

1 **Multiethnic genome-wide meta-analysis of ectopic fat depots identifies loci associated**
2 **with adipocyte development and differentiation**

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4 **AUTHOR BLOCK**

5 Audrey Y Chu^{1,2,48}, Xuan Deng^{3,48}, Virginia A Fisher^{3,48}, Alexander Drong⁴, Yang Zhang^{5,6}, Mary
6 F Feitosa⁷, Ching-Ti Liu³, Olivia Weeks⁶, Audrey C Choh⁸, Qing Duan⁹, Thomas D Dyer¹⁰, John
7 D Eicher¹, Xiuqing Guo¹¹, Nancy L Heard-Costa³, Tim Kacprowski^{12,13}, Jack W Kent Jr¹⁴, Leslie
8 A Lange⁹, Xinggang Liu¹⁵, Kurt Lohman^{16,17}, Lingyi Lu¹⁷, Anubha Mahajan⁴, Jeffrey R
9 O'Connell¹⁵, Ankita Parihar¹⁵, Juan M Peralta¹⁰, Albert V Smith^{18,19}, Yi Zhang²⁰, Georg Homuth¹²,
10 Ahmed H Kissebah^{20,49}, Joel Kullberg²¹, René Laqua²², Lenore J Launer²³, Matthias Nauck^{24,13},
11 Michael Olivier^{14,20}, Patricia A Peyser²⁵, James G Terry²⁶, Mary K Wojczynski⁷, Jie Yao¹¹,
12 Lawrence F Bielak²⁵, John Blangero¹⁰, Ingrid B Borecki⁷, Donald W Bowden^{27,28}, John Jeffrey
13 Carr²⁶, Stefan A Czerwinski²⁹, Jingzhong Ding^{16,30}, Nele Friedrich^{24,13}, Vilmunder Gudnason^{18,19},
14 Tamara B Harris²³, Erik Ingelsson^{31,32}, Andrew D Johnson¹, Sharon LR Kardia²⁵, Carl D
15 Langefeld¹⁷, Lars Lind²¹, Yongmei Liu^{16,33}, Braxton D Mitchell^{15,34}, Andrew P Morris^{35,4}, Thomas
16 H Mosley Jr³⁶, Jerome I Rotter¹¹, Alan R Shuldiner¹⁵, Bradford Towne⁸, Henry Völzke^{37,13,38},
17 Henri Wallaschofski²⁴, James G Wilson³⁹, Matthew Allison⁴⁰, Cecilia M Lindgren⁴¹, Wolfram
18 Goessling^{6,42,43,44,45}, L Adrienne Cupples^{1,3,50}, Matthew L Steinhauser^{5,6,45,46,50}, Caroline S
19 Fox^{1,47,50}

20

21

22 **AFFILIATIONS**

23

- 24 1. NHLBI's Framingham Heart Study, Framingham MA USA
25 2. Division of Preventive Medicine, Brigham and Women's Hospital and Harvard Medical
26 School, Boston MA USA
27 3. Department of Biostatistics, Boston University School of Public Health, Boston MA USA
28 4. Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford UK
29 5. Department of Medicine, Brigham and Women's Hospital and Harvard Medical School,
30 Boston MA USA
31 6. Division of Genetics, Brigham and Women's Hospital and Harvard Medical School,
32 Boston MA USA
33 7. Department of Genetics, Washington University, St. Louis MO USA
34 8. Division of Epidemiology and Biostatistics, Department of Population and Public Health
35 Sciences, Wright State University Boonshoft School of Medicine, Dayton OH USA
36 9. Department of Genetics, University of North Carolina, Chapel Hill NC USA
37 10. South Texas Diabetes and Obesity Institute, University of Texas Health Science Center
38 at San Antonio & University of Texas of the Rio Grande Valley, Brownsville TX USA
39 11. Institute for Translational Genomics and Population Sciences, Department of Pediatrics,
40 LABioMed at Harbor-UCLA Medical Center, Torrance CA USA

- 41 12. Interfaculty Institute for Genetics and Functional Genomics, University Medicine
42 Greifswald, Greifswald Germany
- 43 13. German Centre for Cardiovascular Research (DZHK), Partner Site Greifswald, Germany
- 44 14. TOPS Nutrition and Obesity Research Center, Department of Genetics, Texas
45 Biomedical Research Institute, San Antonio TX USA
- 46 15. University of Maryland School of Medicine, Baltimore MD USA
- 47 16. Wake Forest School of Medicine, Winston-Salem NC USA
- 48 17. Department of Biostatistical Sciences, Wake Forest School of Medicine, Winston-Salem
49 NC USA
- 50 18. Icelandic Heart Association, Kopavogur Iceland
- 51 19. Faculty of Medicine, University of Iceland, Reykjavik Iceland
- 52 20. TOPS Obesity and Metabolic Research Center, Biotechnology and Bioengineering
53 Center, Department of Physiology at the Medical College of Wisconsin, WI USA
- 54 21. Department of Surgical Sciences, Section of Radiology, Uppsala University, Uppsala
55 Sweden
- 56 22. Department of Neuroradiology, University Hospital Berne, Berne Switzerland
- 57 23. National Institute on Aging, Intramural Research Program, National Institutes of Health,
58 Bethesda MD USA
- 59 24. Institute for Clinical Chemistry and Laboratory Medicine, University Medicine Greifswald,
60 Greifswald Germany
- 61 25. Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor
62 MI USA
- 63 26. Departments of Radiology and Radiologic Sciences, Cardiovascular Medicine and
64 Biomedical Informatics, Vanderbilt University Medical Center, Nashville TN USA
- 65 27. Center for Genomics and Personalized Medicine Research, Wake Forest University
66 Health Sciences, Winston-Salem NC USA
- 67 28. Department of Biochemistry, Center for Diabetes Research, and Center for Human
68 Genomics, Wake Forest University School of Medicine, Winston-Salem NC USA
- 69 29. Department of Epidemiology, Human Genetics and Environmental Sciences, University
70 of Texas Health Science Center (UTHealth) School of Public Health Brownsville
71 Campus, Brownsville TX USA
- 72 30. Gerontology and Geriatric Medicine, Wake Forest School of Medicine, Winston-Salem
73 NC USA
- 74 31. Department of Medical Sciences, Molecular Epidemiology and Science for Life
75 Laboratory, Uppsala University, Uppsala Sweden
- 76 32. Department of Medicine, Division of Cardiovascular Medicine, Stanford University
77 School of Medicine, Stanford CA USA
- 78 33. Department of Epidemiology and Prevention, Wake Forest School of Medicine, Winston-
79 Salem NC USA
- 80 34. Geriatrics Research and Education Clinical Center, Baltimore Veterans Administration
81 Medical Center, Baltimore MD USA
- 82 35. Department of Biostatistics, University of Liverpool, Liverpool UK
- 83 36. University of Mississippi Medical Center, Jackson MS USA
- 84 37. Institute for Community Medicine, University Medicine Greifswald, Greifswald Germany

- 85 38. German Centre for Diabetes Research (DZD), Site Greifswald, Germany
86 39. Department of Physiology and Biophysics, University of Mississippi Medical Center,
87 Jackson MS USA
88 40. Division of Preventive Medicine, Department of Family Medicine and Public Health, UC
89 San Diego School of Medicine, San Diego CA USA
90 41. Li Ka Shing Centre for Health Information and Discovery, The Big Data Institute,
91 University of Oxford, Oxford, UK.
92 42. Harvard Stem Cell Institute, Cambridge MA USA
93 43. Gastroenterology Division, Brigham and Women's Hospital, Harvard Medical School,
94 Boston MA USA
95 44. Dana-Farber Cancer Institute, Boston MA USA
96 45. Broad Institute of MIT and Harvard, Cambridge MA USA
97 46. Division of Cardiovascular Medicine, Brigham and Women's Hospital and Harvard
98 Medical School, Boston MA USA
99 47. Division of Endocrinology, Brigham and Women's Hospital and Harvard Medical School,
100 Boston MA USA
101 48. These authors contributed equally to this work
102 49. This author is deceased
103 50. These authors jointly supervised this work

