT LDS sample
1021 buffer (Thermo) and 1x Bolt reducing agent (Thermo) and heated for 10mins at 70°C). Cells
1022 were washed once with PBS and lysed in triton lysis buffer (50 mM Tris pH7.5, 150 mM
1023 NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10
1024 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1% (v/v) triton X-100 and
1025 protease inhibitors (Roche). Lysates were centrifuged at 14,000 rpm for 20 min and the

1026 protein concentration determined using a Bradford assay (BioRad). Equal amounts of
1027 protein were prepared for electrophoresis, as described above. For non-reducing
1028 Immunoblotting, samples were prepared in 1x BOLT LDS sample buffer (without reducing
1029 agent) and heated at 60°C for 5 min. Protein electrophoresis was performed using BOLT gels
1030 (Thermo) and transfer onto nitrocellulose using an iBLOT (Thermo). Membranes were
1031 probed overnight at 4°C using an anti-Flag primary (Sigma) and anti-mouse HRP (DAKO)
1032 secondary antibody or an anti-actin (NEB) primary and anti-rabbit secondary antibody
1033 (DAKO).

1034

1035 **Immunofluorescence and Confocal Microscopy**

1036 80,000 COS7 cells were seeded onto glass coverslips in 12-well plates and transfected. 48 h
1037 after transfection cells were fixed with 4% formaldehyde in PBS for 10 min and washed
1038 three times for 5 min with PBS before membrane staining with 10 µg/ml Alexa Fluor 647
1039 conjugated wheat germ agglutinin for 10 min. Cells were washed three more times,
1040 permeabilised with 0.1% Triton X-100 in PBS for 5 min before being washing again. Cells
1041 were incubated in blocking buffer (3% BSA in PBS) for 1h, then primary antibody (mouse
1042 anti-Flag and Rabbit anti-Calnexin (Cell signaling) diluted in blocking buffer followed by
1043 washing and incubation with secondary antibody (anti-mouse Alexa Fluor 488 and anti-
1044 Rabbit Alexa Fluor 568 (ThermoFisher). Cells were washed and incubated with DAPI (1
1045 µg/ml) for 5 min before being washed again and mounting onto coverslips using mounting
1046 medium (VECTASHIELD with DAPI). Slides were imaged using a Leica SP8 confocal
1047 microscope with a 63x objective (NA 1.4) and images processed using FIJI.

1048

1049

1050

1051 **Structural analysis**

1052 To study how the human *SEMA3-PLXNA-NRP* variants affect signaling, we mapped the
1053 mutated residues onto structural models and assessed their potential effects on stability
1054 and protein-protein interactions. For SEMA3s, all human SEMA3 sequences (SEMA3A-3G)
1055 were first aligned to the sequence of mouse SEMA3A. A homology model encompassing the
1056 first three Sema3 domains, the sema, PSI and Ig domains, was made by combining the sema-
1057 PSI segments of the mouse Sema3A crystal structure at 3.3 Å resolution (PDB 4GZ8)
1058 together with a homology model of the Sema3 Ig domain generated using the 3.0 Å crystal
1059 structure of human Sema4D which includes a more complete Sema-PSI-Ig segment (PDB
1060 1OL2). The human *SEMA3* variants were then mapped onto the equivalent residues in the
1061 mouse Sema3A model. To locate the variants potentially affecting the Sema3-Plxn-Nrp
1062 interfaces, the crystal structure of mouse Sema3A-PlxnA2-Nrp1 (PDB 4GZA) was used as a
1063 reference. Similarly, human PlxnA1-A4 sequences were aligned with mouse PlxnA1 and the
1064 mouse residues corresponding to the human variants were identified. The human *PLXN*
1065 variants on the extracellular segments (domains sema to IPT6) were mapped onto the 4 Å
1066 crystal structure of the mouse PlxnA1 ectodomain (PDB 5L56). Variants in the PlxnA
1067 cytoplasmic domain were mapped onto a homology model of PlxnA1 generated using the
1068 3.3 Å crystal structure of the zebra fish PlxnC1 active dimer (PDB 4M8M). Human *NRP1* and
1069 *NRP2* variants were mapped onto the equivalent positions in the 2.7 Å crystal structure of
1070 mouse Nrp1 (PDB 4GZ9). The MAM domain of the Nrps was not modelled. All protein
1071 sequences were obtained from the UniProt database (<http://www.uniprot.org/>). Protein
1072 sequence alignment was performed using the ClustalW Omega server

1073 (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Homology models were generated and
1074 analysed using Modeller. Structural visualization and image production was performed using
1075 Pymol (<https://pymol.org/2/>).

1076

1077 **STUDIES IN ZEBRAFISH**

1078 Zebrafish homologs of class 3 Semaphorins, class A Plexins and Nrps were identified using
1079 Ensembl GeneTree (ENSGT00760000119048). Phylogenetic trees were constructed from the
1080 zebrafish, human and mouse orthologs using Clustal Omega (Sievers et al., 2011).
1081 Phylogenetic trees were visualized using the Unrooted software ([http://pbil.univ-](http://pbil.univ-lyon1.fr/software/unrooted.html)
1082 [lyon1.fr/software/unrooted.html](http://pbil.univ-lyon1.fr/software/unrooted.html)). Two-five non-overlapping short guide RNAs (gRNA)
1083 targeting each zebrafish gene were identified using the Zebrafish Genomics track data hub
1084 (GA targets) in DanRer 10; gRNA templates were assembled and mRNA produced as
1085 described (Varshney et al., 2015) with the exception that the T7 promoter was substituted
1086 with an SP6 promoter. sgRNAs (200 ng each) were incubated with 600 ng Cas9 protein (New
1087 England Biolabs, #M0646T) for 5 min at 37°C prior to 2 nl being injected into 1-cell stage
1088 zebrafish WIK embryos. For each gRNA, mutagenesis was confirmed using T7 Endonuclease I
1089 (T7E1, New England Biolabs, #M0302), and quantified using TIDE (Brinkman et al., 2014).
1090 gRNA and primer sequences for T7 assays are available from JENM on request. Zebrafish
1091 were reared at equivalent densities. Zebrafish injected with Cas9 protein only were used as
1092 the control group. However, gRNA-only injected zebrafish were also measured and no
1093 difference in somatic growth was observed between Cas9-only fish. Length and weight were
1094 measured as previously described (Parichy et al., 2009). Nile Red staining on postembryonic
1095 larvae was conducted as described (Minchin et al., 2015). Percent body fat was calculated as

1096 previously described (Minchin and Rawls, 2017), in stage matched larvae. For the initial
1097 CRISPR screen, analyses were conducted at 5, 14 and 35 days post fertilisation in three
1098 independent experiments. Between 6 and 25 animals were used for each group within an
1099 experiment. Analyses were conducted prior to any overt sexual differentiation. Standard
1100 length, weight and % body fat were analysed in three additional *nrp2* experiments and used
1101 between 40 and 83 animals in total.

