## A key for hypoxia genetic adaptation in alpaca could be a HIF1A truncated bHLH protein domain

# Una clave para la adaptacion genética de la hypoxia en las alpacas podria ser un dominio de proteina bHLH truncado con HIF1A

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**ABSTRACT:** Animals exposed to hypobaric hypoxia triggers a physiological hypoxia response via Hypoxia Inducible Factor (HIF) proteins that functions as transcriptional complexes. As the South American camelids inhabit at high Andean altitudes we have asked if they have developed genetic adaptations to live at high altitudes. In the present study we investigate genetic structures of the HIF1A proteins carried by members of the superorder Cetartiodactyla. During our investigation we discovered the existence of a genetic event that caused the loss of most of the bHLH domain in the proteins borne by the Alpaca and other members of the Cetartiodactyla superorder; we designate them as bHLH short sequences. Further analysis at the nucleotide level revealed in the 12 short sequences included in the study the presence at the 5'end of the bHLH domains stop codons. Seven out of the 12 short HIF1A proteins, have an identical or almost identical nucleotide sequence at their 5'end with a same TAA stop codon and at the same position. As the mutations affects to both the Artiodactyls and Cetaceans, we postulate that the mutation(s) occurred before their divergence about 55 million years ago. The relevance of these findings for genetic adaptation of Alpacas to hypobaric hypoxia of high altitude conditions is discussed.

KEY WORDS: hypoxia, HIF1A, bHLH domain, alpacas.

#### INTRODUCTION

Camelids and remaining even-toed ungulates (artiodactyls) together with whales and dolphins (cetaceans) are grouped in the superorder Cetartiodactyla (Price *et al.*, 2005). Alpacas (Lama pacos by Linnaeus, 1758), reclassified as Vicugna pacos by Kadwell *et al.* (2001), is one of the four species of South American camelids. Llamas (Lama glama L) along with Alpacas are domestic species, while guanacos (Lama guanicoe by Miller, 1776) and vicuñas (Vicugna vicugna by Molina, 1782) are wild species.

During the evolution of the populations of South American camelids, they developed physiological adaptations to the cold environments and food shortages typical of environments of high-altitude hypoxia above 3000 meters above sea level (Wheeler, 2012). Hypoxia is a situation in which there is a

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reduction in the availability of oxygen to tissues and cells. A different physiological adaptation for living in hypoxia has been described in llamas. These adaptations include higher affinity of hemoglobin for O2, slight increase in blood hemoglobin concentration, high muscle myoglobin concentration, a more efficient O2 extraction at tissue levels and high lactic dehydrogenase activity and less muscularized pulmonary arteries which avoid pulmonary arterial hypertension and cardiac remodeling (Moraga *et al.*, 1996; Llanos *et al.*, 2011a; Llanos *et al.*, 2011b).

Hypoxia is associated with developmental, physiological, environmental and pathophysiological conditions as ischemia, arthritis, inflammation, chronic lung disease, stroke, heart disease and cancer (Semenza, 1999; Maynard & Ohh, 2007). A particular archetype of hypoxia is that associated with high

altitudes (Hypobaric hypoxia). As a consequence of this type of hypoxia, humans and animals triggers an acute (AMS) or chronic (CMS) mountain sickness response depending on the time of exposure. AMS is established when a person is exposed for a short period to hypobaric hypoxia and develops signs of a headache, fatigue, sleep disorder, gastrointestinal disorders or vertigo (Hackett et al., 1976; Davis & Hackett, 2017). CMS also known as Monge's disease, reviewed by Villafuerte and Corante (2016) may be established when a person lives for a long time at high altitude. Around 1.2 to 33% of populations living at high altitude suffer from CMS depending on factors such as age, sex, high and the origin of the population (Azad et al., 2017). One important sign of CMS is the elevation of the hematocrit and the number of erythrocytes (polycythemia). While increasing the amount of hemoglobin in the blood could be a beneficial adaptation to hypoxia, excessive erythrocytosis results in a higher blood viscosity, which affects tissue blood flow and oxygen supply (Prchal, 2010).

The Hypoxia Inducible Factors (HIFs) proteins are transcription factors that play a key role in the homeostasis of oxygen in animals (Rytkonen & Storz, 2011). HIFs proteins are basic Helix-Loop-Helix-PER-ARNT-SIM (bHLH-PAS) proteins distinguish by being both ubiguitously expressed and signal-regulated or constitutively active and tissue specific (Button et al., 2017). HIFs proteins form heterodimeric complexes that are composed of alpha O2-labile subunit, either HIF1A, EPAS1 (HIF2A) or HIF3A and one stable ßsubunit HIF1ß also known as Aryl hydrocarbon receptor nuclear translocator (ARNT). In normoxia conditions in presence of O2, the HIFA subunits are modified by the HIF-specific prolyl-hydroxylases (PHD), causing proteasomal degradation mediated in part by the von Hippel-Lindau suppressor protein (VHL) (Button et al.; Fribourgh & Partch, 2017; Townley et al., 2017).

Ethiopians in Africa, Tibetans in Asia and Aymaras in Andean populations of South America have been the most investigated to elucidate the adaptive mechanisms to CMS of high altitude human populations (Villafuerte & Corante, 2016). Several of these studies have demonstrated the existence of a genetic adaptation to hypoxia in Tibetan populations, for review see Murray *et al.* (2018). Genetic adaptation to hypoxia conditions has also been evidenced in goat, dog, and sheep that live in high-altitudes (Song *et al.*, 2016; Wang *et al.*, 2014; Zhang *et al.*, 2014). In the present study, we investigate the phylogenetic relationships and genetic structures of the HIF1A proteins carried by members of the superorder Cetartiodactyla. During our investigation, we discovered the existence of a genetic event that caused the loss of most of the bHLH domain in the proteins borne by the Alpaca and other members of the Cetartiodactyla superorder. As the mutations affect, both the Artiodactyls and Cetaceans, we postulate that the mutation occurred before their divergence about 55 million years ago (Novacek, 1992; Gingerich & Uhen, 1998). The relevance of these findings for genetic adaptation of Alpacas to hypobaric hypoxia of high altitude conditions is discussed.