104
105 Correspondence should be addressed to AYC (audrey.chu@nih.gov), MLS
106 (msteinhauser@partners.org), or CSF (foxca@nhlbi.nih.gov)

107
108 **ADDRESSES FOR CORRESPONDENCE:**

109 Audrey Y Chu, PHD
110 NHLBI's Framingham Heart Study
111 Framingham MA 01702 USA
112 audrey.chu@nih.gov

113
114 Matthew L. Steinhauser, MD
115 Brigham and Women's Hospital and Harvard Medical School
116 Boston MA 02115 USA
117 msteinhauser@partners.org

118
119 Caroline S Fox, MD MPH
120 NHLBI's Framingham Heart Study
121 Framingham MA 01702 USA
122 foxca@nhlbi.nih.gov

123
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129 **INTRODUCTORY PARAGRAPH**

130 Variation in body fat distribution contributes to the metabolic sequelae of obesity. The genetic
131 determinants of body fat distribution are poorly understood. The goal of this study was to gain
132 new insights into the underlying genetics of body fat distribution by conducting sample-size
133 weighted fixed-effects genome-wide association meta-analyses in up to 9,594 women and
134 8,738 men for six ectopic fat traits in European, African, Hispanic, and Chinese ancestry
135 populations, with and without sex stratification. In total, 7 new loci were identified in association
136 with ectopic fat traits (*ATXN1*, *UBE2E2*, *EBF1*, *RREB1*, *GSDMB*, *GRAMD3* and *ENSA*; $P < 5 \times 10^{-8}$;
137 $FDR < 1\%$). Functional analysis of these genes revealed that loss of function of both *ATXN1*
138 and *UBE2E2* in primary mouse adipose progenitor cells impaired adipocyte differentiation,
139 suggesting a physiological role for *ATXN1* and *UBE2E2* in adipogenesis. Future studies are
140 necessary to further explore the mechanisms by which these genes impact adipocyte biology
141 and how their perturbations contribute to systemic metabolic disease.

142 **MAIN TEXT**

143 Variation in body fat distribution is associated with cardiometabolic risk, including
144 diabetes, hypertension and coronary heart disease,¹⁻⁵ and is at least partially independent of
145 total adiposity. Adipose tissue can be quantified non-invasively using computed tomography
146 (CT) and magnetic-resonance imaging (MRI) to measure fat volume and fat attenuation in
147 different tissue compartments. We previously demonstrated that both indices, in addition to
148 relative fat distribution, are important predictors of cardiometabolic risk.⁶⁻¹¹

149 Several lines of evidence suggest a unique genetic component to body fat distribution.
150 First, indices of body fat distribution are heritable with values ranging from 36-47%, even after
151 adjustment for body mass index (BMI).¹² Second, unique genetic loci exist for body fat
152 distribution. For example, we identified a SNP associated with pericardial fat¹³ that was not
153 associated with visceral fat,¹² BMI or waist-hip-ratio (WHR).^{14,15} Third, several lipodystrophy
154 syndromes, characterized by abnormal body fat distribution, are genetically mediated.¹⁶

155 The current study presents a genome-wide association study and meta-analysis of
156 adipose tissue traits derived from imaging biomarkers (Supplementary Table 1) from 2.6 million
157 SNPs in up to 9,594 women and 8,738 men of European, African, Hispanic and Chinese
158 ancestry (see Supplementary Tables 2, 3 and 4) and uses mouse models to characterize
159 selected loci.

160 Subcutaneous and visceral adipose tissue (SAT, VAT) were previously estimated to
161 have heritabilities of 57% and 36%, respectively^{12,17} (Supplementary Table 5). To assess the
162 genetic contribution to variation in fat attenuation traits, which serve as indirect markers of fat
163 quality (SAT Hounsfield Units [SATHU] and VATHU), heritability (H^2) was estimated in 3,312
164 women and men in the Framingham Heart Study (FHS), and found to be between 29-31%
165 ($P < 1 \times 10^{-15}$). To assess the shared genetic contribution between ectopic fat traits, the genetic
166 correlations were estimated among 3,336 women and men in FHS. Moderate to strong
167 statistically significant correlations were observed between almost all ectopic fat traits pairs

168 (0.35 to 0.67 and -0.74 to -0.35, all $P < 5 \times 10^{-4}$; Supplementary Table 6), suggesting shared loci
169 between ectopic fat traits. However, not all genes were shared between traits ($P < 5 \times 10^{-11}$ for
170 non-overlapping correlations for all pairwise comparisons). The genetic correlations across the
171 ectopic fat traits are also reflected in the phenotypic correlations (Supplementary Table 7).

172 In this combined multiethnic sample-size weighted fixed-effects meta-analysis^{18,19} of up
173 to 18,332 participants, a total of 11 locus-trait associations (7 novel and 4 known) attained
174 genome-wide significance ($P < 5 \times 10^{-8}$) out of 27 genomic scans (from analysis of 9 traits and
175 models in 3 strata – overall, women and men). Of the 7 novel loci, 3 were associated with
176 volumetric subcutaneous (*GSDMB*) and visceral fat traits (*GRAMD3* and *RREB1*), 2 were
177 associated with pericardial fat (*ENSA* and *EBF1*), 1 was associated with fat attenuation
178 (*ATXN1*), and 1 was associated with relative fat distribution (VAT/SAT ratio [*UBE2E2*]) (Table 1;
179 Supplementary Figures 1a-g; with imputation quality in Supplementary Table 8). Associations
180 were robust across ancestry-stratified sensitivity analyses (Supplementary Figures 2a-g and 3a-
181 g; Supplementary Table 9). Manhattan plots and QQ plots for each analysis showed minimal
182 inflation of association test statistics (Supplementary Figures 4a-g). The remaining 4 loci
183 (*LYPLAL1*, *LY86*, *FTO*, *TRIB2*) attaining genome-wide significance were previously
184 identified.^{12,13}

185 rs2123685, located between the 3' untranslated regions of *ZBP2* and *GSDMB*, was
186 associated with SAT in women only ($P_{\text{women}} = 3.4 \times 10^{-8}$, Supplementary Table 10a). Investigation
187 of related ectopic traits among women revealed a direction-consistent nominal association with
188 VAT ($P = 4.8 \times 10^{-4}$). SNPs at *FTO*, the canonical-BMI locus, attained genome-wide significance
189 in association with SAT in the overall sample ($P = 1.4 \times 10^{-9}$).

190 The newly identified association at *RREB1* with VATadjBMI (rs2842895, $P = 1.1 \times 10^{-8}$)
191 was observed in the overall sample and both sexes (Supplementary Table 10b). Examination
192 of related ectopic traits demonstrated nominal associations with VAT and VAT/SAT ratio adjBMI
193 ($P = 4.8 \times 10^{-5}$ and $P = 8.9 \times 10^{-6}$ respectively). The newly identified association of rs10060123 near

194 *GRAMD3* for VATadjBMI was specific to women ($P=4.5\times 10^{-8}$). This locus was nominally
195 associated with VAT and VAT/SAT ratio adjBMI in women (Supplementary Table 10c).

