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Title

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Permalink

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Journal

Nature, 507(7491)

ISSN

0028-0836

Authors

Olalde, Iñigo
Allentoft, Morten E
Sánchez-Quinto, Federico
[et al.](#)

Publication Date

2014-03-01

DOI

10.1038/nature12960

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Peer reviewed

Published in final edited form as:

Nature. 2014 March 13; 507(7491): 225–228. doi:10.1038/nature12960.

Derived Immune and Ancestral Pigmentation Alleles in a 7,000-Year-old Mesolithic European

Iñigo Olalde^{1,*}, Morten E. Allentoft^{2,*}, Federico Sánchez-Quinto¹, Gabriel Santpere¹, Charleston W. K. Chiang³, Michael DeGiorgio⁴, Javier Prado-Martínez¹, Juan Antonio Rodríguez¹, Simon Rasmussen⁵, Javier Quilez¹, Oscar Ramírez¹, Urko M. Marigorta¹, Marcos Fernández-Callejo¹, María Encina Prada⁶, Julio Manuel Vidal Encinas⁷, Rasmus Nielsen⁸, Mihai G. Netea⁹, John Novembre¹⁰, Richard A. Sturm¹¹, Pardis Sabeti^{12,13}, Tomàs Marquès-Bonet^{1,14}, Arcadi Navarro^{1,14}, Eske Willerslev^{2,‡}, and Carles Lalueza-Fox^{1,‡}

¹Institut de Biologia Evolutiva, CSIC-UPF, 08003 Barcelona, Spain ²Center for GeoGenetics, Natural History Museum of Denmark, University of Copenhagen, DK-1350, Copenhagen K, Denmark ³Department of Ecology and Evolutionary Biology, University of California, Los Angeles, California, USA ⁴Department of Integrative Biology, University of California, Berkeley, CA USA ⁵Center for Biological Sequence Analysis, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark ⁶I.E.S.O. 'Los Salados'. Junta de Castilla y León, Spain ⁷Junta de Castilla y León, Servicio de Cultura de León, Spain ⁸Center for Theoretical Evolutionary Genomics, University of California, Berkeley, CA USA ⁹Department of Medicine and Nijmegen Institute for Infection, Inflammation and Immunity, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands ¹⁰Department of Human Genetics, University of Chicago, USA ¹¹Institute for Molecular Bioscience, Melanogenix Group, The University of Queensland, Brisbane, Qld 4072, Australia ¹²Center for Systems Biology, Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA ¹³Broad Institute of the Massachusetts Institute of Technology and Harvard, Cambridge, MA 02142, USA ¹⁴Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Catalonia, Spain

Abstract

Ancient genomic sequences have started revealing the origin and the demographic impact of Neolithic farmers spreading into Europe^{1–3}. The adoption of farming, stock breeding and

‡Corresponding authors: EW (ewillerslev@snm.ku.dk), CL-F (carles.lalueza@upf.edu).

*Both authors equally contributed to this work

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Author Contributions

C.L.-F. and E.W. conceived and lead the project. M.E.P. and J.M.V.E. provided anthropological and archaeological information. O.R. and M.E.A. performed the ancient extractions and library construction, respectively. I.O., M.E.A., F.S.-Q., J.P.-M., S.R., O.R., M.F. and T.M.-B. performed mapping, SNP calling, mtDNA assembly, contamination estimates and different genomic analyses on the ancient genome. I.O., F.S.-Q., G.S., C.W.K.C., M.D.G., J.A.R., J.Q., O.R., U.M.M. and A.N. performed functional, ancestry and population genetic analyses. R.N., and J.N. coordinated the ancestry analyses. M.G.N., R.A.S., and P.S. coordinated the immunological, pigmentation and selection analyses, respectively. I.O., M.E.A., T.M.-B., E.W. and C.L.-F. wrote the majority of the manuscript with critical input from R.N., M.G.N., J.N., R.A.S., P.S. and A.N.

Alignment data are available through the Sequence Read Archive (SRA) (PRJNA230689 and SRP033596).

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The authors declare no competing financial interests.

sedentary societies during the Neolithic may have resulted in adaptive changes in genes associated with immunity and diet⁴. However, the limited data available from earlier hunter-gatherers precludes an understanding of the selective processes associated with this crucial transition to agriculture in recent human evolution. By sequencing a ~7,000-year-old Mesolithic skeleton discovered at the La Braña-Arintero site in León (Spain), we retrieved the first complete pre-agricultural European human genome. Analysis of this genome in the context of other ancient samples suggests the existence of a common ancient genomic signature across Western and Central Eurasia from the Upper Paleolithic to the Mesolithic. The La Braña individual carries ancestral alleles in several skin pigmentation genes, suggesting that the light skin of modern Europeans was not yet ubiquitous in Mesolithic times. Moreover, we provide evidence that a significant number of derived, putatively adaptive variants associated with pathogen resistance in modern Europeans were already present in this hunter-gatherer. Hence, these genomic variants cannot represent novel mutations that occurred during the adaptation to the farming lifestyle.

Next-generation sequencing (NGS) technologies are revolutionizing the field of ancient DNA (aDNA), and have allowed the sequencing of complete ancient genomes^{5,6}, such as that of Ötzi, a Neolithic human body found in the Alps¹. However, very little is known of the genetic composition of earlier hunter-gatherer populations from the Mesolithic period (ca 10,000–5,000 years before present, BP, that immediately preceded the Neolithic period).

The Iberian site called La Braña-Arintero was discovered in 2006 when two male skeletons were found in a deep cave system, 1,500 meters above sea level in the Cantabrian mountain range (León, Northwestern Spain) (Fig. 1a). The skeletons were dated to ~7,000 years BP (7,940–7,690 calibrated BP)⁷. Because of the cold environment and stable thermal conditions in the cave, the preservation of these specimens proved to be exceptional (Fig. 1b). We identified a tooth with high human DNA content (48.4%) and sequenced this specimen to a final 3.40X effective genomic depth-of-coverage (Extended Data Fig. 1).

We undertook several tests to assess the authenticity of the genome sequence and to determine the amount of potential modern human contamination. First, we observed that sequence reads from both the mitochondrial DNA (mtDNA) and the nuclear DNA of La Braña 1 showed the typical ancient DNA misincorporation patterns that arise from degradation of DNA over time⁸ (Extended Data Fig. 2a, b). Second, we showed that the observed number of human DNA fragments was negatively correlated with the fragment length ($R^2 > 0.92$), as expected for ancient degraded DNA, and that the estimated rate of DNA decay was low and in agreement with predicted values⁹ (Extended Data Fig. 2c, d). We then estimated the contamination rate in the mtDNA genome, assembled to a high depth-of-coverage (91X), by checking for positions differing from the mtDNA genome (haplogroup U5b2c1) that was previously retrieved with a capture method². We obtained an upper contamination limit of 1.69% (0.75%–2.6%, 95% CI) (Supplementary Information). Finally, to generate a direct estimate of nuclear DNA contamination, we screened for heterozygous positions (among reads with >4x coverage) in known polymorphic sites (dbSNP-137) at uniquely mapped sections on the X chromosome⁶ (Supplementary Information). We found that the proportion of false heterozygous sites was 0.31%. Together these results suggest low levels of contamination in the La Braña 1 sequence data.