### MATERIAL AND METHOD

#### The species dataset

The complete set of HIF1A protein sequences of the superorder Cetartiodactyla included in the present study belong to 38 species of families from orders Artiodactyla and Cetacea. The species have been identified by a 4-digit acronym, in which the first two letters correspond to the name of the genus and the last two to the name of the species. These acronyms will be used from here on.

The families of the Order Artiodactyla are, Family Camelidae: Vicugna pacos (Vipa); Camelus bactrianus (Caba); Camelus dromedarius (Cadr); Camelus ferus (Cafe). Family Bovidae: Bison bison (Bibi); Bos taurus (Bota); Bos mutus (Bomu); Ovis aries (Ovar); Pantholops hodgsonii (Paho); Capra hircus (Cahi); Bos grunniens (Bogr). Family Cervidae: Odocoileus virginianus texanus (Odvi); Family Suidae: Sus scrofa (Susc).

The families of order Cetacea are Family Balaenopteridae: Balaenoptera acutorostrata scammoni (Baac). Family Phocoenidae: Neophocaena asiaeorientalis (Neas). Family Physeteridae: Physeter catodon (Phca). Family Delphinidae: Orcinus orca (Oror); Tursiops truncates (Tutr). Family Monodontidae: Delphinapterus leucas (Dele). Family Lipotidae: Lipotes vexillifer (Live).

#### Selection of HIF1A proteins

Proteins containing PAS domains borne by Cetartiodactyls species were retrieved from the

NCBI GenBank. We used a PSI-BLAST search (http://www.ncbi.nlm.nih.gov/Blast), with three iterations and the Alpaca EPAS1 protein (accession number XP\_015105262.1) as a query and the sequences were subsequently grouped with the help of the CLANS program (Frickey & Lupas, 2004; Zimmermann *et al.*, 2018; Camacho *et al.*, 2009). Later, only HIF1A proteins were selected and aligned with the help of the Clustal Omega program (Sievers *et al.*, 2011), and further analyzed.

#### **Dendrogram construction**

Evolutionary relationships were evaluated by genetic distance methods using the neighbor-joining algorithm for phylogenetic tree construction of the Mega 7 program (Kumar *et al.*, 2016). The parameters pairwise deletion and p-distance model were used. Bootstrap test of phylogeny was performed with 1000 replicates.

#### RESULTS

As the EPAS1 (HIF2A) protein has been implicated in the genetic adaptation to the hypoxia conditions in humans and animals living in the plateau altitudes (Song *et al.*; Lorenzo *et al.*, 2014; Xu *et al.*, 2014), we first searched in the Vicugna pacos NCBI database for corresponding EPAS1 protein sequences. A single sequence was found (accession number XP\_015105262.1) which then we use as a query, to perform a BLAST search for PAS-containing proteins on the whole Cetartiodactyla NCBI data bank. A total of 959 sequences were identified and retrieved, 46 of which correspond to Alpacas protein sequences. In the present study, we restricted the genetic analysis to the 38 HIF1A proteins.

The set of the 38 HIF1A proteins sequences included in the present study are from the order Artio-