196 PAT represents distinct ectopic fat deposition around the heart. Two findings in the
197 overall sample at the *ENSA* and *EBF1* loci ($P=2.8\times 10^{-9}$ and 1.0×10^{-9} , respectively, Table 1) have
198 not been previously associated with ectopic fat, general adiposity or body fat distribution.
199 Associations at *ENSA* and *EBF1* did not appear to be sex-specific (Supplementary Tables 10d
200 and 10e). Further investigation of the *ENSA* and *EBF1* loci showed no associations with SAT,
201 VAT or VAT/SAT ratio, underscoring their specificity to PAT. *TRIB2* was associated with PAT in
202 this and our prior meta-analysis ($P<5\times 10^{-8}$).¹³

203 Cellular characteristics of fat quality, such as lipid content, vascularity, and adipocyte
204 size and number, may be important factors influencing metabolic risk,^{7,10} but direct assessment
205 is invasive. Fat attenuation traits, assessed with computed tomography, are correlated with fat
206 quality characteristics^{20,21} and thus represent indirect markers of fat quality. *ATXN1* was
207 associated with SATHU among men only ($P=1.4\times 10^{-8}$) with no association among women
208 ($P=0.36$, Supplementary Table 10f). Examination of related ectopic fat traits indicated similar
209 direction of association with VATHU, and opposite direction for SAT and VAT (Supplementary
210 Table 10f) which is consistent with epidemiology findings.⁷

211 The ratio of visceral to subcutaneous fat volumes (VAT/SAT ratio) represents the
212 propensity to store fat viscerally. *UBE2E2* was associated with VAT/SAT ratio ($P=3.1\times 10^{-10}$); a
213 nominal association was also identified with VAT ($P=1.4\times 10^{-3}$) but not SAT, suggesting the
214 finding is mostly driven by the higher relative abundance of VAT. The direction of association in
215 both sex strata was consistent (Supplementary Table 10g). Two known body fat distribution
216 loci, *LYPLAL1* and *LY86*, were also associated with VAT/SAT ratio at genome-wide significance
217 (Table 1), consistent with our prior analyses.^{12,22}

218 Calculation of false discovery rate (FDR) to account for multiple testing across the 27
219 meta-analyses showed all ectopic fat loci that attained genome-wide significance in each
220 individual GWAS ($P < 5 \times 10^{-8}$) also attained an $FDR < 1\%$.

221 To examine the association of the 7 newly identified ectopic fat loci with BMI and WHR,
222 cross-trait evaluations for each lead SNP were performed in the most recent GIANT meta-
223 GWAS, with sample sizes ~10-20 times larger than the current study.^{14,15} Only 2 out of 14 SNP-
224 trait (BMI or WHR) associations were significant after Bonferroni correction for multiple testing
225 ($P < 0.05/14 = 3.6 \times 10^{-3}$; Supplementary Table 10a-g), highlighting the specificity and uniqueness
226 of the ectopic fat loci.

227 To evaluate the relationship between the known 97 BMI and 49 WHR loci^{14,15} and
228 ectopic fat traits, we examined the association for these loci with fat volume and relative fat
229 volume traits among the combined multiethnic sample of women and men. Because the
230 ectopic fat data may be underpowered to determine statistically significant results, we
231 hypothesized that the direction of the BMI and WHR findings would be directionally consistent
232 with abdominal ectopic traits, even if the p-values were not significant (Supplementary Table
233 11). Direction consistent SNP-trait associations between SAT and BMI were observed for 87 of
234 97 loci ($P_{\text{binomial}} = 8.9 \times 10^{-17}$). When restricted to the 27 loci nominally associated with SAT
235 ($P_{\text{SAT}} < 0.05$), all 27 SNP-SAT associations were directionally consistent with BMI
236 ($P_{\text{binomial}} = 7.5 \times 10^{-9}$). SAT is not an ectopic fat depot and may represent a metabolic sink for
237 healthier fat storage that is highly correlated with BMI and shares genetic risk factors (as shown
238 with the enriched number of direction consistent associations), yet also represents a unique
239 metric of fat distribution with unique genetic influences (as shown with the *GSDMB*-SAT
240 association). No other traits showed directionally consistent associations with the BMI or WHR
241 (all $P > 0.05$). These results further underscore how ectopic fat traits are uniquely disparate traits
242 as compared to BMI and WHR.

243 Ectopic fat depots are associated with cardiometabolic risk and cardiovascular events.⁸⁻

244 ¹¹ To gain insight into potential mechanisms linking these conditions, we evaluated the
245 association of the new ectopic fat loci with traits from large-scale genetics consortia. Of 66 pairs
246 of lead SNP-trait associations examined, 3 associations (*UBE2E2*-type 2 diabetes [T2D], *EBF1*-
247 triglycerides, and *EBF1*-HDL cholesterol) were statistically significant after Bonferroni correction
248 for multiple testing ($P < 0.05/66 = 8 \times 10^{-4}$; Supplementary Table 12).

249 To examine if any of the new variants overlap with known regulatory regions in adipose
250 tissue, lead SNPs and variants in linkage disequilibrium (LD) with the lead SNPs ($r^2 > 0.8$) were
251 interrogated using ENCODE Consortium data implemented in HaploReg²³ and RegulomeDB.²⁴
252 Except for *ATXN1*, all other loci contained SNPs in LD with the lead SNP that overlapped with
253 known regulatory regions in adipose tissue. For example, the lead *UBE2E2* variant
254 (rs7374732), and other SNPs in LD, overlapped with a known enhancer region in adipose
255 derived stem cells (Supplementary Table 13).

256 The list of candidate loci was further prioritized based on visual examination of regional
257 association plots (Supplementary Figures 1a-g) and identification of 1) a localized association
258 within a gene body at each locus (*RREB1*, *ATXN1* and *UBE2E2*), or 2) a localized association
259 near the gene body concomitant with the lack of other genes within 1Mbp of the lead SNP
260 (*EBF1*). In applying these criteria, four genes were selected for additional functional study.

261 To test the hypothesis that inter-depot differences in gene expression or their dynamic
262 regulation during adipocyte development would identify candidates with a higher likelihood of
263 functional significance, expression of 4 genes (*Ebf1*, *Rreb1*, *Atxn1*, *Ube2e2*) in murine SAT,
264 VAT, and PAT depots was assessed by qPCR. *Ube2e2* was expressed more highly in the
265 perigonadal VAT of 6 week-old C57BL/6 mice relative to the SAT (2.1 fold, $p < 0.05$, $n = 5$) or PAT
266 (2.6 fold, $p < 0.01$, $n = 5$), but no differences were observed for *Ebf1*, *Rreb1* or *Atxn1* (Figure 1a).
267 Differential gene expression of these 4 genes was also assessed in murine diet-induced
268 obesity. A 2.1 fold induction of *Atxn1* expression in SAT of diet-induced obese mice was

269 observed relative to lean controls ($p < 0.05$, $n = 6$). Significant differences were not observed for
270 *Ebf1*, *Rreb1*, or *Ube2e2* in response to the obesogenic stimulus (Figure 1b).

271 To explore a potential role for the candidate genes in adipocyte development, we
272 examined their regulation during *ex vivo* adipogenic differentiation of progenitor-rich stromal-
273 vascular cell fractions isolated from the subcutaneous and visceral depots of C57BL/6 mice.
274 Candidate gene expression was measured at regular intervals during adipogenic differentiation.
275 In progenitors isolated from both VAT and SAT, we observed a significant down-regulation of
276 *Atxn1*, *Ube2e2*, and *Ebf1* during adipogenesis (Figure 1c and Supplementary Figure 5).
277 However, in all three instances the expression returned to near baseline levels by 96h post-
278 adipogenic induction. In contrast, no significant transcriptional regulation of *Rreb1* after
279 adipogenic induction was observed (Supplementary Figure 5).

