genetic variation within this enhancer is associated with modest impact on TF binding, BCL11A expression, and HbF level. Relatively small effect sizes associated with individual variants may not be surprising given that most single-nucleotide substitutions, even within critical motifs, result in only modest loss of enhancer activity (*31*, *32*). In contrast, loss of the *BCL11A* enhancer results in the absence of BCL11A expression in the erythroid lineage. Most trait-associated SNPs identified by GWAS are noncoding and have small effect sizes (*1*, *33*). The impact of GWAS-identified SNPs on biological processes is often uncertain. Our findings underscore how a modest influence engendered by an individual noncoding variant neither predicts nor precludes a profound contribution of an underlying regulatory element.

Challenges to inhibiting BCL11A for mechanism-based reactivation of HbF include the supposedly "undruggable" nature of transcription factors (*34*) and its important nonerythroid functions (*20*, *30*). With recent developments in their efficiency and precision, sequence-specific nucleases can be designed to exquisitely target genomic sequences of interest (*35*–*37*). We propose the GWAS-identified enhancer of *BCL11A* as a particularly promising therapeutic target for genome engineering in the β-hemoglobinopathies. Disruption of this enhancer would impair BCL11A expression in erythroid precursors with resultant HbF derepression while sparing BCL11A expression in nonerythroid lineages. Rational intervention might mimic common protective genetic variation.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S9
Tables S1 to S6
References (*38*–*54*)

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Ancient DNA Reveals Key Stages in the Formation of Central European Mitochondrial Genetic Diversity

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The processes that shaped modern European mitochondrial DNA (mtDNA) variation remain unclear. The initial peopling by Palaeolithic hunter-gatherers ~42,000 years ago and the immigration of Neolithic farmers into Europe ~8000 years ago appear to have played important roles but do not explain present-day mtDNA diversity. We generated mtDNA profiles of 364 individuals from prehistoric cultures in Central Europe to perform a chronological study, spanning the Early Neolithic to the Early Bronze Age (5500 to 1550 calibrated years before the common era). We used this transect through time to identify four marked shifts in genetic composition during the Neolithic period, revealing a key role for Late Neolithic cultures in shaping modern Central European genetic diversity.

The Central European Neolithic and the subsequent Early Bronze Age (EBA) reflect periods of momentous cultural changes (*1*–*4*). However, the extent to which such prehistoric cultural changes were accompanied by differences in the underlying genetics of local populations (*1*–*5*) and how such population shifts contributed to the present-day genetic diversity of Central Europe (*6*–*9*) are yet to be understood. Ancient DNA studies have revealed genetic discontinuities between indigenous hunter-gatherers and early farmers and between the latter and present-day Europeans (*10*, *11*). Although this confirms the importance of genetic shifts after the arrival of farming, the number and sequence of events and their potential origins and contributions to the genetic composition of modern-day Central Europe remain unclear (*5*, *6*, *12*).

We collected samples from 25 sites of the Mittelelbe-Saale region in Saxony-Anhalt, Germany, attributed to nine archaeological cultures of the Early, Middle, and Late Neolithic period and the EBA, spanning ~4000 years (Fig. 1A, figs. S1 and S2, and table S1) (*13*). Mittelelbe-Saale played a key role in human prehistory in Central Europe (*4*, *13*), and the continuous settlement activity

from the Palaeolithic until today provides a detailed record of Neolithic cultures, including those with expansive European importance, such as the Linear Pottery (LBK), Funnel Beaker (FBC), Corded Ware (CWC), and Bell-Beaker cultures (BBC) (fig. S2) (*1*–*4*, *13*). We genotyped the hypervariable segment I and II of the control region and 22 single-nucleotide coding region polymorphisms from 364 individuals (tables S2 and S3) (*13*), allowing unambiguous haplogroup assignment, in order to characterize changes in the mitochondrial DNA (mtDNA) variability of the Mittelelbe-Saale cultures. To examine genetic affinities of the investigated cultures to prehistoric and modern-day populations, we used 198 mtDNA data from published Mesolithic, Neolithic, and Bronze Age specimens across western Eurasia (Fig. 1B and table S4) (*13*) and a database of 67,996 sequences from present-day Eurasian populations (*13*). We animated our results to illustrate the observed changes in space and time (movie S1).

