Inferring Past Environments from Ancient Epigenomes

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Abstract

Analyzing the conditions in which past individuals lived is key to understanding the environments and cultural transitions to which humans had to adapt. Here, we suggest a methodology to probe into past environments, using reconstructed premortem DNA methylation maps of ancient individuals. We review a large body of research showing that differential DNA methylation is associated with changes in various external and internal factors, and propose that loci whose DNA methylation level is environmentally responsive could serve as markers to infer about ancient daily life, diseases, nutrition, exposure to toxins, and more. We demonstrate this approach by showing that hunger-related DNA methylation changes are found in ancient hunter-gatherers. The strategy we present here opens a window to reconstruct previously inaccessible aspects of the lives of past individuals.

Key words: **ancient DNA, ancient epigenetics, DNA methylation, environmental paleoepigenetics, environmental epigenetics, paleoepigenetics.**

Introduction

In the past tens of thousands of years humans went through dramatic shifts in their life style and environment. Examples include the exodus from Africa, which exposed humans to new climatic conditions, pathogens, and nutritional sources; the transition from hunting and gathering to farming, which altered nutritional composition, culture, and social structure; and the advent of technology (Ermini et al. 2015). Thus, the environment of a human individual thousands of years ago was very different from that of a present-day human. Here, we use the term environment to describe the host of extrinsic and intrinsic conditions affecting an individual, such as nutrition, diseases, social interactions, psychological state, physical activities, ambient temperature, altitude, and exposure to toxins. In order to understand the nature of the challenges that faced ancient humans and the changes and adaptations that followed them, it is critical to develop means to study the environment in which these past individuals lived.

Until recently, methods to investigate the history and life style of an individual were mainly archaeological and paleoanthropological (Klein 2000). Through findings such as bones, ornaments, and tools, researchers have reconstructed aspects of ancient human daily life (Klein 2000). For example, it was shown that Neanderthals used manganese oxides to lower the auto-ignition temperature of wood for fire-making (Heyes et al. 2016); and bone fossils and lithic findings in excavations in Mount Carmel shed light on tool production technology and burial rituals (Bar-Yosef et al. 1992). Another approach which allows inference on ancient life is the study of present-day hunter-gatherers. Often criticized, such studies rely on the idea that some ancient practices survived to this day, or have otherwise been developed in parallel in these populations (Hawkes et al. 1997; Barnard 1998). Though valuable and informative in many ways, the above approaches have some known limitations (Justeson 1973). For example, archaeological analyses rely on preserved artifacts, with a bias towards those which tend to be preserved better. Moreover, the interpretation of findings is sometimes subjective and done through modern eyes (Justeson 1973). Therefore, the study of past environments could benefit from the introduction of new and complementary strategies that will be integrated with the collection of existing methods.

Progress in ancient DNA sequencing technology (Orlando et al. 2015; Krause and Paabo 2016) allowed the development of new ways to study ancient environments. For example, shotgun sequencing of ancient DNA from the calcified dental plaque (calculus) of five Neanderthals allowed inference about variation in Neanderthal diet, health, and microbiome (Weyrich et al. 2017). Similarly, sequencing of 34 early European calculi revealed how the shift from hunting and gathering to farming manifested in alterations in the oral microbiome (Adler et al. 2013). Sequencing of ancient DNA from the *Yersinia pestis* bacteria, which caused recurrent plague pandemics such as the Black Death (14–17th centuries AD), revealed the evolutionary dynamics and epidemiology of these plagues (Schuenemann et al. 2011; Wagner et al. 2014; Rasmussen et al. 2015).