280 Both *Atxn1* and *Ube2Ee2* showed evidence of dynamic regulation of gene expression
281 during adipogenesis with variable depot-specific expression in the murine models providing
282 rationale to further explore their functional significance with a genetic loss-of-function assay.
283 Knock-down of both genes with specific shRNA retroviral constructs during *ex vivo*
284 adipogenesis of SAT progenitors impaired the formation of lipid-containing adipocytes relative to
285 vector control infected cells, whereas only *Ube2e2* knock-down impaired adipogenesis in
286 progenitors isolated from VAT (Figure 1d,e).

287 Our findings provide insight into the genetics of body fat distribution. The scant number
288 of significant associations observed between the ectopic fat loci and more general measures of
289 adiposity, such as BMI and WHR,^{14,15} demonstrates the specificity of the ectopic fat
290 associations, highlights the utility of precise phenotyping of fat distribution, and suggests
291 different mechanisms involved in ectopic fat storage compared to more general adiposity
292 measures. This specificity was particularly notable for PAT loci, which demonstrate no
293 associations with SAT, VAT, VAT/SAT ratio, BMI or WHR.

294 In addition, few cross-trait associations were observed for ectopic fat loci and other
295 cardiometabolic traits, which is striking given the epidemiologic associations between ectopic fat
296 and cardiometabolic risk¹⁻⁵. One notable exception is *UBE2E2*, which is a known T2D locus^{25,26}.
297 The lead T2D SNP does not appear to be in LD with the lead SNP from our study (r^2 [rs7374732,
298 rs7612463]<0.08 across all HapMap2 populations), and therefore likely represents an
299 independent signal. The major allele at rs7374732 is associated with both lower VAT/SAT ratio
300 and lower risk of T2D, suggesting that targeting relative fat distribution may have beneficial
301 downstream effects.

302 Functional studies support a physiologic role for *UBE2E2* and *ATXN1* through regulation
303 of adipocyte differentiation. *ATXN1* encodes a chromatin binding factor involved in the
304 repression of Notch signaling. It has been implicated in neurologic diseases, including
305 spinocerebellar ataxia 1, but there are no reported associations between SNPs in *ATXN1* and
306 adiposity-related traits. In contrast, *UBE2E2* is a known T2D GWAS locus,²⁵⁻²⁷ although the
307 markers are in low LD with the lead SNP in the present study. *UBE2E2* (3p24.2) encodes the
308 ubiquitin-conjugating enzyme E2E2, which is expressed in human pancreas, liver, muscle and
309 adipose tissues. The present GWAS results highlight *UBE2E2* in association with the VAT/SAT
310 ratio, a measure of the relative propensity to store fat in the visceral cavity rather than the
311 subcutaneous compartment. We therefore speculate that SNP-associated modulation of gene
312 expression or function of the protein products may impact adiposity through an effect on
313 adipocyte differentiation and relative impairments in adipocyte development may partially
314 explain a default propensity to deposit viscerally as compared to subcutaneously.

315 Given the uniqueness of the ectopic fat traits, the sample size was limited in comparison
316 to other meta-analyses. Moreover, identification of candidate genes based on proximity to a
317 GWAS signal may miss long distance interactions between genes and regulatory domains. In
318 contrast, multiethnic analyses, such as this study, not only enhance generalizability, but may
319 also boost power for certain traits, particularly in contexts of limited allelic heterogeneity. The

320 possibility of false positive loci is also a consideration, given the absence of external replication.
321 However, all newly identified loci passed FDR<1%. Such statistical limitations are further
322 mitigated in the case of *ATXN1* and *UBE2E2* by functional validation of these loci in murine
323 adipose tissue.

324 Combining large-scale discovery human genetics with the detailed fat phenotyping and
325 experiments in model organisms identified 7 new loci in association with ectopic fat traits, of
326 which *ATXN1* and *UBE2E2* demonstrated a functional effect during adipocyte differentiation.
327 Future studies should further explore the exact mechanism by which modulation of *ATXN1* and
328 *UBE2E2* impact adipocyte differentiation and whether this effect causally impacts systemic
329 metabolic disease.

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332 **Data availability statement:** Summary statistics for all meta-analyses will be made available at
333 the following website <https://www.nhlbi.nih.gov/research/intramural/researchers/ckdgen>.

334

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336 Sources.

337

338 **Author contributions**

339 **Study design:** X Guo, AH Kissebah, J Kullberg, LJ Launer, M Olivier, PA Peyser, IB Borecki,
340 DW Boden, SA Czerwinski, J Ding, V Gudnason, TB Harris, C Langefeld, L Lind, Y Liu, JI
341 Rotter, B Towne, M Allison

342 **Study management:** Yi Zhang, LJ Launer, M Olivier, PA Peyser, JG Terry, IB Borecki, DW
343 Boden, JJ Carr, SA Czerwinski, V Gudnason, TB Harris, L Lind, BD Mitchell, TH Mosely, Jr, JI
344 Rotter, AR Shuldiner, H Völzke, JG Wilson, M Allison

345 **Subject recruitment:** AH Kissebah, J Kullberg, MK Wojczynski, DW Boden, SA Czerwinski, V
346 Gudnason, L Lind, BD Mitchell, TH Mosely, Jr, AR Shuldiner, B Towne, H Völzke

347 **Interpretation of results:** AY Chu, X Deng, VA Fisher, Yang Zhang, MF Feitosa, C Liu, O
348 Weeks, AC Choh, Q Duan, X Guo, NL Heard-Costa, X Liu, L Lu, JR O'Connell, A Parihar, AV
349 Smith, Yi Zhang, AH Kissebah, M Olivier, PA Peyser, JG Terry, MK Wojczynski, LF Bielak, IB
350 Borecki, DW Boden, JJ Carr, SA Czerwinski, J Ding, N Friedrich, SL Kardia, C Langefeld, Y Liu,
351 BD Mitchell, JI Rotter, AR Shuldiner, B Towne, H Wallaschofski, M Allison, CM Lindgren, W
352 Goessling, LA Cupples, ML Steinhauser, CS Fox

353 **Drafting manuscript:** AY Chu, Yang Zhang, MF Feitosa, X Guo, JW Kent Jr., Yi Zhang, AH
354 Kissebah, MK Wojczynski, IB Borecki, CM Lindgren, ML Steinhauser, CS Fox

355 **Critical review:** AY Chu, X Deng, VA Fisher, MF Feitosa, C Liu, O Weeks, AC Choh, X Guo, NL
356 Heard-Costa, JW Kent Jr., X Liu, L Lu, A Mahajan, JR O'Connell, A Parihar, Yi Zhang, G
357 Homuth, AH Kissebah (deceased), J Kullberg, M Nauck, M Olivier, PA Peyser, JG Terry, LF

358 Bielak, J Blangero, IB Borecki, DW Boden, JJ Carr, SA Czerwinski, J Ding, N Friedrich, E
359 Ingelsson, SL Kardia, C Langefeld, L Lind, Y Liu, BD Mitchell, AP Morris, TH Mosely, Jr, JI
360 Rotter, AR Shuldiner, B Towne, H Völzke, H Wallaschofski, M Allison, CM Lindgren, W
361 Goessling, LA Cupples, ML Steinhauser, CS Fox
362 **Statistical methods and analysis:** AY Chu, X Deng, VA Fisher, A Drong, Yang Zhang, MF
363 Feitosa, AC Choh, Q Duan, TD Dyer, JD Eicher, X Guo, NL Heard-Costa, T Kacprowski, JW
364 Kent Jr., LA Lange, X Liu, K Lohman, L Lu, A Mahajan, JR O'Connell, A Parihar, JM Peralta, AV
365 Smith, J Yao, LF Bielak, J Ding, C Langefeld, Y Liu, BD Mitchell, AP Morris, CM Lindgren
366 **Genotyping:** Yi Zhang, G Homuth, M Olivier, DW Boden, SA Czerwinski, E Ingelsson, SL
367 Kardia, Y Liu, AP Morris, JI Rotter, AR Shuldiner, B Towne, CM Lindgren
368 **Bioinformatics:** AY Chu, X Deng, VA Fisher, MF Feitosa, C Liu, AC Choh, JD Eicher, AD
369 Johnson, T Kacprowski, AV Smith, Yi Zhang
370 **Data collection:** Yang Zhang, O Weeks, R Laqua, N Friedrich, W Goessling, ML Steinhauser
371 **Animal work/functional data:** Yang Zhang, ML Steinhauser
372
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374 Laboratories as of December 14, 2015 and July 18, 2016, respectively.
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377 necessarily represent the views of the National Heart, Lung, and Blood Institute; the National
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- 454

455 **FIGURE LEGEND**

456

457 **Figure 1.** Functional characterization of *Atxn1*, *Ebf1*, *Rreb1* and *Ube2e2*.

