UNIVERSITY OF CALIFORNIA SAN DIEGO

Review of the epigenetics of hypoxia

A thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

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Professor Stephanie Mel

2021

The Thesis of Caroline Gaeta is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

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LIST OF ABBREVIATIONS

AHI	Apnea hypopnea index
BP	Base pairs
BMI	Body mass index
CMS	Chronic Mountain Sickness
CpG	Cytosine-guanine
COPD	Chronic obstructive pulmonary disease
DMPs	Differentially methylated positions
DMRs	Differentially methylated regions
EE	Erythrocytosis
EGLN1	Egl-9 homolog 1
EPAS1	Endothelial PAS domain protein 1
GWAS	Genome-wide association studies
HAT	Histone acetyltransferase
HIF	Hypoxia-inducible factor
HIF-1	Hypoxia-inducible factor 1
HIF-2α	Hypoxia-Inducible factor 2-alpha
HMT	Histone methyltransferase
HRE	Hypoxia response element
IHR	Intermittent hypoxia with reoxygenation
LBC	Lothian Birth Cohort
OSA	Obstructive sleep apnea
PCR	Polymerase chain reaction
PHD2	Prolyl hydroxylase domain 2
SAM	S-adenosyl methionine
SaO2	Oxyhemoglobin saturation
SGA	Small for gestational age births
SNP	Single nucleotide polymorphism
SpO2	Oxygen saturation of the blood
WGS	Whole-genome sequencing

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ACKNOWLEDGEMENTS

I would like to acknowledge Professor Tatum Simonson for her support as the chair of my committee. Through our weekly meetings and discussions, her guidance and assistance proved to be indispensable.

I would also like to acknowledge the rest of my committee members, Professor Milton Saier and Professor Stephanie Mel for their support in writing this thesis. Their patience and understanding throughout this process did not go unnoticed.

Lastly, I would like to acknowledge everyone in the Simonson Lab at UCSD and more specifically, Eli Lawrence, Wanjun Gu, and Maianh Phan for their willingness to help in any way they could. Their efforts and cooperation were greatly appreciated.

ABSTRACT OF THE THESIS

Review of the epigenetics of hypoxia

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Master of Science in Biology

University of California San Diego, 2021

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Associations between genetic factors and physical characteristics of populations living more than 3500 m above sea level have been of long-standing interest in studies of high-altitude adaptation. Many present-day highlanders whose ancestors occupied Andean Altiplano have adapted to challenging environmental conditions over many generations, and yet some lack adaptation to this harsh environment. Those who lack adaptations to the hypoxic conditions caused by high-altitude may suffer with health conditions such as chronic mountain sickness

(CMS). Recent technological and analytical advancements have pushed forward efforts to identify the molecular mechanisms that dictate the human's ability to adapt to high-altitude environments, and recent studies suggest the ability to adapt to high-altitude, at least in part, may be due to epigenetic modifications.

Epigenetics is the study of heritable changes acquired through lifestyle and environmental factors that play a role in gene expression without altering DNA sequence. DNA methylation is a type of epigenetic modification that involves the attachment of a methyl group to a part of the DNA molecule. The *EPAS1* and *EGLN1* genes both play key roles in modulating oxygen levels in the hypoxia inducible factor (HIF) pathway and are reported as top targets of selection in both Tibetan and Andean populations, and epigenetic modifications are known to impact the regulation of these genes. Genome-wide epigenetic profiling and targeted bisulfite methylation studies are techniques recently implemented in studies aimed at identifying DNA methylation markers that might explain the differences in altitude-adapted and -maladapted highlanders involving these and other genes.

Introduction

A classic example of natural selection operating on the human genome is high-altitude adaptation (Moore, 2017; Simonson, 2015). The extent to which Peruvian Andean populations are genetically adapted to living in high-altitude conditions has long been of interest. Long-term residents of the Andes mountains have lived more than 4000 m above sea level over many generations; however, some of them appear to lack adaptation to this harsh living environment and develop chronic mountain sickness (CMS), a disease characterized by low oxygen levels in the body, also known as hypoxia. CMS is characterized by excessive production of red blood cells, which is called excessive erythrocytosis (EE). EE is considered maladaptive because it causes an increase in blood viscosity, which impairs blood flow and oxygen delivery to the body's tissues (Villafuerte & Corante, 2016). Some reports suggest people with CMS have lower levels of ventilation than acclimatized newcomers (Julian & Moore, 2019), and CMS is prevalent in ~10% of Andean males above age 30 (Julian, 2017). The prevalence of CMS is highest in the Andeans of Cerro de Pasco at 15.4% in men aged 30-39 (Spielvogel et al., 1981). In Puno, the prevalence is 6%, and 5.2% in men of the same age group in La Paz, Bolivia (Spielvogel et al., 1981). Furthermore, the prevalence of CMS increases as altitude increases (Villafuerte & Corante, 2016).

Transcriptional responses are an essential component of adaptation to hypoxic conditions. Studies have shown that hypoxia-induced transcription factors involve epigenetic modifications, such as DNA methylation, in order to initiate the hypoxic response (Brown & Rupert, 2014). These epigenetic modifications could explain why not all life-long residents of Peru have adapted to the hypoxic conditions in the same way. It is possible that the phenotypic (i.e., hemoglobin concentration and oxygen saturation) differences between altitude-adapted and altitude-maladapted highlanders are explained by epigenetics rather than genetics alone (Brown

& Rupert, 2014). In this review, we comprehensively discuss the breadth of knowledge and research of CMS and high-altitude hypoxia in Peruvian highlanders in the context of genetic and epigenetic studies.

Overview of Peruvian Highlanders

Andeans, similar to other highland populations, live in challenging environmental conditions with decreased oxygen levels, high levels of UV radiation, and low ambient temperatures. These populations have lived in the Andean Altiplano at an average elevation of 4000 m above sea level for up to 11,000 years, displaying unique physiological adaptations including elevated hemoglobin concentration, increased chest circumferences, elevated arterial oxygen saturation, and a low hypoxic ventilatory response in comparison to people living at sea level (Childebayeva et al., 2020).