Technological developments in ancient DNA sequencing were also used to genotype, or even fully sequence, the genomes of the ancient individuals themselves, using DNA extracted from bones, hair, and teeth. As of today, DNA was successfully sequenced from hundreds of individuals that

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lived from medieval times to tens of thousands of years ago (for example see Fu et al. 2014; Gamba et al. 2014; Raghavan et al. 2014; Seguin-Orlando et al. 2014; Allentoft et al. 2015; Haak et al. 2015; Mathieson et al. 2015; Raghavan et al. 2015; Gallego-Llorente et al. 2016; Lazaridis et al. 2016; Skoglund et al. 2016; González-Fortes et al. 2017). Several sequenced individuals belong to extinct human groups such as the Neanderthal (Green et al. 2010; Prüfer et al. 2014) and the Denisovan (Reich et al. 2010; Meyer et al. 2012), see reviews in (Orlando et al. 2015; Krause and Paabo 2016; Skoglund and Reich 2016; Llamas et al. 2017; Nielsen et al. 2017). Using these genomes, researchers were able to reveal historical events affecting human demographics, such as patterns of migrations, the spread of diseases, population admixtures, and replacements, and adaptation to agricultural lifestyle, colder climates, and high altitudes (Huerta-Sánchez et al. 2014; Allentoft et al. 2015; Mathieson et al. 2015; Lazaridis et al. 2016; Racimo et al. 2017).

Yet, the genetic makeup of populations changes slowly, over many generations, and hence tracking down DNA changes may provide information on long-term processes, but can rarely be used as a tool to study responses to new environments (Yona et al. 2015). In contrast, epigenetics—defined as somatically heritable chemical modifications of the DNA that do not entail alterations to the sequence itself—is plastic and responsive to the environment. As epigenetic layers react to external and internal conditions through modulation of their pattern of chemical modifications, they are sometimes referred to as "middleman" between the environment and the DNA (Feil and Fraga 2012; Marsit 2015; Rubin 2015; Yona et al. 2015; Etchegaray and Mostoslavsky 2016). Therefore, identifying environmentally responsive loci (ERLs) of epigenetic layers in ancient individuals has the potential to serve as a novel means to investigate the conditions in which they lived. Here, we define an ERL as any locus whose epigenetic pattern carries information on environmental cues, regardless of whether this locus responds directly to the environment, or is simply associated with it.

Epigenetic patterns play a pivotal role in determining and marking the activity of genes. The term "Epigenetics" refers to several regulatory layers, including histone modifications, chromatin remodeling, nucleosome positioning, and DNA methylation. In this paper, we focus on DNA methylation—the addition of a methyl group to a cytosine nucleotide. In mammals, DNA methylation usually occurs in the context of a cytosine that is followed by a guanine, thus named CpG position. Non-CpG (i.e., CT, CA, or CC) methylation also occurs in mammals, mainly in pluripotent and brain cells, but is otherwise rare (Ziller et al. 2011; Varley et al. 2013). The role of DNA methylation in gene regulation depends on its position and context. In promoters it is generally associated with silencing of genes, whereas in other regions it plays a role in modulating enhancer activity, X-chromosome inactivation, splicing, transposable element silencing, and more (Feil and Fraga 2012; Jones 2012).

Methylation patterns along a genome are determined by two main factors. The first is genetic; much of the methylome is determined directly by the DNA through the binding of methylation-altering factors to specific sequences (Lienert et al. 2011; Teh et al. 2014). Being chiefly dictated by DNA sequence, such methylation patterns persist across generations, and are, by large, less responsive to environmental changes within the lifetime of an individual (Feil and Fraga 2012; Jones 2012; Yuan 2012; Whitaker et al. 2015).

The second factor is external and internal environmental signals that alter the methylome, such as changes in caloric intake, psychological trauma, and *in utero* exposure to toxins. The mechanisms mediating environmental cues and methylation changes are poorly understood, but they are often associated with DNA-binding activity, or affect one-carbon metabolic pathways that dictate the availability of methyl donors (Jaenisch and Bird 2003; Feil and Fraga 2012). Regardless of the underlying mechanisms, the accumulation of whole-genome methylation maps, combined with an increasing interest in the relationship between the environment and the methylome, lead to the identification of a growing number of ERLs and the factors that shape them (Bollati and Baccarelli 2010; Feil and Fraga 2012).