1102

1103 **Immunohistochemical analysis of α MSH and AgRP projections**

1104 Human wild-type *NRP1* and *NRP2* along with the *NRP1* R237Q and *NRP2* A506V variants
1105 were cloned into the pCMV6-Entry vector (Origene), which was then linearized using the
1106 AgeI-HF restriction enzyme (New England Biolabs, #R3552), column cleaned (Zymo Research
1107 Clean & Concentrator, #D4013) and RNA transcribed using the mMessage mMachine T7
1108 Transcription Kit as according to the manufacturers protocol (Thermo Fisher, #AM1344).
1109 Capped RNA was then cleaned using Zymo RNA Clean & Concentrator columns (Zymo
1110 Research Clean & Concentrator, #R1013) and 100 pg injected into one-cell stage wild-type
1111 (WIK) zebrafish embryos. Injected fish were raised at 20 fish/3L until 35 dpf. Thirty-five day
1112 old fish (n=8-9/group) were fixed overnight with 4% paraformaldehyde/borate buffer. The
1113 fish brains were then frozen, sectioned at 20-um thick, and processed for
1114 immunofluorescence using standard procedures (Bouret et al., 2004). Briefly, sections were
1115 incubated with a sheep anti- α MSH antibody (1:40,000, Millipore) and a rabbit anti-AgRP
1116 (1:1,000, Phoenix Pharmaceuticals). The primary antibodies were visualized with Alexa Fluor
1117 568 donkey anti-sheep IgG and Alexa Fluor 488 donkey anti-rabbit IgG (1:200, Millipore).
1118 Sections were counterstained using bis-benzamide (1:10,000, Invitrogen) to visualize cell
1119 nuclei. Two sections through the preoptic area (POA), anterior tuberal nucleus of

1120 hypothalamus (ATN), and lateral hypothalamic nucleus (LH) were acquired using a Zeiss LSM
1121 710 confocal system equipped with a 20X objective. To quantify fibers density, each image
1122 plane was binarized to isolate labeled fibers from the background and to compensate for
1123 differences in fluorescence intensity. The integrated intensity, which reflects the total
1124 number of pixels in the binarized image, was then calculated for each image as previously
1125 described (Bouret et al., 2004). This procedure was conducted for each image stack.

1126

1127 **STUDIES IN MICE**

1128 **Analysis of gene expression**

1129 The hypothalamus of E10, E12, and E14 mouse embryos (n= 3-4/group) as well as the ARH,
1130 DMH, LHA, POA, PVH, SCN, and VMH of P10 male mouse pups and the ARH and PVH of 8-
1131 weeks-old mice (n=5/group) were dissected under a stereomicroscope. In addition, the
1132 hypothalamus of a human fetus at 14 weeks of gestational age and hypothalamic of human
1133 young adults (19.0 years \pm 1.5 year; n = 2/group; generously provided by Dr Prevot, Inserm
1134 U1172, Lille France) was collected. Total RNA was isolated using the Arcturus PicoPure RNA
1135 isolation kit (for mouse tissues) (Life Technologies) or the RNeasy Lipid tissue kit (for human
1136 tissues) (Qiagen). cDNA was generated with the high-capacity cDNA Reverse Transcription
1137 kit (Life Technologies). Quantitative real-time PCR was performed using TaqMan Fast
1138 Universal PCR Mastermix and the commercially available Taqman gene expression primers.
1139 mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the
1140 expression of *Gapdh* housekeeping gene. All assays were performed using an Applied
1141 Biosystems 7900 HT real-time PCR system.

1142

1143 **Real-time PCR primers**

1144 Real-time PCR was performed on Applied Biosystems 7900HT Fast Real-Time PCR System
1145 using TaqMan® Gene Expression Assays (Applied Biosystems): *Pomc* (Mm00435874_m1),
1146 *Npy* (Mm03048253_m1), *Agrp* (Mm00475829_g1), *Leprb* (Mm00440181_m1), *Crh*
1147 (Mm01293920_s1), *Trh* (Mm01963590_s1), *Oxt* (Mm00726655_s1), *Nrp1*
1148 (Mm00435379_m1), *Nrp2* (Mm00803099_m1), *Plxna1* (Mm00501110_m1), *Plxna2*
1149 (Mm00801930_m1), *Plxna3* (Mm00501170_m1), *Plxna4* (Mm00558881_m1), *Sema3a*
1150 (Mm00436469_m1), *Sema3b* (Mm00436477_m1), *Sema3c* (Mm00443121_m1), *Sema3d*
1151 (Mm01224783_m1), *Sema3e* (Mm00441305_m1), *Sema3f* (Mm00441325_m1), *Sema3g*
1152 (Mm01219778_m1), *Gapdh* (Mm99999915_g1).

1153

1154 **Signaling activity of wild-type and mutant SEMA3s in HEK293 cells**

1155 HEK293 cells were grown in monolayers in 5% CO₂ at 37°C, in Dulbecco's modified Eagle's
1156 medium (Life Technologies, Inc.) containing 1 mM sodium pyruvate, 2 mM glutamine, 50
1157 mM glucose, and supplemented with 10% fetal bovine serum (Invitrogen), 100 µg/ml
1158 streptomycin and 100 U/ml penicillin. A cDNA containing the entire coding region of the
1159 human *SEMA3A*, *SEMA3B*, *SEMA3C*, *SEMA3D*, *SEMA3E*, *SEMA3F*, *SEMA3G* was inserted into
1160 a pRK5 plasmid expression vector. Recombinant plasmids containing *SEMA3* cDNAs
1161 harboring the variants identified in obese individuals were then engineered using the
1162 QuickChange mutagenesis protocol (Stratagene). HEK293 cells were transiently transfected
1163 using a fast-forward protocol (Lipofectamine 2000, Invitrogen). Conditioned medium was
1164 collected 48 h after transfection, tested for the presence of Flag by western blot analysis
1165 using an anti-Flag antibody (Sigma-Aldrich).