In order to detect patterns of continuity or discontinuity among and between the archaeological cultures, we conducted a cluster analysis (Fig. 2A and table S5) based on haplogroup frequencies and used sequence data to perform a genetic distance analysis (F_{st}) (Fig. 2, B and C, and table S6) and analyses of molecular variance (AMOVA) (table S7) (*13*). We performed a Mantel test to examine whether the genetic distances correlate with the temporal distances between the ancient cultures, as expected from genetic drift affecting small populations. However, the Mantel test shows no strong correlation with time (Pearson's coefficient $r = 0.3923$, $P = 0.0591$), suggesting more sudden and marked fluctuations in genetic composition. We also developed a test for population continuity (TPC) (Fig. 2D and table S8) to further evaluate whether changes in haplogroup frequencies and composition could be explained by genetic drift or are likely due to other factors such as introgression via migration (introducing new haplogroups) or replacement (*13*). Our detailed transect through time reveals

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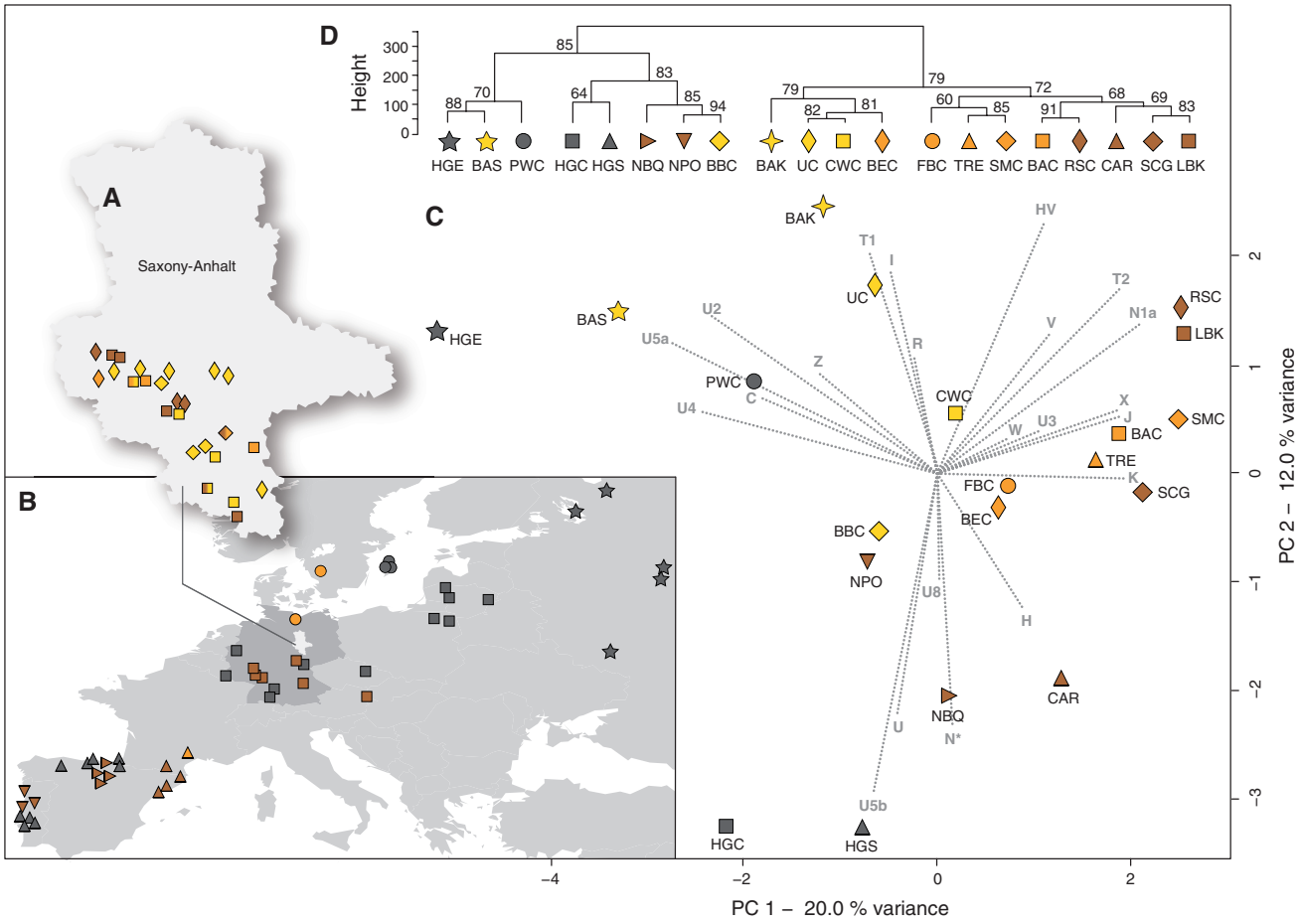