To investigate the relationship to extant European samples, we conducted Principal Component Analysis (PCA)¹⁰ and found the ~7,000-year-old Mesolithic sample was divergent from extant European populations (Extended Data Fig. 3a, b), but was placed in proximity to Northern Europeans (e.g. samples from Sweden and Finland)^{11–14}. Additional PCAs and allele sharing analyses with ancient Scandinavian specimens³ supported the genetic similarity of the La Braña 1 genome to Neolithic hunter-gatherers (Afv70, Ajv52, Ire8) relative to Neolithic farmers (Gok4, Ötzi) (Fig. 1c, Extended Data Fig. 3c and Extended Data Fig. 4). Thus, this Mesolithic individual from Southwestern Europe represents a formerly widespread gene pool that seems to be partially preserved in some modern-day Northern European populations, as suggested previously with limited genetic data^{2,3}. We subsequently explored the La Braña affinities to an ancient Upper Palaeolithic genome from Mal'ta site near Lake Baikal in Siberia¹⁵. Outgroup f_3 and D statistics^{16,17}, using different modern reference populations, support that Mal'ta is significantly closer to La Braña 1 than to Asians or modern Europeans (Extended Data Fig. 5 and Supplementary Information). These results suggest that despite the vast geographical distance and temporal span, La Braña 1 and Mal'ta share common genetic ancestry, indicating a genetic continuity in ancient Western and Central Eurasia. This observation matches findings of similar cultural artifacts across time and space in Upper Paleolithic Western Eurasia and Siberia, particularly the presence of anthropomorphic “Venus” figurines which have been recovered from several sites in Europe and Russia, including the Mal'ta site¹⁵. We also compared the genome-wide heterozygosity of the La Braña 1 genome to a dataset of modern humans with similar coverage (3–4X). The overall genomic heterozygosity was 0.042%, lower than the values observed in present day Asians (0.046–0.047%), Europeans (0.051–0.054%), and Africans (0.066–0.069%) (Extended Data Fig. 6a). The effective population size, estimated from heterozygosity patterns, suggests a global reduction in population size of ~20% relative to extant Europeans (Supplementary Information). Moreover, no evidence of tracts of autozygosity suggestive of inbreeding was observed (Extended Data Fig. 6b).

To systematically investigate the timing of selection events in the recent history of modern Europeans, we compared the La Braña genome to modern populations at loci that have been categorized as of interest for their role in recent adaptive evolution. With respect to two recent well-studied adaptations to changes in diet, we found the ancient genome to carry the ancestral allele for lactose intolerance⁴ and ~5 copies of the salivary amylase (*AMY1*) gene (Extended Data Fig. 7 and Supplementary Information), a copy number compatible with a low-starch diet¹⁸. These results suggest the La Brana hunter-gatherer was poor at digesting milk and starch, supporting the hypotheses that these abilities were selected for during the later transition to agriculture.

To expand the survey, we analyzed a catalog of candidate signals for recent positive selection based on whole-genome sequence variation from the 1000 Genomes Project¹³, which included 35 candidate non-synonymous variants – ten of which were detected uniquely in the CEU sample (Utah residents with Northern and Western European ancestry)¹⁹. For each variant we assessed whether the Mesolithic genome carried the ancestral or derived (putatively adaptive) allele.

Of the ten variants, the Mesolithic genome carried the ancestral and non-selected allele as a homozygote in three regions: *CI2orf29* (a gene with unknown function), *SLC45A2* (rs16891982) and *SLC24A5* (rs1426654) (Table 1). The latter two variants are the two strongest known loci affecting light skin pigmentation in Europeans^{20–22} and their ancestral alleles and associated haplotypes are either absent or segregate at very low frequencies in extant Europeans (3% and 0% for *SLC45A2* and *SLC24A5* respectively) (Fig. 2). We subsequently examined all genes known to be associated with pigmentation in Europeans²², finding ancestral alleles in *MC1R*, *TYR* and *KITLG* and derived alleles in *TYRP1*, *ASIP* and *IRF4* (Supplementary Information). Although the precise phenotypic effects cannot currently be ascertained in a European genetic background, results from functional experiments²⁰ indicate that the allelic combination in this Mesolithic individual is likely to have resulted in dark skin pigmentation and dark/brown hair. Further examination revealed that this individual carried the rs12913832*C single nucleotide polymorphism (SNP) and the associated homozygous haplotype spanning the *HERC2/OCA2* locus that is strongly associated with blue eye color²³. Moreover, a prediction of eye color based on genotypes at additional loci using HIrisPlex²⁴ yielded a 0.823 maximal and 0.672 minimal probability for being non-brown eyed (Supplementary Information). The genotypic combination leading to a predicted phenotype of dark skin and non-brown eyes is unique and no longer present in contemporary European populations. Our results indicate that the adaptive spread of light skin pigmentation alleles was not complete in some European populations by the Mesolithic, and that the spread of alleles associated with light/blue eye color may have preceded changes in skin pigmentation.

For the remaining loci, La Braña 1 displayed the derived, putatively adaptive variants in five cases, including three genes, *PTX4*, *UHRF1BP1*, and *GPATCH1*¹⁹, involved in the immune system (Table 1 and Extended Data Fig. 8). The latter is associated with the risk of bacterial infection. We subsequently determined the allelic states in 63 SNPs from 40 immunity genes with previous evidence for positive selection and for carrying polymorphisms shown to influence susceptibility to infections in modern Europeans (Supplementary Information). La Braña 1 carries derived alleles in 24 genes (60%) that have a wide range of functions in the immune system: pattern recognition receptors, intracellular adaptor molecules, intracellular modulators, cytokines and cytokine receptors, chemokines and chemokine receptors and effector molecules. Interestingly, four out of six SNPs from the first category are intracellular receptors of viral nucleic acids (*TLR3*, *TLR8*, *IFIH1/MDA5* and *LGP2*)²⁵.

Finally, to explore the functional regulation of the genome, we also assessed the La Braña 1 genotype at all expression quantitative trait loci (eQTL) regions associated to positive selection in Europeans (Supplementary Information). The most interesting finding is arguably the predicted overexpression of eight immunity genes (36% of those with described eQTLs), including three Toll-like receptor genes (*TLR1*, *TLR2* and *TLR4*) involved in pathogen recognition²⁶.