1166

1167 **Explant co-culture assays**

1168 Brains were collected from P4 Pomc-Cre; TdTomato male mice (for ARH explants) and P8-
1169 P12 wild-type male mice (for PVH, DMH, and VMH explants) and sectioned at a 200-um
1170 thickness with a vibroslicer as previously described. In addition, *Nrp2*^{loxP/loxP} mice received
1171 stereotaxic bilateral injections of AAV5-CMV.HI.eGFP-Cre.WPRE.SV40, in the ARH at P0.
1172 Control group consisted of WT mice that received bilateral stereotaxic injections of AAV5-
1173 CMV.HI.eGFP-Cre.WPRE.SV40 virus into the ARH at P0. Brains were collected for co-culture
1174 experiments at P6. The ARH, PVH, DMH, and VMH were then carefully dissected out of each
1175 section under a stereomicroscope. ARH explants were co-cultured onto a collagen matrix
1176 (BD Bioscience) with either PVH, DMH, or VMH explants (n = 4-12 explants/group from 3-6
1177 independent experiments) or HEK293 cells transfected with Sema3-encoding vectors
1178 described above (n = 6-28 explants/group from 3-6 independent experiments). Control
1179 experiments included co-cultures with control HEK293 cell aggregates and cortical explants.
1180 For the heterochronic cultures, beginning on the first day *in vitro*, each explant were
1181 transferred to fresh modified Basal Medium Eagle (Invitrogen) containing either Nrp1 or
1182 Nrp2 neutralizing antibodies (1.5 ug/ml, R&D Systems) or control goat IgGs. After 48 h, the
1183 explants were fixed in paraformaldehyde and stained with betaIII tubulin (TUJ1 monoclonal
1184 antibody, 1:5,000, Covance) or NPY (1:3,000, Chemicon). Pomc-TdTomato⁺, TUJ1⁺ and NPY⁺
1185 neurites extending from the ARH explants were analyzed as followed. Confocal image stacks
1186 of the co-cultures, that included the proximal and distal edges of the ARH explant, were
1187 acquired using a Zeiss LSM 710 confocal system equipped with a 10X objective. Slides were
1188 numerically coded to obscure the treatment group. Image analysis was performed using
1189 ImageJ analysis software (NIH). For the *in vitro* experiments, each image plane was
1190 binarized, and the total density of Pomc⁺, TUJ1⁺ and Npy⁺ neurites were measured in the

1191 proximal (P) and distal (D) parts of the explant. The P/D ratio is a measure of growth
1192 towards or away from the explant, with a ratio >1 indicating net increase in projections and
1193 <1 indicating decreased axon growth.

1194

1195 **Immunohistochemical analysis of α MSH projections**

1196 15-17-weeks old male mice (n = 4/group) were perfused transcardially with 4%
1197 paraformaldehyde. The brains were then frozen, sectioned at 30-um thick, and processed
1198 for immunofluorescence using standard procedures (Bouret et al., 2004). Briefly, sections
1199 were incubated with a sheep anti- α MSH antibody (1:40,000, Millipore). The primary
1200 antibody was visualized with Alexa Fluor 488 donkey anti-sheep IgG (1:200, Millipore).
1201 Sections were counterstained using bis-benzamide (1:10,000, Invitrogen) to visualize cell
1202 nuclei. Two sections through various anatomical compartments of the PVH (PVHmpd and
1203 PVHpml, neuroendocrine; and postPVH, autonomic) were acquired using a Zeiss LSM 710
1204 confocal system equipped with a 20X objective. For the quantitative analysis of fibers
1205 density, each image plane was binarized to isolate labeled fibers from the background and
1206 to compensate for differences in fluorescence intensity. The integrated intensity, which
1207 reflects the total number of pixels in the binarized image, was then calculated for each
1208 image. This procedure was conducted for each image stack.

1209

1210

1211 **Analysis of Pomc cell numbers**

1212 15-17-weeks old male mice (n = 4/group) were perfused transcardially with 4%
1213 paraformaldehyde. The brains were then frozen, sectioned at 30-um thick, and processed
1214 for fluorescent *in situ* hybridization using standard procedures. Two sections through the

1215 ARH were acquired using a Zeiss LSM 710 confocal system equipped with a 20X objective.
1216 For the quantitative analysis of cell number, the number of Pomc⁺ cell bodies in the ARH
1217 were manually counted using Image J analysis software (NIH). The average number of cells
1218 counted in two ARH hemi-sections from each mouse was used for statistical comparisons.

1219

1220 **Physiological measurements in mice**

1221 Male mice (n ≥ 9/group) were weighed every 2 days from P4 to P22 (weaning) and weekly
1222 from 4 to 14 weeks using an analytical balance. Body composition analysis (fat/lean mass)
1223 was performed in 16-week-old mice (n = 7-8/group) using NMR (Echo MRI). Food intake,
1224 energy expenditure, and locomotor activity were monitored at 17-19 weeks of age using a
1225 combined indirect calorimetry system (TSE Systems) (n =7-8/group). Mice were acclimated
1226 in the monitoring chambers for 2 days then data were collected for 3 days. These
1227 physiological measures were performed at the Rodent Metabolic Core of Children's Hospital
1228 of Los Angeles. Glucose tolerance tests (GTTs) were conducted in 10- to 11-week-old mice
1229 (n= 9-11/group) through i.p. injection of glucose (1.5 mg/g body weight) after overnight
1230 fasting. Blood glucose levels were measured at 0, 15, 30, 45, 60, 90, 120, and 150 min post-
1231 injection. Serum leptin, insulin, T3, T4 levels were assayed in 18-21-week-old fed mice and
1232 corticosterone levels were assayed in 18-21-week-old fed mice (n = 8-11/group) using ELISA
1233 kits (Millipore, Calbiotech and Enzo Life Sciences, respectively).

1234

1235 **Fluorescent *in situ* hybridization**

1236 Antisense digoxigenin-labeled riboprobes were generated from plasmids containing PCR
1237 fragments of *Pomc*. Briefly, sections were postfixed for 10 minutes in 4% paraformaldehyde.
1238 Then, sections were incubated with 1ug/ml Proteinase K (Promega) for 5 min at 37°C and 15

1239 min at RT, respectively. They were then incubated for 10 min in 0.1 M triethanolamine
1240 (TEA), pH 8.0, and then for 5 min at room temperature in 100 mL 0.1 M triethanolamine
1241 (TEA) with 500 μ L glacial acetic acid. Tissue was pre-hybridized for two hours at 62°C in
1242 hybridization buffer (66% (v/v) deionized formamide, 13% (w/v) dextran sulfate, 260 mM
1243 NaCl, 1.3 X Denhardt's Solution, 13 mM Tris pH 8.0, 1.3 mM EDTA pH 8.0). Probes were
1244 denatured at 80°C for 5 minutes before being added to the hybridization buffer, along with
1245 tRNA and DTT for a final concentration of 0.5mg/ml tRNA and 10mM DTT. Tissue was
1246 hybridized overnight at 62°C. After washes in stringency solutions, sections were blocked in
1247 TNB solution (0.5% blocking reagent, Roche). Tissue sections were then incubated for 1 hr at
1248 RT in a horseradish peroxidase-conjugated sheep anti-DIG antibody (1:400, Roche Applied
1249 Sciences). DIG was visualized using a TSA PLUS Biotin Kit (Perkin Elmer). After washes,
1250 sections were incubated for 30 minutes in a 1:50 dilution of the Biotin Amplification Reagent
1251 working solution (prepared following manufacturer's instructions), before incubating for 1
1252 hr in 1:200 streptavidin conjugated to cyanin 2 (Jackson Immunoresearch). Sections were
1253 counterstained using bisbenzamide (1:10,000, Invitrogen), to visualize cell nuclei, and
1254 coverslipped with buffered glycerol (pH 8.5).

