KLB is associated with alcohol drinking, and its gene product β-klotho is necessary for FGF21 regulation of alcohol preference

Gunter Schumann[1]*, Chunyu Liu[2-4]*, Paul F. O'Reilly[5]*, He Gao[6]*, Parkyong Song[7]*, Bing Xu[1], Barbara Ruggeri[1], Najaf Amin[8], Tianye Jia[1], Sara R. Preis[4], Marcelo P Segura Lepe[6], Shizuo Akira[9], Caterina Barbieri[10], Sebastian E. Baumeister[11,12], Stephane Cauchi[13], Toni-Kim Clarke[14], Stefan Enroth[15], Krista Fischer[16], Jenni Hällfors[17], Sarah E. Harris[18,19], Saskia Hieber[20], Edith Hofer[21,22], Jouke-Jan Hottenga[23], Åsa Johansson[15], Peter K. Joshi[24], Niina Kaartinen[25], Jaana Laitinen[26], Rozenn N. Lemaitre[27], Anu Loukola[17], Jian'an Luan[28], Leo-Pekka Lyytikäinen[29], Massimo Mangino[30,31], Ani Manichaikul[32], Hamdi Mbarek[23], Yuri Milaneschi[33], Alireza Moayyeri[6,34,35], Kenneth Mukamal[36], Christopher P. Nelson[37,38], Jennifer A. Nettleton[39], Eemil Partinen[40], Rajesh Rawal[41], Antonietta Robino[42], Lynda M. Rose[43], Cinzia Sala[10], Takashi Satoh[9], Reinhold Schmidt[21], Katharina E. Schraut[24], Robert Scott[44], Albert Vernon Smith[45], John M. Starr[18,46], Alexander Teumer[11,47], Stella Trompet[48,49], André G. Uitterlinden[50,51], Cristina Venturini[30], Anne-Claire Vergnaud[6], Niek Verweij[52], Veronique Vitart[53], Dragana Vuckovic[54], Juho Wedenoja[17], Loic Yengo[13], Bing Yu[55], Weihua Zhang[6,56], Jinghua Zhao[44], Dorret I. Boomsma[23], John C. Chambers[6,56,57], Daniel I. Chasman[43,58], Toniolo Daniela[10], Eco J.C. de Geus[23], Ian J. Deary[18,59], Johan G. Eriksson[25,60-63], Tõnu Esko[16], Volker Eulenburg[64], Oscar H. Franco[51], Philippe Froguel[13,65], Christian Gieger[41], Hans J. Grabe[66], Vilmundur Gudnason[67], Ulf Gyllensten[15], Tamara B. Harris[68], Anna-Liisa Hartikainen[69-71], Andrew C. Heath[72], Lynne J.Hocking[73], Albert Hofman[51], Cornelia Huth[74], Marjo-Riitta Jarvelin[6,71,75,76], J. Wouter Jukema[48], Jaakko Kaprio[17,25,77], Jaspal S Kooner[56,57,78], Zoltan Kutalik[79], Jari Lahti[62,80,81], Claudia Langenberg[28], Terho Lehtimäki[29], Yongmei Liu[82], Pamela A.F. Madden[83], Nicholas G. Martin[84], Alanna C. Morrison[55], Brenda W.J.H. Penninx[33], Nicola Pirastu[54], Bruce M. Psaty[27,85-87], Olli T. Raitakari[88], Paul M. Ridker[43,58], Richard J. Rose[89], Jerome I. Rotter[90], Nilesh J. Samani[37,38], Helena Schmidt[91], Tim Spector[30], David J. Stott[92], David P. Strachan[93], Ioanna Tzoulaki[6], Pim van der Harst[52,94,95], Cornelia M. van Duijn[8], Pedro Marques Vidal[96], Peter Vollenweider[96], Nick Wareham[44], John B. Whitfield[84], James F. Wilson[24,97], Bruce H.R. Wolffenbuttel[98], Georgy Bakalkin[99], Evangelos Evangelou[6,100], Yun Liu[101], Kenneth Rice[102], Sylvane Desrivières[1]*, Steven Kliewer[7,103]*, David Mangelsdorf[7]*, Christian P. Müller[20]*, Daniel Levy[2,3]*, Paul Elliott[6]*

* These authors contributed equally to this work

Affiliations

1 Medical Research Council - Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, Psychology & Neuroscience, King's College London, United Kingdom.
2 The Framingham Heart Study, Framingham, Massachusetts 01702, USA.
3 The Population Sciences Branch, Division of Intramural Research, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20824, USA.
4 Boston University School of Public Health, 715 Albany St, Boston, MA 02118, USA.