These observations suggest that the Neolithic transition did not drive all cases of adaptive innovation on immunity genes found in modern Europeans. Several of the derived haplotypes seen at high frequency today in extant Europeans were already present during the Mesolithic, either as neutral standing variation and/or due to selection predating the

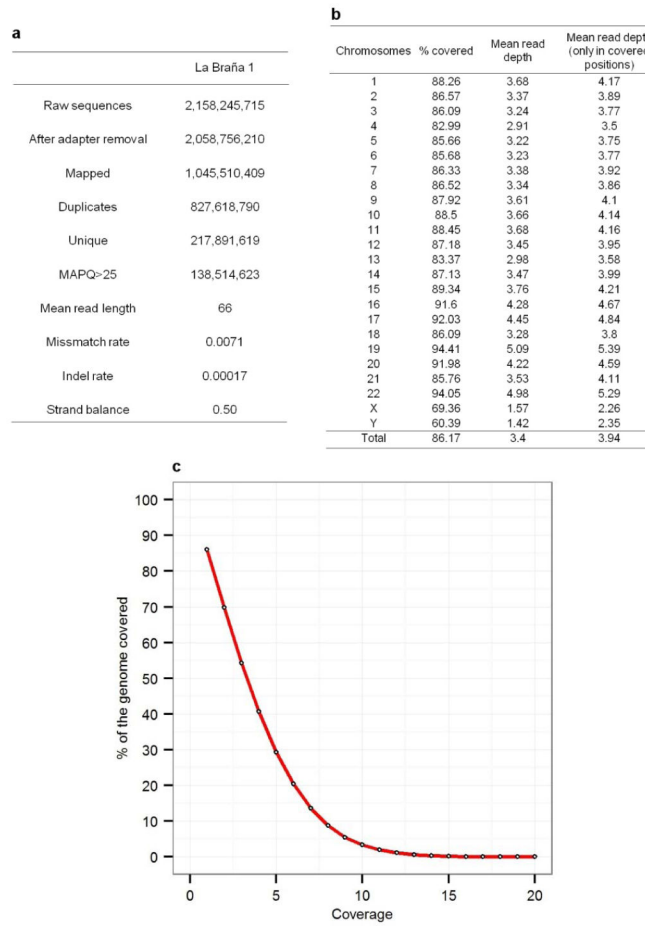
Neolithic. *De novo* mutations that increased in frequency rapidly in response to zoonotic infections during the transition to farming should be identified among those genes where La Braña 1 carries ancestral alleles.

To confirm if the genomic traits seen at La Braña can be generalized to other Mesolithic populations, analyses of additional ancient genomes from Central and Northern Europe will be needed. Nevertheless, this genome sequence provides the first insight as to how these hunter-gatherers are related to contemporary Europeans and other ancient peoples in both Europe and Asia, and suggests how ancient DNA can shed light on the timing and nature of recent positive selection.

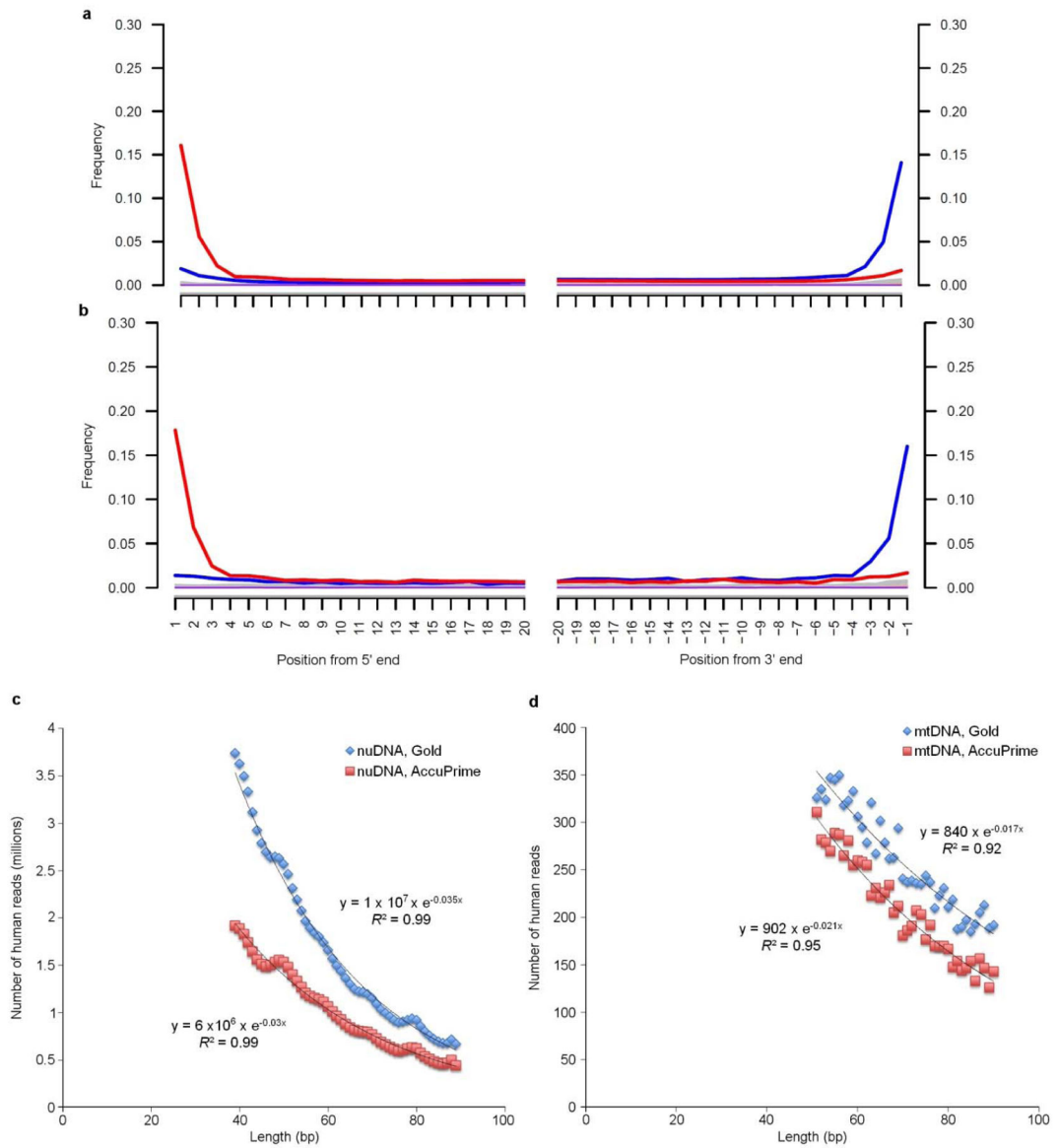
Methods Summary

DNA was extracted from the La Braña 1 tooth specimen with a previously published protocol². Indexed libraries were built from the ancient extract and sequenced on the Illumina HiSeq platform. Reads generated were mapped with BWA²⁷ to the human reference genome (NCBI 37, hg19) after primer trimming. A metagenomic analysis and taxonomic identification was generated with the remaining reads using BLAST 2.2.27+ and MEGAN4²⁸ (Extended Data Fig. 9 and Supplementary Information). SNP calling was undertaken using a specific bioinformatic pipeline designed to account for ancient DNA errors. Specifically, the quality of misincorporations likely caused by aDNA damage was rescaled using the mapDamage2.0 software²⁹, and a set of variants with a minimum read depth of 4 was produced with GATK³⁰. Analyses including PCA¹⁰, Outgroup f_3 ¹⁶ and D statistics¹⁷ were performed to determine the population affinities of this Mesolithic individual (Supplementary Information).