Therefore, given a sufficiently long list of ERLs, and their methylation levels in ancient individuals, we could identify "environmental signatures" that would shed light on the conditions in which these individuals lived. Such inference requires precise knowledge of ERLs and the environmental changes they are associated with (environmental epigenetics), as well as an ability to infer accurate epigenetic patterns from ancient DNA (paleoepigenetics). As of today, both of these fields are in their infancy. Environmental epigenetics still lacks sufficient knowledge about the mechanisms and factors that shape the methylome, and faces challenges in designing well-controlled experiments. Paleoepigenetics still relies on a small set of high-quality methylomes that come from a limited repertoire of tissues. However, as both fields are rapidly expanding, we see great value in combining them into what we term environmental paleoepigenetics. The development of environmental paleoepigenetics is a forward-looking research goal that could contribute to the study of past environments by integrating multiple lines of evidence from archeological, paleontological, microbial, and paleoepigenetic approaches.

In this review, we examine the use of paleoepigenetic techniques to reconstruct premortem methylation maps from ancient DNA. We then offer guidelines how to identify ERLs that could be useful for environmental paleoepigenetics. Next, we survey works that reported such potentially useful ERLs, mainly in response to diseases, changes in nutrition, and exposure to toxins. Finally, we point out the presence of hunger-related ERLs in some ancient individuals, and discuss how this may be interpreted.

Reconstructing Ancient Methylation Maps

The spontaneous deamination of cytosines is a central chemical degradation process of ancient DNA. This process produces distinct degradation signatures for methylated and for unmethylated cytosines, whereby methylated cytosines are deaminated into thymines, whereas unmethylated cytosines

are deaminated into uracils. During library preparation uracils are removed, leading to enrichment of thymines in premortem methylated positions. Using this signal, premortem methylation maps of ancient individuals can be reconstructed (Briggs et al. 2010; Gokhman et al. 2014; Pedersen et al. 2014; Hanghøj et al. 2016). We have previously used this idea to reconstruct the full methylomes of our closest extinct relatives—the Neanderthal and the Denisovan (Gokhman et al. 2014). Comparing these maps to a modern bone methylation map, we identified ~2,000 differentially methylated regions (DMRs), providing information on the genes that are differentially regulated between these human groups.

Which ERLs Could Be Useful in Environmental Paleoepigenetics?

Using ancient DNA methylation patterns to infer about the environment of an individual that died thousands of years ago must address the fact that DNA methylation is tissue-specific. DNA does not survive long in most tissues, and the majority of ancient DNA material comes from bone, hair, and teeth (Orlando et al. 2015; Gokhman et al. 2016). This means that we can reconstruct the methylomes of a limited number of tissues, which are not necessarily those whose methylation had changed in response to an environmental cue. For instance, an individual exposed to dietary stress may exhibit DNA methylation alterations in the brain or adipose tissues, but not necessarily in bone tissue.

At first glance, this renders the use of bones, teeth, and hair for inference about environmental signatures in other tissues inadvisable. However, it has been shown that some genomic regions establish their methylation patterns early in embryogenesis (Roemer et al. 1997; Rakyan et al. 2002; Dolinoy et al. 2007). Therefore, if an environmentally induced methylation change happened early during embryogenesis, prior to the differentiation of tissues, then this change would be carried on to the daughter cells and tissues (Rakyan et al. 2002; Dolinoy et al. 2007; Gokhman et al. 2016). Thus, timing is critical to the information-content of methylation in ERLs: the earlier in development an epigenetic alteration occurred, the more cell types it is expected to affect.

A special case of methylation changes following early exposure is that of metastable epialleles. These are loci that are differentially methylated between genetically identical individuals, but show low variability between tissues of the same individual. These properties suggest that their methylation state is established early in development, and that they are particularly influenced by environmental conditions during early pregnancy. Metastable epialleles may be identified by comparing DNA methylation maps in different tissues across individuals, for example comparing monozygotic twins or comparing individuals from different controlled environments. The methylation state of metastable epialleles was described as an environmentally dependent stochastic event that results in cell variegation, and in some cases affects gene expression and phenotypes. Metastable epialleles have been shown to affect coat color, transgene expression in myocytes, neural tube development, and more (Rakyan et al. 2002; Dolinoy et al. 2007; Waterland and Michels 2007; Alan Harris et al. 2013). Regardless of their phenotypic effect, the combination of high susceptibility to environmental conditions, low intertissue variability, and lifelong stability makes metastable epialleles good candidates to serve as loci whose methylation state in bones, teeth, and hair could be a proxy to the conditions during early embryogenesis of an archaic individual.