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XP_007193021.1_Baac_S												
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NP_001116596.1_Susc_L XP_007471761.1_Live_S XP_007133021.1_Baac XP_007103966.2_Phca_S XP_015105262.2_Phca_S XP_01415631.1_Cafe_S XP_014415631.1_Cafe_S XP_014415631.1_Cafe_S XP_010974976.1_Cafe_S XP_010974976.1_Cafe_S XP_00275277.1_Odvi_S XP_00275277.1_Odvi_S XP_00116596.1_Susc_L XP_007193021.1_Baac_S XP_007193021.1_Baac_S XP_007193021.1_Baac_S XP_00197656.2_Phca_S XP_016105262.1_VipaS	241 2 DSKTFLSRBS	51 24 LDMRFSYCDE	51 2' RITELMGYEP 	71 21 EELLCRSTYE 	81 2: YYHALDSDHL	91 3 TKTHHDMFTK	01 3: GUVTTGYRM 21 4: TETDDOQLEE E. 	L1 3 LAKRGGYVWV IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	21 3 ETQATVIYNT 	31 34 KNSQPQCIVC	11 38 VNYVVSGIIO	51 HDLIFSLOOT
NP_001116596.1_Susc_L XP_007471761.1_Live_S XP_007193021.1_Baac_S XP_007193021.1_Baac_S XP_007109956.2_Phca_S XP_0150526.1_Tutr_S XP_0150526.1_Tutr_S XP_0105262.1_Vipa_S XP_01074976.1_Cadr_S XP_010974976.1_Cadr_S XP_010845948.1_Bibi_S NP_001116596.1_Susc_L XP_007471761.1_Live_S XP_00719956.2_Phca_S XP_00719956.2_Phca_S XP_00719956.2_Phca_S XP_007195262.1_Vipa_S XP_014415631.1_Cafe_S XP_01071676.1_Cafe_S	241 2 DSKTFLSRB5	51 24 LDMRFSYCDE	51 2' RITELMGYEP 	71 21 EELLCRSTYE S. S. S. S. S.	81 2: YYHALDSDHL	91 3 TKTHHDMFTK	01 3: GUVTTGYRM 21 4: TETDDQULEE E. 	11 3 LAKRGGYVWV 	21 3 ETQATVIYNT 41 4 SSNEMO(NIN 5	31 34 KNSQPQCIVC 51 44 LAMSPLPASE 	11 38 VNYVVSGIIO	51 HDLIFSLOOT R 71 DPALNOEVAL
NP_001116596.1_Susc_L XP_007471761.1_Live_S XP_007193021.1_Baac_S XP_007193021.1_Baac_S XP_007109966.2_Phca_S XP_01418666.1_Tutr_S XP_014415631.1_Cafe_S XP_014415631.1_Cafe_S XP_0127527.1_Cahi_L XP_017909034.1_Cahi_S XP_00207257.1_Cahi_S XP_001116596.1_Susc_L XP_001116596.1_Susc_L XP_001116596.1_Susc_L XP_001199766.1_Tutr_S XP_001199766.2_Phca_S XP_016105262.1_Vipa_S XP_0109415631.1_Cafe_S XP_010947651_1_Cahe_S	241 2 DSKTFLSRBS	51 24 LDMRFSYCDE	51 2' RITELMGYEP 	71 21 EELLCRSTYE	81 2: YYHALDSDHL	91 3 TKTHHDMFTK	21 4 TETTDQULEE E E 	L1 3 LAKRGGYVWV	21 3 ETQATVIYNT 	31 34 KNSQPQCIVC 51 44 LAMSPLPASE 	11 38 VNYVVSGIID	51 HDLIFSLOOT
NP_001116596.1_Susc_L XP_007471761.1_Live_S XP_007193021.1_Baac_S XP_007193021.1_Baac_S XP_007109956.2_Phca_S XP_0150566.1_Tutr_S XP_01505262.1_Vipa_S XP_0109761.1_Catr_S NP_001272657.1_Catr_L XP_017909034.1_Catr_S XP_010845948.1_Bibi_S NP_001116596.1_Susc_L XP_007471761.1_Live_S XP_007193021.1_Baac_S XP_0019956.2_Phca_S XP_00199562.1_Vipa_S XP_00199562.2_Phca_S XP_001976561.1_Catr_S NP_001272657.1_Catr_S NP_001272657.1_Catr_S NP_001272657.1_Catr_S	241 2 DSKTFLSRB5	51 24 LDMRFSYCDE	51 2' RITELMGYEP 	71 21 EELLCRSTYE 	81 2: YYHALDSDHL	91 3 TKTHHDMFTK	01 3: GUVTTGYRM 21 4: TETDDQQLEE .E. .A. .A. .A.	L1 3 LAKRGGYVWV IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	21 3 ETQATVIYNT 	31 34 KNSQPQCIVC 51 44 LAMSPLPASE 53 5. 51 5. 51 4.	11 38 VNYVVSGIIO	51 HDLIFSLOOT
NP_001116596.1_Susc_L XP_007471761.1_Live_S XP_007133021.1_Baac_S XP_007139866.1_Tutr_S XP_007109986.2_Phca_S XP_0145056.1_Tutr_S XP_014415631.1_Cafr_S XP_010974976.1_Cadr_S NP_001272657.1_Cahi_L XP_017909034.1_Cahi_S XP_010845948.1_Bibi_S NP_0101416596.1_Susc_L XP_007471761.1_Live_S XP_007199866.1_Tutr_S XP_0071099666.1_Tutr_S XP_0071099666.1_Virs_S XP_007109966.2_Phca_S XP_0115105262.1_Vipa_S XP_010974976.1_Cadr_S XP_010974976.1_Cadr_S XP_0109708267.1_Cahi_L XP_017909034.1_Cahi_S	241 2 DSKTFLSRBS	51 24 LDMRFSYCDE	51 2' RITELMGYEP 	71 21 EELLGRSTYE	81 2: YYHALDSDHL	91 3 TKTHHDMFTK	01 3: GUVTTGYRM 21 4: TETDQULEE E. A. A. A. A.	L1 3 LAKRGGYVWV	21 3 ETQATVIYNT 41 4 SSNEMQNIN 	31 34 KNSQPQCTVC 51 44 LAMSPLPASE 	11 38 VNYVVSGIIO	31 HDLIFSLOOT
NP_001116596.1_Susc_L XP_007471761.1_Live_S XP_007133021.1_Baac SXP_007133021.1_Baac XP_01718656.1_Tutr_S XP_0115105262.1_Vipa_S XP_014415631.1_Cafe_S XP_014415631.1_Cafe_S XP_001272657.1_Cahi_L XP_002725677.1_Odvi_S XP_002752077.1_Odvi_S XP_001116596.1_Susc_L XP_007471761.1_Live_S XP_001116596.1_Susc_L XP_007471761.1_Live_S XP_001193021.1_Baac_S XP_011970865.1_Tutr_S XP_011970865.1_Tutr_S XP_011970865.1_Tutr_S XP_011970865.1_Tutr_S XP_011970865.1_Tutr_S XP_011970865.1_Cafe_S XP_011970865.1_Cafe_S XP_011970865.1_Cafe_S XP_011970861.1_Cafe_S XP_011970934.1_Cahi_S XP_01272677.1_Cahi_S XP_010849484_1_Bibi_S	241 2 DSKTFLSRBS	51 24 LDMRFSYCDE	51 2' RITELMGYEP 	71 21 EELLCRSTYE 	81 2: YYHALDSDHL	91 3 TKTHHDMFTK	21 4 TETDDOQLEE 	11 3 LAKRGGYVWV	21 3 ETQATVIYNT 	31 34 KNSQPQCIVC	11 38 VNYVVSGIIO	51 HDLIFSLQQT

Figure 1. Alignment of Cetartiodactyla HIF1A proteins. Dots indicate identity with the sequence of the top of the figure. Dashes from site 1 to position 62 in short sequences indicate deleted amino acids in the bHLH domain of the HIF1A proteins. Dashes at the end of the proteins indicate unavailable information. Name of the species after accession numbers corresponds to the mention in the material and methods section.