Extended Data

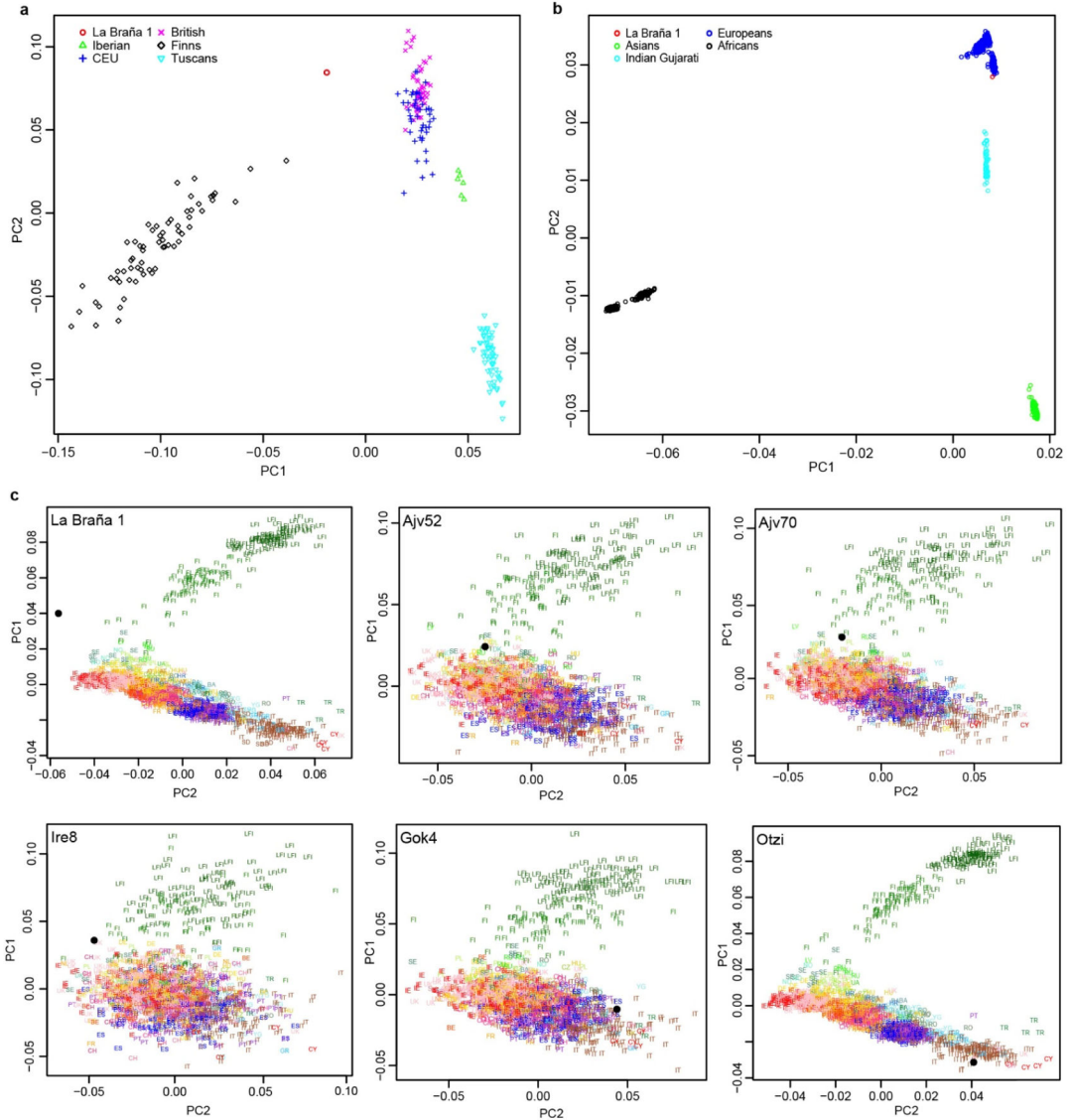


Extended Data Figure 1. Alignment and coverage statistics of the La Braña 1 genome
a, Alignment summary of La Braña 1 sequence data to hg19 assembly. **b**, Coverage statistics per chromosome. The percentage of the chromosome covered by at least one read is shown, as well as the mean read depth of all positions and positions covered by at least one read. **c**, Percentage of the genome covered at different minimum read depths.



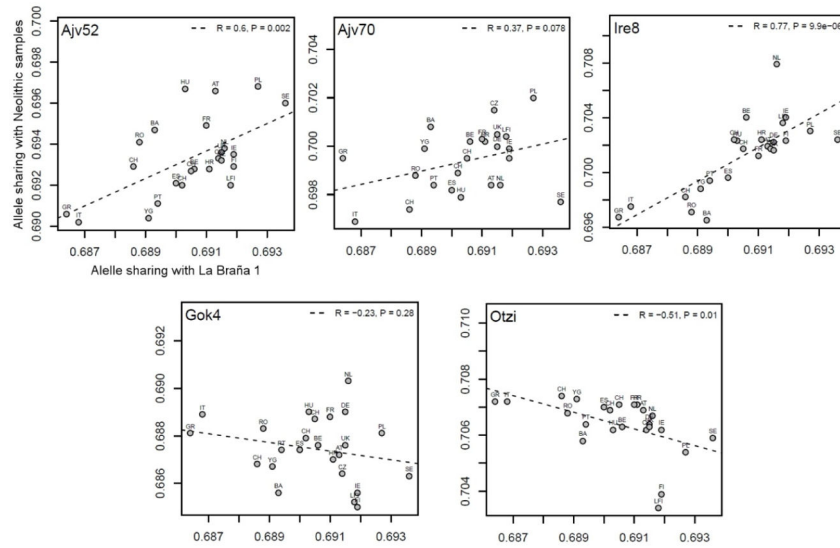
Extended Data Figure 2. Damage pattern of La Braña 1 sequenced reads

Frequencies of C to T (red) and G to A (blue) misincorporations at the 5' end (left) and 3' end (right) are shown for the nuclear DNA (a) and mtDNA (b). Fragment length distribution of reads mapping to the nuclear genome (c) and mtDNA genome (d). Coefficients of determination (R^2) for an exponential decline and damage fractions (λ) are provided for the four different datasets.