Fig. 1. Location of Mittelelbe-Saale and prehistoric comparative data, as well as PCA and Ward clustering. (**A**) The locations of study sites in the Mittelelbe-Saale region in Saxony-Anhalt, Germany, of the Early Neolithic (LBK; RSC, Rössen culture; and SCG, Schöningen group), Middle Neolithic (BAC, Baalberge culture), SMC, Salzmünde culture; and BEC), Late Neolithic (CWC and BBC), and Early Bronze Age (UC, Unetice culture) cultures. (**B**) The locations of published data from 11 Mesolithic (HGC, hunter-gatherer Central Europe; HGS, hunter-gatherer South Europe; HGE, hunter-gatherer East Europe; and PWC, Pitted Ware culture), Neolithic [CAR, (Epi)Cardial; NPO, Neolithic Portugal, NBQ, Neolithic Basque Country and Navarre; FBC; TRE, Treilles culture], and Bronze Age [BAS, Bronze Age Siberia; BAK, Bronze Age Kazakhstan (not shown)] populations. Symbols indicate populations from Central Europe (squares and diamonds), southern Scandinavia (circles), the Iberian Peninsula (triangles), and East Europe/Asia (stars). Color shading of data points denote to hunter-gatherer (gray), Early Neolithic (brown), Middle Neolithic (orange), and Late Neolithic/EBA (yellow) samples [for further information see (*13*), figs. S1 and S2, and tables S1 to S4]. The haplogroup frequencies of these populations (table S9) were used to perform PCA (**C**) and Ward clustering (**D**). The first two principal components of the PCA display 32.8% of the total genetic variation. We superimposed each haplogroup as component loading vectors (gray), proportionally to their contribution. *P* values of the clusters are given in percent of reproduced clusters based on 10,000 bootstrap replicates.

a complex pattern of both genetic continuity and discontinuity (Fig. 2, A to D, and tables S5 to S8), based on the assumption that haplogroups are monophyletic and neutral, that is, not evolving into new haplogroups via mutations from an existing haplogroup or resulting from selection. Indigenous Central European hunter-gatherers (*10*, *14*) are set apart from the Neolithic Mittelelbe-Saale cultures on the basis of both cluster analysis (Fig. 2A) and significantly different F_{st} values (F_{st} = 0.0845 to 0.21358, P = 0.00000 to 0.03292) (Fig. 2B), because of mutually exclusive haplogroup compositions (fig. S3 and movie S1). The results of the TPC show that the transition from hunter-gatherers to the LBK farmers cannot be explained by genetic drift alone (P = 0.000001) (Fig. 2D), consistent with previous findings (*10*, *11*).

The Mittelelbe-Saale cultures themselves can be further differentiated into distinct Early/Middle Neolithic and Late Neolithic/EBA clusters (Fig. 2A), as shown by significantly higher F_{st} values (F_{st} = 0.02776 to 0.05605, P = 0.00000 to 0.016616) (Fig. 2, B and C). The two groupings are also strongly supported in AMOVA tests, where 289 different combinations of the ancient cultures were examined. We found the highest among-group variance, and low variation within the groups, when the Mittelelbe-Saale cultures were separated into two groups of Early/Middle Neolithic and Late Neolithic/EBA cultures (among groups 3.06%, F_{st} = 0.03061, P = 0.00683; within groups 0.45%, F_{st} = 0.00468, P = 0.18891) (table S7). Similarly, TPC also indicates that changes in the mtDNA profiles between most of the Early/Middle Neolithic cultures and the Late Neolithic/EBA (P = 0.000007 to 0.049428) as well as between the BBC and EBA (P = 0.000803) (Fig. 2D) cannot be explained by drift alone. These results suggest multiple population genetic shifts: the first during the introduction of farming, followed by further changes during the later Neolithic.