#### Supplementary material A

	1	11	21	31	41	51	61	71	B1 :	91 1	01 1:	11
NP 001116596.1 Susc L	MEGAGGA	NDKKKISSER	RKEKSRDAAR	SRRSKESEVF	YELAHOLPLP	HNVSSHLDKA	SVMRLTISYL	RVRKLLDAGD	LDIEDEMKAQ	MNCFYLKALD	GEVMVLTDDG	DMIYISDNVN
XP 007471763.1 Live L												
XP 007471762.1 Live L												
XP 007471761.1 Live S												
XP 007193020.1 Baac L												
XP 007193021.1 Baac S												
AHN85600.1 Neas L												
XP 004262152.1 Oror L												
XP 019778656.1 Tutr S												
AIB53793.1 Dele L												
ATB53792 1 Phca L		м										
XP 007109955.2 Phca L												
XP 007109956.2 Phca S												
EPY80386 1 Cafe L		MG		1.								
XP 015105262 1 Vipa S												
XP 010960539 1 Caba L												
XP 014415631 1 Cafe S												
XP 010974976 1 Cadr S												
XP 005960147 1 Paho L	ALASOKRAFN	LEST										
NP 001272657 1 Cabi L		G		P								
ARL79603 1 Paho L	G											
AAX89137 1 Paho L												
AGM38929 1 Cabi L												
VD 014964262 1 Orran L	MT ADOLTVCC	WEW										
VD 017909034 1 Cabi S	MINISHICS	V10. H										
AFW10558 1 Cabi L												
VD 012002642 1 Orran I												
VD 020752076 1 0drei I												
VD 020752077 1 0dvi S												
00DCC7 1 Born I												
ADBOGST.I BOGT L	MI COCI TYCC	UTC W										
ADHOGSSO I BOGT L	MIGROLITCO	V10. N										
VD 005000756 1 Dame T												
COVERAGE 1 Date T												
VD 010045047 1 Dibi T	MI CDCI TVCC	UTC W										
AF 010040347.1 BIDI L	MUGRALITICS	V15.W										
NF //6/64.2 Bota L		TND										
BLROUU49.1 BORU L		LNR										
AP_010845948.1_B1D1_S												

Figure 2. Alignment of Cetartiodactyla HIF1A proteins. Dots indicate identity with the sequence of the top of the figure. Dashes from site 1 to position 62 in short sequences indicate deleted amino acids in the bHLH domain of the HIF1A

dactyla, (1 Vipa sequence; 1 Caba; 2 Cafe; 1 Cadr; 2 Bibi; 2 Bota; 2 Bomu; 2 Ovar; 3 Paho; 4 Cahi; 3 Bogr; 2 Odvi; 1 Susc) and from the order Cetacea (2 Baac; 1 Neas; 3 Phca; 1 Oror; 1 Tutr; 1 Dele; 3 Live).

The alignment of the sequences is shown in Figure 1 and in the Figure 2. In Figure 1 the alignment include the first 480 amino acids of 12 representative HIF1A protein sequences encompassing the bHLH, PAS and PAS 3 domains. In the Figure 2, the whole set of the 38 full length HIF1A proteins is included. The alignment of the protein sequences reveals that they can be divided according to their length, into two categories which we designate as long and short (L or S in Figure 1 and Figure 2). The former start with a Met amino acid at position 1, 4 or 14 of the alignment, the latter with a Met amino acid at position 63. Of the total of the sequences, 28 belong to the L type, while the remaining 10 correspond to the type S. Sequences of type L or S are present in both, Artiodactyla and Cetacea orders.

For example, among the S-type sequences are those borne by the Artiodactyls Vipa, Cafe, Cadr, Cahi, Susc and Bibi and by Cetacean Baac, Tutr, Phca, Odvi, and Live. A similar situation occurs with the L type sequences. When in the alignment more than 1 HIF1A proteins are borne by members of one single species, the sequences may be either of the same types or from a different one. Examples from the first category are the 4 Paho sequences which all belong to the L type. Alternatively, out of the four Cahi sequences, 3 are L and 1 S, or the Phca sequences (2 L and 1 S) and Baac sequences (1L and 1 S).

In a characteristic HIF1A protein, four domains are present. A basic Helix-Loop-Helix amino-terminal domain (bHLH), is followed by two PER-ARNT-SIM domains (PAS) and then by two transactivation domains, one NH2- terminal domain and one COOH- terminal domain. In the set of sequences that we have analyzed, we have realized that the short sequences differ from the long ones, in that they are devoid of most of the bHLH domain. In fact, only the second helix region is present. In the long type, the entire bHLH domain contained 50 amino acids, while the short type contains only 13 residues. Thus, in the short, only amino acids corresponding to the second helix region are present (numbering according to Zhou *et al.* (1997)).

Sequence analysis at the DNA level allows getting an insight into the genetic events that generated the short HIF1A proteins. The alignment of the sequences is shown in Figure 3 and in the Figure 4.

In Figure 3 the alignment include the full bHLH domain of 15 representative HIF1A protein