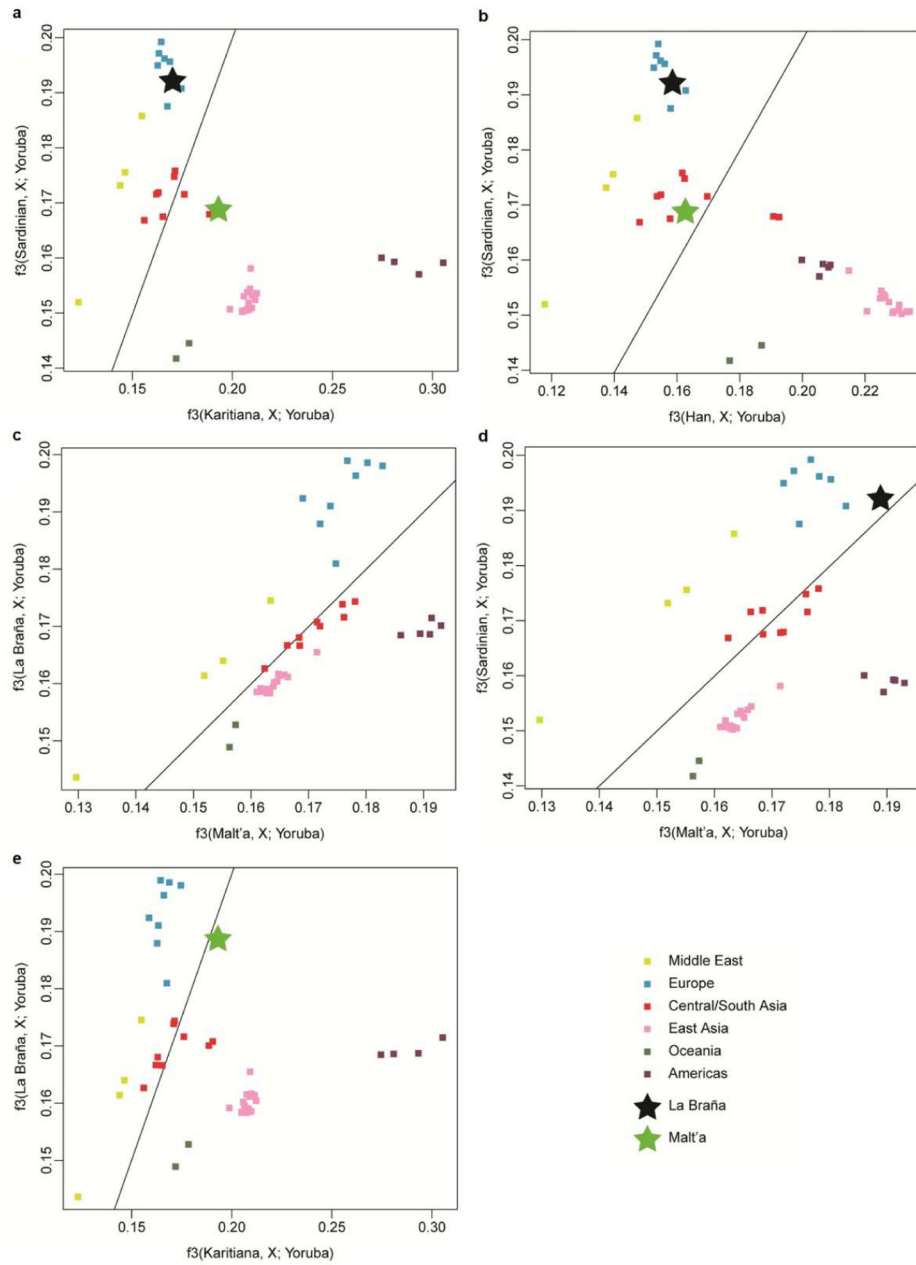
Extended Data Figure 3. Genetic affinities of the La Braña 1 genome

a, Principal Component Analysis (PCA) of the La Braña 1 SNP data and the 1000G Project European individuals. **b**, PCA of La Braña 1 vs Omni world-wide data. Continental terms refer to each Omni population groupings as follows. Africans: Yoruba and Luyha. Asians: Chinese (Beijing, Denver, South, Dai), Japanese and Vietnamese. Europeans: Iberians, Tuscans, British, Finns and CEU; and Indian Gujarati from Texas. **c**, Each panel shows PC1 and PC2 based on the PCA of one of the ancient samples with the merged POPRES +FINHM sample, prior to Procrustes transformation. The ancient samples include the La Braña 1 sample, four Neolithic samples from Skoglund et al.³ and Keller et al.¹.



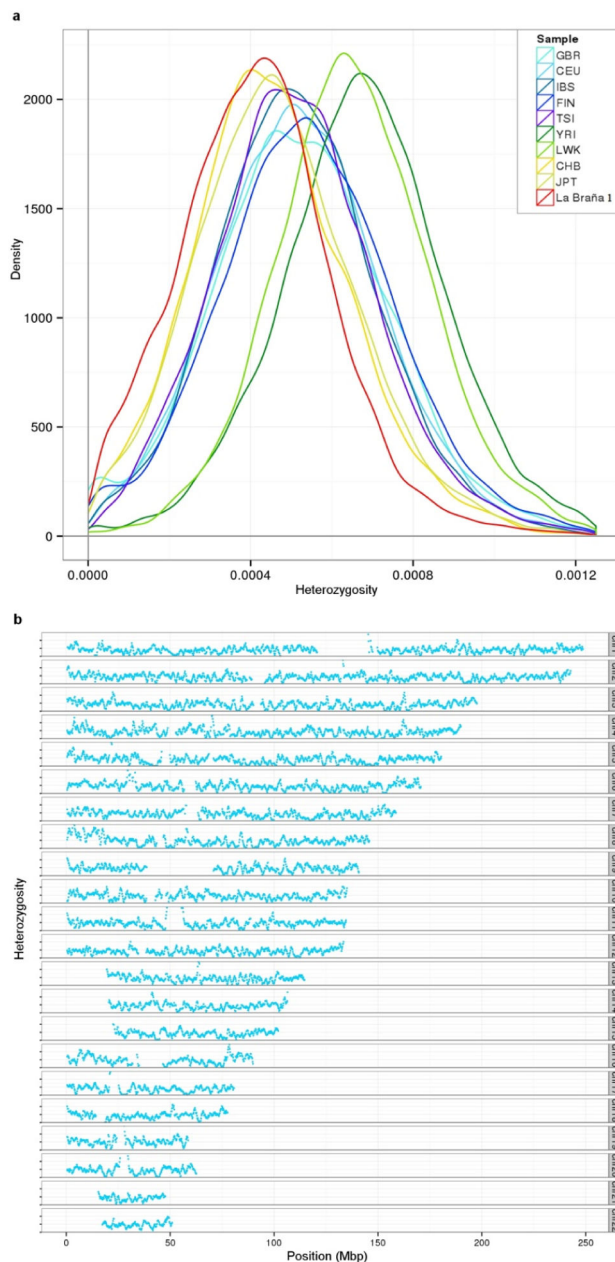
Extended Data Figure 4. Allele sharing analysis

Each panel shows the allele sharing of a particular Neolithic sample from Skoglund et al.³ and Keller et al.¹ with the La Braña 1 sample. The sample IDs are presented in the upper left of each panel (Ajv52, Ajv70, Ire8, Gok4, and Ötzi). In the upper right of each panel, the Pearson’s correlation coefficient is given with associated p-value.



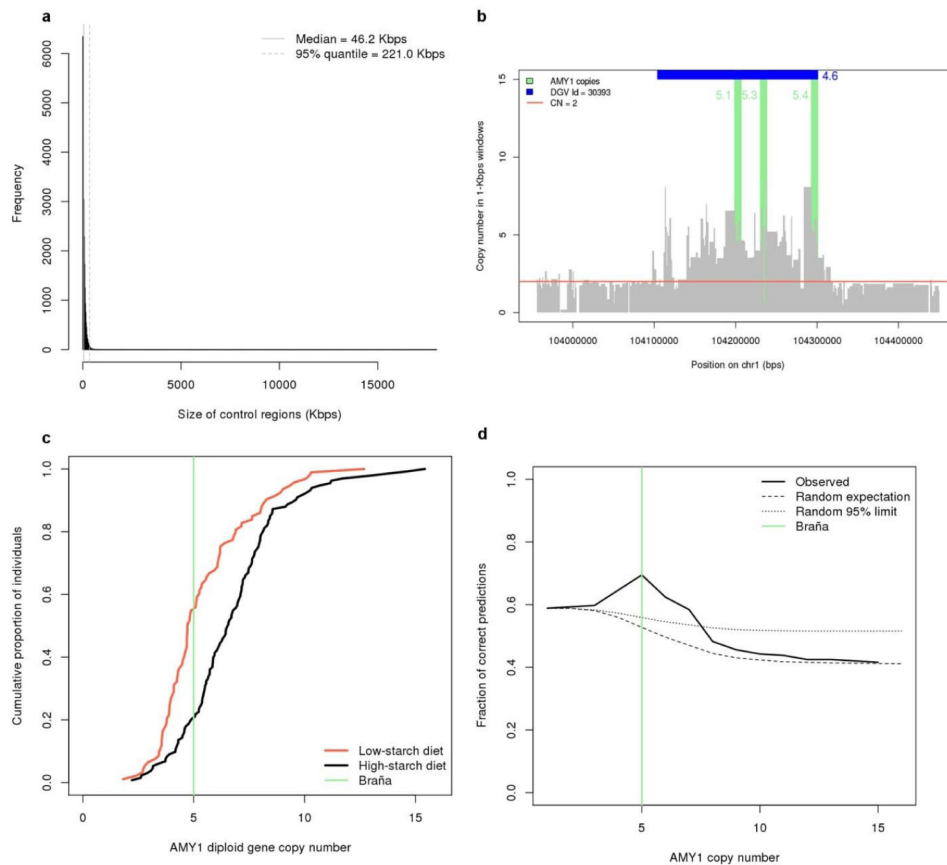
Extended Data Figure 5. Pairwise outgroup f_3 statistics