5 MRC Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, Psychology and Neuroscience, King's College London, London, United Kingdom.
6 Department of Epidemiology and Biostatistics, MRC-PHE Centre for Environment and Health, School of Public Health, Imperical College London, London W2 1PG, UK.
7 Department of Pharmacology and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, USA 75390.
8 Genetic Epidemiology Unit, Department of Epidemiology, Erasmus MC, Rotterdam, the Netherlands.
9 Laboratory of Host Defense, World Premier International Immunology Frontier Research Center, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.
10 Division of Genetics and Cell Biology, San Raffaele Research Institute, Milano, Italy.
11 Institute for Community Medicine, University Medicine Greifswald, Greifswald, 17475, Germany.
12 Department of Epidemiology and Preventive Medicine, University of Regensburg, Germany.
13 CNRS UMR 8199, Lille Pasteur Institute, Lille 2 University, European Genomic Institute for Diabetes (EGID), Lille, France.
14 Divison of Psychiatry, University of Edinburg, Edinburgh, UK.
15 Science for Life Laboratory, Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden.
16 Estonian Genome Center, University of Tartu, Estonia.
17 Department of Public Health, University of Helsinki, Helsinki, Finland.
18 Centre for Cognitive Ageing and Cognitive Epidemiology, University of Edinburgh, Edinburgh, UK, EH8 9JZ.
19 Centre for Genomic and Experimental Medicine, University of Edinburgh, Edinburgh, UK, EH4 2XU.
20 Department of Psychiatry and Psychotherapy, Friedrich-Alexander-University Erlangen-Nuremberg, Schwabachanlage 6, 91054 Erlangen, Germany.
21 Clinical Division of Neurogeriatrics, Department of Neurology, Medical University Graz, Austria.
22 Institute of Medical Informatics, Statistics and Documentation, Medical University Graz, Austria.
23 Department of Biological Psychology, VU Amsterdam and EMGO Institute for Health and Care Research, Amsterdam, The Netherlands.
24 Usher Institute for Population Health Sciences and Informatics, University of Edinburgh, Teviot Place, Edinburgh, EH8 9AG, Scotland.
25 National Institute for Health and Welfare, Helsinki, Finland.
26 Finnish Institute of Occupational Health, Helsinki, Finland.
27 Cardiovascular Health Research Unit, Department of Medicine, University of Washington, Seattle, WA, USA.
28 MRC Epidemiology Unit, University of Cambridge School of Clinical Medicine, Institute of Metabolic Science, Cambridge, CB2 0QQ, UK.
29 Department of Clinical Chemistry, Fimlab Laboratories and University of Tampere School of Medicine, Tampere 33520, Finland.
30 Department of Twin Research and Genetic Epidemiology, King's College London.
31 NIHR Biomedical Research Centre at Guy's and St. Thomas' Foundation Trust.

32 Center for Public Health Genomics and Biostatistics Section, Department of Public Health Sciences, University of Virginia, Charlottesville, Virginia 22903, USA.
33 Department of Psychiatry, EMGO Institute for Health and Care Research and Neuroscience Campus Amsterdam, VU University Medical Center/GGZ inGeest, Amsterdam, The Netherlands.
34 Institute of Health Informatics, University College London, 222 Euston Road, London NW1 2DA, UK.
35 Farr Institute of Health Informatics Research, 222 Euston Road, London NW1 2DA, UK.
36 Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA.
37 Department of Cardiovascular Sciences, University of Leicester, Glenfield Hospital, Leicester, UK.
38 National Institute for Health Research Leicester Cardiovascular Biomedical Research Unit, Glenfield Hospital, Leicester, UK.
39 Division of Epidemiology, Human Genetics, and Environmental Sciences, University of Texas Health Sciences Center, Houston, TX, USA.
40 Faculty of Medicine, University of Tartu, Estonia.
41 Department of Molecular Epidemiology, Institute of Epidemiology II, Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany.
42 Institute for Maternal and Child Health - IRCCS "Burlo Garofolo", Trieste, Italy.
43 Division of Preventive Medicine, Brigham and Women's Hospital, Boston, MA 02215, USA.
44 MRC Epidemiology Unit, University of Cambridge School of Clinical Medicine, Cambridge, UK.
45 Icelandic Heart Association, Kopavogur, Iceland.
46 Alzheimer Scotland Research Centre, University of Edinburgh, Edinburgh, UK, EH8 9JZ.
47 Interfaculty Institute for Genetics and Functional Genomics, University Medicine Greifswald, Greifswald, 17475, Germany.
48 Department of Cardiology, Leiden University Medical Center, leiden, the Netherlands.
49 Department of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, the Netherlands.
50 Department of internal Medicine, Erasmus MC, Rotterdam, the Netherlands.
51 Department of Epidemiology, Erasmus MC, Rotterdam, the Netherlands.
52 University of Groningen, University Medical Center Groningen, Department of Cardiology, 9700RB Groningen, The Netherlands.
53 Medical Research Council Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK.
54 Department of Medical Sciences, University of Trieste, Italy.
55 Human Genetics Center and Div. of Epidemiology, University of Texas Health Science Center at Houston, Houston, TX, USA.
56 Department of Cardiology, Ealing Hospital, Uxbridge Road, Middlesex UB1 3HW, UK.
57 Imperial College Healthcare NHS Trust, London W12 0HS, UK.
58 Harvard Medical School, Boston, MA 02115, USA.
59 Psychology, University of Edinburgh, Edinburgh, UK, EH8 9JZ.
60 Department of General Practice and Primary health Care, University of Helsinki, Finland.
61 Helsinki University Central Hospital, Unit of General Practice, Helsinki, Finland.