	1									10										20						
XM 022570429.1 Dele S																				ATA	CTG	ACT	TTC	ATC	ACC	TTC
XM 007109894.2 Phca S																				ATA	CTG	ATT	TTC	ATC	ACC	TTC
XM 019923097.1 Tutr S																				ATA	CTG	ATT	TTC	ATC	ACC	TTC
XM 018053545.1 Cahi S																				ATA	CTG	ATT	TTC	ATC	ACC	TTC
XM 010847647.1 Bibi S																				ATA	CTG	ATT	TTC	ATC	ACC	TTC
XM 020896418 1 Odvi S																				AAA	CTG	ATT	TTC	ATC	ACC	TTC
ABBB02138382 1 Vipa S	ATG	TAA	AAT	GAG	TTT	TAT	TCA	AGC	AAA	GGC	AAA	AAA	TGT	ATA	TAA	CTT	CCA	TCT	TTT	TTC	TCT	CCT	CCA	CCT	CCT	CCT
JDVD01013070 1 Cadr S	ATG	TAA	AAT	GAG	TTT	TAT	TCA	AGC	121	GGC	AAC	222	TGT	ATA	AAT	TTT	CCA	TCT	TTT	TTC	TCT	CCT	CCA	CCT	CCT	CCT
ACV701032341 1 Cafe S	ATC	TAA	AAT	GRG	TTT	TAT	TCA	ACC	121	CCC	110	222	TGT	ATE	DAT	TTT	CCA	TOT	TTT	TTC	TCT	CCT	CCA	CCT	CCT	CCT
VM 010847646 1 Bibi S				0110							Ano						COA			CTT	CTT	CAT	CTA	ACT	TAC	CTT
XM 007471699 1 Live S																				CCC	TTC	TTA	CCA	CAC	TCC	TAA
VM 016097105 1 0108 5																	ATC	CTC	CCA	202	TCC	TTA	3CA	TAC	TCC	TCA
VM 010047645 1 Dibi T																	ATC	OTO	CCA	AGA	TCC	TTA	ACA 3.CA	TAC	TOC	TCA
DOCODOLO 1 Dame I																	ATG	OTO	COR	AGA	TCC	TTA	LCA	TAC	TGC	TCA
DU838048.1_Bogr_L																	AIG	CIG	GGA	AGA	TUC	ALL	ACA	TAC	TGC	ICA
XM_005960085.1_Paho_L																				TCC	CAA	AAG	GAG	GCA	TIT	AAT
				10.00										12123						***	***	***	***	***	***	***
				30										40										50		
XM_022570429.1_Dele_S	TTC	ATC	TAA	TTT	AGG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAG	AAG	TCT	AGA	GAT	GCA	GCC	AGA	TCT	CGA	CGA	AGT	AAA	GAG
XM 007109894.2 Phca S	TTC	ATC	TAA	TTT	AGG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAG	AAG	TCT	AGA	GAT	GCA	GCC	AGA	TCT	CGA	CGA	AGT	AAA	GAG
XM 019923097.1 Tutr S	TTC	ATC	TAA	TTT	AGG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAG	AAG	TCT	AGA	GAT	GCA	GCC	AGA	TCT	CGA	CGA	AGT	AAA	GAG
XM 018053545 1 Cabi S	TTC	ATC	TAA	TTT	AGG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAG	AAG	TCT	AGA	GAT	GCA	GCC	AGA	TCT	CGT	CGA	AGT	AAA	GAG
XM 010847647 1 Bibi S	TTC	ATC	TAA	CTT	ACC	ATA	AGT	TCT	CAR	CGT	CGA	222	GAG	220	TCT	ACA	GAT	CCA	GCC	AGA	TCT	CGT	CGA	ACT	222	GAG
VM 020096419 1 Odri 6	TTC	ATC	1721	TTT	200	ATA	ACT	TOT	C2.2	COT	CCA	3.5.7	CAC	330	TOT	2.02	CAT	CCA	000	202	TOT	COT	CCA	ACT	2.2.2	CAC
APD 020896418.1 0001 5	110	TTO	TAA	111	AGG	ATA	AGI	TOT	CAR	CGI	CGA	333	GAG	ANG	TOT	AGA	GAL	GCA	000	AGA	TOT	CGI	CGA	AGI	333	GAG
ABRRU2138382.1_Vipa_S	111	TIG	ALL	AAI	AGG	AIA	AGI	ICI	GAA	CGI	CGA	AAA	GAG	AAA	ICI	AGA	GAI	GCA	GUU	AGA	TGI	CGA	CGA	AGI	AAA	GAG
JDVD01013070.1_Cadr_S	TTT	TTG	TTA	AAT	AGG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAG	AAA	TCT	AGA	GAT	GCA	GCC	AGA	TCT	CGA	CGA	AGT	AAA	GAG
AGVR01032341.1_Cafe_S	TTT	TTG	TTA	AAT	AGG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAG	AAA	TCT	AGA	GAT	GCA	GCC	AGA	TCT	CGA	CGA	AGT	AAA	GAG
XM 010847646.1 Bibi S	TGT	CAA	CIC	TTG	AGG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAG	AAG	TCT	AGA	GAT	GCA	GCC	AGA	TCT	CGT	CGA	AGT	AAA	GAG