Some of the first studies of adaptation were focused primarily on physiological changes -- specifically changes in the oxygen transport system that occur with acclimatization in newcomers versus long-term residents of the Andes (reviewed in Julian & Moore, 2019). These initial studies alongside more recent studies have found that Andeans exhibit larger lungs, greater diffusion from the air into the lung, decreased hypoxic pulmonary vasoconstriction, greater uterine blood flow in pregnancy, and greater cardiac oxygen utilization (Julian & Moore, 2019).

Chronic Mountain Sickness

Chronic mountain sickness (CMS), also known as Monge's disease, is a high-altitude disease characterized by excessive production of red blood cells, or erythrocytosis (EE) and severe hypoxemia caused by lifelong exposure to hypoxia. It is a progressive incapacitating syndrome prevalent in most high-altitude regions around the world (Villafuerte & Corante,

2016). CMS develops because at higher altitudes, such as the Andes mountains, air pressure is lower; therefore, less oxygen is able to enter the body through the lungs, causing hypoxemia. Over an extended period of time, the body attempts to make up for low oxygen levels by producing more red blood cells leading to erythrocytosis. On average, 5%-10% of the 140 million people living above 2500 m worldwide are at risk of developing CMS (Leon-Velarde et al., 2005). CMS is considered a maladaptive because it causes an increase in blood viscosity, which impairs blood flow and oxygen delivery to the body's tissues (Colleen, 2017). CMS is associated with pulmonary hypertension, and, in severe cases, the condition may cause cor pulmonale or congestive heart failure (Villafuerte & Corante, 2016). People with CMS have lower levels of ventilation than acclimatized newcomers do (Villafuerte & Corante, 2016). Currently, it is believed that a component of CMS prevalence involves a genetic predisposition, but there are also risk factors and comorbidities that may trigger or worsen the condition (Villafuerte & Corante, 2016). In order to provide a diagnosis and adequate healthcare to Peruvian highlanders, appropriate research and medical information on CMS is needed.

The prevalence of CMS has shown differences between different ethnic groups. Many ethnic groups have inhabited high-altitude locations at different time points in human history (Villafuerte & Corante, 2016). This has shown different periods of adaptation to chronic hypoxia -- those who have lived longer at high-altitude locations are often considered "better" or "more" adapted to the harsh environmental conditions (Villafuerte & Corante, 2016). For example, Ethiopian and Tibetan highlanders exhibit a lower hemoglobin concentration and lower prevalence of CMS and EE relative to Andeans and Han immigrants to high-altitude (Moore, 2001; Villafuerte & Corante, 2016).

Primary CMS is diagnosed by the absence of a medical health condition that may worsen hypoxemia and lead to the development of EE (Villafuerte & Corante, 2016). Secondary CMS is diagnosed by preexisting chronic medical conditions such as chronic bronchitis, cystic fibrosis, or lung cancer, which worsens hypoxemia (Villafuerte & Corante, 2016). Diagnosis for primary CMS is based on clinical symptoms such as dizziness, headache, shortness of breath, fatigue, sleep disturbances, and loss of appetite (Villafuerte & Corante, 2016). The Qinghai Score is used to assess the severity of CMS and it is based on hemoglobin concentration with the presence or absence of EE, alongside the presence of CMS additional symptoms such as headaches and shortness of breath (Leon-Velarde et al., 2005).

Epigenetic Modifications

Epigenetics is a natural and heritable external change in gene expression that does not affect the underlying DNA base sequence but will influence the way genes are transcribed (Goldberg et al., 2007). Epigenetic modifications influence DNA transcription by regulating the DNA-binding proteins that control which genes will be active or silenced (Gibney & Nolan, 2010). This regulates gene expression and thereby changes the phenotype of the organism, which is relevant under environmental conditions of hypoxia (Brown & Rupert, 2014).

Epigenetic modifications can also be established following mitosis, allowing epigenetic markers to be passed down through generations and can even allow transgeneration transfer of regulatory cues (D'Urso & Brickner, 2014). Epigenetic information is tissue-specific and can change according to endogenous and exogenous alterations (Kanherkar et al., 2014). Through epigenetics, organisms are capable of genetically changing themselves and their future offspring in order to optimize gene expression and adapt to a changing environment, such as high-altitude, which is of great interest for studies at high-altitude (Brown & Rupert, 2014).

Epigenetic change occurs naturally but it can also be influenced by age, environment and or lifestyle, and diseases. These modifications are regular and as common as cells in the body differentiating to become skin cells, brain cells, lung cells, and every other cell. However, epigenetics can also have damaging effects to the body, in some cases leading to cancer. The environment and human lifestyle can cause epigenetic change that can be passed down from one generation to the next. An example of epigenetic change being passed down to the next generation is seen in Holocaust survivors and their offspring (Yehuda et al., 2015). It was found that Holocaust survivors that underwent extreme trauma had epigenetic change (DNA methylation) in *FKBP5*, a gene responsible for modulation stress, which was passed down to their offspring, increasing vulnerability to psychological consequences of childhood adversity (Yehuda et al., 2015). This provides insight into how severe psychophysiological trauma can have intergenerational effects (Yehuda et al., 2015).

The environment at high-altitude can have a direct effect on the human genome and influence epigenetic change in order to adapt. DNA methylation, histone modification, and non-coding RNA-associated gene silencing are three types of epigenetic change (Watson et al., 2010). DNA methylation has been seen in long-term Peruvian highlanders who are better adapted to live in such harsh living conditions. However, epigenetic differences between different cells, individuals, or populations have yet to be thoroughly characterized for traits such as altitude acclimatization and limited data exist for populations under long-term environmental stress at high-altitude (Brown & Rupert, 2014; Childebayeva et al., 2019; Childebayeva et al., 2020).