Importantly, the idea of using epigenetics to infer about past environments does not rely on whether the epigenetic modifications are heritable across generations. The extent to which DNA methylation in mammals is heritable across generations is debated (Heard and Martienssen 2014), but an ERL could serve as an environmental marker regardless of whether it emerged during the lifetime of an individual or passed to him/her from past generations. It is important to emphasize that if an ERL was shown to be transgenerationally inherited, it could probably serve as a good marker for environmental paleoepigenetics, because its methylation state exists in the predifferentiated cells of the early embryo and is therefore likely to be found in bones, teeth, and hair as well.

The Effects of Nutrition, Toxins, and Diseases on the Methylome

Environmental epigenetics investigates how different factors such as diseases, exposure to toxins, and nutrition shape the epigenome. We suggest environmental paleoepigenetics build upon this growing body of knowledge to address the reverse process—analyzing patterns of ancient epigenomes and deducing what environmental factors might underlie them (fig. 1).

Using information embedded in ancient methylation maps to infer on nonenvironmental factors has already been achieved with regard to age and tissue type. Pedersen et al. (2014) used the reconstructed methylation map of an ancient Saqqaq Eskimo in order to infer his age at death, and we have previously used the reconstructed Neanderthal and Denisovan methylomes in order to show that their DNA came from bone cells (Gokhman et al. 2014).

Below, we survey studies in environmental epigenetics that reported links between DNA methylation changes and environmental factors. We use two criteria to determine the scope of studies we cover. First, we focus on nutrition, toxins, and diseases, as these are extensively studied factors which are particularly relevant to environmental reconstruction. Second, we spotlight ERLs that might be useful in environmental paleoepigenetics, namely ERLs that are either 1) established early in development; 2) transmitted transgenerationally; or 3) influenced by environmental factors in tissues from which ancient DNA usually originates. As transgenerational inheritance of DNA methylation in mammals is still contested, and as environmental epigenetics rarely looks at tissues such as bones, teeth, and hair, the vast majority of ERLs reported here stem from environmental changes that affect the early embryo. In addition, we include studies involving nonhuman mammals, especially rodents and primates.