1255

1256 **Histomorphological assessment of white adipose tissue**

1257 Male mice were anesthetized at 16-17 weeks of age (n = 5/group). Epididymal adipose
1258 tissue was collected, fixed in a 4% paraformaldehyde solution, sectioned at 5 μ m, and then
1259 stained with a Perilipin A antibody (1:1,000, Sigma) using standard procedures. Images were
1260 taken with a Zeiss LSM 710 confocal microscope with a 20X objective. Determination of
1261 mean size (μm^2) was measured using Image J software (NIH, Image J 1.39 T). The average

1262 adipocyte size measured from three sections in each mouse was used for statistical
1263 comparisons.

1264

1265

1266 **QUANTIFICATION AND STATISTICAL ANALYSIS**

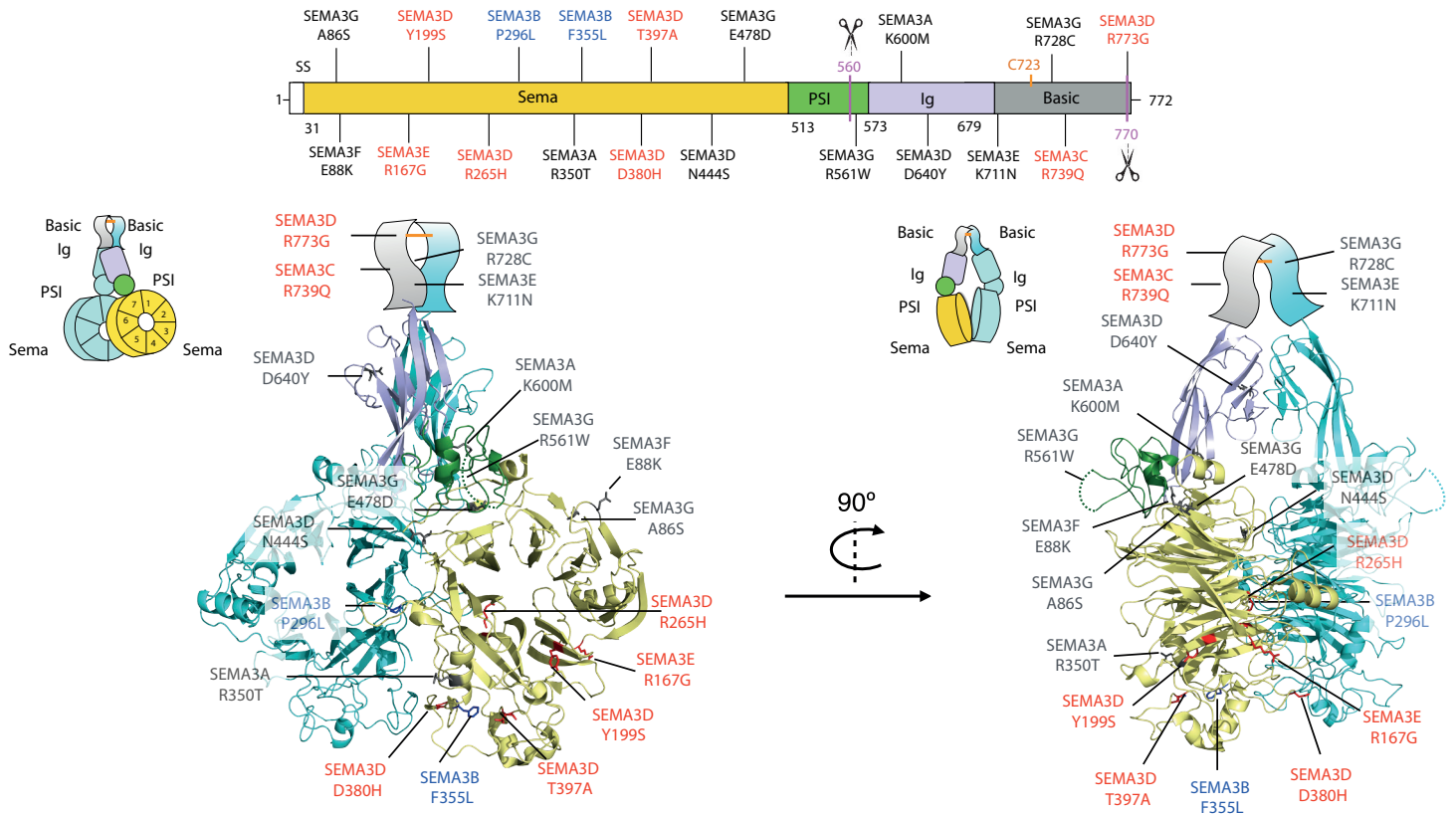
1267 All values were represented as the mean \pm SEM. Studies in cellular models are from at least
1268 3 independent experiments. Numbers for every experiment are found in the relevant part of
1269 the Methods. Explant co-culture assays are derived from 4-28 explants in 3-6 independent
1270 experiments. Mice studies are from 3–11 animals per group. Statistical analyses were
1271 conducted using GraphPad Prism (version 6.07). Data sets with only two independent
1272 groups were analyzed for statistical significance using unpaired two-tailed Student's t test.
1273 Data sets with more than two groups were analyzed using one-way analysis of variance
1274 (ANOVA) followed by the Bonferroni posthoc test. For statistical analyses of body weight
1275 and GTT (mice), we performed two-way ANOVAs followed by Bonferroni's posthoc test.
1276 Statistically significant outliers were calculated using Grubb's test for outliers. $P \leq 0.05$ was
1277 considered statistically significant. Statistical significance is represented as * $P < 0.05$,
1278 ** $P < 0.01$ and *** $P < 0.001$.