To further explore these patterns, we used a principal component analysis (PCA) and a cluster analysis (Fig. 1, C and D, and table S9) to describe the characteristic haplogroups of each culture and to identify genetic affinities to other prehistoric populations (*13*). We then examined affinities to present-day Eurasian populations to inform on potential geographic origins of the different cultures. We performed multidimensional scaling (MDS) (fig. S4, A to I, and table S10) based on continuous sequence data, which is sensitive to shared haplotypes between populations (*13*). In parallel, we also used PCA (fig. S5, A to I, and table S11), Procrustes and cluster analyses (fig. S6, A to I, and table S12), and genetic distance mapping (fig. S7, A to I, and table S13) based on discrete haplogroup frequencies (*13*).

Detailed investigation of the mtDNA composition of each culture reveals a series of haplogroup frequency changes because of different genetic profiles for hunter-gatherers, the Early/Middle Neolithic group, and individual cultures of the later Neolithic/EBA including the Bernburg culture (BEC) and the temporally overlapping BBC, CWC, and EBA (Fig. 3, fig. S3, and movie S1). The latter suggests that this period was heterogeneous, with genetically differentiated cultures resulting in a separation in the PCA (Fig. 1C). These shifts are also visible in the genetic distance maps and Procrustes-projected PCAs, where the Near Eastern affinity of the LBK and its subsequent regional derivatives switches to a clear European affinity in later Neolithic/EBA cultures, with distinct geographic orientations (see below; movie S1; and figs. S6, A to I, and S7, A to I).

We synthesized the different lines of evidence from our comparative genetic analyses to reconstruct a series of four prominent population dynamic events (termed A to D; Fig. 3 and movie S1), which we reconcile with known European cultural expansions (*1*–*5*). Overall, these analyses reveal a pattern of relative genetic continuity for the first 2500 years after the introduction of farming