62 Folkhälsan Research Centre, Helsinki, Finland.
63 Vasa Central Hospital, Vasa, Finland.
64 Institute for Biochemistry and Molecular Medicine, Friedrich-Alexander-University Erlangen-Nuremberg, Fahrstrasse 17, 91054 Erlangen, Germany.
65 Department of Genomics of Common Disease, School of Public Health, Imperial College London, London, UK.
66 Department of Psychiatry and Psychotherapy, University Medicine Greifswald, 17475 Greifswald, Germany.
67 National Institute on Aging; National Institutes of Health, Bethesda, MD, USA.
68 Faculty of Medicine, University of Iceland, Reykjavik, Iceland.
69 Department of Obstetrics and Gynecology, Oulu University Hospital, Oulu, Finland
70 Medical Research Center, University of Oulu, Oulu, Finland.
71 Unit of Primary Care, Oulu University Hospital, Oulu, Finland.
72 Department of Psychiatry, Washington University School of Medicine in St. Louis, 660 S. Euclid Ave., St. Louis, MO 63110.
73 Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK.
74 Institute of Epidemiology II, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany.
75 Center for Life Course Epidemiology, Faculty of Medicine, P.O.Box 5000, FI-90014 University of Oulu, Finland.
76 Biocenter Oulu, P.O.Box 5000, Aapistie 5A, FI-90014 University of Oulu, Finland.
77 Institute for Molecular Medicine (FIMM), University of Helsinki, Helsinki, Finland.
78 Faculty of Med, National Heart & Lung Institute, Cardiovascular Science, Hammersmith Campus, Hammersmith Hospital, Hammersmith Campus, Imperial College London, London W12 0NN, UK.
79 Institute of Social and Preventive Medicine, Centre Hospitalier Universitaire Vaudoise (CHUV), Lausanne 1010, Switzerland.
80 Collegium for Advanced Studies, University of Helsinki, Finland.
81 Institute of Behavioural Sciences, University of Helsinki, Finland.
82 Wake Forest School of Medicine, Department of Epidemiology & Prevention, Public Health Sciences, Wake Forest School of Medicine, Winston-Salem, NC 27157.
83 Washington University School of Medicine, Saint Louis, MO, USA
84 Genetic Epidemiology, QIMR Berghofer Medical Research Institute, Bancroft Centre, 300 Herston Road, Herston, Brisbane.
85 Department of Epidemiology, University of Washington, WA, USA.
86 Department of Health Services, University of Washington, WA, USA.
87 Group Health Research Institute, Group Health Cooperative, Seattle, WA, USA.
88 Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, and Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku 20520, Finland.
89 Department of Psychological and Brain Sciences, Indiana University, Bloomington, Indiana, USA.
90 Institute for Translational Genomics and Population Sciences, Los Angeles Biomedical Research Institute at Harbor–UCLA Medical Center, Torrance, CA, USA.
91 Institute of Molecular Biology and Biochemistry, Centre for Molecular Medicine, Medical University of Graz, Austria.

92 Institute of Cardiovascular and Medical Sciences, Faculty of Medicine, University of Glasgow, United Kingdom.
93 Population Health Research Institute, St George's, University of London, London SW17 0RE, UK.
94 University of Groningen, University Medical Center Groningen, Department of Genetics, 9700RB Groningen, The Netherlands.
95 Durrer Center for Cardiogenetic Research, ICIN - Netherlands Heart Institute, 3511GC Utrecht, The Netherlands.
96 Department of Internal Medicine , University Hospital, 1011 Lausanne, Switzerland.
97 MRC Human Genetics Unit, Institute for Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, Scotland.
98 University of Groningen, University Medical Center Groningen, Department of Endocrinology, 9700RB Groningen, The Netherlands.
99 Division of Biological Research on Drug Dependence, Department of Pharmaceutical, Biosciences, Uppsala University, Uppsala, Sweden.
100 Department of Hygiene and Epidemiology, University of Ioannina Medical School, Ioannina, Greece.
101 Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education; Department of Biochemistry and Molecular Biology, Fudan University Shanghai Medical College, Shanghai, P.R. China
102 Department of Biostatistics, University of Washington, WA, USA.
103 Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA 75390.

Alcohol drinking is a major public health problem worldwide. We conducted a genome-wide association meta-analysis and replication study among 105,898 individuals of European ancestry, and identify a novel locus associated with alcohol consumption in β-Klotho *(KLB)* (rs11940694; $P=9.2x10^{-12}$), a component of Fibroblast-Growth-Factor-Receptors for FGF19/21. We show genotype-dependent alcohol preference in *klb* brain-specific knock-out mice compared with controls, and demonstrate that the effect of FGF21 on alcohol drinking depends on β-Klotho.