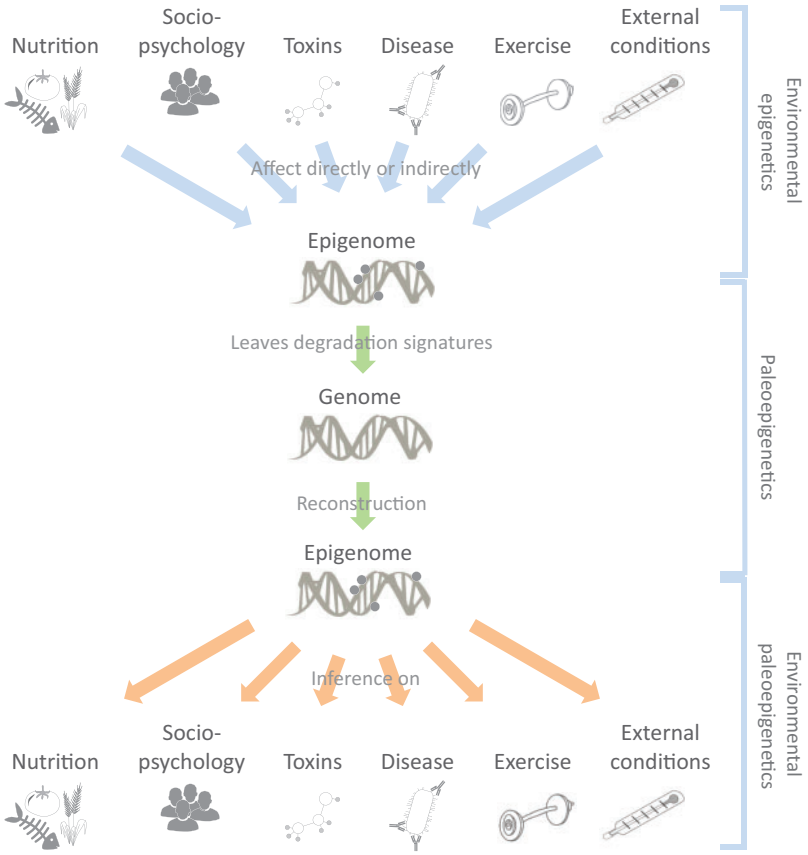

Fig. 1. Environmental paleoepigenetics builds on environmental epigenetics and paleoepigenetics. In environmental epigenetics, researchers study how extrinsic and intrinsic factors affect the epigenome (blue arrows). Paleoepigenetics harnesses degradation signals in ancient DNA to reconstruct premortem DNA methylation maps (green arrows). Environmental paleoepigenetics would use the reconstructed methylation maps of ancient individuals to infer on the unknown extrinsic and intrinsic factors that shaped them (orange arrows).

Although it is still to be determined which of these nonhuman ERLs also exist in humans, they provide a more comprehensive view of the way environmental factors shape the methylome. Additionally, these nonhuman ERLs could be used in the future to study the environments of additional ancient organisms, as nonhuman methylation data (Seguin-Orlando et al. 2015) and high-quality nonhuman ancient genomes (Palkopoulou et al. 2015) are starting to emerge.

Most studies to date examined the nutritional factor. One of the most well-known examples in humans described how periconceptional exposure to famine (the Dutch Hunger Winter of 1944–1945) was associated with hypomethylation of the 5′-UTR of the *IGF2* gene (Heijmans et al. 2008). Later, it was shown that many other loci change their methylation in response to famine, including regions in the *INSIGF*, *IL10*, *ABCA1*, *GNASAS*, *MEG3*, and *LEP* genes (Tobi et al. 2009). On the other end, high-fat diet (HFD) was found to significantly increase methylation levels in mice at 30 CpGs in intron 2 of *igf2r* (Gallou-Kabani et al. 2010). In rats, paternal HFD drove hypermethylation of a CpG site upstream of *Il13ra2* in female offspring (Ng et al. 2010). As may be expected, the level of methyl-donor compounds in the diet was also shown to affect methylation: Waterland et al. supplemented the diet of pregnant female mice with extra folic acid, vitamin B_{12}, choline, and betaine—all methyl-donor-rich foods—resulting in hypermethylation of several loci in the offspring (Waterland and Jirtle 2003; Waterland et al. 2006). See supplementary table 1, Supplementary Material online, for additional studies of nutrition–methylation relationship.

A recent study separated the effects of genetic background and environment on the methylome of human populations, by comparing Central African populations that differ in genetic background, lifestyle, or both (Fagny et al. 2015). The study looked at the genotypes and whole blood methylomes of hundreds of individuals from five populations. Among the investigated populations were West African rainforest hunter-gatherers, West African agrarians, and West African agrarians that readopted life in the rainforest. Although the first and second populations differ by both ancestry and lifestyle, the second and third populations differ mostly by lifestyle. The authors identified >3, 000 differentially methylated sites that could be attributed to the recent change of habitat, and showed that they are overrepresented in genes involved in immune response, host–pathogen interactions, and various cellular processes. The developmental stage at which these environmental cues leave a mark on the methylome is still to be determined.

Another factor that was shown to alter DNA methylation at various sites was exposure to toxins and pollutants. *In utero* exposure to smoking was associated with alterations in

methylation across various genes, including hypermethylation in *BNDF* (Toledo-Rodriguez et al. 2010), *AXL*, and *PTPRO* (Breton et al. 2009) and hypomethylation in CYP1A1 (Suter et al. 2010) and in the repetitive element AluYb8 (Breton et al. 2009). Other compounds that triggered local changes in methylation include ethanol (Kim et al. 2014), arsenic (Intarasunanont et al. 2012), and cadmium (Vidal et al. 2015).