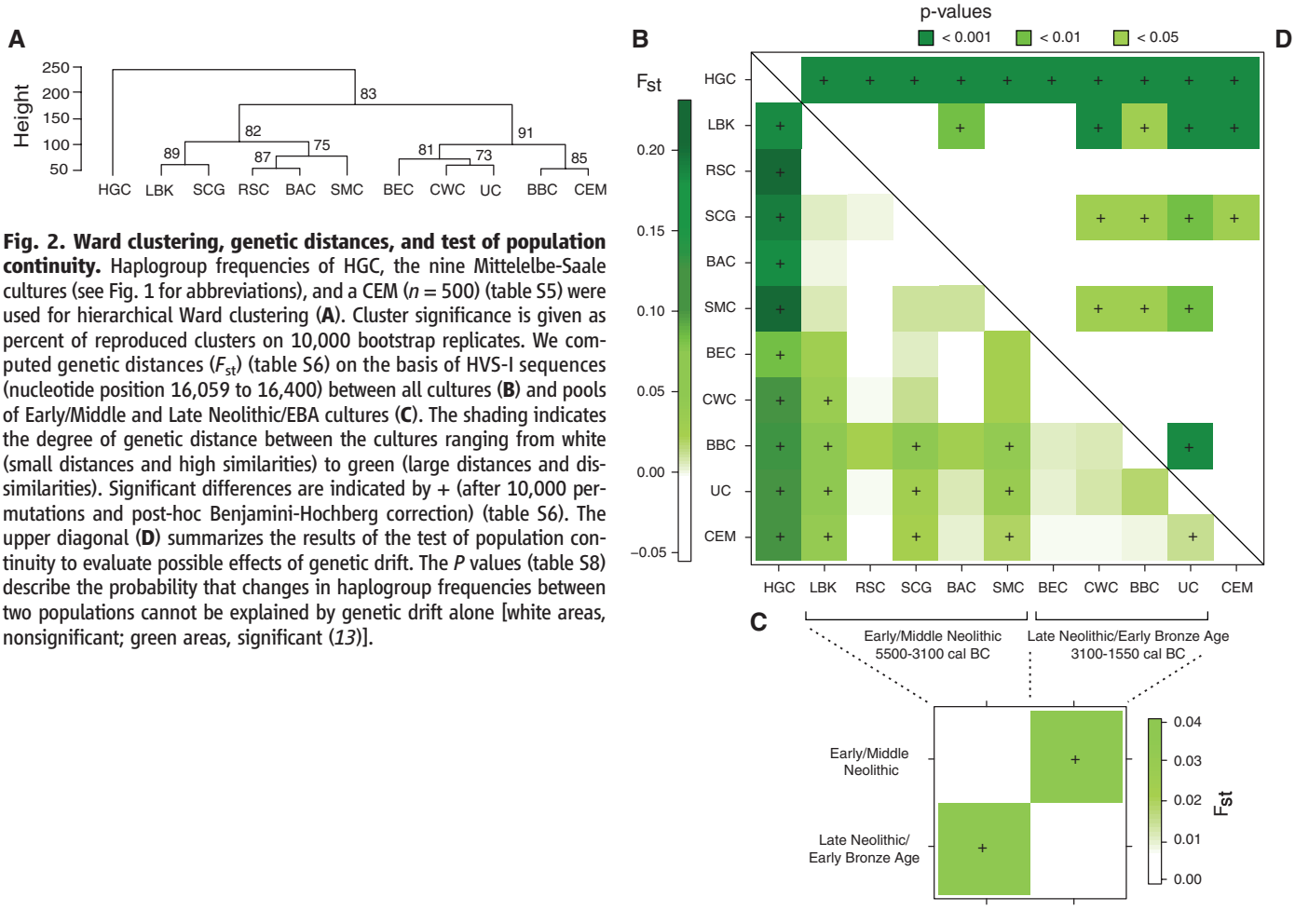

Fig. 2. Ward clustering, genetic distances, and test of population continuity. Haplogroup frequencies of HGC, the nine Mittelelbe-Saale cultures (see Fig. 1 for abbreviations), and a CEM (n = 500) (table S5) were used for hierarchical Ward clustering (**A**). Cluster significance is given as percent of reproduced clusters on 10,000 bootstrap replicates. We computed genetic distances (F_{st}) (table S6) on the basis of HVS-I sequences (nucleotide position 16,059 to 16,400) between all cultures (**B**) and pools of Early/Middle and Late Neolithic/EBA cultures (**C**). The shading indicates the degree of genetic distance between the cultures ranging from white (small distances and high similarities) to green (large distances and dissimilarities). Significant differences are indicated by + (after 10,000 permutations and post-hoc Benjamini-Hochberg correction) (table S6). The upper diagonal (**D**) summarizes the results of the test of population continuity to evaluate possible effects of genetic drift. The P values (table S8) describe the probability that changes in haplogroup frequencies between two populations cannot be explained by genetic drift alone [white areas, nonsignificant; green areas, significant (*13*)].

in Central Europe, followed by a series of discontinuities in the later Neolithic.

Event A marks the transition from foraging to farming introduced by the LBK, which reached Central Europe ~5500 calibrated years before the common era (cal BCE) (movie S1) (*1*–*3*). MtDNA data from Central European hunter-gatherers comprises exclusively U lineages (U, U4, U5, and U8) (*10*, *14*), whereas the LBK is characterized by a distinct haplogroup profile including N1a, T2, K, J, HV, V, W, and X (Fig. 1C) (*11*). These haplogroups can be denoted as a mitochondrial "Neolithic package" and comprise around 79.4% of the diversity in the LBK, whereas hunter-gatherer lineages are rare (2.9%) (Fig. 3). This marked shift suggests a rapid transition process, with the comparative analyses indicating a genetic influx from the Near East, Anatolia, and the Caucasus (movie S1 and figs. S4A to S7A) (*1*–*3*, *11*). The subsequent Early/Middle Neolithic cultures closely resemble the mtDNA haplogroup composition of the LBK (Figs. 1, C and D, and 2, A and D, and table S7) with similar affinities to present-day Near East populations (figs. S4, B to E, and S7, B to E), suggesting a period of genetic continuity over 2500 years.