458 (a,b,e) Data is displayed as box/whisker plots where the center line represents the median, box
459 limits contain the 25th-75th percentiles, and whiskers span max/min values.

460 (a) Gene expression measured by qPCR in murine subcutaneous (SAT), perigonadal visceral
461 (VAT), and pericardial (PAT) adipose tissues (n=6 mice). Statistical significance was assessed
462 using ANOVA and Sidak's correction for multiple comparisons.

463 (b) Gene expression measured by qPCR in murine adipose tissues after 8 weeks of high fat
464 feeding compared to normal chow fed controls (n=5 mice per group). Statistical significance was
465 assigned using a two-sided T-test.

466 (c) Gene expression measured by qPCR in cultured adipocyte progenitors isolated from the
467 subcutaneous (SAT) or perigonadal visceral (VAT) depots (n=4 replicates). Cells were
468 expanded to confluence and then collected at intervals after induction of adipogenic
469 differentiation. Data displayed as mean, error bar=s.e.m. Statistical significance was assessed
470 using ANOVA and Sidak's correction for multiple comparisons to time 0.

471 (d) Oil-red-o staining of progenitors isolated from subcutaneous adipose and exposed to
472 retroviral delivery of shRNA constructs during *ex vivo* expansion and induction of adipogenesis.
473 Relative to control vector carrying a scramble sequence, shRNA constructs specific for *Atxn1*
474 and *Ube2e2* impaired adipogenic differentiation. Scale=1mm.

475 (e) Oil-red-o stain was alcohol extracted and quantified at OD₅₂₀ (n=9 technical replicates).
476 Statistical significance was assessed using ANOVA and Sidak's correction for multiple
477 comparisons to control (Scramble). Data representative of 3 independent experiments.

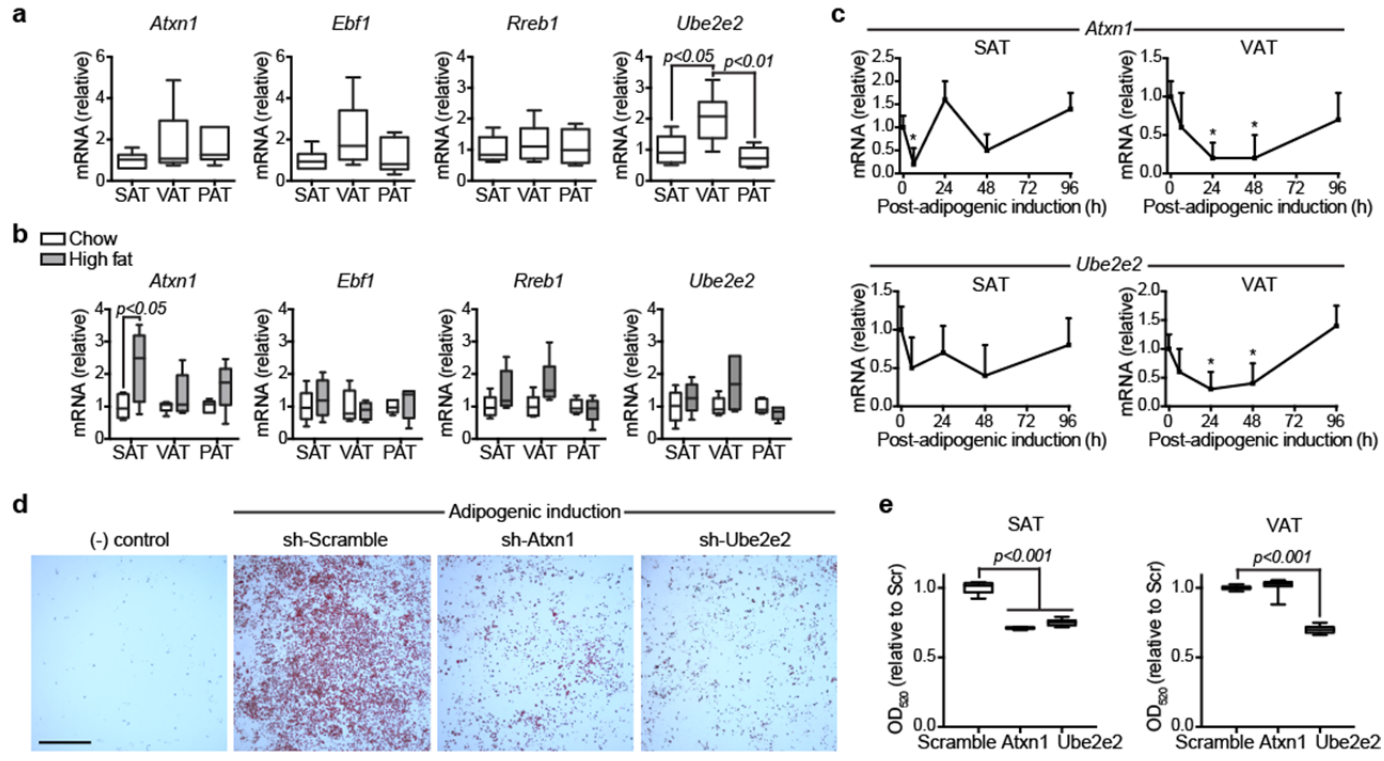
478 **Table 1.** SNPs associated with ectopic fat traits ($p < 5 \times 10^{-8}$)¹. Association statistics were obtained using a sample-size weighted fixed-
 479 effects meta-analysis implemented in METAL.^{18,19}