The last group of factors that interacts with DNA methylation is diseases and disorders. In this regard, the interaction between methylation and diseases is bidirectional. On one hand, many studies have shown how changes in methylation mark or even drive a wide variety of disorders (Robertson 2005; Bergman and Cedar 2013; Chen et al. 2016; Li et al. 2016; Martino et al. 2016; Reynard 2016; Wüllner et al. 2016). On the other hand, it was shown how maternal diseases leave epigenetic marks in the offspring. In the latter case, most studies have focused on diabetes and showed localized changes in specific genes, as well as genome-wide differences affecting thousands of genes. For example, children of type 2 diabetic mothers had over 4, 000 differentially methylated regions (Del Rosario et al. 2014), and offspring to mothers with gestational diabetes mellitus had lower methylation levels in several CpG sites in the lipoprotein lipase gene (*LPL*) (Houde et al. 2014), hypermethylation in leptin promoters (Lesseur et al. 2014), and in over 1, 000 additional genes (Ruchat et al. 2013). Regardless of the directionality of effect (methylation changes that drive diseases or diseases that drive methylation changes), these studies open a window to investigate the health profile of past individuals.

Perhaps the most intriguing factors that affect DNA methylation are psychological and social. Most studies that looked into these factors examined methylation patterns associated with behavioral, psychological, or social events at late stages of life (supplementary table 1, Supplementary Material online). However, studies investigating prenatal maternal stress, such as the Great Ice Storm of 1998 (Cao-Lei et al. 2014), war stress in the Democratic Republic of Congo (Mulligan et al. 2012), intimate partner violence (Radtke et al. 2011), and depression during pregnancy (Liu et al. 2012), provide insight into methylation changes that are associated with stress exposure in early stages of development. Finally, physical exercise, as well as external conditions such as temperature, humidity, and altitude, has been repeatedly associated with various ERLs. However, to our knowledge, these studies have all been conducted on adults, and on tissues other than teeth, hair, and bone, and thus, their relevance environmental paleoepigenetics is still to be determined.

Additional studies that investigated ERLs and effects of the environment on global DNA methylation level are summarized in supplementary table 1, Supplementary Material online.

Methylation Patterns of Hunter-Gatherers and Archaic Humans Are Consistent with Low Caloric Intake

Environmental epigenetics is still a young field, and as of today the number of ERLs that can be used for inference on past environments is very small. One of the goals of this review is to encourage researchers in the field to dedicate efforts to identifying ERLs suitable to environmental paleoepigenetics. As a further encouragement, we provide here a small-scale demonstration of the potential of environmental paleoepigenetics to enrich our understanding of the conditions under which humans lived thousands of years ago.

In a study by Dominguez-Salas et al., researchers focused on a rural Gambian population where caloric intake varies considerably according to season, with a "hungry season" followed by a "harvest season." The "hungry season" is the rainy season, characterized by restricted protein-energy availability, whereas the dry "harvest season" does not hold any nutritional stress. The oscillations in nutrient availability have been shown to affect *in utero* development and growth (Rayco-Solon et al. 2005). These studies found that children conceived during the "hungry season" showed hypermethylation at six metastable epialleles, residing near or within the following genes: *LOC654433*, *EXD3*, *RBM46*, *BOLA3*, *ZNF678*, and *ZFYVE28* (Waterland et al. 2010; Dominguez-Salas et al. 2014). In follow-up work on these data, we crossed these six ERLs with a list of DMRs found between archaic humans (Neanderthal and Denisovan) and 21 present-day humans (Gokhman et al. 2014). We found that three of the ERLs partially or completely overlap DMRs (in *EXD3*, *RBM46*, and *ZNF678*) that are significantly hypermethylated in the Neanderthal, the Denisovan, or both (fig. 2*a*–*c*). The observed overlap is significantly higher than would be expected by chance, even when controlling for GC content ($P < 10^{-6}$, randomization test, 1, 000, 000 iterations). Also, these methylation differences are unlikely to be driven by sequence changes, as the closest single nucleotide change or indel that differentiate the hominin groups are found tens of thousands of bases away (23 kb for *RBM46*, 84 kb for *EXD3*, and 125 kb for *ZNF678*), whereas methylation-affecting polymorphisms tend to be much closer to DMRs (with a peak enrichment at 45 bp) (Gibbs et al. 2010). Since the ERLs were found within metastable epialleles, they are likely to reflect not only the methylation state in the tissues where they were measured, but rather across tissues, including bones and teeth (Rakyan et al. 2002; Dolinoy et al. 2007; Waterland and Michels 2007; Waterland et al. 2010; Alan Harris et al. 2013; Dominguez-Salas et al. 2014). It is important to note that the present-day methylation maps used in order to identify these DMRs (Gokhman et al. 2014) were produced by reduced representation bisulfite sequencing (RRBS), and thus provide information for only ~10% of CpGs in the genome. Therefore, further research is needed in order to fully characterize the extent of these DMRs.