1279

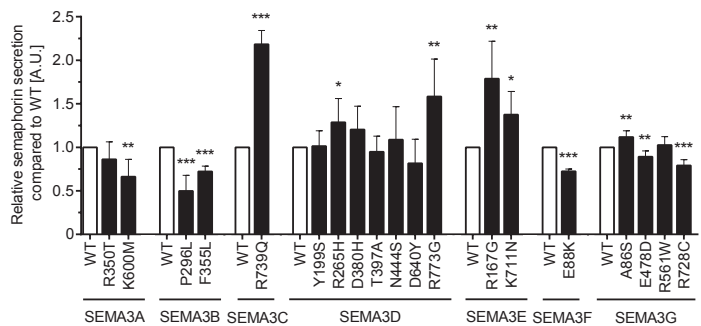
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Figure 1

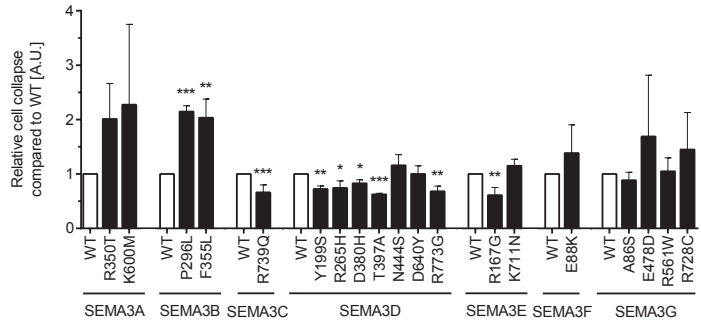
A



B



C



D

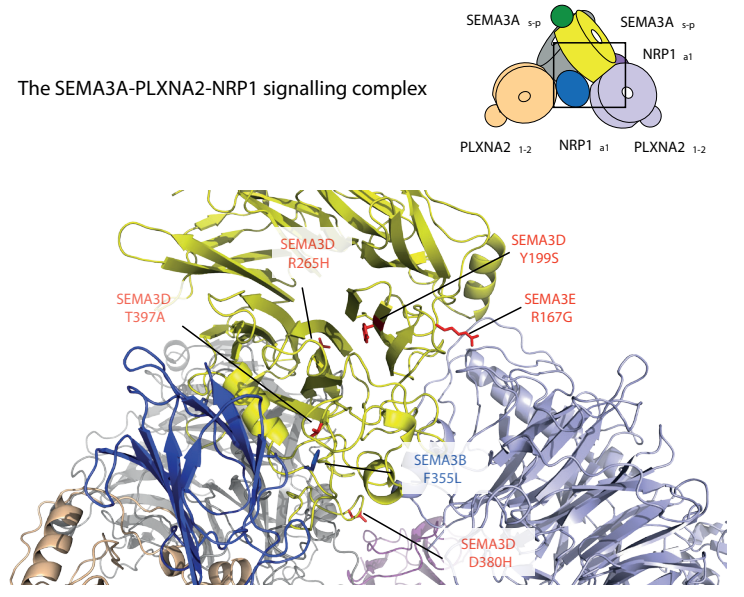


Figure 2

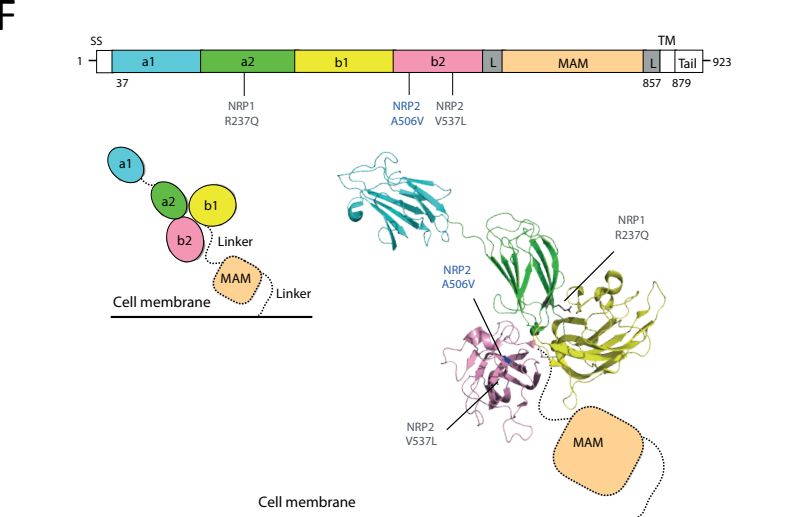
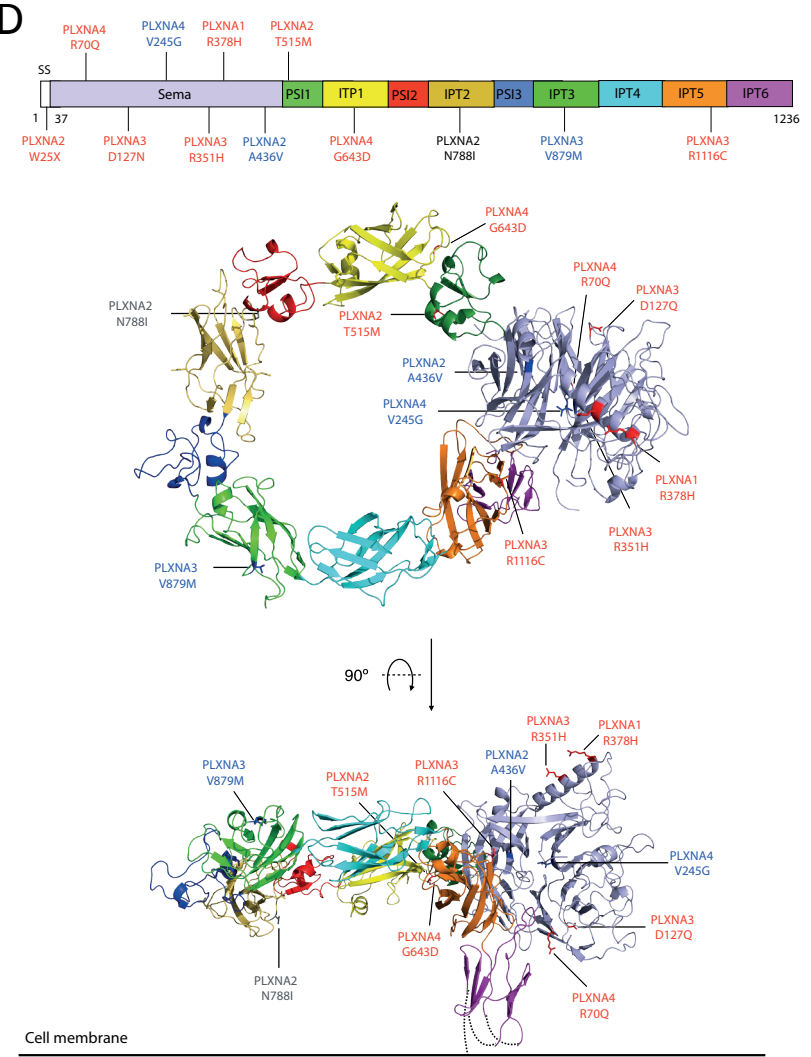
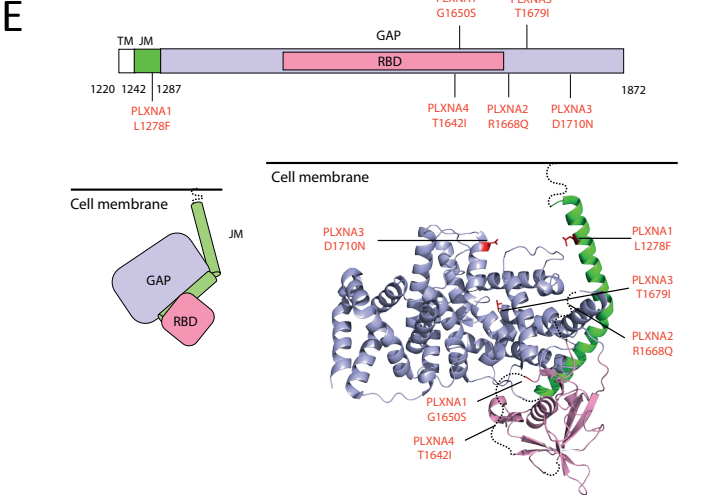
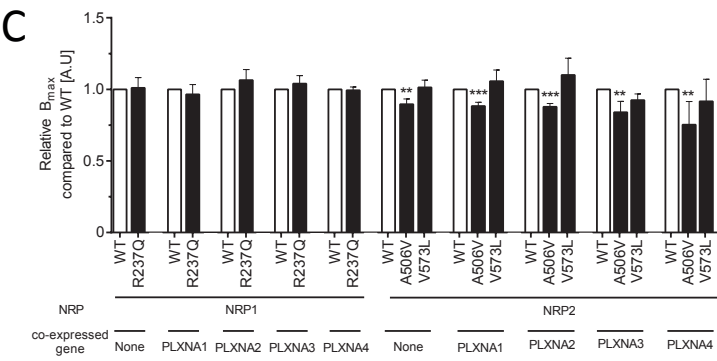
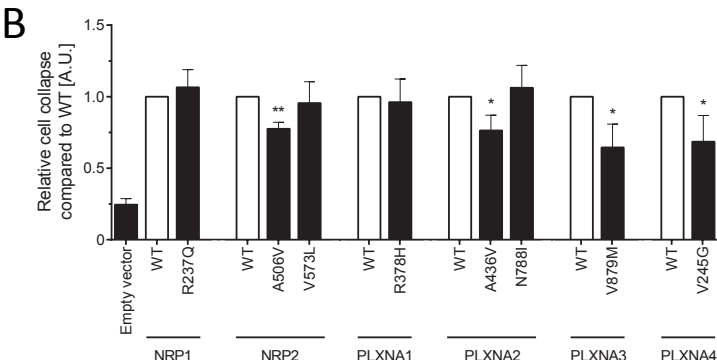
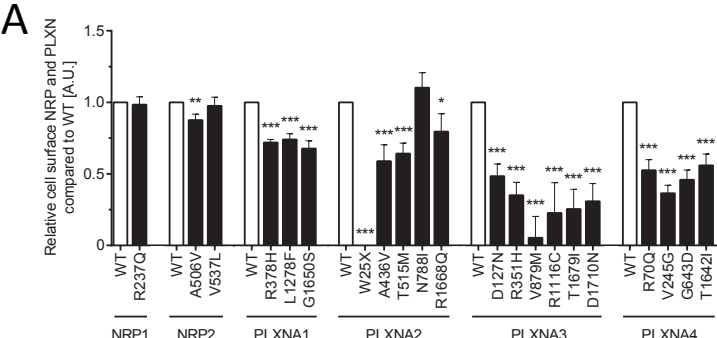