	Locus ²	Trait	Strata	Lead SNP	Chr	SNPID	Position	A1 ³	A2 ⁴	Freq A1 ⁵	N	Z score	P-value ⁶
Fat Volume Traits^{7,8}													
NEW													
	<i>ENSA</i>	PATadjHtWt	ALL	rs6587515	1	rs6587515	148875512	a	g	0.09	11027	-5.94	2.8×10^{-9}
	<i>GRAMD3</i>	VATadjBMI	WOMEN	rs10060123	5	rs10060123	125711809	a	c	0.23	9623	5.47	4.5×10^{-8}
	<i>EBF1</i>	PATadjHtWt	ALL	rs1650505	5	rs1650505	157962312	a	g	0.24	11566	-6.10	1.0×10^{-9}
		PAT	ALL		5	rs2434264	157954781	t	g	0.61	11614	5.93	3.0×10^{-9}
	<i>RREB1</i>	VATadjBMI	ALL	rs2842895	6	rs2842895	7051315	c	g	0.50	17297	5.72	1.1×10^{-8}
	<i>GSDMB</i>	SAT	WOMEN	rs2123685	17	rs2123685	35307415	t	c	0.94	7137	5.52	3.4×10^{-8}
KNOWN													
	<i>TRIB2</i>	PATadjHtWt	ALL	rs10198628	2	rs10198628	12881948	a	g	0.42	11572	-8.88	6.7×10^{-19}
		PATadjHtWt	MEN							0.43	5466	-6.68	2.4×10^{-11}
		PATadjHtWt	WOMEN							0.42	6106	-6.02	1.8×10^{-9}
		PAT	ALL							0.42	11605	-7.87	3.7×10^{-15}
	<i>FTO</i>	SAT	ALL	rs7185735	16	rs7185735	52380152	a	g	0.58	17812	-6.05	1.4×10^{-9}
Fat Attenuation Traits^{7,8}													
NEW													
	<i>ATXN1</i>	SATHU	MEN	rs2237199	6	rs2237199	16538000	a	g	0.11	5780	5.67	1.4×10^{-8}
Relative Fat Distribution Traits^{7,8}													
NEW													
	<i>UBE2E2</i>	VAT/SAT ratio	ALL	rs7374732	3	rs7374732	23178458	t	c	0.69	18205	-6.29	3.1×10^{-10}
		VAT/SAT ratio adjBMI	ALL							0.69	18190	-5.64	1.7×10^{-8}
KNOWN													
	<i>LYPLAL1</i>	VAT/SAT ratio	ALL	rs6689335	1	rs6689335	217695305	t	c	0.59	15214	-5.59	2.3×10^{-8}
		VAT/SAT ratio adjBMI	ALL		1	rs6689335	217695305	t	c	0.59	15199	-5.53	3.2×10^{-8}
	<i>LY86</i>	VAT/SAT ratio	ALL	rs912056	6	rs912056	6681196	a	t	0.35	17387	-5.96	2.5×10^{-9}
		VAT/SAT ratio adjBMI	ALL							0.35	17372	-5.98	2.3×10^{-9}

480 ¹ SNPs are grouped by ectopic fat trait and are listed by new discoveries and then previously identified loci. Any association attaining
 481 genome-wide significance ($p < 5 \times 10^{-8}$) is listed.

482 ² Conventional locus name based on closest gene in the region
483 ³ A1 is the coded allele
484 ⁴ A2 is the non-coded allele
485 ⁵ FreqA1 is the allele frequency of Allele1
486 ⁶ P-values are double genomic control corrected
487 ⁷ European and African ancestry cohorts contributed to all ectopic fat traits; Chinese and Hispanic ancestry cohorts contributed only
488 to pericardial volume traits
489 ⁸ Abbreviations:
490 SAT - Subcutaneous Adipose Tissue Volume
491 VAT - Visceral Adipose Tissue Volume
492 PAT - Pericardial Adipose Tissue Volume
493 SATHU - Subcutaneous Adipose Tissue Attenuation
494 VATHU - Visceral Adipose Tissue Attenuation
495 VAT/SAT ratio - Visceral to Subcutaneous Adipose Tissue Volume Ratio
496 adjBMI - Model Adjusted for BMI
497 adjHtWt - Model Adjusted for Height and Weight

498 **Figure 1.**



499

500 **Online Methods**

501 *Study Participants*

502 Up to 18,332 participants from 13 cohorts of European and African ancestry were
503 available for analysis of subcutaneous and visceral adipose tissue volumetric traits, up to
504 11,596 from 6 cohorts of European, African, Asian, and Hispanic ancestry were available for
505 analysis of pericardial adipose volumetric traits, up to 12,519 participants from 5 cohorts of
506 European and African ancestry were available for analysis of attenuation traits, and up to
507 18,191 participants from 6 cohorts of European and African ancestry were available for analysis
508 of relative fat distribution traits. This epidemiological sample constitutes the largest known
509 collection of participants with radiologically derived ectopic fat measures and genetic data at the
510 inception of this project. Supplementary Table 2 and 3 contain information regarding imaging
511 modality used by each cohort, distribution by sex and ancestry per cohort for each trait analyzed
512 and cohort descriptive information. All participants provided informed consent and each study
513 was approved by their governing ethics committee.

514

515 *Trait assessment*

516 The traits measured in this study can be categorized into three groups: 1) fat volume
517 measurements: subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT) and
518 pericardial adipose tissue (PAT); 2) fat attenuation measurements: subcutaneous adipose
519 tissue attenuation (SATHU) and visceral adipose tissue attenuation (VATHU); and 3) relative fat
520 distribution measurements: visceral-to-subcutaneous adipose tissue volume ratio (VAT/SAT
521 ratio). All volume-based measures were assessed by computed tomography (CT) or magnetic
522 resonance imaging (MRI) following study-specific protocols; attenuation-based measures were
523 assessed by CT following study specific protocols. Please see Supplementary Table 2 and
524 Supplementary Note for further detail.

525 The following traits were created by each cohort in the overall sample, women and men:
526 volume-based traits - SAT, VAT, VAT adjusted for BMI, PAT, PAT adjusted for height and
527 weight; attenuation-based traits - SATHU and VATHU; relative-distribution traits - VAT/SAT
528 ratio, VAT/SAT ratio adjusted for BMI pericardial traits. The rationale for including the ectopic
529 fat traits, the adjustment models, and the sex-stratified analyses was 4-fold. First, ectopic fat
530 measures are correlated with each other and with general adiposity and we wished to adjust for
531 these factors as potential confounders or intermediates and to examine the genetic associations
532 independent of the adjustment factor. Please see refer to Supplementary Table 7 for pairwise
533 correlations of all traits within FHS, the largest participating cohort. For example, the correlation
534 between VAT and BMI is 0.71 to 0.75 and adjusting for BMI when examining VAT provides the
535 relative amount of VAT controlling for degree of general adiposity. Although the correlations
536 between VAT/SAT ratio and BMI are modest, adjusting for BMI allowed us to examine the
537 propensity to store fat viscerally compared to subcutaneously independent of general adiposity.
538 Second, adjustment of covariates reduces the residual variance of the trait associated with the
539 given covariate and thus increases power to detect genetic associations. Third, in the adiposity
540 genetics literature there is evidence of sexually dimorphic loci in which the variance explained is
541 larger in women versus men²⁸ and association of the loci is markedly stronger in women
542 compared to men, and vice versa.^{14,22} Lastly, we adjusted PAT for height and weight to be
543 consistent with our prior work¹³ (see Supplementary Table 1 for guide to nomenclature for traits
544 and adjustment models).

545 Due to the known differences in body fat distribution by sex, each cohort created sex-
546 and ancestry-specific residuals adjusted for age, age-squared, smoking status, measures of
547 subpopulation stratification and family structure (if necessary). Family-based studies created an
548 additional set of residuals from all participants (both women and men) to account for family
549 structure when analyzing the overall sample. Participants with missing genotype, phenotype or
550 covariate data were excluded from analysis as pre-specified in the analysis plan.

551 *Study Specific Protocol*

552 Trait measurements and descriptions from each cohort are available in Supplementary
553 Material under “Cohort Specific Information and Protocols”.

554

555 *Genotyping and Imputation*

556 Each cohort was genotyped as specified in Supplementary Table 4 and performed
557 ancestry-specific imputation up to ~2.6 million SNPs based on the HapMap Project Phase 2
558 haplotypes (<http://hapmap.ncbi.nlm.nih.gov/index.html.en>). All newly identified loci were
559 imputed with imputation qualities >0.8 in each cohort. Imputation quality by locus and cohort
560 are available in Supplementary Table 8.

561

562 *Heritability Analysis*

563 Heritability was estimated from the Framingham Heart Study using variance components
564 analysis in SOLAR.¹⁶

565

566 *Genetic Correlation Analysis*

567 Pairwise genetic correlations between subcutaneous fat (volume and attenuation),
568 visceral fat (volume and attenuation), ratio of visceral-to-subcutaneous fat and BMI were
569 calculated using SOLAR¹⁶ in the Framingham Heart Study among 3,312 participants. We used
570 residuals adjusted for age and sex. Two separate hypotheses were tested: 1) $Rho_G=0$ is the
571 test for overlapping genetic correlations, and 2) absolute value (Rho_G)=1 is the test for non-
572 overlapping genetic correlations.