DNA Methylation

DNA methylation is a common type of epigenetic modification that occurs when a methyl group is added to the carbon 5 position of the cytosine ring within cytosine-guanine (CpG) dinucleotides, as seen in Figure 1 (Moore et al., 2013). It occurs naturally in DNA and plays a role in controlling the expression potential of regions of the genome (Laird, 2010). DNA methylation is a heritable and reversible epigenetic change that silences gene transcription by changing the accessibility for RNA polymerase and transcription factors in the gene promoter region and either silences or activates genes (Chen et al., 2019).

Methylation plays a vital role in gene expression, embryonic development, cellular proliferation, differentiation, and chromosome stability (Li & Tollefsbol, 2011). Abnormal DNA methylation can lead to the loss of DNA homeostasis and ultimately result in diseases such as cancer (Li & Tollefsbol, 2011). Current research has shown that epigenetics plays an important role in the cell's hypoxic response. Transcriptional responses are an essential component of adaptation to hypoxic conditions with the hypoxia inducible factor (HIF) pathway influencing the expression of thousands of genes (Semenza, 2019). Studies have shown that hypoxia-induced transcription factors involve epigenetic modifications, such as DNA methylation, in order to initiate the hypoxic responses (Brown & Rupert, 2014). Techniques to comprehensively describe DNA methylation patterns have been developed because DNA methylation encodes important biological information (Laird, 2010). Bisulfite arrays and sequencing are methods of detecting DNA methylation.

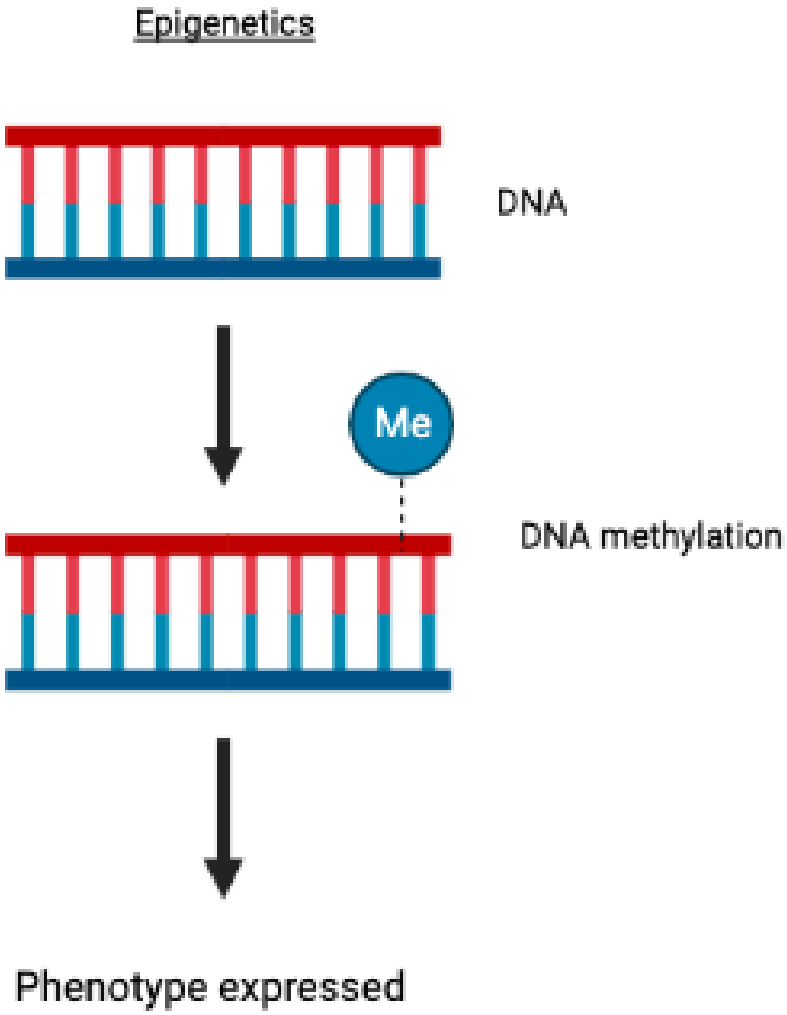


Figure 1. DNA Methylation
This image depicts how DNA methylation, a form of epigenetic modification, does not affect the underlying DNA sequence. A methyl group (Me) attaches to the DNA strand and affects how a gene is transcribed and ultimately how the phenotype is expressed.

Histone Modification

DNA in eukaryotic cells is compacted and organized by chromatin, a nucleoprotein structure (Rossetto et al., 2012). Chromatin consists of 146 base pairs of DNA wrapped around small basic proteins called histones, which are present in pairs at the histone core (Rossetto et al., 2012). The four histones present at the histone core are H2A, H2B, H3 and H4 (Rossetto et al., 2012). Due to the chromatin being tightly wrapped around DNA, the chromatin structure must be modulated in order to access the DNA (Rossetto et al., 2012). One mechanism that modifies chromatin compaction is through post-translational modifications (PTMs) of histones, such as methylation, acetylation, and phosphorylation, which are all considered epigenetic changes (Rossetto et al., 2012).

Histone methylation adds methyl groups at the lysine residues of histone H3 and H4 (Zhang et al., 2021). This methylation occurs by the histone methyltransferase (HMT) transferring methyl groups onto the lysine residues of histones (Zhang et al., 2021). These histone lysine residues can be methylated one to three times to activate or silence marks of gene expression (Zhang et al., 2021).

Histone acetylation adds a negatively charged acetyl group to the lysine residues on histone proteins and it is regulated by the opposing action of histone acetyl transferases (HATs) (Gräff & Tsai, 2013). It is associated with increasing the likelihood of gene transcription by manipulating the folding properties of the chromatin fiber (Gräff & Tsai, 2013; Verdone et al., 2006).

Histone phosphorylation adds a negatively charged phosphoryl group to serine, threonine, and tyrosine residues in the N-terminal histone tails (Ellenbroek et al., 2016). It is also associated with increasing gene expression through transcriptional activation (Ellenbroek et al., 2016).

Histone phosphorylation is also involved in chromatin remodeling and DNA damage repair (Ellenbroek et al., 2016).