Figure 3

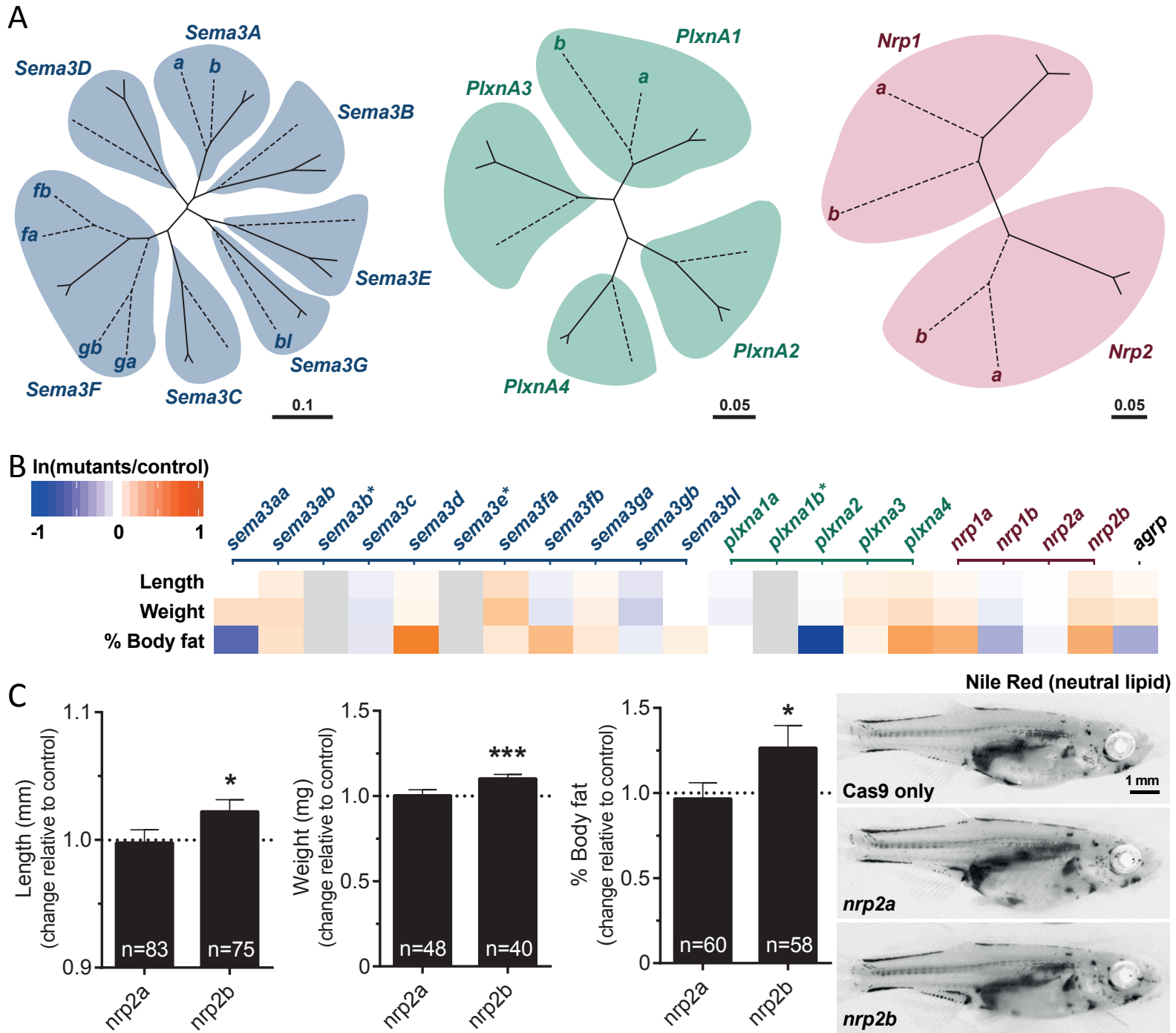


Figure 4

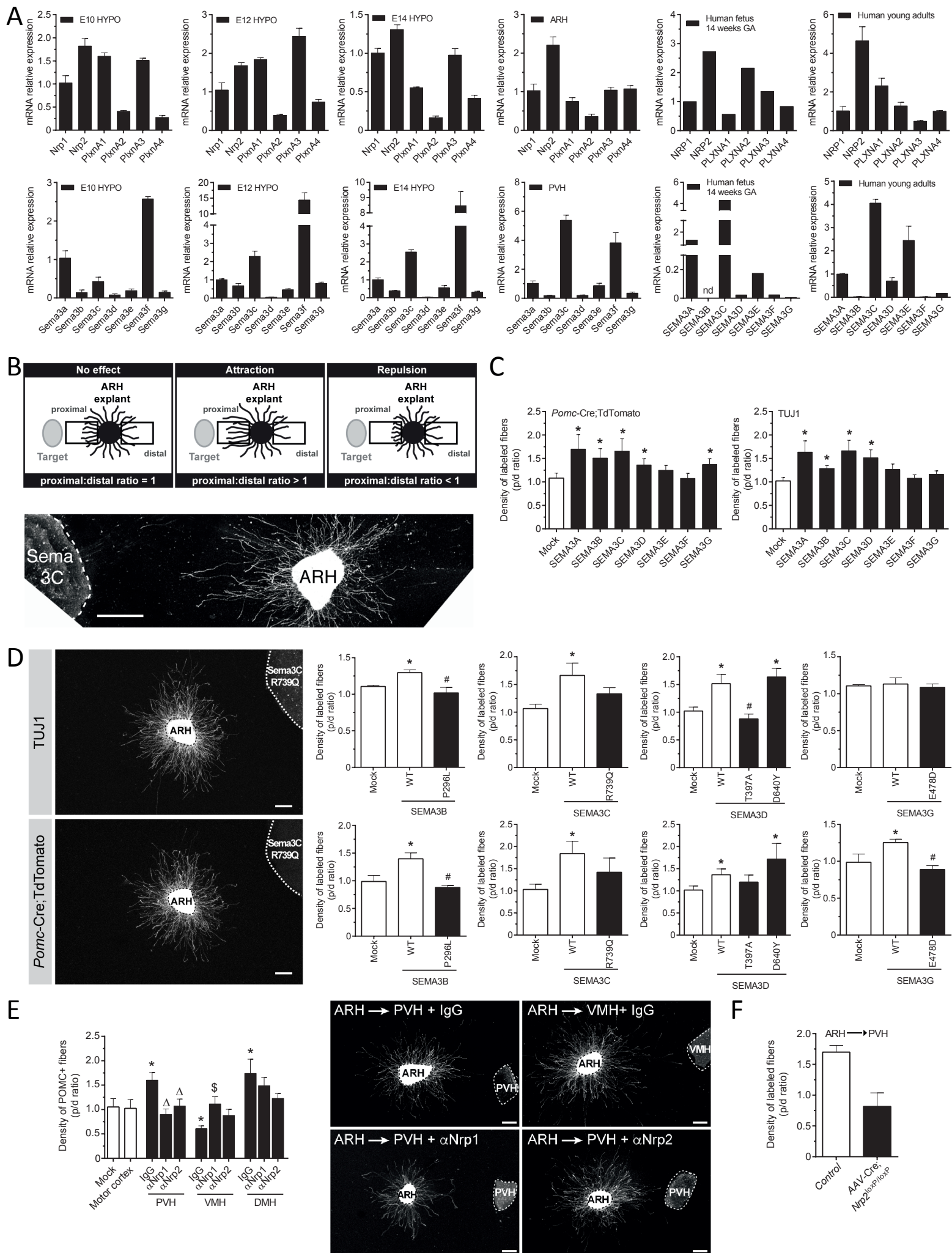