Alcohol drinking is a major public health problem worldwide causing an estimated 3.3 million deaths in 2012[1]**.** Much of the behavioral research associated with alcohol has focused on dependent patients. However, the burden of alcohol associated disease largely reflects the amount of alcohol consumption in a population, not alcohol dependence[2]; it has long been recognized that small shifts in the mean of a continuously distributed behavior such as alcohol drinking can have major public health benefits[3]. For example, a shift from heavy to moderate drinking could have beneficial effects on cardiovascular disease risk[4].

Alcohol drinking is a heritable complex trait[5]. Genetic variants in the alcohol and aldehyde-dehydrogenase gene family *(ADH/ALDH*) can result in alcohol intolerance mediated by peripheral metabolism of alcohol. However, genetic influences on brain functions affecting drinking behavior have been more difficult to detect because, like for many complex traits, the effect of individual genes is small, so large sample sizes are required to detect the genetic signal[6]. Here we report a genome-wide association (GWAS) and replication study of over 100,000 individuals of European

descent and functional characterization in a mouse knock-out. We identify a variant in a novel gene in a pathway previously described to regulate macronutrient preference. We then functionally characterize this pathway in a knock-out mouse model.

We carried out GWAS of quantitative data on alcohol intake among up to 70,460 individuals (60.9% women) of European descent from 30 cohorts. We followed up the most significantly associated SNPs (6 sentinel SNPs $P<1.0x10^{-6}$ from independent regions) among up to 35,438 individuals from 14 additional cohorts (see Supplementary Note and Supplementary Table 1). We analyzed both continuous data on daily alcohol intake in drinkers (g/day, log transformed) and a dichotomous variable of heavy vs light or no drinking (see Online Methods and Supplementary Table 1). Average alcohol intake in drinkers across the samples was 14.0 g/day in males and 6.0 g/day in females. We performed per cohort sex-specific and combined-sex single SNP regression analyses under an additive genetic model, and conducted meta-analysis across the sex-specific strata and cohorts using an inverse variance weighted fixed effects model.

Results of the primary GWAS for log g/day alcohol are shown in Table 1, Supplementary Figure 1, and Supplementary Table 2A. We identified five SNPs for replication at $P<1x10^{-6}$ (Supplementary Table 2A). In addition to rs10950202 in *AUTS2* ($P=2.9x10^{-7}$), we took forward SNP rs6943555 in *AUTS2* ($P=1.4x10^{-4}$) which was previously reported in relation to alcohol drinking[6]. Combining discovery and replication data, we report genome-wide significance for SNP rs11940694 (A/G) in *KLB* ($P=9.2x10^{-12}$) (Table1, Supplementary Figure 2), for which the minor allele A was associated with reduced drinking. In the dichotomous analysis primary GWAS

(Supplementary Table 2B), we took forward two SNPs for replication, but neither replicated (Supplementary Table 3).

KLB is localized on human chromosome 4p14 and encodes a transmembrane protein, β–Klotho, which is an essential component of oligomeric receptors for FGF19 and FGF21[7,8]. β–Klotho is abundantly expressed in liver and adipose tissue, and is also expressed in discrete regions of the brain[9]. In mice FGF15 (homologue of FGF19) is expressed at high levels in the brain during embryogenesis and has been shown to promote neurogenesis and early brain structural development[10,11]. In adult mice, FGF21 is secreted from the liver in response to nutritional stress such as starvation and high-carbohydrate diets and acts co-ordinately on multiple tissues, including the brain, to regulate metabolism and related behaviors[7,8]. Among its actions, FGF21 suppresses sweet preference by acting on the brain[12,13]. FGF21 has been associated with macronutrient preference in man[14].

SNP rs11940694 is localized in intron 1 of the *KLB* gene. We found no association of rs11940694 with gene expression in peripheral blood of 5,236 participants of the Framingham study[15] (Supplementary Table 4).

To examine whether β-Klotho affects alcohol drinking in mice, and whether it does so through actions in the brain, we measured alcohol intake and the alcohol preference ratio of brain-specific β-Klotho-knockout (Klb^{Camk2a}) mice and control floxed Klb ($Klb^{fl/fl}$) mice. We used a voluntary two-bottle drinking assay performed with water and alcohol. Since we previously showed that FGF21-transgenic mice, which express FGF21 at pharmacologic levels, have a reduced alcohol preference[12],