Hypoxia

Hypoxia occurs when oxygen levels in tissues are below what is needed for normal physiological functions (Span & Bussink, 2015). It can arise due to poor oxygen delivery or if tissues are not utilizing oxygen as they should (Sarkar et al., 2017). Hypoxia can be chronic, acute, or cycling (intermittent) and can occur at different severity levels (Span & Bussink, 2015). Common causes of hypoxia include asthma, respiratory distress, chronic obstructive pulmonary disease (COPD), lung infection, damage, scarring, or collapse of the lungs, and fluid accumulation in the lungs, to name a few (Span & Bussink, 2015).

Hypoxia and hypoxemia are not synonymous and do not always coexist -- there can be one without the other. Hypoxemia is the decrease in partial pressure of oxygen in the body (Sarkar et al., 2017). It is difficult to establish a single threshold value for severe hypoxia because of the altitude dependence of SpO2 and the range of difference of hemoglobin concentration with SpO2 (Villafuerte & Corante, 2016). However, there have been some cut-off values established for certain regions and altitudes. Studies in Peru at 4340 m elevation have proposed a threshold for diagnosing hypoxemia at SpO2 < 83% (Monge et al., 1992).

Consequences of Chronic Hypoxia

Chronic hypoxia has been established as the primary reason CMS develops in people living at high-altitude. When hypoxia is sustained for long periods of time, new blood cells and blood vessels are generated (Pouyssegur & Bareno, 2016). Chronic hypoxia can also lead to pulmonary hypertension, as seen in people with chronic obstructive pulmonary disease (COPD)

(Ziello et al., 2007). The severity of COPD is often associated with how low oxygen levels are within the body (Leverve, 1998).

Human Adaptation to High Altitude

At higher altitudes, air pressure is lower which allows less oxygen to enter the lungs. Over extended periods of time, the body makes up for the low oxygen levels by changing breathing patterns and producing more red blood cells and blood vessels. This, however, is considered a maladaptive adaptation because it causes erythrocytosis, an excessive amount of red blood cells and increased hemoglobin concentration, which causes the blood to become thicker and can lead to other health complications (Julian & Moore, 2019). Various genes have been found in genomic studies to be involved in various high-altitude populations' ability to adapt to this environment with specific associations between hemoglobin concentration and putatively adaptive genetic signatures identified through patterns detected across thousands of single nucleotide polymorphisms (SNPs) genotyped on microarrays (Simonson et al., 2010; Yi et al., 2010; Beall et al., 2010; Simonson, 2015; Moore et al., 2017).

Whole-genome sequencing to identify genomic regions of interest targeted by natural selection

Whole-genome sequencing (WGS) is a method for the analysis of entire genomes. Research conducted on the human genome has been key in identifying inherited diseases and disorders and discovering mutations that create cancer. Some advantages of whole-genome sequencing include high-resolution view of the genome, capturing large and small variants that might be missed with narrower approaches, and delivering a large volume of data in a short time ("Whole-Genome Sequencing"). It is also a quick and affordable way to obtain information about the DNA in question. Whole-genome sequencing involves these steps: 1) DNA shearing--

using molecular scissors to cut the DNA, 2) DNA bar-coding--small DNA tags are added to identify DNA sections, 3) whole-genome sequencing--the bar-coded DNA are combined and placed in the whole genome sequencer, and 4) data analysis--computer analysis tools used to compare sequences and observe differences ("Whole-Genome Sequencing,").

Few studies of whole genome sequence analyses have been completed in high-altitude studies of genetic adaptation relative to SNP array studies. Whole genome sequence data and subsequent selection analyses have been completed in Tibetans (Hu et al. 2017; Ouzhuluobu et al., 2020). A single Andean study by Zhou et al. performed whole-genome sequencing (WGS) on 20 Andean highlanders, 10 with and 10 without CMS phenotypes, and they identified 11 regions with substantial haplotype frequency differences between the CMS and non-CMS individuals (Zhou et al., 2013). Their study provided a foundation to identify the genetic basis of high-altitude adaptation and identify a potential CMS treatment mechanism (Zhou et al., 2013). Additional WGS studies in Andeans revealed different genetic factors associated with adaptation to high-altitude (Crawford et al., 2017), suggesting additional studies are needed to better understand maladaptive and adaptive genetic loci.

HIF Pathway of Hypoxia

Oxygen is essential for life and humans cannot survive without it for more than a few minutes (Sarkar et al., 2017). The hypoxia-inducible factor (HIF) protein complex plays an important role in the human body's ability to adapt to changing oxygen levels. It controls several important genes associated with the production of red blood cells, the formation of new blood vessels, and it is involved in cell division ("EPAS1 gene"; Semenza, 2020). HIFs control the rate of gene transcription by responding to the changing levels of oxygen and binding to specific DNA sequences (Ziello et al., 2007). The hypoxia-inducible factor 1 (HIF-1) is a dimer made up

of HIF-1α and HIF-1b subunits. HIF-1α is present at low levels in all organs under normal oxygen conditions and HIF-1b is continuously expressed (Ziello et al., 2007). Under hypoxic conditions, the oxygen needed for HIF-1α to be ubiquitinated is missing (Zepeda et al., 2013; Ziello et al., 2007). Because of this, HIF-1α continues intact and moves to the nucleus where it binds with HIF-1b (Ziello et al., 2007). There, HIF-1α gathers coactivator proteins to the HIF binding site with the hypoxia response element (HRE) (Ziello et al., 2007). This results in the up-regulation of a number of genes that help in the adaptation of hypoxia, such as the erythropoietin gene that is involved in the production of more red blood cells (Semenza, 2019; Ziello et al., 2007).