XM 007471699.1 Live S	GTG	CTC	AAT	AAA	TGG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAG	AAG	TCT	AGA	GAT	GCA	GCC	AGA	TCT	CGA	CGA	AGT	AAA	GAG
XM 015097105.1 Ovar L	GTG	CTT	AGT	AAA	TGG	ATA	AGT	TCT	GAA	CGT	CGA	AAA	GAG	AAG	TCT	AGA	GAT	GCA	GCC	AGA	TCT	CGT	CGA	AGT	AAA	GAG
XM 010847645 1 Bibi L	CTC	CTT	AGT	222	TGG	ATA	AGT	TCT	GAA	CGT	CGA	222	GAG	AAG	TCT	ACA	GAT	CCA	GCC	ACA	TCT	CGT	CGA	AGT	222	CAG
DO020040 1 Bogs I	CTC	CTT	ACT	333	TCC	ATA	ACT	TOT	C33	CCT	CCA	7.7.7	CAC	330	TOT	3.03	CAT	CCA	ccc	3.03	TOT	CCT	CCR	ACT	222	CAC
VM ODERCOORE 1 Daba I	CTT	TTT	TCC	AAA	100	ATA	AGI	TOT	CAA	COT	COA	333	CAC	333	TOT	AGA	CAT	CCA	000	AGA	TOT	COT	COA	AGI	333	CAC
MI_003360085.1_Pano_L	***	***	***	ALC	ARG	AIA	AGI	101	GAM	CGI	CGM	AAA	GAG	AAA	101	AGA	GAI	GCM	GUU	AGA	101	CGI	CGM	AGI	AAA	GAG
								60										70								
104 000000000 1 D-1- C	TOT		OTT	mmm	-	03.0	OTT	COT		~~~	-	~~~	-	~~~	-		-	200	-	-	OTT		330		TOT	-
XM_022570429.1_Dele_5	TCI	GAA	GII	111	TAT	GAG	CII	GCI	CAL	CAG	TIG	CCA	CIT	CCC	CAL	AAI	GIG	AGC	TCA	CAI	CII	GAI	AAG	GCI	TCT	GIT
XM_007109894.2_Phca_S	TCT	GAA	GIT	TTT	TAT	GAG	CIT	GCT	CAT	CAG	TTG	CCA	CIT	CCC	CAT	AAT	GTG	AGC	TCA	CAT	CIT	GAT	AAG	GCT	TCT	GIT
XM_019923097.1_Tutr_S	TCT	GAA	GTT	TTT	TAT	GAG	CTT	GCT	CAT	CAG	TTG	CCA	CTT	CCC	CAT	AAT	GTG	AGC	TCA	CAT	CTT	GAT	AAG	GCT	TCT	GTT
XM_018053545.1_Cahi_S	TCT	GAA	GTT	TTT	TAT	GAG	CTT	GCT	CAT	CAG	TTG	CCG	CTC	CCC	CAT	AAT	GTA	AGC	TCG	CAT	CTT	GAT	AAG	GCT	TCT	GTT
XM 010847647.1 Bibi S	TCT	GAA	GTT	TTT	TAT	GAG	CTT	GCT	CAT	CAG	TTG	CCA	CTC	CCC	CAT	AAT	GTA	AGC	TCG	CAT	CTT	GAT	AAG	GCT	TCT	GTT
XM 020896418.1 Odvi S	TCT	GAA	GTT	TTT	TAT	GAG	CTT	GCT	CAT	CAG	TTG	CCA	CTC	CCT	CAT	AAT	GTA	AGC	TCG	CAT	CTT	GAT	AAG	GCT	TCT	GTT
ABRR02138382.1 Vipa S	TCT	GAA	GTT	TTT	TAT	GAG	CTT	GCT	CAT	CAG	CTG	CCA	CTT	CCC	CAT	AAT	GTG	AGC	TCG	CAT	CTT	GAT	AAG	GCT	TCT	GTT
TDUD01012070 1 Cody C	TOT	C3.3	OTT	TTT	TAT	CAC	OTT	COT	CAT	CAC	OTC	CCA	OTT	000	CAT	AAT	CTC	ACC	TCC	CAT	OTT	CAT	330	COT	TOT	OTT
300001013070.1 Cade 3	TOT	CAR	OTT		TAT	CAG	OTT	COT	CAL	CAG	CTG	COA	OTT	000	CAL	AAI	CTC	AGC	100	CAL	OTT	CAT	ANG	COT	TOT	OTT
AGVRUIU32341.1 Care_S	TOT	GAA	GIT	111	TAT	GAG	CII	GUI	CAT	CAG	CIG	CCA	ome	000	CAT	AAT	GIG	AGC	TUG	CAT	CII	GAT	AAG	GCI	TUL	GII
XM_010847646.1_B1bi_S	TCT	GAA	GIT	TTT	TAT	GAG	CIT	GCT	CAT	CAG	TTG	CCA	CTC	CCC	CAT	AAT	GTA	AGC	TCG	CAT	CIT	GAT	AAG	GCT	TCT	GIT
XM_007471699.1_Live_S	TCT	GAA	GIT	TTT	TAT	GAG	CIT	GCT	CAT	CAG	TTG	CCA	CIT	CCC	CAT	AAT	GIG	AGC	TCA	CAT	CIL	GAT	AAG	GCT	TCT	GTT
XM_015097105.1_Ovar_L	TCT	GAA	GTT	TTT	TAT	GAG	CTT	GCT	CAT	CAG	TTG	CCA	CTC	CCC	CAT	AAT	GTA	AGC	TCG	CAT	CTT	GAT	AAG	GCT	TCT	GTT
XM 010847645.1 Bibi L	TCT	GAA	GTT	TTT	TAT	GAG	CTT	GCT	CAT	CAG	TTG	CCA	CTC	CCC	CAT	AAT	GTA	AGC	TCG	CAT	CTT	GAT	AAG	GCT	TCT	GTT
DQ838048.1 Bogr L	TCT	GAA	GTT	TTT	TAT	GAG	CTT	GCT	CAT	CAG	TTG	CCA	CTC	CCC	CAT	AAT	GTA	AGC	TCG	CAT	CTT	GAT	AAG	GCT	TCT	GTT
XM 005960085.1 Pabo L	TCT	GAA	GTT	TTT	TAT	GAG	CTT	GCT	CAT	CAG	TTG	CCA	CTC	CCC	CAT	AAT	GTA	AGC	TCG	CAT	CTT	GAT	AAG	GCT	TCT	GTT