To explore whether the differences between archaic and present-day humans could reflect the transition from hunting and gathering to farming, we looked at additional published DNA methylation maps of modern and ancient individuals, totaling five hunter-gatherers and four sedentary individuals (Gokhman et al. 2017). We found that these ERLs are hypermethylated by 7% on average in skeletal tissues of hunter-gatherers ($P = 7.3 \times 10^{-5}$, *t*-test), similarly to the effect size detected in the Gambian population study

Fig. 2. Methylation patterns in the Neanderthal and the Denisovan point to a low-calorie diet. Methylation maps are shown for a present-day human, a Neanderthal, and a Denisovan. Each lines represents a CpG position. Methylation levels are color-coded from green (unmethylated) to red (methylated). Present-day human maps are partial because the protocol used to produce the maps was reduced representation bisulfite sequencing (RRBS), which provides information for ~10% of CpG positions. Reconstructed ancient methylation and DMRs were taken from (Gokhman et al. 2014). ERLs were taken from Dominguez-Salas et al. (2014). (*a*) Archaic humans are hypermethylated in the *EXD3* gene compared with the present-day human. The DMR completely overlaps the ERL, where hypermethylation is associated with low-calorie diet. (*b*) The Neanderthal is hypermethylated in the *RBM46* gene compared with the present-day human. The DMR partially overlaps with the ERL, where hypermethylation is associated with low-calorie diet. (*c*) The Denisovan is hypermethylated upstream of the *ZNF678* gene compared with the present-day human. The DMR completely overlaps the ERL, where hypermethylation is associated with low-calorie diet. (*d*) Box plots of methylation levels of hunter-gatherers and sedentary individuals within the six hunger-related ERLs. In LOC654433, RBM46, and EXD3 hunter-gatherers are significantly hypermethylated compared with sedentary individuals, reflecting possible low caloric intake. Within BOLA3, however, hunter-gatherers are hypomethylated.

(Dominguez-Salas et al. 2014). Particularly, we found that *LOC654433*, *RBM46*, and *EXD3* are significantly hypermethylated in hunter-gatherers compared with sedentary individuals, compatible with their hypermethylation in Gambian individuals conceived during the hungry season, whereas *BOLA3* presents the opposite trend, incompatible with hunger state (fig. 2*d*).

Though further research is needed in order to fully understand the precise factors that trigger such changes in methylation, these preliminary findings point to the possibility that *in utero*, archaic humans, and possibly hunter-gatherers in general, experienced conditions that are somewhat similar to those experienced by present-day individuals conceived during a hungry season. More importantly, this highlights

the potential of using ERLs that are established during early development to investigate the environment in which ancient individuals lived.

Summary

Although some ERL studies analyzed metastable epialleles or ERLs that are established early in development, many others focused on a specific tissue of interest and on regions that have not been tested for stability across tissues. In this review, we have focused on studies that identified ERLs in early stages of development, and are therefore likely to be shared by many tissues. However, in order to understand the extent to which these ERLs persist in different cell types, and specifically, to be able to test them in tissues from which ancient DNA samples are collected, it is imperative to conduct further studies which cover a large array of tissues and individuals.