Event B describes a bidirectional interaction along a north-south axis during the Early and Middle Neolithic, which saw the introduction of the Neolithic package to southern Scandinavia by Central European cultures (B_1 ~4100 cal BCE), followed by a reflux of hunter-gatherer lineages to Central Europe (B_2 ~3100 cal BCE) (movie S1). The Neolithic transition of southern Scandinavia was closely linked to the FBC, which replaced local foragers that had retained the Mesolithic lifestyle for ~1500 years after farming arrived in Central Europe (*1*–*3*). FBC individuals from Scandinavia (*10*, *15*, *16*) have yielded high frequencies of hunter-gatherer haplogroups (30%) alongside a large amount of Neolithic package haplogroups (60%) (table S9), leading to an intermediate position between hunter-gatherers and the Early/Middle Neolithic Mittelelbe-Saale cultures in the PCA (Fig. 1C). This suggests that pioneer groups from Central Europe had interacted with local hunter-gatherers who adopted farming (movie S1) (*1*–*4*), a view also supported by ancient genomic data (*16*). Subsequently, around a millennium later in Mittelelbe-Saale, a genetic shift associated with the BEC (Fig. 1, A to D, and table S7), a late representative of the FBC in Central Europe (*4*), saw an increase in hunter-gatherer lineages (29.4%) and a decrease in farmer lineages (47.1%) (Fig. 3), resulting in a haplogroup composition similar to that of the Scandinavian FBC (Fig. 1C) (*10*, *15*). Although previous populations show affinities to the Near East, the BEC marks a clear shift toward those in present-day North Europe (movie S1 and figs. S4F to S7F).

In the Late Neolithic, we identify two independent events (C and D), each associated with major contemporary Pan-European phenomena. Event C (~2800 cal BCE) is marked by the emergence of the CWC (movie S1), whose subgroups were widespread across Central and Eastern Europe (fig. S2) (*2*–*4*). The CWC is characterized by haplogroups I and U2 (4.6%), which are new maternal elements in Mittelelbe-Saale (Fig. 1C and fig. S3) and appear alongside other Late Neolithic/EBA lineages such as T1 (6.8%) and hunter-gatherer haplogroups U4 and U5 (20.5%), whereas Early/Middle Neolithic haplogroups further decrease (45.5%) (Fig. 3). The binomial probability that we missed I and U2 in 211 individuals of preceding cultures is very low ($P = 0.00$). Haplogroup U2 has been reported exclusively from Paleolithic, Mesolithic, and Bronze Age samples from Russia (*17*–*19*), and PCA and cluster analyses reveal similarities of the CWC to two ancient Kurgan groups of South Siberia (*19*) and Kazakhstan (*20*) (Fig. 1, C and D), in which haplogroups I, U2, and T1 are frequent (18.2 to 37.5%) (table S9). Intriguingly, the Y chromosomal haplogroup R1a1a, frequent in ancient Siberian populations (*19*), has previously been detected in