Figure 3. Nucleotide alignment of Cetartiodactyla bHLH domain of HIF1A proteins. Dashes at the beginning of the sequences were introduced for optimal alignment. Asterisk at the bottom of sequences between position sites 20-29 indicate the Thymine rich stretch of the sequences. Stop codons are in bold letters. Name of the species after accession numbers corresponds to that mention in the material and methods section.

sequences. In the Figure 4, the whole set of the full length bHLH domain sequences is included. In the alignment, the conserved sequence CGAAAAGAG in position 37-39, encodes for the first 3 residues (RKE in Figure 1), of the bHLH domain of HIF1A proteins. Although the next 5 'upstream 16 positions are also well preserved, then the sequences are segregated into two clusters. One of the clusters, in which all short sequences are grouped is distinguish from the other group in having a high nucleotide variability and been very Thymine rich at its 5'end.

In the alignment, the Cetacean sequences Dele, Phca, Tutr, Odvi and Baac and the Artiodactyls Cahi and Bibi conform an identical or nearly identical compact group and in all seven sequences a TAA stop codon is present at position 29 of the alignment. All these sequences are short and the presence of the stop codon by itself explain the fact of the shortening of the HIF1A protein that they borne. Sequence Live, which also have the TAA stop codon in the Thymine rich stretch is different from all other short nucleotide sequences. Finally, camelids Vipa, Cadr and Cafe conform a second conserved cluster, with a stop codon TAA at position 2 in the alignment.

#### DISCUSSION

When animals are acute or chronically exposed to hypobaric hypoxia, they trigger a hypoxic response via Hypoxia Inducible Factor (HIF) proteins that function as transcriptional complexes. HIF proteins are heterodimers with 2 chains, HIF-alfa

Supplementary mater	ial	в																								
	1									10										20						
XM 007192959.1 Baac S	_																			ATA	CTG	ATT	TTC	ATC	ACC	TTC
XM 022570429.1 Dele S																				ATA	CTG	ACT	TTC	ATC	ACC	TTC
XM 007109894.2 Phca S																				ATA	CTG	ATT	TTC	ATC	ACC	TTC
XM 019923097.1 Tutr S																				ATA	CTG	ATT	TTC	ATC	ACC	TTC
XM 018053545.1 Cahi S																				ATA	CTG	ATT	TTC	ATC	ACC	TTC
XM 010847647.1 Bibi S																				ATA	CTG	ATT	TTC	ATC	ACC	TTC
XM 020896418.1 Odvi S																				AAA	CTG	ATT	TTC	ATC	ACC	TTC
ABRR02138382.1_Vipa_S	ATG	TAA	AAT	GAG	TTT	TAT	TCA	AGC	AAA	GGC	AAA	AAA	TGT	ATA	AAT	CTT	CCA	TCT	TTT	TTC	TCT	CCT	CCA	CCT	CCT	CCT
JDVD01013070.1 Cadr S	ATG	TAA	AAT	GAG	TTT	TAT	TCA	AGC	AAA	GGC	AAC	AAA	TGT	ATA	AAT	TTT	CCA	TCT	TTT	TTC	TCT	CCT	CCA	CCT	CCT	CCT
AGVR01032341.1 Cafe 5	ATG	TAA	AAT	GAG	TTT	TAT	TCA	AGC	AAA	GGC	AAC	AAA	TGT	ATA	AAT	TTT	CCA	TCT	TTT	TTC	TCT	CCT	CCA	CCT	CCT	CCT
XM_010847646.1_Bibi_S																				CTT	CTT	CAT	CTA	ACT	TAG	GTT
XM_007471699.1_Live_S																				CGG	TTC	TTA	GCA	CAG	TGC	TAA
XM_015097105.1_Ovar_L																	ATG	CTG	GCA	AGA	TCC	TTA	ACA	TAC	TGC	TCA
XM_010847645.1_Bibi_L																	ATG	CTG	GGA	AGA	TCC	TTA	ACA	TAC	TGC	TCA
DQ838048.1 Bogr_L																	ATG	CTG	GGA	AGA	TCC	TTA	ACA	TAC	TGC	TCA
XM_005960085.1_Paho_L																				TCC	CAA	AAG	GAG	GCA	TTT	AAT
AY971808.1_Paho_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
XM_012147252.2_Ovar_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
XM_020896417.1_0dvi_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
KC700026.1_Cahi_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
NM_174339.3_Bota_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
XM_019969024.1_Boin_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
XM_006054432.1_Bubu_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
AY621118.1_Bogr_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
XM_005890694.1_Bomu_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
XM_010962237.1_Caba_L																				ATG	GAG	GGC	GCC	GGC	GGC	GCG
NM_001123124.1_Susc_L																				ATG	GAA	GGC	GCC	GGC	GGC	GCG
KJ144249.1_Neas_L																				ATG	GAG	GGC	GCC	GGG	GGC	GCG
KJ619998.1_Phca_L																				ATG	GAG	GGC	GCC	GGC	GGC	GCG
XM_019923093.1_Tutr_L																				ATG	GAG	GGC	GCC	GGC	GGC	GCG
XM_022570428.1_Dele_L																				ATG	GAG	GGC	GCC	GGC	GGC	GCG
XM_004262104.1_Oror_L																				ATG	GAG	GGC	GCC	GGC	GGC	GCG
XM_007471701.1_Live_L																				ATG	GAG	GGC	GCC	GGC	GGC	GCG
XM_007192958.1_Baac_L																				ATG	GAG	GGC	GCC	GGC	GGC	GCG
																				444	4.4.4		4.4.4	4.4.4	4.4.4	4.4.4

Figure 4. Alignment of Cetartiodactyla HIF1A proteins. Dots indicate identity with the sequence of the top of the figure. Dashes from site 1 to position 62 in short sequences indicate deleted amino acids in the bHLH domain of the HIF1A

and HIF-beta. A full hypoxic response requires dimerization, nuclear translocation and binding of HIF proteins to p300-CBP proteins, important for maximal transcriptional activation (Arany *et al.*, 1996).

The analysis of the sequences HIF1A proteins in Cetartiodactyla reveals that the Alpaca protein lacks most of the bHLH domain, present in the generality of bHLH -PAS family proteins (Wang *et al.*; Wang *et al.*, 1995). These proteins share a common structure that includes DNA Binding domain (bHLH), followed in tandem by PAS domains (PAS-A and PAS-B) and variable domains of transactivation or transrepression.

The alignment in Figure 3 provides several distinctive features which give some insight into the evolution of the nucleotide bHLH encoding domain. First, all but one of the seven sequences at the top part of the Figure 3 is identical in 5'thymine rich stretch. The only exceptions are the Odvi and Dele sequences. Second, all the seven sequences contain the stop codon TAA at position 29 of the alignment. Third, in the Neighbor joining (NJ) tree shown in Figure 5, the seven sequences are grouped into two branches. One contains the three artiodactyl sequences (Cahi, Odvi and Bibi) and the second all the four cetacean bHLH sequences.