EPAS1 Gene

The endothelial PAS domain protein or *EPAS1* gene (also known as *HIF2A*) codes for a protein called hypoxia-inducible factor 2-alpha (HIF-2α), which is a subunit of a larger protein complex called the hypoxia inducible factor (HIF) ("EPAS1 gene"). HIF plays an important role in the body's ability to adapt to changing oxygen levels and regulates a hormone called erythropoietin, which controls the production of red blood cells ("EPAS1 gene"). HIF-2α is constantly produced in the body, and it is broken down to avoid build up when an appropriate amount of oxygen is sensed ("EPAS1 gene"). However, when there is not enough oxygen in the body (under conditions of hypoxia), HIF-2α is broken down slower than usual which results in higher levels of HIF that stimulate the formation of new blood vessels, as well as the production of red blood cells ("EPAS1 gene"). Both occurrences help deliver adequate oxygen to the body's tissues and organs ("EPAS1 gene"). Figure 2 shows the locations of epigenetic modifications that have been reported at the *EPAS1* locus, including CpG islands, which are sections of DNA

rich in CpG methylation, as well as various histone modifications in particular cell types as determined by the Encyclopedia of DNA Elements (ENCODE) Project.

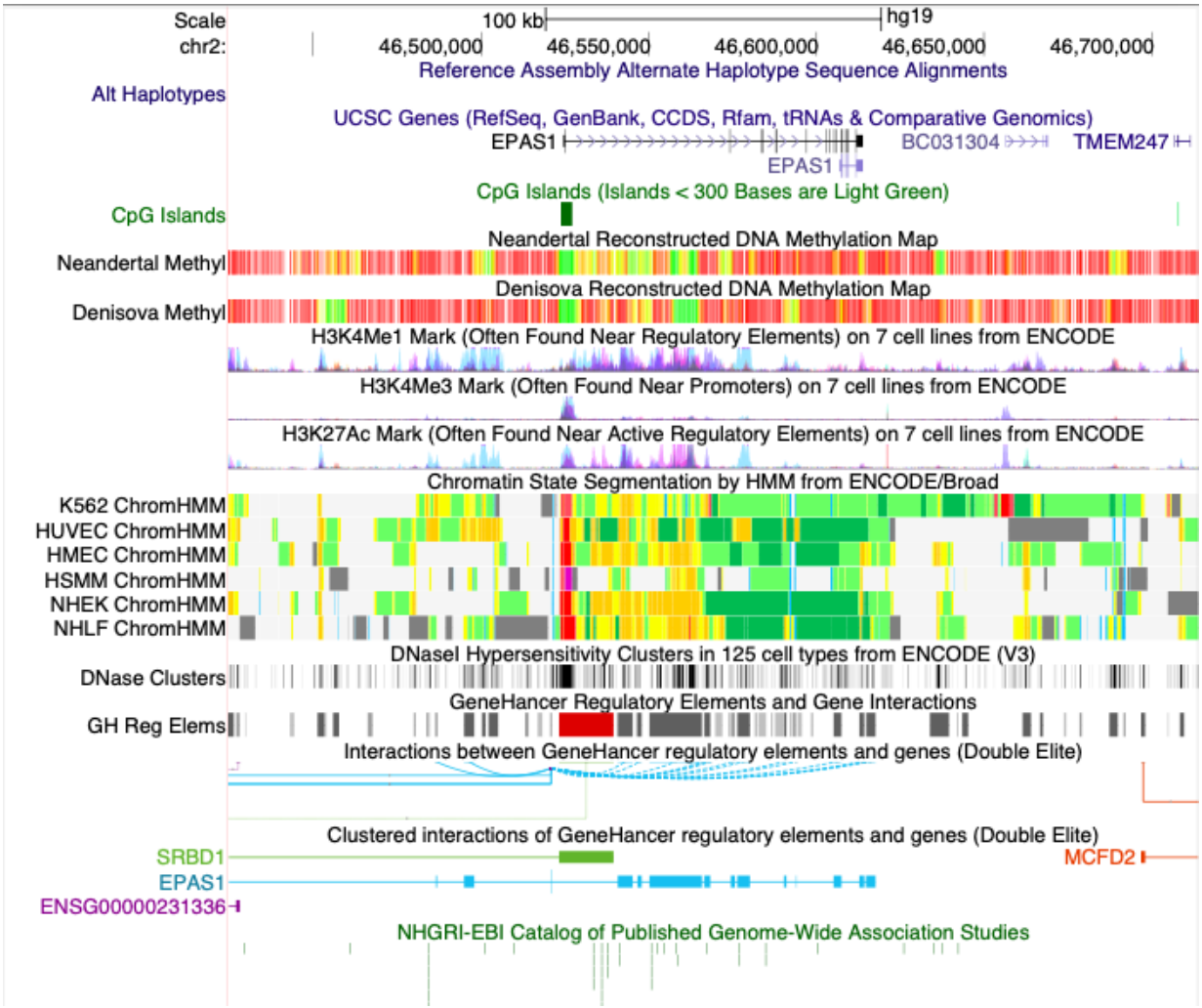
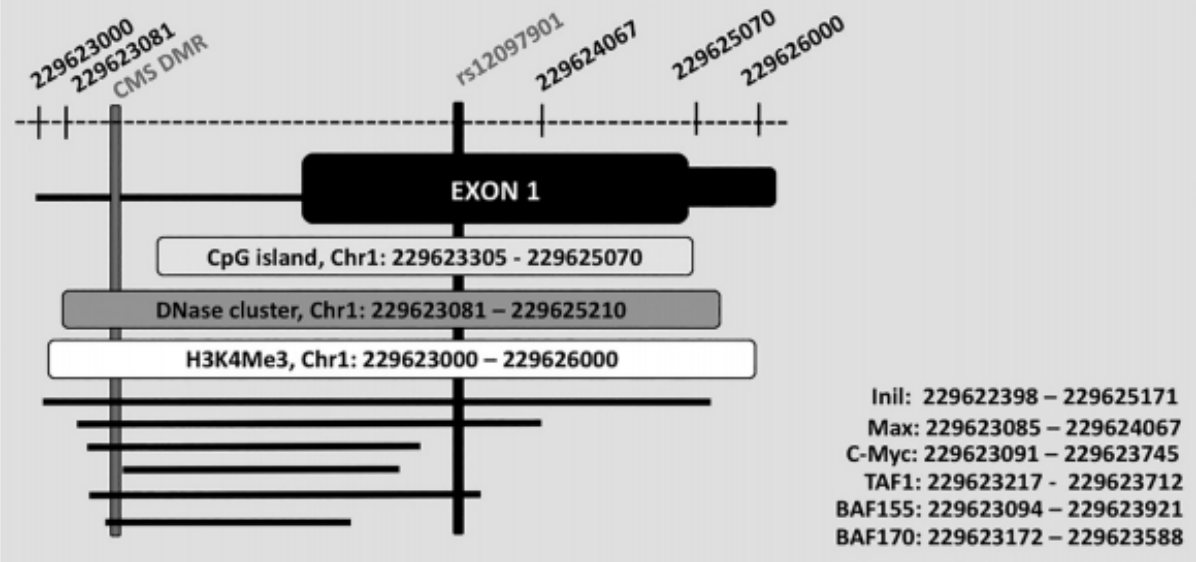