Importantly, although the mechanisms that drive the changes in methylation and their resulting phenotypic effects are of great interest, they are practically irrelevant for environmental reconstruction. These ERLs serve only as markers for the conditions to which an individual was exposed. Thus, in this regard, it is irrelevant whether they are only correlated with specific environmental conditions, or directly driven by them, and whether they have phenotypic consequences, or are completely neutral.

A single ERL may respond to more than one environmental cue. For example, *NR3C1*, *SLC6A4*, and *IGF2* have all been shown to be influenced by multiple factors (supplementary table 1, Supplementary Material online). It is therefore critical to identify a full complement of ERLs that respond to each extrinsic or intrinsic factor, so as to obtain an "ERL signature" to each factor. Such signatures would allow reconstruction of past environments, whereas taking into account pleiotropic effects as well as correlation between various factors affecting the same locus. In this way, each ERL may be viewed as a broadly tuned environmental sensor, and each environmental cue may be associated with a specific pattern of ERL responses.

It is also important to consider effect size. Although some studies report considerable effect sizes, for example 24.4% methylation change in AHRR (Zeilinger et al. 2013), and 30% change in GFI1(Elliott et al. 2014), both in response to smoking, most effect sizes are smaller, sometimes as little as 1% (Fagny et al. 2015). This poses a particular challenge when analyzing reconstructed methylation maps, where signals are smoothed using a sliding window, and where the variance in predicting methylation level depends on how well we can estimate the deamination rate of methylated cytosines (Gokhman et al. 2016). For this reason, environmental paleoepigenetics should focus on higher effect sizes or longer ERLs where the smoothing effect is mitigated. Environmental inference where effect sizes are small would probably require the use of methylation maps from many individuals, which would increase statistical power.

DNA sequence can directly affect methylation states through factors such as CpG density and the activity of binding proteins (Lienert et al. 2011). Genetic changes affecting DNA methylation patterns may be detected through various means, for example by identifying methylation quantitative trait loci (meQTLs) (ZhaNg et al. 2010). The genetic background of an individual can therefore affect the way his/her ERLs respond to environmental cues (Teh et al. 2014). Genotype–methylation–environment interplay is well demonstrated in the *FKBP5* gene, where the risk for childhood trauma-dependent demethylation is increased by a polymorphism that affects the chromatin interaction between the enhancers of *FKBP5* and its transcription start site (Klengel et al. 2012). Therefore, methylation-based environmental inferences, and specifically those that include samples from different ancestries, should consider potential genetic effects, which tend to reside close to the site of differential methylation (Gibbs et al. 2010).

Our focus in this perspective was on humans. However, the use of ERLs in order to reconstruct past environments could be implemented on additional methylation maps, such as those of nonhuman mammals (Llamas et al. 2012) and even plants (Smith et al. 2014). Many studies have demonstrated how environmental factors, such as temperature, alter DNA methylation patterns in plants (Feil and Fraga 2012). Thus, we anticipate that ancient plant methylation maps (Smith et al. 2014), as well as nonhuman mammalian methylation maps (Seguin-Orlando et al. 2015), could be analyzed to infer the environmental conditions to which they were exposed thousands of years ago.

The motivation behind many of the above studies was mainly medical—to understand how different environmental factors affect our physical and mental health. This field is growing rapidly, and our understanding of how the environment shapes our epigenome is becoming deeper and more complex. In parallel, the fields of paleogenetics and paleoepigenetics are gaining momentum (Hanghøj et al. 2016), with ancient DNA samples being sequenced in increasing numbers, to higher quality and from older periods (Orlando et al. 2015; Krause and Paabo 2016). We believe that the integration of these two fields will have a synergistic effect, opening new angles to explore how the lives of archaic individuals looked like. Once enough knowledge is gained in both fields, we believe that the epigenomes of ancient individuals could reveal aspects of their daily life, diseases from which they suffered, substances to which they were exposed, and possibly even their psychosocial state.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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