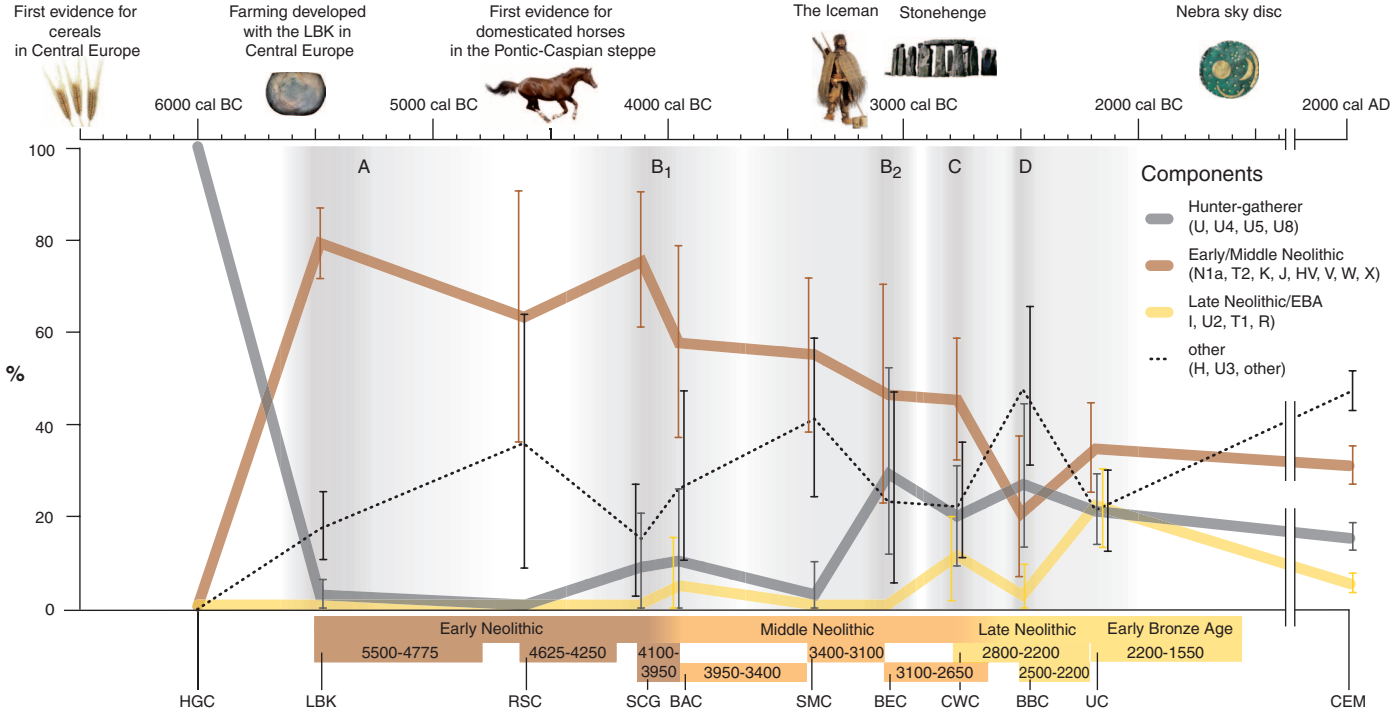

Fig. 3. Development of mtDNA components from the Late Mesolithic to present day. Population data from HGC, the nine Mittelelbe-Saale cultures (see Fig. 1 for abbreviations), and a CEM ($n = 500$) were placed in chronological order (*x* axis), and the amounts of lineages ascribed to particular time periods were evaluated in each population. The characterizing haplogroups of the hunter-gather (U, U4, U5, and U8; gray), Early/Middle Neolithic (N1a, T2, K, J, HV, V, W, and X; brown), and Late Neolithic/EBA (I, U2, T1, and R; yellow) periods were summarized into three respective components (*y* axis) (table S5) accordingly to the differentiation in the PCA (Fig. 1C). Haplogroups that could not be ascertained unambiguously to one of the three components were reported as "other" (H, U3, and other African and Asian lineages of the CEM) (*13*). Error bars of component frequencies indicate the 95% confidence interval of 10,000 bootstrap replicates (table S5). Horizontal shading denotes the population dynamic events (A, B_1, B_2, C, and D) inferred from the synthesis of all population genetic analyses (see main text).

our CWC data set (*21*), suggesting additional paternal genetic links to Kurgan cultures. Together with the affinities of the CWC to present-day populations of Eastern Europe, the Baltics, and the Caucasus (figs. S4G to S7G), this suggests a genetic influx into Central Europe from the East, likely influenced by Kurgan cultures (movie S1) (*2*, *3*).