we performed these studies while administering either recombinant FGF21 or vehicle by osmotic minipump. Alcohol preference vs. water was significantly increased in vehicle-treated Klb^{Camk2a} compared to $Klb^{fl/fl}$ mice at 16 vol. % alcohol (Figure 1A). FGF21 suppressed alcohol preference in $Klb^{fl/fl}$ mice, but not in Klb^{Camk2a} demonstrating that the effect of FGF21 on alcohol drinking depends on β-Klotho expressed in the brain (Figure 1A). There was a corresponding decrease in plasma alcohol levels immediately after 16 vol. % alcohol drinking, which reflects the modulation of the drinking behavior (Figure 1B). However, plasma FGF21 levels were comparable in $Klb^{fl/fl}$ and Klb^{Camk2a} mice administered recombinant FGF21 at the end of the experiment (Figure 1C). Alcohol bioavailability was not different between FGF21 treated $Klb^{fl/fl}$ and Klb^{Camk2a} mice (Figure 1D). We have previously shown that FGF21 decreases the sucrose and saccharine preference ratio in $Klb^{fl/fl}$ but not Klb^{Camk2a} mice, and has no effect on the quinine preference ratio[12]. To rule out a potential perturbation of our findings as a result of the experimental procedure, we independently measured preference and consumption of 16 vol. % alcohol in $Klb^{fl/fl}$ and Klb^{Camk2a} mice without implantation of an osmotic minipump. Again, Klb^{Camk2a} mice showed significantly greater alcohol consumption and increased alcohol preference compared to $Klb^{fl/fl}$ mice (Figure 2A,B), thus replicating our findings above. Alcohol bioavailability after an i.p. injection was not different between $Klb^{fl/fl}$ and Klb^{Camk2a} mice after 1 and 3 hours (Figure 2C).

Increased alcohol drinking in humans and mice may be motivated by its rewarding properties or as a means to relieve anxiety[16]. FGF21 increases corticotrophin release factor and catecholamine release in mice[17], which is linked to heightened anxiety. We therefore tested $Klb^{fl/fl}$ and Klb^{Camk2a} mice in behavioral paradigms measuring anxiety, including novelty suppressed feeding (Supplementary Figure 4A), elevated plus maze (Supplementary Figure 4B), and open field activity

tests (Supplementary Figure 4C) but did not find differences between *Klb*$^{fl/fl}$ and *Klb*^{Camk2a} mice in any of the anxiety measures, or in general locomotor activity. Our finding of increased alcohol preference in *Klb*^{Camk2a} mice may thus be caused by alteration of alcohol-associated reward mechanisms. This notion is consistent with our previous results showing *Klb* expression in areas important for alcohol reinforcement, specifically the nucleus accumbens and the ventral tegmental area[12].

Here we report from observations in over 100,000 individuals that SNP rs11940694 in *KLB* associates with alcohol consumption. In functional animal experiments we show that β-Klotho controls alcohol drinking through a central nervous system mechanism involving the action of FGF21 that is secreted in the liver. Whereas most previous studies investigating the mechanisms underlying alcohol drinking behavior have focused on investigations of brain (or liver) functions alone, our results suggest the possibility of a coordinated action across the two organ systems, liver and brain. This *FGF21-KLB* axis may be involved in regulation of complex adaptive behaviors involving alcohol drinking.

ONLINE METHODS

Alcohol phenotypes

Alcohol intake in grams of alcohol per day was estimated by each cohort based on information about drinking frequency and type of alcohol consumed. For cohorts that collected data in 'drinks per week', standard ethanol contents in different types of alcohol drinks were provided as guidance to convert the data to 'grams per week', which was further divided by 7 to give intake as 'grams per day'. Adjustment was made if cohort-specific drink sizes differed from the standard. For cohorts that collected alcohol use in grams of ethanol per week, the numbers were divided by 7 directly into 'grams per day'. Cohorts with only a categorical response to the question for drinks per week used mid-points of each category for the calculation. All non-drinkers (individuals reporting zero drinks per week) were removed from the analysis. The 'grams per day' variable was then $\log_{10}$ transformed prior to the analysis. Sex-specific residuals were derived by regressing alcohol in $\log_{10}$(grams per day) in a linear model on age, age-square, weight, and if applicable, study site and principal components to account for population structure. The sex-specific residuals were pooled and used as the main phenotype for subsequent analyses.

Dichotomous alcohol phenotype was created based on categorization of 'drinks per week' variable. Heavy drinking was defined as >=21 drinks per week in men, or >=14 drinks per week in women. Light (or zero) drinking was defined if male participants had <=14 drinks per week, or female participants had <=7 drinks per week. Drinkers having >14 to <21 drinks for men, or >7 to <14 drinks for women were excluded. Where information was available, current non-drinker who was former drinker of >14 drinker per week in men, and >7 drinks per week in women, as well as current non-drinker who was a former drinker of unknown amount were

excluded; whereas current non-drinkers who were former drinkers of <=14 for men or <=7 for women were included. Further exclusion was made if there were missing data on alcohol consumption or on the covariates. The analyses only included participants of European origin.