Figure 2. *EPAS1* gene Methylation Markers
This is an image captured using the UC Santa Cruz Genome Browser showing 50kb upstream and 50kb downstream of the *EPAS1* gene. It shows the methylation markers located at CpG islands, histone modifications in various cell types, and gene enhancer regulatory elements.

EGLN1 Gene

The egl nine homolog 1 or *EGLN1* gene (also known as HIF polyhydroxylase 2 and PHD2) codes for an enzyme called prolyl hydroxylase domain 2 (PHD2), which interacts with the alpha subunit of HIF and causes it to be targeted for degradation so that it does not build up when not needed ("EGLN1 gene"). Therefore, when there is enough oxygen in the body for normal functions, PHD2 enzyme becomes active and breaks down HIF-α ("EGLN1 gene"). When oxygen levels drop to hypoxic conditions, the PHD2 enzyme becomes less active and HIF-α is degraded at a slower rate, leaving more HIF available to start transcription of hundreds to thousands of genes (Semenza, 2020). This process helps the body's organs and tissues maximize the amount of oxygen delivered ("EGLN1 gene").

DNA methylation has also been shown to impact the *EGLN1* gene, which may play a role in why some highlanders are better adapted to living in such harsh environments. Supplementary Figure 1 from Colleen Julian's 2017 review on epigenomics and human adaptation to high-altitude shows the differentially methylated regions in the *EGLN1* gene of Andeans with and without EE (Julian, 2017). Figure 3 shows the methylation markers located at CpG islands, histone modifications in various cell types, and gene enhancer regulatory elements determined by the Encyclopedia of DNA Elements (ENCODE) Project.



Supplementary Figure 1. Differentially Methylated region in *EGLN1* of Andeans with EE
This figure shows a differentially methylated region of the *EGLN1* gene identified in Andeans with EE. The gray vertical bar, Chr1229,623,151-229,623,259 represents the EE-associated differentially methylated region in the Andean highlanders survey (Julian, 2017). It is located 46 base pairs (bp) from the CpG island, Chr1: 229,623,305-229,625,070 that includes the *EGLN1* promoter region and less than 700 bp from the rs12097901, represented by the black vertical bar, Chr1: 229623259 (Julian, 2017). This suggests a potential function importance of this differentially methylated region (Julian, 2017). This is further supported by this differentially methylated region overlapping with a DNase cluster (depicted by the dark gray horizontal line) a promoter associated with H3K4Me3 (depicted by the white horizontal bar), and regulator elements/transcriptional binding sites (depicted by the black horizontal lines) (Julian, 2017).