573

574 *Statistical Analysis*

575 Within each cohort, by ancestry and by sex, genome-wide linear regression analyses
576 were conducted on the 11 trait and model combinations assuming an additive genetic model

577 using allele dosages. All traits approximated a normal distribution and untransformed traits
578 were used for analysis. To prevent the undue influence of rare variants and/or of poorly
579 imputed SNPs, we included variants with a minor allele count >10 and imputation quality >0.4
580 (for MaCH²⁹) or >0.3 (for IMPUTE³⁰) in each cohort.

581 For multiethnic analysis, we combined all cohort-specific results using a sample size-
582 weighted fixed-effects meta-analysis (Stouffer's method) as implemented in METAL^{18,19} to allow
583 for differences in trait measurement and scaling due to different imaging modalities across
584 cohorts. European and African ancestry cohorts contributed to all ectopic fat traits; Chinese and
585 Hispanic ancestry cohorts contributed only to pericardial volume traits (Supplementary Table 3).
586 All analyses were performed for the overall sample (ALL), among women only (WOMEN) and
587 among men only (MEN). All analyses were corrected for genomic control at the cohort-level.
588 We excluded variants with minor allele frequency (MAF)<5% due to the low power to detect
589 associations of such variants. We set a traditional genome-wide significance threshold at
590 $P<5\times 10^{-8}$, the Bonferroni correction for the number of independent and common variants across
591 the genome (~1 million SNPs). All p-values represent two-sided p-values unless otherwise
592 specified. All regional association plots, Manhattan plots, and QQ plots were created using R
593 version 3.1.1 (<https://cran.r-project.org/>). Linkage disequilibrium plots were created using
594 SNAP³¹ and the gap R package (<https://www.jstatsoft.org/article/view/v023i08>).

595 To correct for multiple testing, false discovery rate (FDR) was calculated across the 27
596 ectopic fat GWAS scans using the qvalue R package (<http://github.com/jdstorey/qvalue>).
597 FDR<1% was set as the multiple testing corrected significance threshold.

598 For mouse studies, individual cages of mice were randomly assigned in an un-blinded
599 fashion to normal chow or high fat diet. Each *in vivo* study was conducted one time and no mice
600 were excluded from the analyses. In the absence of *a priori* data regarding the variance of gene
601 expression in the tissues of interest, we applied sample sizes that have in our experience been
602 of sufficient size to detect a two-fold increase in gene expression. For normally distributed data

603 from more than two groups (Shapiro-Wilk), an ANOVA test followed by Sidak's correction for
604 multiple testing was conducted (Figures 1a,c,e). For non-normal data a Kruskal-Wallis test was
605 used. For comparisons between two normally distributed groups (Figure 1b: chow versus high
606 fat) a two-sided T-test was used, unless the data was non-normal, in which case a Mann-
607 Whitney test was used. Data were expressed as mean, s.e.m. Significance was assigned for
608 two-sided $p < 0.05$. Data were analyzed and graphed using JMP 10.0 (SAS institute) and Prism 6
609 (Graphpad).

610

611 *Sensitivity Analyses*

612 To ensure the newly identified loci from our multiethnic analysis were robust and not
613 driven by statistical outliers related to ancestry, ancestry-specific meta-analysis results were
614 compared with each other with respect to the minor allele, the minor allele frequency and
615 direction of the Z-score association statistic (Supplementary Table 9). Due to the scaling
616 differences in imaging modalities across each cohort and use of the sample size weighted meta-
617 analysis heterogeneity statistics cannot be calculated.

618 The lead SNP for the *GSDMB* locus associated with SAT in women was not observed in
619 non-European ancestry cohorts and thus was not included in this analysis. For each of the
620 remaining 6 lead SNPs from the newly identified ectopic fat loci, Z scores were directionally
621 consistent across ancestry-specific meta-analyses (please see Supplementary Figure 2 for
622 forest plots of each locus and Supplementary Figure 3 for linkage disequilibrium [LD] plots
623 across ancestry). For 5 of these loci, the minor allele was identical across ancestries; only the
624 minor allele of rs2842895 (*RREB1*) differed between the European ancestry and African
625 ancestry cohorts. This observation may explain the slight attenuation in the association of
626 *RREB1* and VATadjBMI after combining European and African ancestries in the multiethnic
627 meta-analysis ($P_{\text{European-ancestry}} = 5.8 \times 10^{-9}$ to $P_{\text{multiethnic}} = 1.1 \times 10^{-8}$), although the multiethnic result
628 remains genome-wide significant.

629 *Analyses of Related Traits*

630 For each SNP attaining genome-wide significance in association with any ectopic fat
631 trait, we extracted association results in each strata of analysis (ALL, WOMEN, and MEN) for
632 related ectopic fat traits within our study.

633 To investigate the association of the new ectopic fat loci with measures of generalized
634 adiposity (BMI) and central obesity (WHR) - two traits that are strongly correlated with, but
635 distinct from ectopic fat - we evaluated the lead genome-wide significant SNPs in publically
636 available datasets from the most recent GIANT meta-analyses of BMI and WHR.^{14,15}

637 To investigate associations of new loci with cardio-metabolic traits that are
638 epidemiologically associated with ectopic fat, cross-trait evaluations for the lead SNPs only were
639 performed in the publically available datasets from the MAGIC (Meta-Analyses of Glucose and
640 Insulin Consortium for fasting glucose and insulin³²), GLGC (Global Lipids Genetics Consortium
641 for high-density lipoprotein cholesterol, triglycerides and total cholesterol³³),
642 CARDIoGRAM+CAD consortium (Coronary ARtery Disease Genome wide Replication and
643 Meta-analysis [CARDIoGRAM] plus The Coronary Artery Disease [C4D] Genetics for coronary
644 artery disease and myocardial infarction^{34,35}), ICBP (International Consortium for Blood
645 Pressure for systolic and diastolic blood pressure³⁶), and DIAGRAM (DIAbetes Genetics
646 Replication And Meta-analysis²⁵).

647

648 *Analysis of general adiposity and central adiposity loci*

649 To evaluate the relationship between the known 97 BMI and 49 WHR loci^{14,15} with
650 ectopic fat traits, we examined the association for these loci with fat volume and relative fat
651 volume traits among the combined multiethnic sample of women and men. Because the ectopic
652 fat data may be underpowered to determine statistically significant results, we hypothesized that
653 the direction of the BMI and WHR findings would be directionally consistent with the ectopic fat
654 traits, even if the p-values were not significant. Binominal tests were used to test the

655 significance of direction consistent associations (1-sided p-values). If the binominal test across
656 the BMI or WHR loci was significant, a second 1-sided binominal test was performed evaluating
657 consistency of associations restricting to SNPs with nominally significant associations ($P < 0.05$).

658

659 *Functional Profiling - Bioinformatics and Annotation*

660 To further characterize novel genome-wide significant loci, the following bioinformatics
661 databases were queried for the lead ectopic fat loci: GWAS Catalog
662 (<https://www.ebi.ac.uk/gwas/>; access date: 10/15/2015) to investigate other traits associated
663 with newly identified loci, and HaploReg²³ and RegulomeDB²⁴ to identify regulatory elements
664 overlapping the loci for the index SNP and SNPs in LD with the index SNP ($r^2 > 0.8$;
665 Supplementary Table 13). To contextualize the newly identified ectopic loci and the surrounding
666 genes, SNIPPER (<https://github.com/welchr/Snipper.git>) was used to search for biologically
667 relevant mechanisms (Supplementary Table 14).