Discovery GWAS in AlcGen and CHARGE+ and replication analyses

Genotyping methods are summarized in Supplementary Table 1B, 1C and 1F. SNPs were excluded if: HWE $P < 1x10^{-6}$ or based on cohort-specific criteria; MAF < 1%; imputation information score < 0.5; if results were only available from 2 or fewer cohorts, or total N < 10,000. Population structure was accounted for within cohorts via principal components analysis (PCA). LD score regression[18] was conducted on the GWAS summary results to examine the degree of inflation in test statistics, and genomic control correction was considered unnecessary (λGC=1.06 and intercept=1.00; λ=0.99 to 1.06 for individual cohorts, Supplementary Table 1B, 1C). SNPs were taken forward for replication from discovery GWAS if they passed the above criteria and if they had $P < 1x10^{-6}$ (one SNP with the smallest *P* taken forward in each region, except for *AUTS2* for which two SNPs were taken forward based on previous results[6]). Meta-analyses were performed by METAL[19] or R (v3.2.2).

Gene Expression Profiling in Framingham study

In the Framingham study, gene expression profiling was undertaken for the blood samples of a total of 5,626 participants from the Offspring (N=2,446) at examination eight and the Third Generation (N=3,180) at examination two. Fasting peripheral whole blood samples (2.5ml) were collected in PAXgene™ tubes (PreAnalytiX, Hombrechtikon, Switzerland). RNA expression profiling was conducted using the Affymetrix Human Exon Array ST 1.0 (Affymetrix, Inc., Santa Clara, CA) for samples that passed RNA quality control. The expression values for ~ 18,000 transcripts were obtained from the total 1.2 million core probe sets. Quality control procedures for

transcripts have been described previously. All data used herein are available online in dbGaP (http://www.ncbi.nlm.nih.gov/gap; accession number phs000007).

The *cis*- Expression Quantitative Trait Loci Analysis in Framingham study

To investigate possible effects of rs11940694 in *KLB* on gene expression, we performed *cis*-eQTL analysis. The SNP in *KLB* was used as the independent variable in association analysis with the transcript of *KLB* measured using whole blood samples in the FHS (n=5,236). Affymetrix probe 2724308 was used to represent the *KLB* overall transcript levels. Age, sex, BMI, batch effects and blood cell differentials were included as covariates in the association analysis. Linear mixed model was used to account for familial correlation in association analysis.

Mouse studies

klb knock-out: All mouse experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center. Male littermates (2 to 4-month-old) maintained on a 12 hr light/dark cycle with *ad libitum* access to chow diet (Harlan Teklad TD2916) were used for all experiments. The *Klb* gene was deleted from brain by crossing $Klb^{fl/fl}$ mice with *Camk2a*-Cre mice on a mixed C57BL/6J;129/Sv background as described[9].

Alcohol drinking in mice

For voluntary two-bottle preference experiments, male mice (n=9-13 per group) were given access to two bottles, one containing water and the other containing 2-16% ethanol (vol/vol) in water. After acclimation to the two-bottle paradigm, mice were exposed to each concentration of ethanol

for 4 days. Total fluid intake (water + ethanol-containing water), food intake and body weight were measured each day. Alcohol consumption (g) was calculated based on EtOH density (0.789 g/ml). To obtain accurate alcohol intake that corrected for individual differences in littermate size, alcohol consumption was normalized by body weight per day for each mouse. As a measure of relative alcohol preference, the preference ratio was calculated at each alcohol concentration by dividing total consumed alcohol solution (ml) by total fluid volume. Two-bottle preference assays were also performed with sucrose (0.5 and 5%) and quinine (2 and 20 mg/dl) solutions. For all experiments, the positions of the two bottles were changed every two days to exclude position effects.

Mouse experiments with native FGF21

For FGF21 administration studies, recombinant human FGF21 protein provided by Novo Nordisk was administered at a dose of 0.7 mg/kg/day by subcutaneous osmotic mini-pumps (Alzet 1004). Mice were single caged following mini-pump surgery, which was conducted under isoflurane anesthesia and 24 hour buprenorphine analgesia. Mice were allowed to recover from mini-pump surgery for 4 days prior to alcohol drinking tests. After experiments, mice were sacrificed by decapitation and plasma was collected using EDTA or heparin after centrifugation for 15 minutes at 3000 rpm. Plasma FGF21 concentrations were measured using the Biovendor FGF21 ELISA Kit according to manufacturer's protocol.

Plasma ethanol concentration and clearance

For alcohol bioavailability tests, mice (n=4-5 per group) were injected i.p. with alcohol (2.0 g/kg, 20% w/vol) in saline, and tail vein blood was collected after 1 and 3 hours. Plasma alcohol concentrations were measured using the EnzyChrom™ Ethanol Assay Kit.

Emotional behavior in mice

For open field activity assays, naïve mice were placed in an open arena (44 cm^2, with the center defined as the middle 14 cm^2 and the periphery defined as the area 5 cm from the wall), and the amount of time spent in the center versus along the walls and total distance traveled were measured. For elevated plus maze activity assays, mice were placed in the center of a plus maze with 2 dark enclosed arms and 2 open arms. Mice were allowed to move freely around the maze, and the total duration of time in each arm and the frequency to enter both the closed and open arms was measured. For novelty suppression of feeding assays, mice fasted for 12 hours were placed in a novel environment and the time to approach and eat a known food was measured.