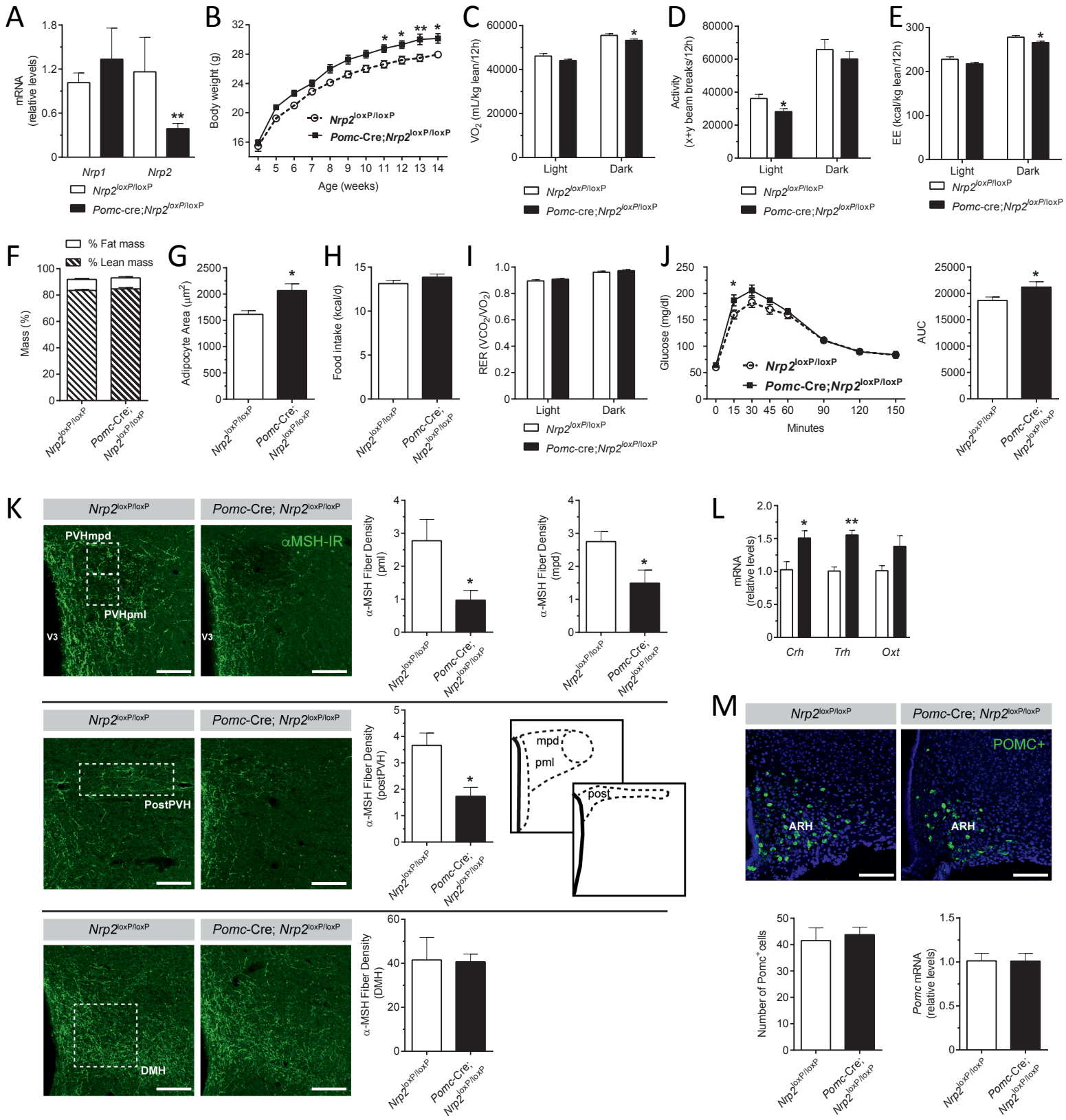
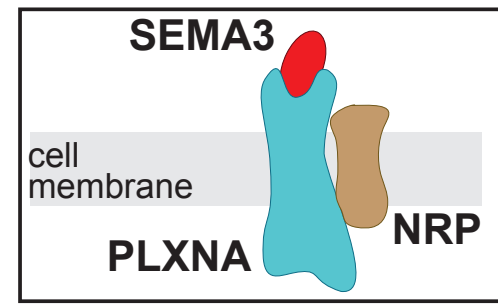
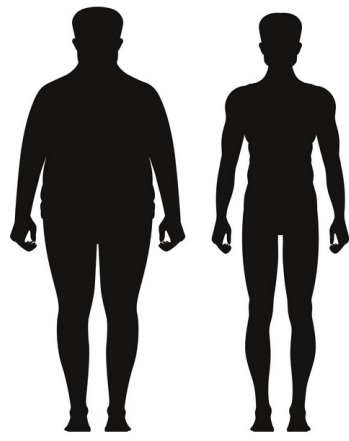