Event D (~2500 cal BCE) is defined by the BBC (movie S1), the western counterpart of the CWC (fig. S2) (*2*–*4*). BBC groups appeared ~300 years later in Mittelelbe-Saale and coexisted alongside the CWC for more than 300 years (*4*). The BBC is distinguished from the CWC by the absence of haplogroup I and U2 and an overwhelmingly dominant genetic signature of haplogroup H (48.3%) (fig. S3), leading to a separation of the BBC from all other Mittelelbe-Saale cultures in PCA and cluster analysis (Fig. 1, C and D). H remains the most frequent haplogroup in West European populations today (~40%) (*8*, *9*) and was absent in Central European hunter-gatherers (*10*, *14*) but prevalent in ancient populations of the Iberian Peninsula since Mesolithic times (20.7 to 70.7%) (table S9) (*22*–*24*). As a result, the BBC clusters with these Iberian populations (Fig. 1, C and D), whereas the results from Procrustes and MDS were less informative. However, genetic links between the BBC and modern Iberian populations were supported by genetic distance maps accounting for H subhaplogroups (fig. S7H) and ancient mitochondrial H genomes (*12*). These suggest the BBC was associated with a genetic influx from southwest Europe (movie S1), which is consistent with the oldest archaeological signs of this culture being found in Portugal ~2800 cal BCE (*2*, *3*).

The onset of the EBA in Mittelelbe-Saale (~2200 cal BCE) was characterized by socially and economically stratified societies associated with the emerging metallurgies (*2*–*4*). All of the analyses show close genetic links between the EBA and the CWC (Figs. 1, C and D, and 2A) on the basis of elevated frequencies of Late Neolithic/EBA haplogroups such as I, U2, and T1 (22.3%) (Figs. 1C and 3 and fig. S3), and both appear to have similar affinities to modern-day East European populations (movie S1 and figs. S5I to S8I). TPC (Fig. 2D) indicate a minimal contribution of the BBC to the EBA in Central Europe. Thus, the Late Neolithic/EBA in Mittelelbe-Saale appears to have witnessed rapid and dynamic changes in mtDNA composition at the crossroads of distinct Eastern and Western European influences (movie S1).

To investigate the potential impact of the geographically widespread archaeological cultures and events examined here (fig. S2) on the demography and genetic variation of present-day Central Europeans, we compared the ancient data with a Central European metapopulation (CEM) consisting of 500 randomly selected individuals (*13*). AMOVA supports a model of continuity from the Late Neolithic/EBA to the CEM with the best inter- and intragroup variance observed when all Late Neolithic/EBA samples are pooled with the CEM into one group and the Early/Middle Neolithic specimens into another (among groups 2.57%, F_{st} = 0.02572, P = 0.00891; within groups 0.50%, F_{st} = 0.00511, P = 0.08089) (table S14). TPC analyses also support continuity since the Late Neolithic/EBA (P = 0.134672 to 0.418949) (Fig. 2D). Similarly, Bayesian coalescent-based simulations (*13*) support a demographic model involving exponential population growth since the Neolithic with a contribution of at least 50% migrants to Mittelelbe-Saale during the Early Neolithic. This is followed by a constant ratio of gene flow/admixture between Early/Middle and incoming Late Neolithic/EBA components and, after this fusion, a genetic continuity until the present day (Akaike Information Criterion 99.9%) (fig. S8 and table S15). The fact that continuity since the Late Neolithic/EBA could not be rejected confirms that the succeeding events B to D, despite their differing geographic affinities, had formed today's mtDNA diversity. Notably, the CEM clusters with the Late Neolithic cultures and individuals of the BBC in particular (Fig. 2A), suggesting that the Western European mtDNA variability had a stronger influence than the contemporaneous eastern CWC/EBA complex, implying yet another shift after the EBA.

We evaluated the amount of lineages in the CEM that can be attributed to particular time periods by characteristic haplogroups (*13*) and found that a total of 53% can currently be assigned to the Palaeolithic/Mesolithic (16%), Early/Middle Neolithic (31.2%), and Late Neolithic periods (5.8%) (Fig. 3). The remaining proportion of lineages (47%, mainly haplogroup H) requires further resolution (*12*). The presence of all major mtDNA haplogroups by the end of the Neolithic makes it increasingly difficult to discern recent demographic changes and would require larger population events to have an observable effect and/or full mitochondrial genome sequencing to detect more subtle changes.

The detailed genetic analyses of this transect through Neolithic Central Europe demonstrate the key role of Late Neolithic cultures at the dawn of metallurgy and stratified societies in the formation of modern Central European mtDNA diversity. The four successive genetic shifts highlight the biological cohesiveness of archaeological cultures such as the LBK, FBC, CWC, and BBC cultures and the importance and dynamics of genetic input from different geographic regions.

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Supplementary Materials

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