Statistical Analysis

All data are expressed as means ± S.E.M. Statistical analysis between the two groups was performed by unpaired two-tailed Student's t test using Excel or GraphPad Prism (GraphPad Software, Inc.). For multiple comparisons, one-way analysis of variance (ANOVA) with post-hoc Tukey was done using SPSS.

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Main Figures and Tables

Table 1. Associations of single nucleotide polymorphisms* with alcohol intake (log g/day) in the Genome-wide association analysis (GWAS).

SNP	Chr	Position (hg 19)	Nearest gene	Effect / other alleles	EAF[#]	Discovery GWAS		Replication		Combined		
						Beta (SE)	*P*-value	Beta (SE)	*P*-value	Beta (SE)	*P*-value	N
rs780094	2	27741237	*GCKR*	T/C	0.40	-0.0155 (0.0026)	$3.6x10^{-9}$	0.0035 (0.0029)	0.238	-0.0102 (0.0019)	$1.6x10^{-7}$	98,679
rs350721	2	52980427	*ASB3*	C/G	0.18	0.0206 (0.0040)	$3.2x10^{-7}$	-0.0000 (0.0042)	0.994	0.0109 (0.0029)	$1.9x10^{-4}$	100,859
rs197273	2	161894663	*TANK*	A/G	0.49	-0.0141 (0.0026)	$9.8x10^{-8}$	-0.0058 (0.0028)	0.040	-0.0103 (0.0019)	$7.4x10^{-8}$	97,631
rs11940694	4	39414993	*KLB*	A/G	0.42	-0.0137 (0.0027)	$3.2x10^{-7}$	-0.0135 (0.0030)	$5.2x10^{-6}$	-0.0136 (0.0020)	$9.2x10^{-12}$	98,477
rs6943555	7	69806023	*AUTS2*	A/T	0.29	-0.0115 (0.0030)	$1.4x10^{-4}$	-0.0070 (0.0033)	0.032	-0.0094 (0.0022)	$1.9x10^{-5}$	104,282
rs10950202	7	69930098	*AUTS2*	G/C	0.16	-0.0194 (0.0038)	$2.9x10^{-7}$	-0.0015 (0.0042)	0.720	-0.0113 (0.0028)	$5.9x10^{-5}$	105,639

[*] One SNP with smallest *P*-value taken forward per region

[#] Effect Allele Frequency, in Discovery GWAS

Figure 1

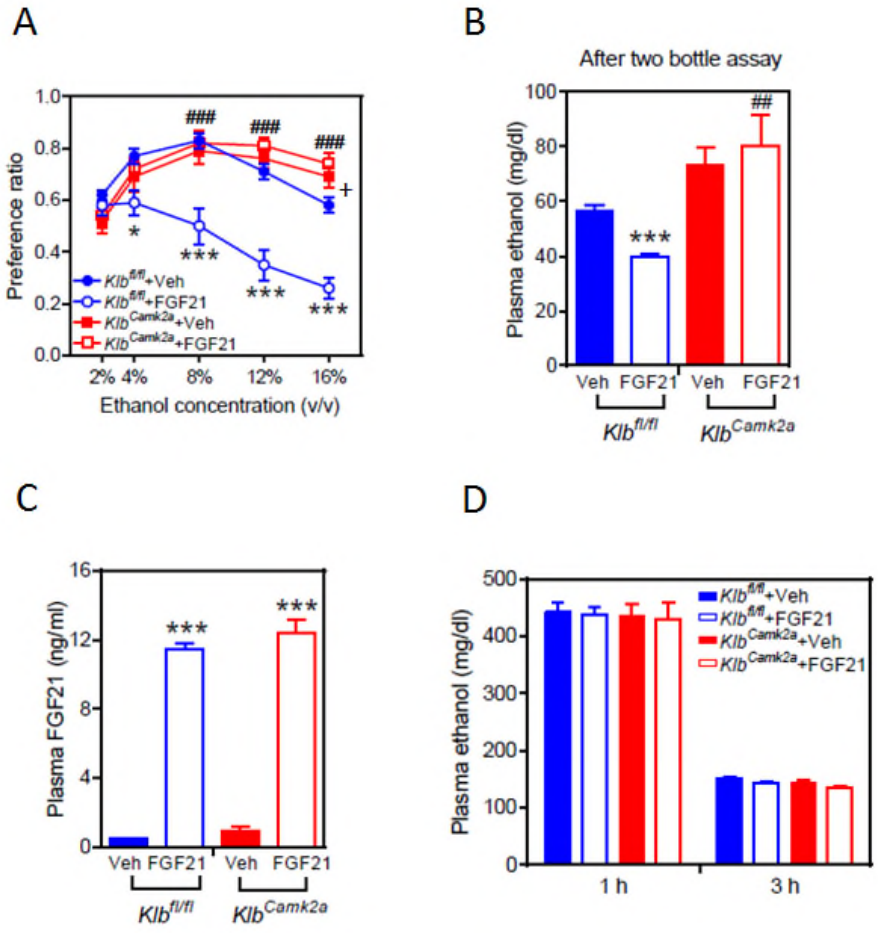
A
Preference ratio
Ethanol concentration (v/v)
2% 4% 8% 12% 16%
Klb^fl/fl+Veh
Klb^fl/fl+FGF21
Klb^Camk2a+Veh
Klb^Camk2a+FGF21
B
After two bottle assay
Plasma ethanol (mg/dl)
Veh FGF21 Veh FGF21
Klb^fl/fl
Klb^Camk2a
C
Plasma FGF21 (ng/ml)
Veh FGF21 Veh FGF21
Klb^fl/fl
Klb^Camk2a
D
Plasma ethanol (mg/dl)
Klb^fl/fl+Veh
Klb^fl/fl+FGF21
Klb^Camk2a+Veh
Klb^Camk2a+FGF21
1 h
3 h