668

669 *Variance Explained*

670 The variance explained for each of the loci was approximated using the following
671 formula $R^2 = \beta^2 \text{var}(SNP) / \text{var}(\text{ectopic fat trait})$, where β^2 is the estimated effect of the SNP on the
672 ectopic fat trait, and $\text{var}(SNP) = 2 * MAF_{SNP} * (1 - MAF_{SNP})$. Because sample-size weighted fixed-
673 effect meta-analysis does not estimate effect sizes, the beta-coefficient for the association
674 between the SNP and ectopic fat trait and the variance of the ectopic fat trait were obtained
675 from cohort level analysis per contributing study. The mean of the variance explained per locus
676 across all contributing cohorts ranges from 0.1% to 4.4% (Supplementary Table 15).

677

678

679

680 *Power Calculations*

681 Power for discovery in the ectopic fat genomewide scan was calculated using
682 GWAPower³⁷ using the range of sample size in this study (5,842-18,332 participants) and
683 setting $\alpha = 5 \times 10^{-8}$. For the smallest sample size analyzed (N=5,842) we had $\geq 80\%$ power to
684 detect loci explaining at least 0.64% of the trait variance. For the largest sample size analyzed
685 (N=18,332), we had $\geq 80\%$ power to detect loci explaining at least 0.20% of the trait variance.
686 For example, our novel loci explained from 0.15-4.4% of the trait variance for ectopic fat as seen
687 in Supplementary Table 15.

688 To address the power to detect associations for the lookup analyses, we used
689 GWAPower³⁷ with the maximum sample sizes from the each of the quantitative trait datasets
690 (52,000-94,000 participants), a modest range of variance explained (0.01-0.05%; based on the
691 variance explained for each locus [0.1-4.4%] and the age- adjusted correlations between
692 ectopic fat and the cardiometabolic trait of interest [$R^2=0.02-0.46$]) and a Bonferroni corrected α
693 $= 7.4 \times 10^{-4}$ ($\sim 0.05/66$ pairs of SNP-trait associations). For the smallest dataset (Fasting Insulin,
694 N~52,000), we had 80% power to detect loci explaining at least 0.030% of the variance in
695 fasting insulin. For the largest dataset (HDL-C and total cholesterol, N~94,000), we had 80%
696 power to detect loci explaining 0.018% of the variance in HDL-C or total cholesterol. These
697 calculations indicate that we largely had adequate power for a large portion of the SNP-trait
698 associations.

699

700 *eQTL analysis*

701 Using a curated collection of 6 eQTL datasets in adipose-related tissues, index SNPs at
702 newly identified ectopic fat loci were examined in association with transcript expression.
703 Datasets were collected through publications, publically available sources, or private
704 collaboration. The eQTL datasets met criteria for statistical thresholds for SNP-gene transcript

705 associations as described in the original papers and were limited to index SNPs and SNPs in
706 LD with the index SNP ($r^2 > 0.8$) across all ancestries available in the 1000 Genomes Project pilot
707 (SNAP³¹). A general overview of the larger collection of more than 50 eQTL studies from which
708 the adipose-related datasets (omental, visceral and subcutaneous adipose,³⁸⁻⁴²) were derived
709 from has been published.⁴³ Additional eQTL data was integrated from online sources including
710 ScanDB, the Broad Institute GTEx Portal, and the Pritchard Lab (eqtl.uchicago.edu). Results for
711 GTEx Analysis V4 for subcutaneous adipose tissue were downloaded from the GTEx Portal and
712 then additionally filtered as described below (www.gtportal.org⁴¹). Splicing QTL (sQTL) results
713 generated with sQTLseeker with false discovery rate $P \leq 0.05$ were retained. For all gene-level
714 eQTLs, if at least 1 SNP passed the tissue-specific empirical threshold in GTEx, the best SNP
715 for that eQTL was always retained. All gene-level eQTL SNPs with $P < 1.67 \times 10^{-11}$ were also
716 retained, reflecting a global threshold correction of $P = 0.05 / (30,000 \text{ genes} \times 1,000,000 \text{ tests})$.

717 Cis-eQTL analysis showed SNPs at *ENSA* (a locus identified in association with PAT)
718 was correlated with multiple transcripts (*MRPS21*, *CTSK* and *LASS2*, $P < 10^{-4}$) in subcutaneous
719 and omental adipose tissue (Supplementary Table 16), suggesting these may be the relevant
720 transcripts at this locus and not *ENSA*, the closest gene to the lead association signal.
721 However, the *ENSA* locus was not selected for functional validation, as there were too many
722 genes in the region to practically follow up. No other eQTLs were identified.

723

724 *Characterization in Model Organisms*

725 *Selection of Loci for Characterization*

726 For functional follow-up and characterization of ectopic fat loci, four gene-trait
727 associations were selected based on visual examination of regional association plots
728 (Supplementary Figures 1a-g) for a localized association within a gene body at each locus
729 (*RREB1*, *ATXN1* and *UBE2E2*) or localized association near the gene body and the lack of

730 other genes within 1Mbp of the lead SNP (*EBF1*) to increase the probability of experimentally
731 testing the likely causal gene in murine models.

732

733 *Mouse studies*

734 Experiments were approved by and in compliance with the ethical regulations of the
735 Harvard Medical Area Standing Committee on Animals. Male C57BL/6 mice were purchased
736 from Charles River and housed at 22 ± 2°C, with a 12h light (0700-1900 h), 12h dark (1900-
737 0700 h) cycle and *ad libitum* access to food and water. With the exception of the data shown in
738 Supplementary Figure 6, experiments were conducted in male mice. Diet-induced obesity was
739 modeled with high fat (D12492) and control chow (D12450J) matched for sucrose content
740 (Research Diets, Inc.). Adipose tissue was harvested, homogenized in Trizol (Life
741 Technologies), and RNA extracted according to the manufacturers protocol. cDNA was
742 synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies).
743 qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) on
744 an iCycler (Bio-Rad) instrument. See Supplementary Table 17 for primer sequences used in
745 these analyses. Gene expression was normalized to 18S. The delta-delta CT method was
746 utilized to calculate fold change in transcript levels.

747

748 *Comparison of baseline adipose-specific expression of *Atxn1**

749 Given that the SNP-ectopic fat association for *ATXN1* was confined to men, we
750 assessed gender-specific effects in mice of *Atxn1* expression. There was no detectable gender
751 effect on the baseline, adipose-specific expression of *Atxn1* (Supplementary Figure 6).

752

753 *Adipogenesis assay*

754 Adipose tissue from C57BL/6 mice was minced and digested with collagenase D
755 (Roche) in a shaking water bath (37C, 225rpm, 40min). The digest was centrifuged at 400g for

756 10 min. Pelleted stromal vascular cells were filtered (40µm) and then washed with PBS and
757 subjected to additional negative selection (CD31⁻ / lineage⁻) adapted from previously performed
758 methods⁴⁴ using antibody coated microbeads (Miltenyi Biotec). Cells were cultured to
759 confluence in collagen-coated plates and stimulated with dexamethasone, insulin and 3-
760 isobutyl-1-methylxanthine to induce adipogenic differentiation. For genetic loss of function
761 assays, validated shRNA sequences (Broad, *Ube2e2*: TRCN0000040962; *Atxn1*:
762 TRCN0000240655) or scramble sequence were subcloned into a retroviral vector (pMKO.1).
763 Gene knock-down efficiency was confirmed by qPCR in 3T3L1 cells, in each instance
764 reproducibly achieving a minimum of 60% reduction of transcriptional activity. Differentiation into
765 mature lipid-containing adipocytes was determined by oil-red-o (ORO) staining and quantified
766 by measuring alcohol-extracted ORO dye at optical density 520 nm (OD₅₂₀).

767

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771

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