As a whole, these three characteristics seem to indicate that in the evolution of HIF1A protein, the advent of the short protein version was a unique mutational event which gave rise to a bHLH pseudogene. Also, the data indicate that this mutational event occurred before the divergence of artiodactyls and cetaceans, about 55 Mya (Novacek, 1992; Gingerich & Uhen, 1998).

Additional information is provided by the camelids species, one Alpaca (Vipa) and the two camel sequences (Cadr and Cafe), which all three share the 5'thymine richness nucleotide stretch with the other short sequences. The three sequences also show a TAA stop codon, but this is located 27 codons upstream of one detected in the previous 7 ones. All three camelids sequences are almost identical each other which indicate that their differentiation with the rest of the sequences occur during the 40-45 Millions year of evolution in the plains of North America and before the separation of North and South American camelids, which occurs some 3 Mya (Wheeler). The short Bibi sequence is also 5'thymine rich and also contains a stop codon, but this is instead TAG and is located 4 codons upstream of the TAA in sequences at the top of Figure 3. Finally, the Live S sequence is similar to all other short sequences in having both the TAA stop codon and in being Thymine rich



Figure 5. Phylogenetic trees based on selected nucleotide bHLH domain of Cetartiodactyla HIF1A sequences. The numbers shown on the interior nodes are bootstrap probabilities in percent. The parameters pairwise deletion and p-distance model were used.

at its 5'end. A particular feature of the sequence is the fact that it shows at the 5'end some similarities with the three of the long sequences Ovar, Bibi and Bogr. Taking this similarity into account, we can speculate that perhaps the original mutation originating the bHLH pseudogene could have occurred in a long sequence which was rich in thymine at its 5'end.

A phylogenetic tree based on bHLH nucleotide sequences shows that they are grouped with two exceptions, into two major clades depending on whether they are derived from coding sequences for long or short HIF1A proteins (Figure 5). In one of the clades are found all but one of the long sequences. In the other clade are found all but one of the short sequences. The exceptions are Paho long and the Live short sequences, which incongruous locations can be explained by their differences at a stretch from codon 20 to 30 in the alignment.

In the clade of the long sequences, three different branches can be found. In the first are all Bovidae

sequences, such as Bota and Ovar and one of the two sequences of Paho. The second branch contains the long sequences derived from the Camelidae Caba, the Cervidae Neas, the Suidae Susc and all the long sequences derived from cetaceans. In a third branch are the 5'Thymine rich long Bovidae Ovar, Bibi and Bogr sequences and the incongruous Lipotidae Live short sequence. In the clade of the short sequences, two main branches can be found. One contains the Camelids, Cadr, Cafe and Vipa, the Bovids Bibi and the incongruous Paho long sequence. The others are all the seven sequences described above.

Initial studies showed that cutting the bHLH domain does not affect the dimerization and nuclear translocation of HIF1A, but it diminished its binding to DNA, impelling the normal hypoxic response (Reisz-Porszasz et al., 1994). Further studies have corroborated these conclusions (Semenza et al., 1997; McGuire et al., 2001; Huang et al., 2012). Then, according to these findings, we conclude that the hypoxic-response in Alpacas is diminished due to the adaptive loss of part of bHLH domain of HI-F1A proteins. Consequently, Alpacas are genetica-Ily adapted to live in a low oxygen tension environment, without the negative consequences of a full hypoxic response, such as AMS and CMS. Interestingly, we have found that the absence of most the bHLH domain in the HIF1A protein of the only South American camelid included in the present study also seems to exist in other members of the Cetartiodactyla superorder. Thus, in addition to the Alpaca, truncated bHLH domain in HIF1A proteins, are also possible to be found among goats, old world camelids and cetaceans. The main conclusion of this finding is that the mutation(s) that gave rise to truncated bHLH domains in some Cetartiodactyla HIF1A proteins occurred around 55 Mya, before the divergence of Artiodactyla and Cetaceans (Novacek, 1992; Gingerich & Uhen, 1998).

MORAGA, F. A.; FIGUEROA, F.; CARRASCO, R. & MORAGA, D. Una clave para la adaptacion genética de la hypoxia en las alpacas podria ser un dominio de proteina bHLH truncado con HIF1A. *J. health med. sci.*, 6(2):97-106, 2020

**ABSTRACT:** Los animales expuestos a hypoxia hipobárica generan una respuesta hipóxica fisiológica debido a unas proteinas de Factor-Hipoxia Inducible (HIF) que funcionan como complejos transcripcionales. Debido a que los camelidos Americanos habitan en las grandes alturas andinas, nos hemos preguntado si han desarrollado una adaptación genética para vivir a grandes alturas. En este estudio hemos investigado la estructura genética de las proteinas HIF1A que llevan consigo los miembros de la superorden de los cetartiodáctilos. Durante nuestra investigación, descubrimos la existencia de un evento genético que causó la perdida de la mayoría del dominio bHLH en las proteinas transmitidas por la alpaca y otros miembros de la superorden de los cetartiodáctilos; las hemos designado como secuencias cortas de bHLH. Análisis posteriores a nivel nucleótido revelaron que en la doceava secuencia corta incluida en el studio, hubo presencia de codones de terminación en el extreme 5' del dominio de bHLH. Siete de las doce proteinas cortas HIF1A, tiene una secuencia idéntica o casi idéntica de nucleotidos en su extremo 5', con el mismo codón de terminación TAA y en la misma posición. Debido a que la mutación afecta tanto a Artiodáctilos como Cetáceos, proponemos que la mutación(es) ocurrió antes de su divergencia hace unos 55 millones de años. Analizamos la relevancia de estos descubrimientos sobre la adaptación genética de las alpacas a la hipoxia hipobárica en condiciones de grandes alturas.

PALABRAS CLAVE: hipoxia, HIF1A, dominio bHLH, alpacas.

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