Figure 2

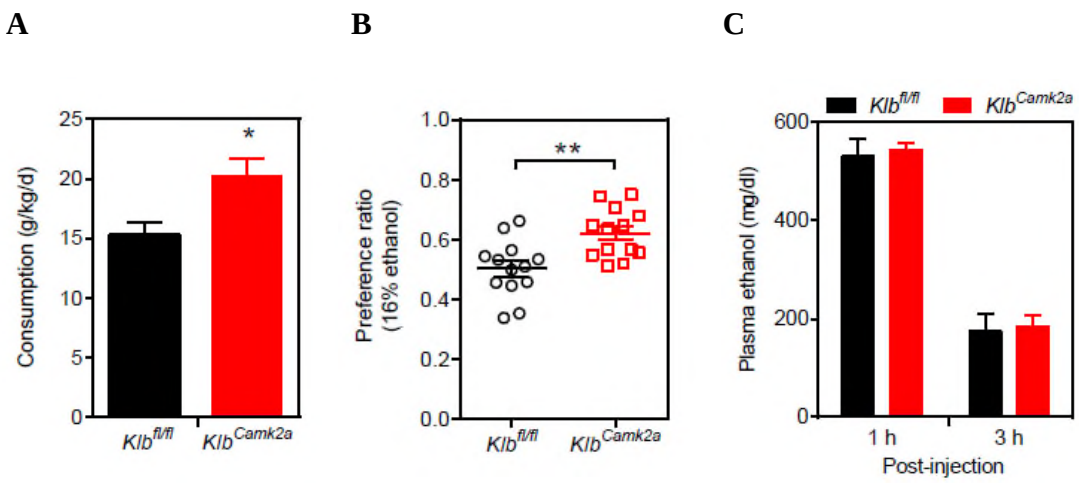
A
B
C
Consumption (g/kg/d)
25
20
15
10
5
0
*
Klb^fl/fl
Klb^Camk2a
Preference ratio (16% ethanol)
1.0
0.8
0.6
0.4
0.2
0.0
**
Klb^fl/fl
Klb^Camk2a
Plasma ethanol (mg/dl)
600
400
200
0
Klb^fl/fl
Klb^Camk2a
1 h
3 h
Post-injection

Figure Legends:

Figure 1: FGF21 reduces alcohol preference by acting on the brain in mice. **(A)** Alcohol preference ratios determined by two-bottle preference assays with water and the indicated ethanol concentrations for control (*Klb*$^{fl/fl}$) and brain-specific β-Klotho knockout (*KlbCamk2a*) mice administered either FGF21 (0.7 mg/kg/day) or vehicle (n=10/ group). **(B)** Plasma ethanol and **(C)** FGF21 concentrations at the end of the 16% ethanol step of the two-bottle assay. **(D)** Plasma ethanol concentrations 1 and 3 hours after i.p. injection of 2 g/kg alcohol (n=4/each group). **(E)** Preference ratios for sucrose and quinine (n=4/each group). Values are means ±S.E.M, $^{+}$p<0.05 for *Klb*$^{fl/fl}$ + vehicle versus *Klb*^{Camk2a} + vehicle groups; *p<0.05; **p<0.01; ***p<0.001 for *Klb*$^{fl/fl}$ + vehicle versus *Klb*$^{fl/fl}$ + FGF21 groups; and $^{\#}$ p<0.05; $^{\#\#}$ p<0.01; $^{\#\#\#}$ p<0.001 for *Klb*$^{fl/fl}$ + FGF21 versus *Klb*^{Camk2a} + FGF21 groups as determined by one-way ANOVA followed by Tukey's post-tests.

Figure 2: β-Klotho in brain regulates alcohol drinking in mice. **(A)** Consumption of 16% ethanol (g/kg/d) and **(B)** alcohol preference ratios in two-bottle preferences assays performed with control (*Klb*$^{fl/fl}$) and brain-specific β-Klotho-knockout (*KlbCamk2a*) mice. Alcohol preference was measured by volume of ethanol/total volume of fluid consumed (n=13/group). **(C)** Plasma ethanol concentrations 1 and 3 hours after i.p. injection of 2 g/kg alcohol (n=5/group).