a, Sardinian vs. Karitiana. **b**, Sardinian vs. Han. **c**, La Braña 1 vs. Mal'ta. **d**, Sardinian vs Mal'ta. **e**, La Braña 1 vs. Karitiana. The solid line is the $y = x$ line.



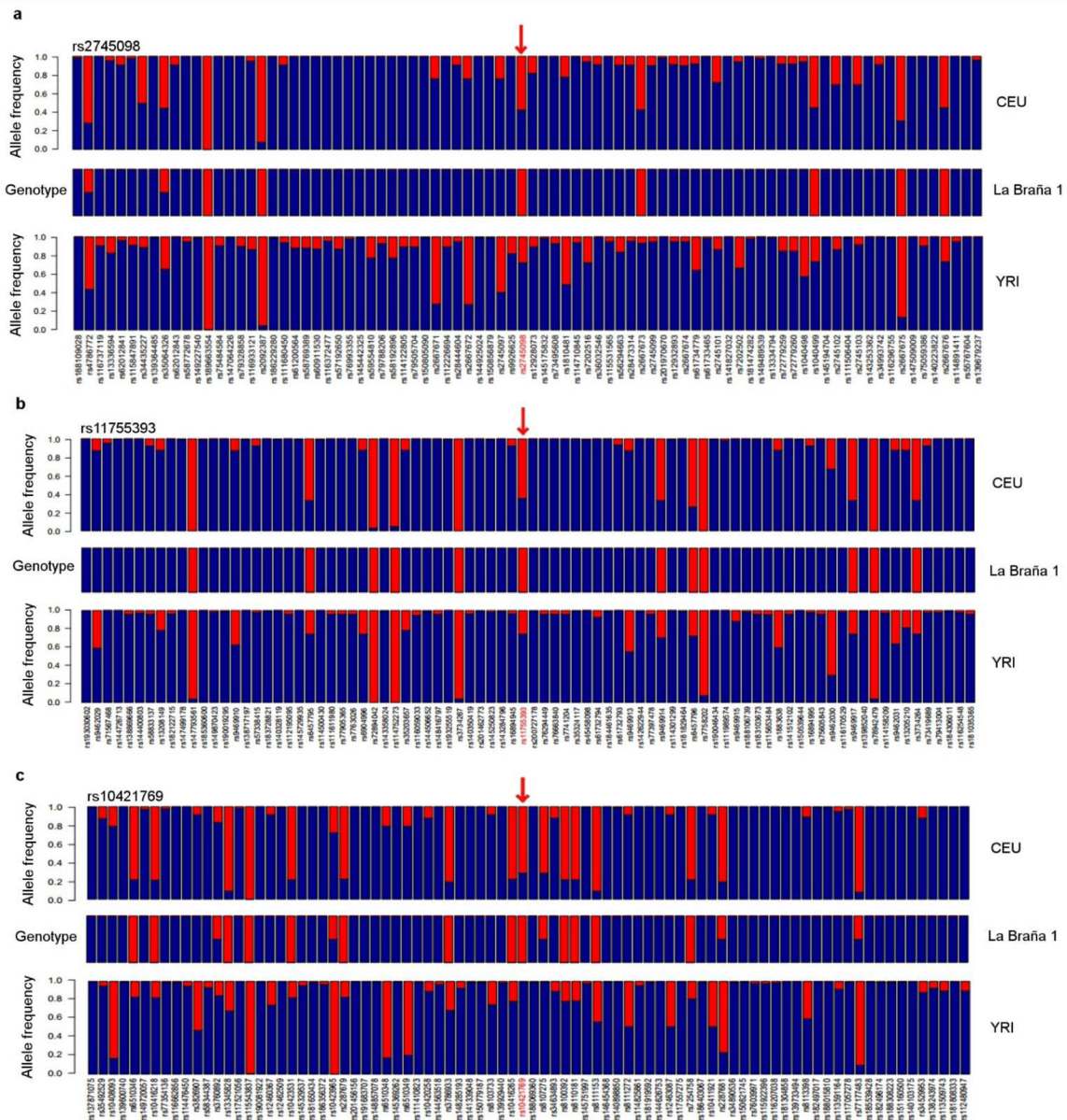
Extended Data Figure 6. Analysis of heterozygosity

a, Heterozygosity distributions of La Braña 1 and modern individuals with similar coverage from the 1,000 Genomes Project (using 1 Mb-windows with 200 Kb overlap). GBR: Great Britain, CEU: Northern-Western European ancestry, IBS: Iberians, FIN: Finns, TSI: Tuscans, YRI: Yorubans, LWK: Luhya, CHB: Han Chinese, JPT: Japanese. b, Heterozygosity values in 1-Mb windows (with 200 kb overlap) across each chromosome.