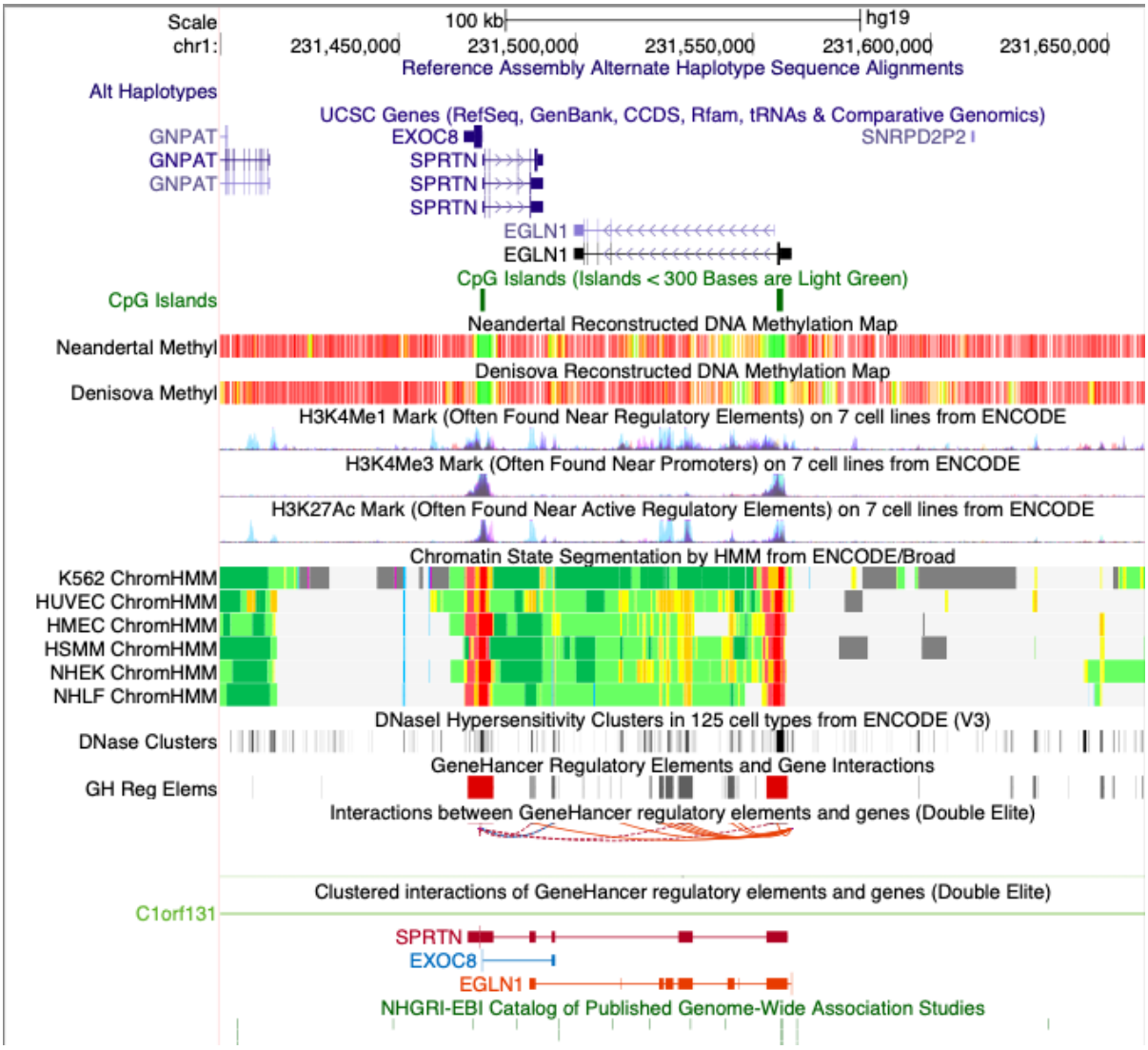


Figure 3. *EGLN1* gene Methylation Markers
This is an image taken using the UC Santa Cruz Genome Browser showing 50kb upstream and 50kb downstream of the *EGLN1* gene. It shows the methylation markers located at CpG islands, histone modifications in various cell types, and gene enhancer regulatory elements.

Bisulfite Sequencing

Bisulfite sequencing is a technique developed by Frommer and colleagues used for large-scale high-resolution DNA methylation analysis in relatively even coverage (Meissner et al., 2005; Li & Tollefsbol, 2011). It is considered the "gold standard" method because it is able to distinguish 5-methylcytosine from cytosine using sodium bisulfite, making it useful for both qualitative and semiquantitative measurement of DNA methylation (Al Harrasi et al., 2017; Li & Tollefsbol, 2011). Bisulfite sequencing treats DNA with sodium bisulfite and converts unmethylated cytosine into uracil by deamination, and methylated cytosine remains untouched (Bibb et al., 2018). The steps for bisulfite sequencing of DNA include: the DNA being extracted from samples, purified, treated with sodium bisulfite, polymerase chain reaction (PCR) amplified, or restriction digested, followed by PCR amplification, and finally the DNA is sequenced (Bibb et a., 2018). De Cubas and colleagues (2015) analyzed methylation levels of CpG islands in both paragangliomas and pheochromocytomas (de Cubas et al., 2015). They used bisulfite sequencing to locate specific methylated areas of the genome and validate their results (de Cubas et al., 2015).

Methylation Microarray

Illumina's Infinium MethylationEPIC Kit is a methylation profiling microarray that provides extensive coverage of genes, CpG islands, and enhancers. With this kit, researchers can look at over 850,000 methylation sites across the genome at single-nucleotide resolution. Multiple samples (up to 8) can be analyzed side-by-side to provide high-throughput power while keeping the cost per sample low ("Infinium MethylationEPIC Kit"). This method has proven effective when looking at DNA methylation in blood samples and can open up the door to new epigenetic discoveries.

Candidate Gene Methylation Studies

A gene whose chromosomal location is associated with a particular disease or phenotype to be studied is called a candidate gene ("Candidate gene"). These genes are chosen based on ideas about their possible role in the processes that cause disease (Shabalin et al., 2015). These candidate gene/gene region methylation studies are often chosen for hypothesis-driven studies, affordability, and/or for the ease of interpretation in terms of lab techniques and/or statistical procedures (Shabalin et al., 2015). For example, Egl-9 homolog 1 gene (*EGLN1*) is a gene shown to be targeted by natural selection in high-altitude populations and is used as a candidate gene for examining genotype-phenotype relationships (Brutsaert et al., 2019). DNA methylation profiles have revealed differentially methylated candidate genes in the *EGLN1* gene (Bick et al., 2012), and illustrate its use as a candidate gene for epigenetic DNA methylation studies.

Clinical Studies

While limited data exists for studies of epigenetics and hypoxia in high-altitude populations, there are various studies in highlanders that suggest epigenetics may play an important role in hypoxia responses and other literature from clinical studies of hypoxia that provide direct evidence for epigenetic modifications in varying levels of hypoxia exposure. These relevant studies and reviews are summarized below.

LINE-1 and EPAS1 DNA methylation associations with high-altitude exposure (Childebayeva et al., 2019).

Childebayeva and colleagues (2019) focused their study on epigenetic changes that occur in lowland individuals who visit high-altitude. In order to characterize if Andean adaptive responses to high-altitude involve epigenetics, Childebayeva and colleagues analyzed DNA

methylation of the promoter region of *EPAS1* and *LINE-1* repetitive element in 572 Quechua individuals from high-4,388m-to low-altitude (0m) (Childebayeva et al., 2019). The individuals recruited at high-altitude showed lower *EPAS1* DNA methylation and higher *LINE-1* methylation (Childebayeva et al., 2019). Altitude of birth was not associated with higher *EPAS1* methylation, but it was associated with higher *LINE-1* methylation (Childebayeva et al., 2019). The number of years the individuals lived at high-altitude was positively associated with *LINE-1* methylation, but negatively associated with *EPAS1* methylation (Childebayeva et al., 2019). Their results demonstrate that current and lifetime exposure to high-altitude hypoxia affect *EPAS1* and *LINE-1* methylation among Quechua individuals, which suggests that epigenetic modifications may play a role in high-altitude adaptation (Childebayeva et al., 2019).