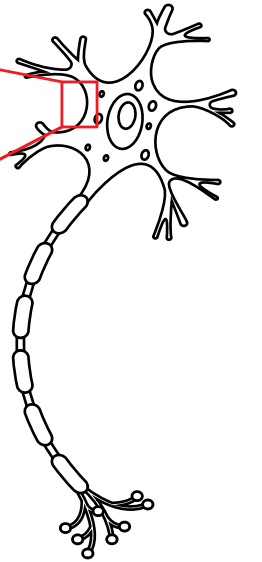
Figure 5



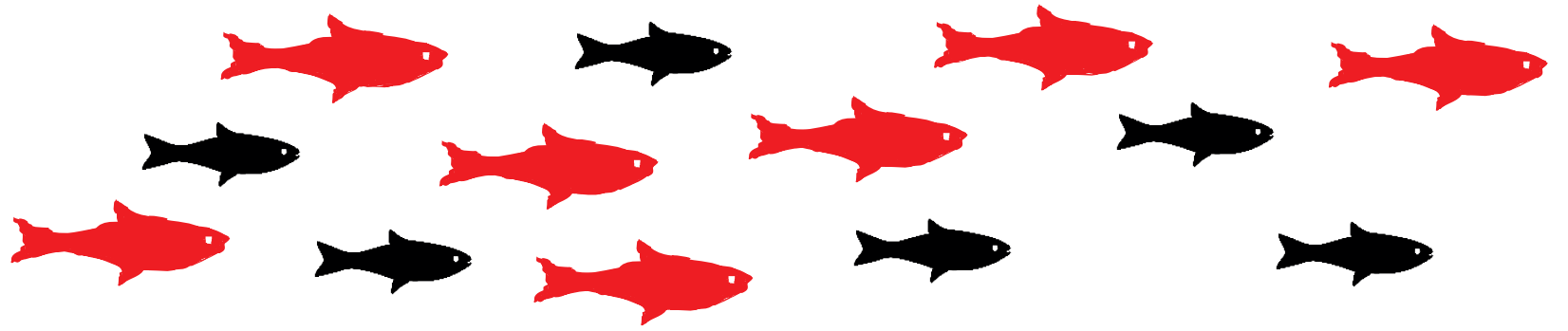
Rare variants in Semaphorin 3s, Neuropilins and Plexins are enriched in severe obesity



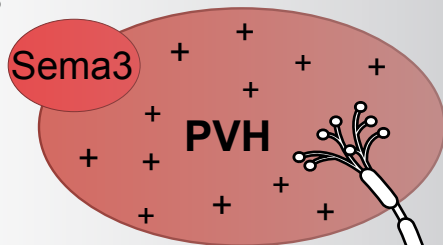
Neuronal development



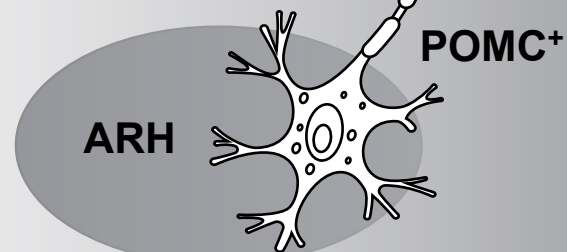
Deletion of 7 out of 13 genes increases somatic growth and/or adiposity in zebrafish



Hypothalamus



Semaphorin 3-NRP signaling directs the development of POMC⁺ projections to the PVH



POMC⁺-specific deletion of Neuropilin 2 leads to weight gain