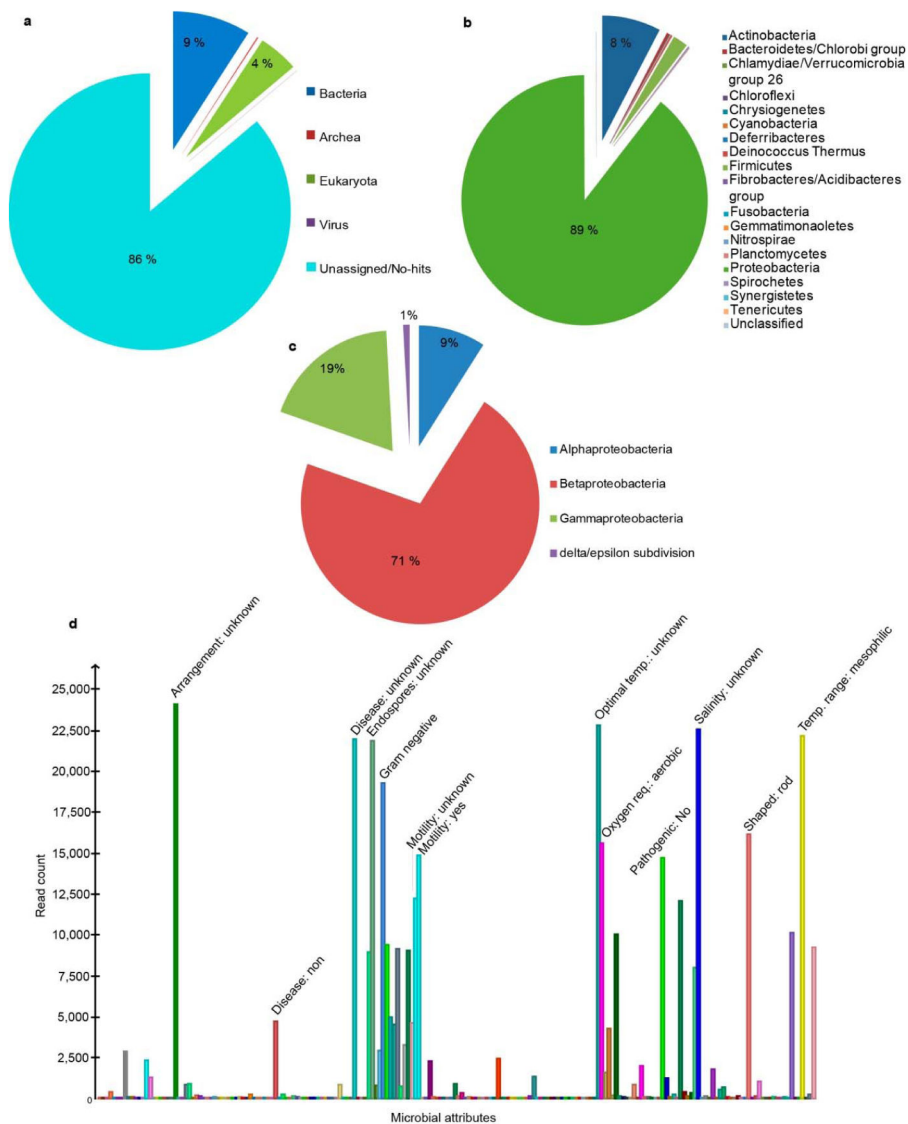


Extended Data Figure 7. Amylase copy number analysis

a, Size distribution of diploid control regions. b, *AMY1* gene copy number in La Braña 1. Abbreviations: DGV = Database of Genomic Variation; CN = copy number. c, La Braña 1 *AMY1* gene copy number in the context of low-and high-starch diet populations. d, Classification of low-and high-starch diet individuals based on *AMY1* copy number. Using data from¹⁸, individuals were classified as in low-(less or equal than) or high-starch (higher than) categories and the fraction of correct predictions (g) was calculated. In addition, we calculated the random expectation and 95% limit of low-starch diet individuals classified correctly at each threshold value.



Extended Data Figure 8. Neighboring variants for three diagnostic SNPs related to immunity (a) rs2745098 (*PTX4* gene), (b) rs11755393 (*UHRF1BP1* gene) and (c) rs10421769 (*GPATCH1* gene). For *PTX4*, *UHRF1BP1* and *GPATCH1*, La Braña 1 displays the derived allele and the European-specific haplotype, indicating that the selective sweep was already present in the Mesolithic. Blue: ancestral, red: derived.



Extended Data Figure 9. Metagenomic analysis of the non-human reads
a, Domain attribution of the non-human reads. **b**, Proportion of different Bacteria groups. **c**, Proportion of different types of Proteobacteria. **d**, Microbial attributes of the microbes present in the La Braña 1 sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Luis Alfredo Grau Lobo (Museo de León), for access to the La Braña specimen and Maanasa Raghavan for early access to Mal'ta genome data. Sequencing was performed at the Danish National High-Throughput DNA-Sequencing Centre, University of Copenhagen. The POPRES data were obtained from dbGaP (accession number # 2038). The Danish National Research Foundation, ERC Starting Grant (260372) to TM-B, and (310372) to MGN, FEDER and Spanish Government Grants BFU2009-13409-C02-02, BFU2012-38236 to AN, BFU2011-28549 to

TM-B, BFU2012-34157 to CL-F, ERC (Marie Curie Actions) to MEA, NIH NRSA postdoctoral fellowship (F32GM106656) to CWKC, NIH (R01-HG007089) to JN, NSF postdoctoral fellowship (DBI-1103639) to MDG, the Australian NHMRC to RAS and a predoctoral fellowship from the Basque Government (DEUI) to IO for economic support.

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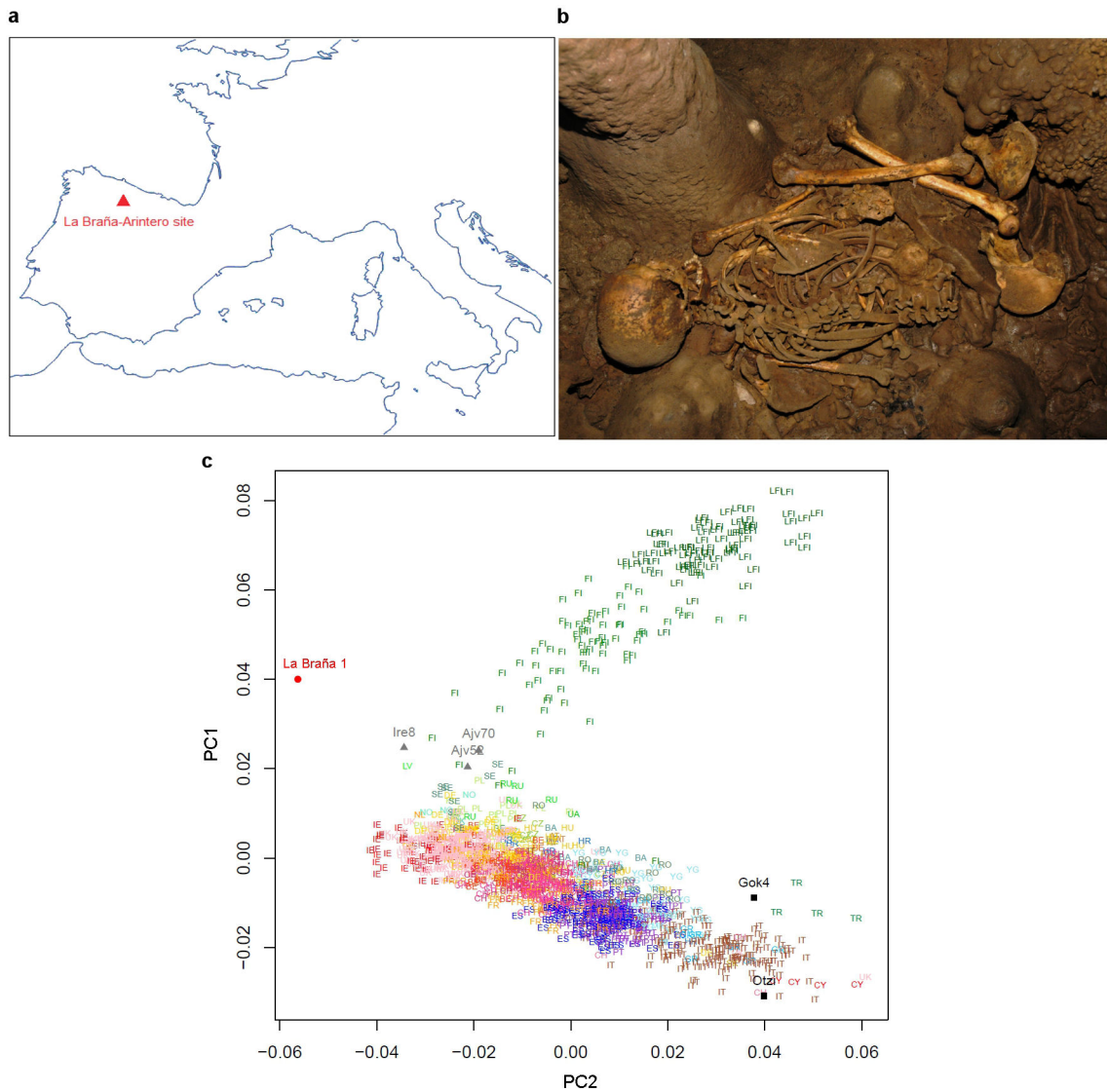