Genome-Wide Epigenetic Signatures of Adaptive Developmental Plasticity in the Andes (Childebayeva et al., 2020)

Childebayeva and colleagues (2020) also performed an epigenome-wide DNA methylation association study based on whole blood from 113 Peruvian Quechua with differential lifetime exposure to high-altitude (>2,500m) (Childebayeva et al., 2020). They identified two significant differentially methylated positions (DMPs) and 62 differentially methylated regions (DMRs) associated with high-altitude developmental and lifelong exposure statuses (Childebayeva et al., 2020). DMPs and DMRs were found in genes associated with hypoxia-inducible factor pathway, red blood cell production, blood pressure, and others (Childebayeva et al., 2020). They also found a significant association between *EPAS1* methylation and *EPAS1* SNP genotypes, suggesting that local genetic variation influences patterns of methylation (Childebayeva et al., 2020). Their findings demonstrated that DNA methylation is associated with early development and lifelong high-altitude exposures among

Peruvian Quechua as well as altitude-adaptive phenotypes (Childebayeva et al., 2020). Together, these findings suggest that epigenetic mechanisms might be involved in adaptive developmental plasticity to high-altitude (Childebayeva et al., 2020).

High-altitude ancestry protects against hypoxia-associated reductions in fetal growth (Julian et al., 2007)

While no epigenetic data were examined, a study published in 2007 by Julian and colleagues focused on developmental aspects to determine why multigenerational high-altitude (>2500 m) populations appeared to be protected against decreased birth weight due to hypoxia compared to newcomer groups (Julian et al., 2007). They used a two-way analysis of variance and x^2 tests to exam and compare maternal and infant characteristics from 3,551 medical records from Andean, European or Mestizo (i.e. admixed) women at high, intermediate and low altitudes from Bolivia (Julian et al., 2007). The maternal and infant characteristics they focused on were birth weight and the frequency of small for gestational age births (SGA) (Julian et al., 2007). They found that the higher the altitude, the lower the birth weight and increased SGA in all ancestry groups (Julian et al., 2007). Julian and colleagues also found Andean infants weighed more and had a lower rate of SGA than European or Mestizo infants at high-altitude (Julian et al., 2007). They concluded that Andean ancestry compared to European ancestry protects against high-altitude negative effects on birth weight (Julian et al., 2007).

In addition to the limited studies of individuals at high-altitude, recent reports of epigenetic modifications in other populations or clinical cohorts provide important insight into techniques that may be applied to assess epigenetic changes that occur across the lifespan. We hypothesize distinct epigenetic shifts occur over time in individuals who developed *in utero*, were born, raised, and lived at high-altitude for many generations. For example, individuals of

Han Chinese ancestry who move to high altitude exhibit a blunted ventilatory response after many years at altitude (Zhuang et al., 1993), which may be due to changes in epigenetic profiles although this remains to be tested. We expect DNA methylation profiles, when examined in the same individuals over time, will provide important clues into the progression of maladaptive phenotypes in highlanders and other populations exposed to various environmental factors. For example, as summarized below from a birth cohort study, the quantification of DNA methylation is associated with BMI, disease, and poor health. This provides an example of how epigenetic profiles may predict and provide information about key pathways underlying individual variation and disease progression in a specific environmental context.

An epigenetic score for BMI based on DNA methylation correlates with poor physical health and major disease in the Lothian Birth Cohort (Hamilton et al., 2019)

A major risk factor for OSA is obesity, and a recent study by Hamilton and colleagues examined the contribution of DNA methylation to obesity-related health outcomes by analyzing data from the Lothian Birth Cohort (LBC) 1936 (Hamilton et al., 2019). Hamilton et al. used the weights for the epigenetic body mass index (BMI) score of the LBC to analyze health-related, psychosocial, lifestyle, and cognitive outcomes by using a regression on methylation (Hamilton et al., 2019). Amongst all their results, they found that a higher epigenetic BMI score was associated with higher BMI, greater body weight, poorer general physical health, and lower health-related quality of life (Hamilton et al., 2019). Studies like this one demonstrate the importance of understanding epigenetics and the implications they have on health.

Epigenetics: A Potential Mechanism Involved in the Pathogenesis of Various Adverse Consequences of Obstructive Sleep Apnea (Chen et al., 2019)

Individuals residing at low altitude may be exposed to conditions of hypoxia as a result of numerous cardiopulmonary diseases, such as chronic obstructive lung disease or cardiometabolic diseases, such as sleep apnea. Yung-Che Chen and colleagues (2019) focused their review on the epigenetic processes that are involved in the development of various adverse consequences of obstructive sleep apnea (OSA) (Chen et al., 2019). OSA is a condition where an individual has recurrent episodes of upper airway collapse which ultimately leads to sleep fragmentation and chronic intermittent hypoxia with reoxygenation (IHR) injury (Chen et al., 2019). While it is largely undiagnosed, recent reports suggest that OSA affects about 25% of men and 13% of women worldwide and, if left untreated, can lead to hypertension, diabetes mellitus, ischemic heart disease, dementia, and depression (Benjafield et al., 2019; Chen et al., 2019). Epigenetic modifications have been shown to contribute to the phenotypic variability of the development of hypoxic pulmonary hypertension in OSA (Chen et al., 2019). Increased DNA methylation has been found in the *FOXP3* gene, which regulates the expression of T regulatory lymphocytes (Chen et al., 2019). This has been found in children with OSA who have had increased systemic inflammatory responses, which suggests that the epigenetically mediated down-regulation of T regulatory lymphocytes may play an important role in the morbidity and inflammatory phenotype in OSA (Kim et al., 2012). Our studies of the control of breathing and sleep apnea in Andean highlanders have identified substantial variation within this population (Heinrich et al., 2020) that may be linked to epigenetic profiles.

Summary

To summarize, some highlanders like those living in the Andean Altiplano, are better adapted to physiological challenges at high-altitude while others develop chronic health conditions, such as chronic mountain sickness. Signatures of adaptation have been identified at genes involved in the hypoxia inducible factor pathway, including *EPAS1* and *EGLN1*. The extent to which epigenetic modifications underlie variation in response to hypoxia are largely unknown and may involve changes in DNA methylation at *EPAS1* and *EGLN1* genes, in addition to other hypoxia-response and downstream genes. Additional research that aims to understand how individual epigenetic profiles relate to and could be used to predict a person's response to low oxygen could be valuable at high-altitude and various clinical settings. Such research can push forward modern medicine that could treat individuals suffering with maladaptive responses to harsh high-altitude living conditions.

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