Figure 1. Geographic location and genetic affinities of the La Braña 1 individual
a, Location of the La Braña-Arintero site (Spain). **b**, The La Braña 1 skeleton as discovered in 2006. **c**, Principal Component Analysis (PCA) based on the average of the Procrustes transformations of individual PCAs with La Braña and each of the five Neolithic samples^{1,3}. The reference populations are the Finnish HapMap, FINHM, and POPRES. Population labels with labeling of ¹² with the addition of FI (Finns) or LFI (Late-settlement Finns). Ajv70, Ajv52, Ire8 and Gok4 are Scandinavian Neolithic hunter-gatherers and a farmer, respectively³. Ötzi is the Tyrolean Ice Man¹.

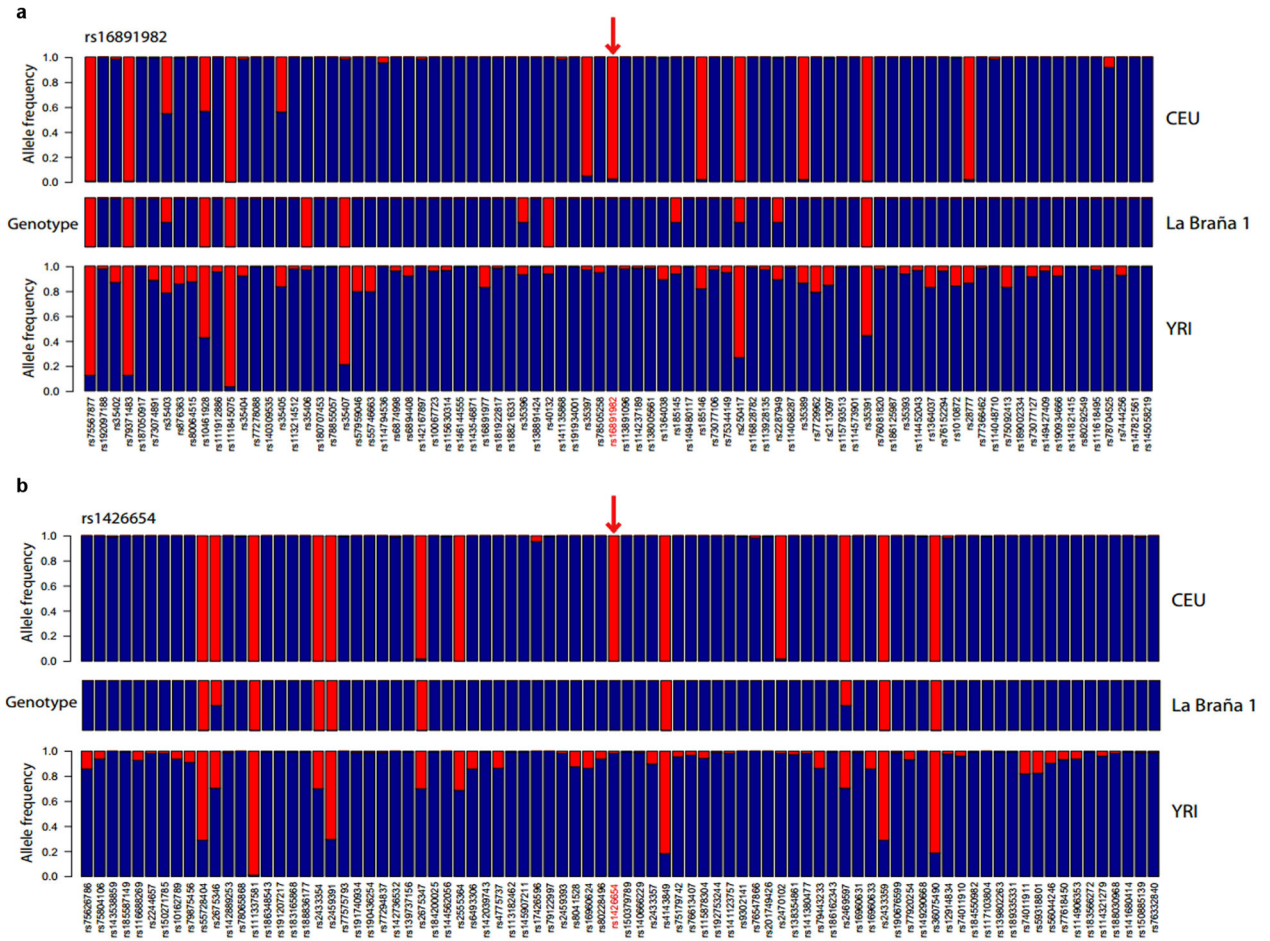


Figure 2. Ancestral variants around the *SLC45A2* (rs16891982, above) and *SLC24A5* (rs1426654, below) pigmentation genes in the Mesolithic genome (blue: ancestral, red: derived)
 SNPs around the two diagnostic variants in these two genes were analyzed. The resulting haplotype comprises neighboring SNPs that are also absent in modern Europeans (CEU) but present in Yorubans (YRI). This pattern confirms that the La Braña 1 sample is older than the selective sweep in these regions.

Table 1
Mesolithic genome allelic state at 10 nonsynonymous variants recently selected in Europeans

Gene	Name	SNP	Amino acid change	Function
La Braña 1 carries the derived allele	<i>PTX4</i>	rs2745098	R281K	May be involved in innate immunity
	<i>UHRF1BP1</i>	rs11755393	Q454R	Risk locus for systemic lupus erythematosus
	<i>GPATCH1</i>	rs10421769	L520S	Receptor for OmpA expressed by <i>E. coli</i>
	<i>WWOX</i>	rs12918952	A179T	Acts as a tumor suppressor and plays a role in apoptosis
	<i>CCDC14</i>	rs17310144	T365P	Unknown
La Braña 1 carries both the ancestral and the derived allele	<i>SETX</i>	rs1056899	V2587I	Involved in Spinocerebellar Ataxia and Amyotrophic lateral sclerosis
	<i>TDRD12</i>	rs11881633	E413K	Unknown
La Braña 1 retains the ancestral allele	<i>C12orf29</i>	rs9262	V238L	Unknown
	<i>SLC45A2</i>	rs16891982	L374F	Associated to skin pigmentation
	<i>SLC24A5</i>	rs1426654	A111T	Associated to skin pigmentation